Clontech Laboratories, Inc.

iDimerize™ Reverse Dimerization System

PT5180-1 (PR123824)
Cat. No(s). 635066, 635054 & 635053
Published 3/30/2011

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I. Introduction

A. Summary

ARGENT cell signaling regulation kits from ARIAD are now available exclusively from Clontech, as the iDimerize Inducible Dimerization Systems.

The **iDimerize Reverse Dimerization System** (Cat. No. 635066), is used to create and express 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 the addition of a cell-permeant ligand (the D/D Solubilizer).

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.

The iDimerize Reverse Dimerization System consists of components identical to those previously supplied in the ARGENT Regulated Secretion/Aggregation Kit from ARIAD. The names of the plasmids, dimerization domains, and dimerization ligands have been changed by Clontech but are identical to those previously supplied in the ARGENT kit. 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 and forms the basis of our four iDimerize Inducible Dimerization Systems. 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 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 **iDimerize Inducible Homodimer System** (Cat. No. 635068), 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 **iDimerize Inducible Heterodimer System** (Cat. No. 635067).
- Another dimerization system, our **iDimerize Inducible Expression System** (Cat. No. 635065), places the transcription of a target gene under the control of a "dimerizer", which causes the assembly of a functional transcription factor in order to achieve tightly regulated conditional expression of genes of interest.

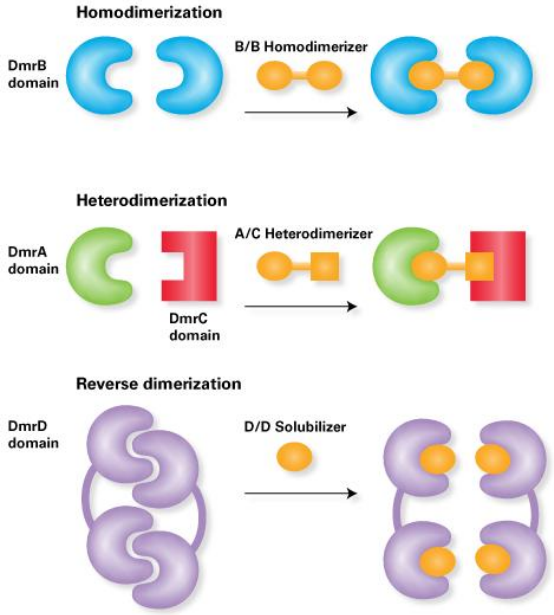

Figure 1. The different types of dimerization include homodimerization, heterodimerization, and reverse dimerization. Separate kits are available from Clontech.

C. iDimerize Reverse Dimerization System Overview

The 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 (prHom-1), three (prHom-Nuc1), or four copies (prHom-Sec1) of a DmrD self-dimerizing domain, each of which bind D/D Solubilizer. The resulting fusion proteins are localized to the cytoplasm (prHom-1), nucleus (prHom-Nuc1), or endoplasmic reticulum (prHom-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. The plasmids in this kit provide an assortment of components, such as self-dimerization domains, an HA epitope tag, and localization sequences. prHom-Sec1contains a signal sequence that causes your protein of interest to be secreted, and a furin cleavage site that results in cleavage of the DmrD domains from this protein in the trans-golgi network prior to secretion. The components in the different vectors can easily be customized and exchanged to generate the fusion protein whose aggregation state you wish to control (Figure 2).

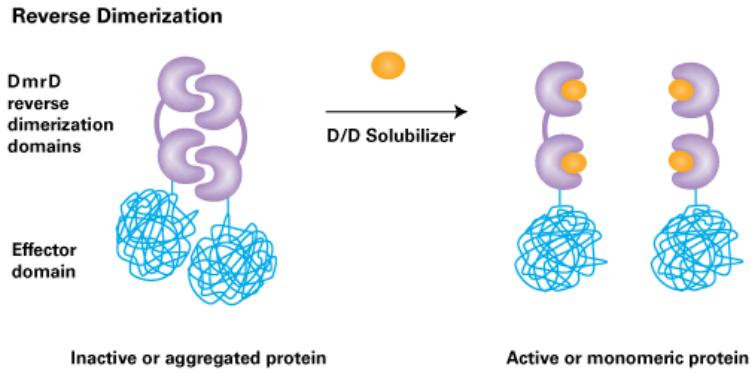

Figure 2. Controlling protein activity using regulated aggregation.

D. Controlling Secretion Using the iDimerize Reverse Dimerization System

The 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.

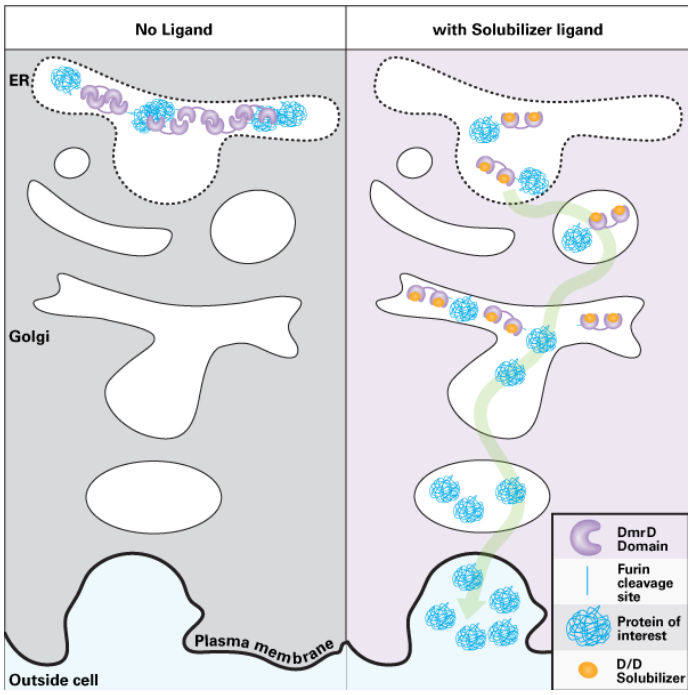

Figure 3. Induction of protein secretion by addition of D/D Solubilizer.

II. List of Components

Store all components at -20°C.

- 1 each iDimerize Reverse Dimerization Vector Set 1
  - 20 µl prHom-1 Vector (500 ng/µl)
  - 20 µl prHom-Nuc1 Vector (500 ng/µl)
  - 20 µl prHom-Sec1 Vector (500 ng/µl)
  - 40 µl Linear Hygromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631625)
  - 40 µl Linear Puromycin Marker (50 ng/µl) (also sold separately as Cat. No. 631626)
- 500 µl D/D Solubilizer (0.5 mM)
  (also sold separately as Cat. Nos. 635054 & 635053—see Section III.A)

The iDimerize Reverse Dimerization System components are identical to those previously supplied in the ARGENT Regulated Reverse dimerization Kit from Ariad—only the names have been changed (Table 1).

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

Plasmid Name in ARIAD Kit	Plasmid Name in Clontech Kit
$pC_4EN\text{-}F_M2E$ $pC_4EN\text{-}F_M3$ $pC_4S_1\text{-}F_M4\text{-FCS-hGH}$	prHom-1 prHom-Nuc1 prHom-Sec1
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in Clontech Kit**
F_M	DmrD

NOTES:

- 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 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

- 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)
- Dulbecco's phosphate buffered saline (DPBS; VWR, Cat. No. 82020-066 or Sigma, Cat. No. D8662)

C. Antibiotics for Selecting Stable Cell Lines

Cat. No.	Antibiotic
631306	Puromycin (100 mg)
631305	Puromycin (25 mg)
631309	Hygromycin B (1 g)

D. Xfect™ Transfection Reagents

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types. Xfect mESC Transfection Reagent is optimized for mouse embryonic stem cells.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)
631320	Xfect mESC Transfection Reagent (100 rxns)
631321	Xfect mESC Transfection Reagent (300 rxns)

E. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that greatly simplifies 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)

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, replace the SpeI-BamHI fragment of prHom-Sec1 with an SpeI-BamHI fragment that contains a furin cleavage sequence followed by the mature coding sequence of your protein of interest (Figure 4). This is most easily accomplished by using Clontech's In-Fusion HD Cloning System (see Section III.E). 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 prHom-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.

NOTE: The stuffer sequence in the SpeI-BamHI fragment of prHom-Sec1 encodes human growth hormone.

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

- Fusion proteins that localize to the cytoplasm and nucleus are created by cloning signaling proteins of interest into either the XbaI site or the SpeI site of the prHom-1 and prHom-Nuc1 vectors, respectively (Figure 4). Cloning into the XbaI site places the DmrD domains at the C-terminus and cloning into the SpeI site places the DmrD domains at the N terminus of your protein of interest.
- If the sequence encoding your protein is cloned into the N-terminal XbaI site of prHom-1, it must lack a stop codon, be in-frame with the start codon (ATG), just upstream of the Xba I site, and be in-frame with the downstream DmrD domains. If your protein is cloned into the Xba I site of prHom-Nuc1, this protein must be in-frame with the N-terminal nuclear localization signal. The coding sequence of a protein that is cloned into the Xba1 site must not contain a stop codon, and must be in-frame with the downstream DmrD domains.
- Fusion proteins localize to the cytoplasm when created using prHom-1 (which contains no targeting signal), and to nucleus when created using prHom-Nuc1 (which contains an N-terminal nuclear localization signal between the EcoRI and XbaI sites).
- A hemagglutinin (HA) epitope tag located between the SpeI and BamHI sites in the prHom-1 and prHom-Nuc1 vectors is expressed at the C-terminus of fusion proteins created using either vector. This tag is useful for determining subcellular protein localization, facilitating protein purification, identifying associated proteins, and characterizing new proteins by immunoprecipitation (HA-Tag Polyclonal Antibody, Cat. No. 631207).

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.

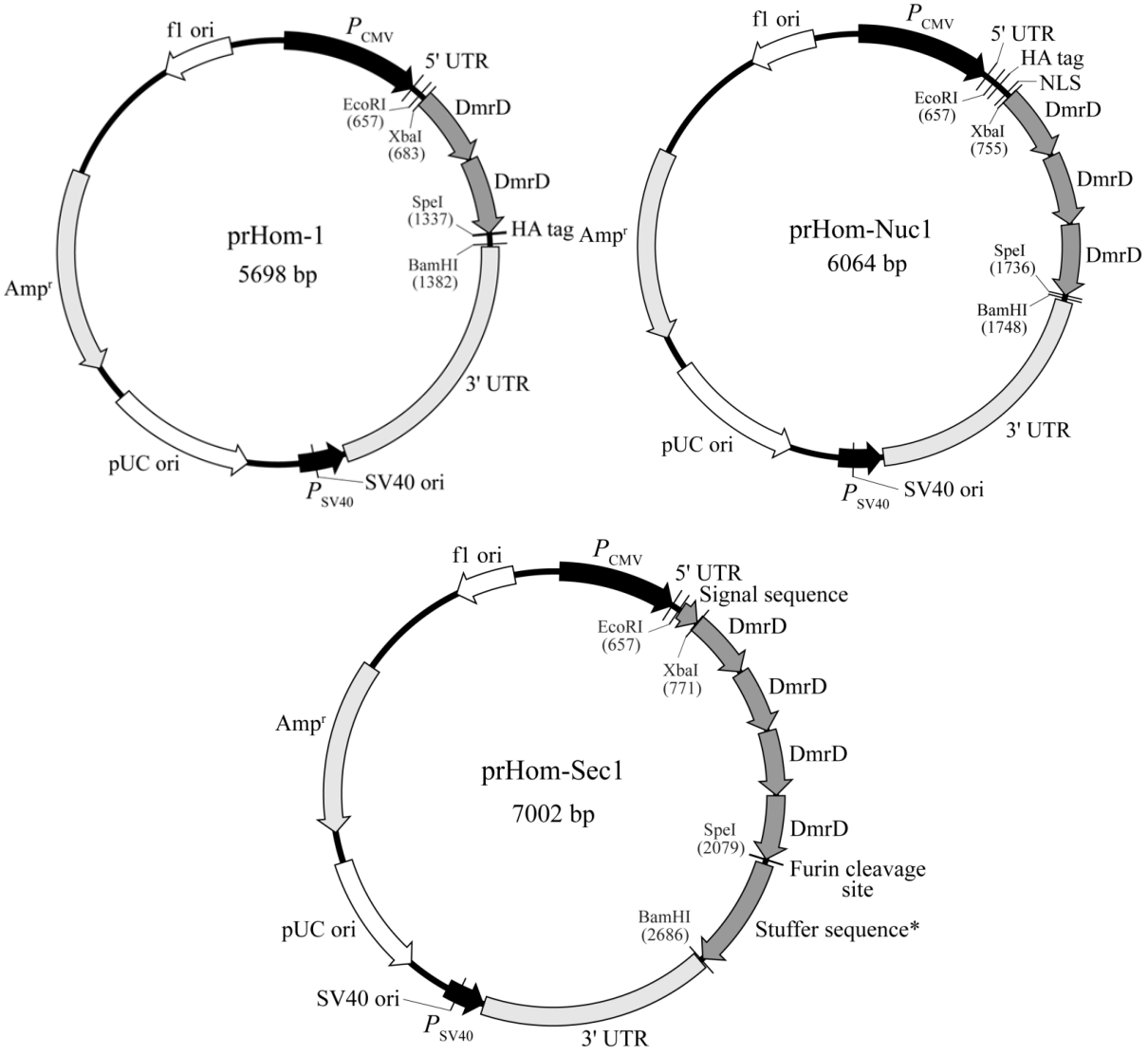

Figure 4. prHom-1, prHom-Nuc1 and prHom-Sec1 Vector Maps.

B. Protocol: Cloning Strategy for Creating Fusion Proteins

Create fusion proteins containing the DmrD domain and your protein of interest with the prHom-Sec1, prHom-1 and/or prHom-Mem1 vectors, using the following cloning strategies (For vector map information (see Figure 4).

In-Fusion HD cloning is generally recommended over ligation-based cloning because In-Fusion HD cloning is directional, is not affected by internal SpeI and XbaI sites, and is highly efficient (most clones contain the correct insert). For In Fusion HD ordering information; see Section III.E.

1. Cloning options for prHom-Sec1

For inducible secretion studies, we recommend cloning your sequence of interest into the SpeI-BamHI sites in prHom-Sec1. You will replace the stuffer fragment (hGH gene) already cloned into these sites. The DmrD tags that are added will be removed later, via cleavage at the furin cleavage site, when your protein passes through the secretory pathway.

- **SpeI-BamHI Site**
  - Clone here to place four DmrD domains and a signal sequence at the N-terminus of your protein.
  - Linearize the vector, remove the stuffer sequence using SpeI and BamHI, and directionally clone using Clontech's In-Fusion HD Cloning System. Alternatively, amplify your gene with flanking SpeI and BamHI sites and use traditional restriction/ligation-based cloning.
  - The furin cleavage site present on the vector will be removed when replacing the stuffer fragment, so this 24 bp sequence should be added to your forward primer when amplifying your gene.
  - Make sure that the coding region of your furin cleavage site/gene is in frame with the last codon of the DmrD sequence (nucleotides 2075–2077). Tip: If your sequence is in-frame with the SpeI site in your primers, it will automatically be in the correct reading frame when cloned.
  - Include a stop codon at the end of your gene.

2. Cloning options for prHom-1

- **XbaI Site**
  - Clone here to place two DmrD domains at the C-terminus of your protein.
  - Linearize the vector at the XbaI site and directionally clone using the In-Fusion HD Cloning System. Alternatively, amplify your gene with flanking XbaI and SpeI sites (SpeI and XbaI have compatible cohesive ends) and clone the digested fragment into the XbaI site.
  - Do not include a ATG start codon in your gene, the ATG will be supplied by the vector at nucleotides 676–678.
  - Do not include a stop codon in your gene.
  - Make sure that the coding region of your gene is in frame with the ATG at nucleotides 676–678. Tip: If your gene is in frame with the XbaI site in your primers, it will automatically be in the correct reading frame when cloned.
  - The expressed protein will contain a C-terminal HA-tag.

3. Cloning options for prHom-Nuc1

- **XbaI Site**
  - Clone here to place three DmrD domains at the C-terminus of your protein. An HA-Tag and nuclear localization sequence (NLS) will be placed at the N-terminus of your protein
  - Linearize the vector at the XbaI site and directionally clone using the In-Fusion HD Cloning System. Alternatively, amplify your gene with flanking XbaI and SpeI sites (SpeI and XbaI have compatible cohesive ends) and clone the digested fragment into the XbaI site.
  - Do not include a ATG start codon in your gene; the ATG will be supplied by the vector at nucleotides 673–675.
  - Do not include a stop codon in your gene.
  - Make sure that the coding region of your gene is in frame with the ATG at nucleotides 673–675. Tip: If your gene is in frame with the XbaI site in your primers, it will automatically be in the correct reading frame when cloned.

4. Creating Fusion Proteins with Multiple DmrD Domains

You may choose to add additional dimerization domains to your protein. Additional DmrD domains can be added to the XbaI (or SpeI) site of any of the vectors by using In-Fusion HD or via traditional cloning of an XbaI-SpeI fragment. Since the flanking XbaI and SpeI sites are maintained after cloning, additional DmrD domains can be fused if desired.

V. *In Vitro* Reverse Dimerization

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

A. Protocol: Transient Transfection of Reverse Dimerizer Constructs & Initial Testing of D/D Solubilizer

Prior to establishing a stable cell line that expresses the prHom-1, prHom-Nuc1, or prHom-Sec1constructs containing your genes of interest, your constructs should be transiently transfected and tested for disaggregation 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 one well of a 6-well plate, use Xfect Transfection Reagent (Section III.D) to transfect your target cell line with with 5 µl of the prHom-1, prHom-Nuc1, or prHom-Sec1construct containing your gene of interest. Follow the Xfect Protocol (Type PT5003-2 in the keyword field at www.clontech.com/manuals).
2. At 12 hours after transfection, split transfected cells into different plates, 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 cells reattach after the split before removing the medium.

   a. Recommended D/D Solubilizer Concentrations and Time Points

   - Try D/D Solubilizer concentrations between 10 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 medium 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 dimerization can be analyzed with an assay that is appropriate for your experiment.

NOTE: Since the cells in both plates/wells originated from the same transfection, they should display similar transfection efficiencies.

B. Protocol: Stable Expression of Reverse Dimerizer Constructs

To select for stable clones that express the prHom-1, prHom-Nuc1, or prHom-Sec1 constructs containing your gene of interest (and have been shown to be responsive to D/D Solubilizer in Section V.A), cotransfect the construct into your target cell line along with a linear selection marker (Pur^r or Hyg^r), and select stable transfectants by screening for hygromycin or puromycin resistance, as follows:

1. Seed your target cells in a single well of a 6-well plate at a density sufficient to reach near confluence at 48 hr after transfection.
2. Transfect the prHom-1, prHom-Nuc1, or prHom-Sec1 constructs into your target cells using Xfect Transfection Reagent. Follow the Xfect Protocol (PT5003-2 from www.clontech.com/manuals), except use 2 µg of your prHom-1, prHom-Nuc1, or prHom-Sec1 constructs per well together with 100 ng of one of the supplied linear selection markers (puromycin or hygromycin).

NOTE: We use less DNA for stable transfections than is required by the general Xfect protocol, to ensure that individual colonies are well-separated after puromycin or hygromycin selection.

3. After 48 hr, split the confluent well into 4 x 10 cm dishes (do not add the selective antibiotic yet).
4. After an additional 48 hr, add either puromycin or hygromycin at the selection concentration that is optimal for your cell line.(Table 2).

Table 2. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

Cat. No.	Antibiotic	Recommended Concentration (µg/ml)	
		Selecting Colonies[1]	Maintenance
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631309	Hygromycin B (1 g)	50–400	100

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

5. Replace medium with fresh complete medium plus hygromycin or puromycin every four days, or more often if necessary.
6. Cells that have not integrated the plasmid should begin to die after ~3–5 days.

NOTE: Avoid passaging the cells a second time, since replating cells under selection may result in plates containing too many colonies for effective colony isolation (because individual colonies are not well-separated).

7. After ~2 weeks, resistant colonies should begin to appear.

8. When the colonies are large enough to transfer, use cloning cylinders or disks to harvest (i.e., pick) large, healthy colonies, and transfer each into a separate well of a 24-well plate.
9. Culture 3-4 clones in a maintenance concentration of antibiotic (Table 2). Expand and test clones using your preferred assay, after treating them with D/D Solubilizer according to the concentrations and incubation conditions determined in Section V.A.

C. Protocol: Washout Experiment—Removing D/D Solubilizer from Cells

Perform this experiment to compare target cells before and after D/D Solubilizer treatment. Instructions are provided for both adherent and suspension cells.

1. Adherent cells

a. Remove the media from your transfected and compound treated cells.

b. Rinse cells with warm PBS with Ca^{2+} and Mg^{2+}.

c. Detach cells by your method of choice (trypsin, cell dissociation buffer, etc.).

d. Split cells into at least two new cell culture plates.

e. Culture cells in one plate in the presence of D/D Solubilizer in medium at a concentration of your choice (positive control) and culture the second plate without D/D Solubilizer (negative control).

f. Collect cells at a specific time after splitting that is defined by your needs, in order to analyze and compare cells cultured under the different conditions described above.

2. Suspension cells

a. Collect the cells via centrifugation.

b. Resuspend one portion of the cells in medium with D/D Solubilizer and another portion of the cells in medium without D/D Solubilizer.

c. Analyze the cells using an assay that is appropriate for your experiment.

D. Results Obtained Using *In Vitro* Reverse Dimerization

The example in Figure 5 shows induction of secretion by increasing concentrations of D/D Solubilizer in HeLa cells transiently transfected with a construct expressing a fusion protein consisting of *Metridia* luciferase fused to 4 copies of the DmrD domain at its N-terminus. This fusion protein contains a human growth factor-derived, N-terminal signal sequence, which targets the protein to the endoplasmic reticulum (ER).

In the absence of D/D Solubilizer, the DmrD domains self-associate, forming large luciferase-DmrD complexes which accumulate in the ER. As a result, only a very low level of *Metrid*ia luciferase is secreted into the medium. However, treatment of the cells with increasing concentrations of D/D Solubilizer (ranging from 10–1,000 nM) causes the protein complexes to dissociate, so the luciferase fusion protein can pass through the secretory pathway, allowing luciferase to be secreted in a concentration-dependent manner.

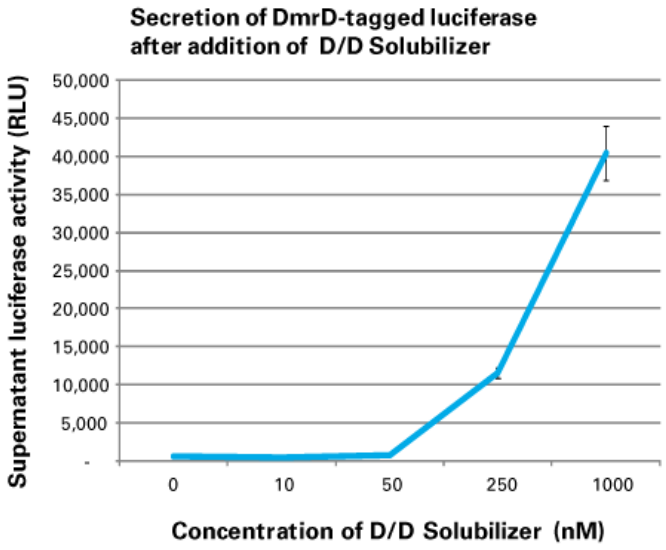

Figure 5. Secretion of DmrD-tagged luciferase after addition of D/D Solubilizer. 7 hr after transfection with a DmrD-tagged *Metridia* luciferase construct, cells were split into wells of a 6 well plate. The medium was removed and fresh medium lacking D/D Solubilizer or containing D/D Solubilizer in concentrations of 10 nM, 50 nM, 250 nM, or 1,000 nM was added. 18 hr later, the media was collected and analyzed for the presence of *Metridia* luciferase using Clontech's **Ready To Glow™ Secreted Luciferase Reporter System** (Cat. No. 631731).

VI. References

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VII. Troubleshooting

Description of Problem	Possible Explanation	Solution
Disaggregation is observed in the absence of D/D Solubilizer	The DmrD tag in your construct may not be functioning efficiently enough. and might need to be extended by adding additional copies of DmrD.	Clone in one to two more copies of the DmrD domain to allow formation of a stronger protein aggregate in the absence of D/D solubilizer.
Addition of D/D Solubilizer does not result in any of the expected effects	• The D/D Solubilizer concentration is too low. • The monitoring assay is not sensitive enough. • The volume of D/D Solubilizer used causes cells to die due to high solvent concentration.	• Increase the amount of D/D Solubilizer added. • Make sure to include a positive control when performing your assay. • Prepare a more concentrated stock solution.

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This document has been reviewed and approved by the Clontech Quality Assurance Department.