Clontech Laboratories, Inc.

iDimerize™ Inducible Homodimer System User Manual

PT5178-1 (111115)
Cat. Nos. 635068, 635058, 635059, 635069, 635088, 635090, 632622

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: tech@clontech.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.543.6116

Table of Contents

I. Introduction 3
II. List of Components 5
III. Additional Materials Required 5
A. B/B Homodimerizer 5
B. B/B Washout Ligand 5
C. DmrB Monoclonal Antibody 6
D. Mammalian Cell Culture Supplies 6
E. Antibiotics for Selecting Stable Cell Lines 6
F. Xfect™ Transfection Reagents 6
G. In-Fusion® HD Cloning System 6
IV. Creating Fusion Proteins Containing Dimerization Domains 6
A. General Considerations 6
B. Protocol: Cloning Strategy for Creating Fusion Proteins 8
V. *In Vitro* Inducible Homodimerization 10
A. Protocol: Transient Transfection of Homodimerizer Constructs 10
B. Protocol: Stable Expression of Homodimerizer Constructs 11
C. Results Obtained Using *In Vitro* Homodimerization 12
D. Protocol: Dissociating Dimerized Proteins with the B/B Washout Ligand 12
VI. *In Vivo* Inducible Homodimerization 14
A. General Guidelines 14
B. Results Obtained Using *In Vivo* Homodimerization 14
VII. References 15
VIII. Troubleshooting 15

Table of Figures

Figure 1. Different iDimerize kits use different dimerization strategies 4
Figure 2. Controlling signal transduction using regulated homodimerization. 4
Figure 3. pHom-1 and pHom-Mem1 Vector Maps 7
Figure 4. Apoptosis resulting from B/B Homodimerizer-induced dimerization of Fas 12
Figure 5. B/B Washout Ligand can be used to disrupt protein interactions that were induced by the B/B Homodimerizer 12
Figure 6. B/B Washout Ligand has a much more dissociative effect than simple removal of the B/B Homodimerizer 13
Figure 7. Activation of Fas signaling after intravenous administration of B/B Homodimerizer 14

Table of Tables

Table 1. ARGENT and Clontech Nomenclature for iDimerize Inducible Homodimer System Components 5
Table 2. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines 11

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 **iDimerize Inducible Homodimer System** (Cat. No. 635068), which contains reagents for bringing together two molecules of an engineered fusion protein by adding a small molecule "dimerizer". 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 iDimerize Inducible Homodimer System consists of components identical to those previously supplied in the ARGENT Regulated Homodimerization 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 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 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 recognized by the heterodimerizer. For applications requiring heterodimerization, we provide a separate kit, the **iDimerize Inducible Heterodimer System** (Cat. No. 635067), 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 **iDimerize Reverse Dimerization System** (Cat. No. 635066).
- Another dimerization system, our **iDimerize Regulated Transcription System** (Cat. No. 635081), 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.

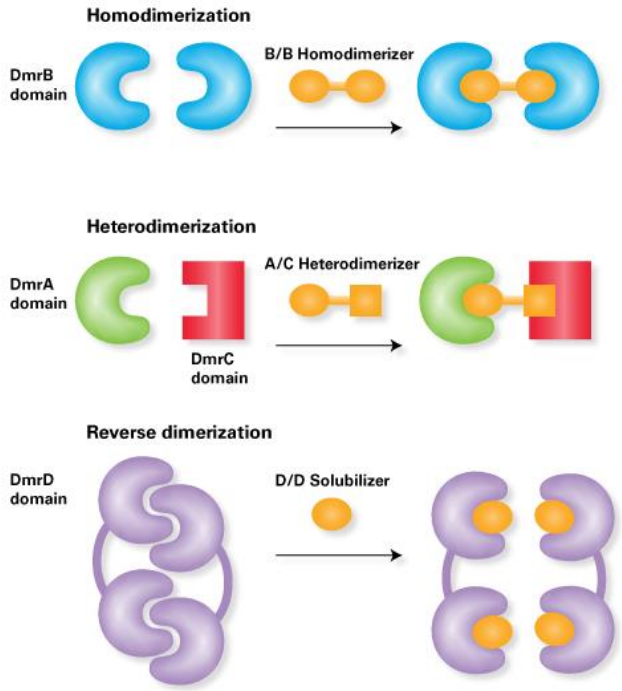

Figure 1. Different iDimerize kits use different dimerization strategies: homodimerization, heterodimerization, and reverse dimerization. Separate kits are available from Clontech.

C. iDimerize Inducible Homodimer System

The 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 two vectors used to create fusion proteins that contain DmrB dimerization domains—and either lack a subcellular localization domain (pHom-1) or include a plasma membrane targeting domain (pHom-Mem1). 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 (Clackson *et al.*, 1998). The plasmids in this kit provide an assortment of components (i.e., multiple DmrB domains, an HA epitope tag, and localization sequences) that can easily be customized and exchanged to generate fusion proteins whose activity and localization can be controlled by the dimerizer (Figure 2).

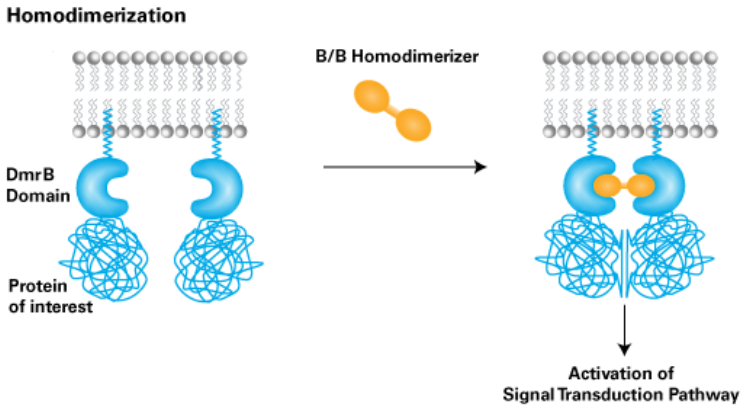

Figure 2. Controlling signal transduction using regulated homodimerization.

II. List of Components

Store all components at -20°C.

- 1 each iDimerize Inducible Homodimer Vector Set 1
  - 20 µl pHom-1 Vector (500 ng/µl)
  - 20 µl pHom-Mem1 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 B/B Homodimerizer (0.5 mM)
  (Also sold separately as Cat. Nos. 635059, 635058, 635069 & 632622—see Section III.A)

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

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

Ligand Name in ARIAD Kit	Ligand Name in Clontech Kit
AP20187	B/B Homodimerizer
Plasmid Name in ARIAD Kit	**Plasmid Name in Clontech Kit**
pC_4-F_V1E	pHom-1
pC_4M-F_V2E	pHom-Mem1
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in Clontech Kit**
F_V	DmrB
$F_{V'}$	DmrB

NOTES:

- The B/B Homodimerizer is so named because it induces dimerization of two proteins that contain the DmrB dimerization domain.
- The amino acid sequences for the F_V and $F_{V'}$ domains are identical. However, there are significant differences at the nucleotide sequence level to reduce the potential for recombination between the repeating domain within the pC_4M-F_V2E plasmid (pHom-Mem1).

III. Additional Materials Required

A. B/B Homodimerizer

Each 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 dimerization 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

- 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)

E. Antibiotics for Selecting Stable Cell Lines

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

F. 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)

G. 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 Dimerization Domains

A. General Considerations

1. Controlling localization of fusion proteins

- Fusion proteins are created by cloning signaling proteins of interest into either the XbaI site or the SpeI site of the pHom-1 and pHom-Mem-1 vectors (Figure 3). Cloning into the XbaI site places the DmrB domain(s) at the C-terminus and cloning into the SpeI site places the DmrB domain(s) at the N terminus of your protein of interest.

- If the sequence encoding your protein is cloned into the XbaI site of the pHom-1 vector, it must not contain a stop codon, and must be in-frame with the start codon (ATG) located upstream of the Xba I site and with the downstream DmrB domain. If your protein of interest is cloned into the Xba I site of the pHom-Mem1 vector, it must not contain a stop codon, and must be in-frame with the N-terminal myristoylation signal and with the downstream DmrB domains.
- Fusion proteins localize to the cytoplasm when created using pHom-1 (which contains no targeting signal), and to the inner leaflet of the plasma membrane when created using pHom-Mem1 (which contains an N-terminal myristoylation signal between the EcoRI & XbaI sites).
- A hemagglutinin (HA) epitope tag located between the SpeI and BamHI sites in the pHom-1 and pHom-Mem-1 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).

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.

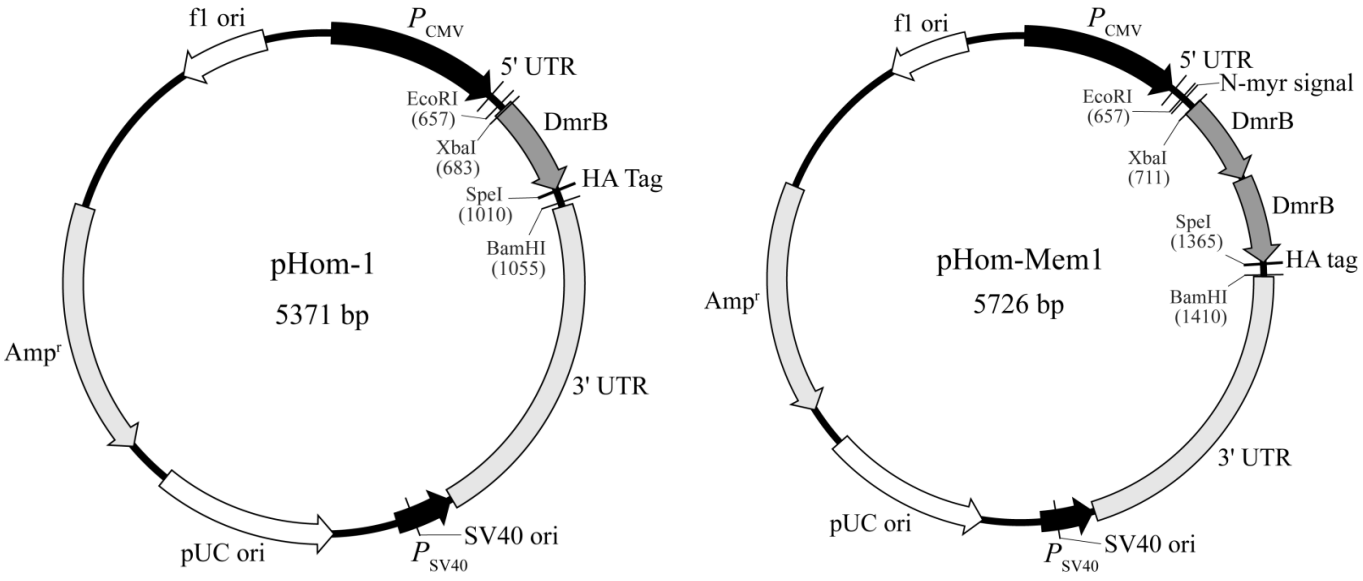

Figure 3. pHom-1 and pHom-Mem1 Vector Maps: Cloning Strategy. Use the XbaI site to clone your gene of interest upstream of the dimerizing domain(s), and the SpeI site to clone your gene of interest downstream of the domain(s) as described in Section IV.B.

B. Protocol: Cloning Strategy for Creating Fusion Proteins

Create fusion proteins containing the DmrB domain and your protein of interest with the pHom-1 and/or pHom-Mem1 vectors using the following cloning strategy (For vector map information, see Figure 3).

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.G.

1. Cloning options for pHom-1

- **XbaI Site**
  - Clone here to place the DmrB domain 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 an 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.
- **SpeI Site**
  - Clone here to place the DmrB domain at the N-terminus of your protein.
  - Linearize the vector at the SpeI site and directionally clone using Clontech's In-Fusion HD Cloning System. Alternatively, amplify your gene with flanking XbaI and SpeI sites (SpeI and XbaI have compatible cohesive ends) and clone into the SpeI site.
  - Make sure that the coding region of your gene is in frame with the last codon of the DmrB sequence (nucleotides 1006–1008). Tip: If your gene is in frame with the SpeI site in your primers, it will automatically be in the correct reading frame when cloned.
  - If you wish to retain the HA-Tag, do not include a stop codon at the end of your gene.
  - If you do not wish to retain the HA-Tag, you must include a stop codon at the end of your gene.

2. Cloning options for pHom-Mem1

- **XbaI Site**
  - Clone here to place two DmrB domains at the C-terminus and an N-myr signal 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 an ATG start codon in your gene; the ATG will be supplied by the vector at nucleotides 668–670.
  - 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 668–670. Tip: if your gene sequence 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.
- **SpeI Site**
  - Clone here to place two DmrB domains and the N-myr signal at the N-terminus of your protein.
  - Linearize the vector at the SpeI site and directionally clone using Clontech's In-Fusion HD Cloning System. Alternatively, amplify your gene with flanking XbaI and SpeI sites (SpeI and XbaI have compatible cohesive ends) and clone into the SpeI site.
  - Make sure that the coding region of your gene is in frame with the last codon of the DmrB sequence (nucleotides 1364–1366). Tip: If your gene sequence is in frame with the SpeI site in your primers, it will automatically be in the correct reading frame when cloned.
  - If you wish to retain the HA-Tag, do not include a stop codon at the end of your gene.
  - If you do not wish to retain the HA-Tag, include a stop codon at the end of your gene.

3. Creating Fusion Proteins with Multiple DmrB Domains

You may choose to add additional dimerization domains to your protein to allow for higher order oligomerization. Additional DmrB domains can be added to the XbaI (or SpeI) site of either vector 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 DmrB domains can be fused if desired.

V. *In Vitro* Inducible Homodimerization

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 establishing a stable cell line that expresses the pHom-1 or pHom-Mem1 construct containing your gene of interest, your construct should be transiently transfected and 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.F) to transfect your target cell line with 5 µl of the pHom-1 or pHom-Mem1 construct containing your gene of interest. Follow the Xfect Protocol (type Xfect in the keyword field 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: Stable Expression of Homodimerizer Constructs

To select for stable clones that express the pHom-1 or pHom-Mem1 construct containing your gene of interest (and have been shown to be responsive to B/B Homodimerizer 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 pHom-1 or pHom-Mem1 construct into your target cells using Xfect Transfection Reagent. Follow the Xfect Protocol (type Xfect in the keyword field at www.clontech.com/manuals), except use 2 µg of your pHom-1 or pHom-Mem1 construct 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 B/B Homodimerizer according to the concentrations and incubation conditions determined in Section V.A.

C. Results Obtained Using *In Vitro* Homodimerization

The example in Figure 4 shows the activation of Fas signaling by increasing concentrations of B/B Homodimerizer in cells transduced with a construct containing the death domain of Fas cloned into pHom-Mem1 (Clackson *et al.*, 1998). In these experiments, maximal Fas-induced cell death upon overnight treatment with dimerizer was seen with concentrations below 1 nM of B/B Homodimerizer. In other applications, concentrations of dimerizer as high as 10–100 nM were required to obtain the maximal effect. In initial experiments we recommend that B/B Homodimerizer be tested across a broad range of concentrations (e.g., 0.01–100 nM) to provide a complete dose-response profile.

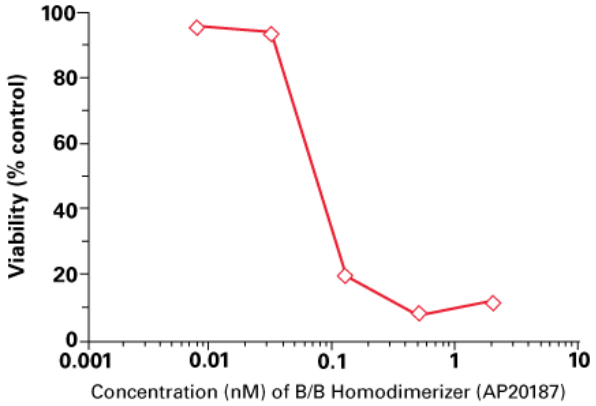

Figure 4. Apoptosis resulting from B/B Homodimerizer-induced dimerization of Fas. Data courtesy of ARIAD Pharmaceuticals, Inc. (Clackson *et al.*, 1998).

D. 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.

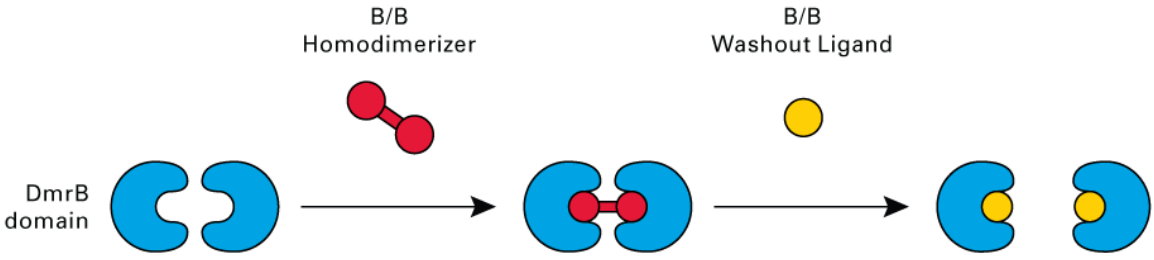

Figure 5. B/B Washout Ligand can be used to disrupt protein interactions that were induced by the B/B Homodimerizer.

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).

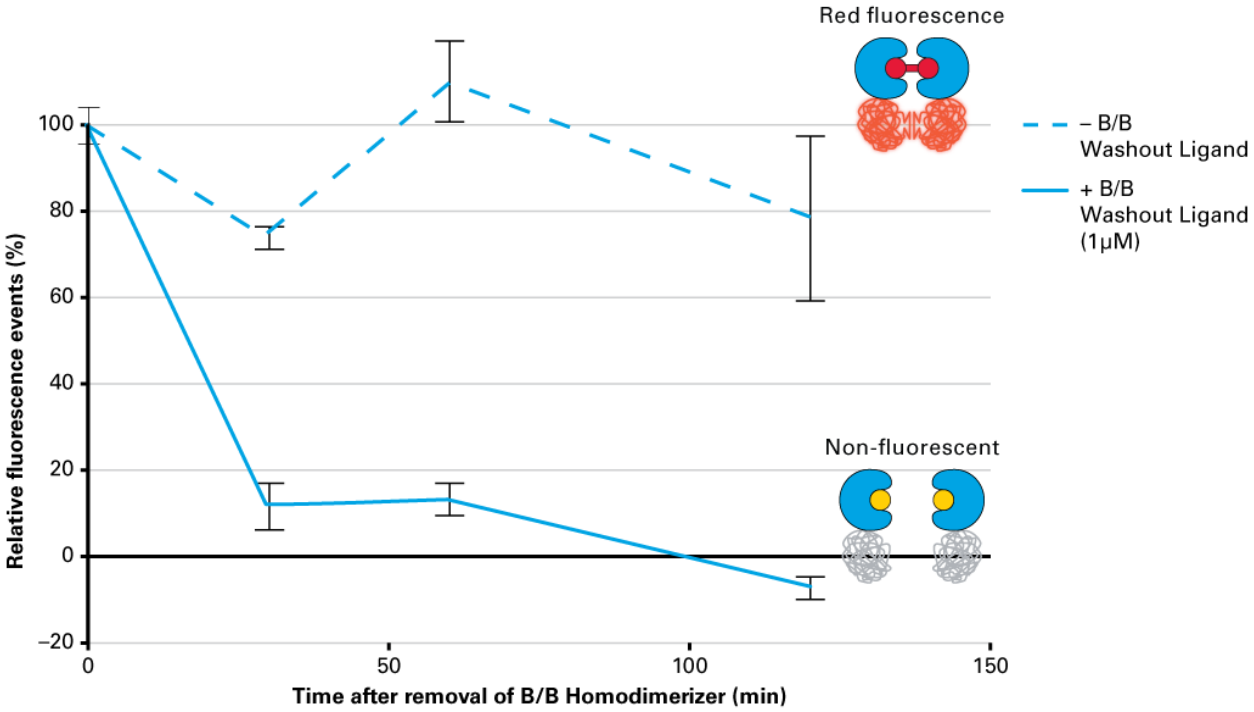

Figure 6. B/B Washout Ligand has a much more dissociative effect than simple removal of the B/B Homodimerizer. A stable HeLa cell line expressing DmrB-tagged versions of the two parts of a split red fluorescent protein was treated with 0.1 µM B/B Homodimerizer for 3 hr. The medium was then removed and 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. *In Vivo* Inducible Homodimerization

A. General Guidelines

For *in vivo* use, reconstitute the 5 mg (Cat. No. 635058), 25 mg (Cat. No. 635069), or 4 x 25 mg (Cat. No. 632622) format of B/B Homodimerizer following your established injection protocol (for 5 mg, 25 mg, and 4 x 25 mg formats—see Section III.A). The ligand has been successfully used in mice with maximal effects seen at doses in the range of 0.5–10 mg/kg delivered intravenously.

B. Results Obtained Using *In Vivo* Homodimerization

The example in Figure 5 shows the activation of Fas signaling in mice after intravenous (i.v.) administration of increasing doses of B/B Homodimerizer (AP20187). Cells were transduced with two constructs, one containing the death domain of Fas cloned into a vector similar to pHom-Mem1 (Clackson *et al.*, 1998) and the other containing a human growth hormone (hGH) cDNA expressed constitutively from the CMV promoter. These cells were then implanted intramuscularly into mice. Twenty-four hours later, the mice were administered increasing doses of B/B Homodimerizer. Measurement of serum hGH levels provided a surrogate measurement of the number of viable Fas-transduced cells.

As shown in the figure, maximal killing of engineered cells was seen with doses as low as 0.5 mg/kg. In other applications, concentrations of dimerizer as high as 10 mg/kg have been required to see the maximal effect (Jin *et al.*, 2000). In initial experiments we recommend that B/B Homodimerizer be tested across a broad range of concentrations (e.g., 0.005–10 mg/kg) to provide a complete dose-response profile and to allow determination of the minimal effective dose.

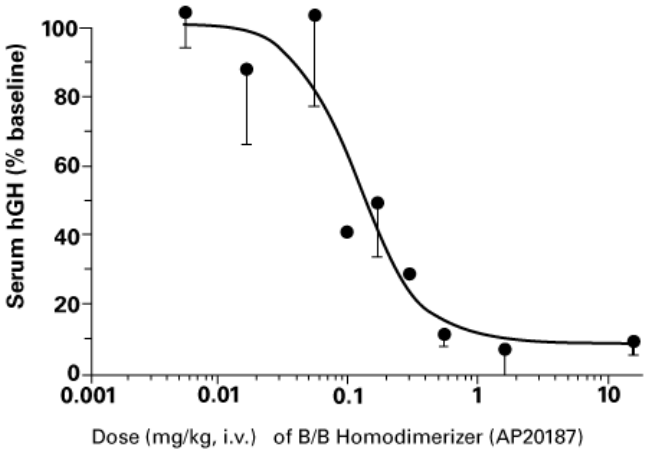

Figure 7. Activation of Fas signaling after intravenous administration of B/B Homodimerizer. Data courtesy of ARIAD Pharmaceuticals, Inc. (Clackson *et al.*, 1998).

VII. References

Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F. Jr., Gilman, M. & Holt, D. A. (1998) Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. *Proc. Natl. Acad. Sci. USA* **95**(18): 10437–10442.

Crabtree, G. R. & Schreiber, S. L. (1996) Three-part inventions: intracellular signaling and induced proximity. *Trends Biochem. Sci.* **21**(11): 418–422.

Jin, L., Zeng, H., Chien, S., Otto, K. G., Richard, R. E., Emery, D. W. & Anthony Blau, C. (2000) *In vivo* selection using a cell-growth switch. *Nat. Genet*. **26**(1): 64–66.

VIII. Troubleshooting

Description of Problem	Possible Explanation	Solution
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) or establish stable clones and screen for the lowest background.
Addition of B/B Homodimerizer does not result in any of the expected effect(s).	• The B/B Homodimerizer concentration is too low. • The monitoring assay is not sensitive enough. • The volume of B/B Homodimerizer used causes cells to die due to high solvent concentration. • Low expression level	• Increase the amount of B/B Homodimerizer added. • Make sure to include a positive control when performing your assay. • Prepare a more concentrated stock solution. • Monitor protein expression in your cells by Western blot using the anti-DmrB antibody (Section III.C).

Contact Us For Assistance	
Customer Service/Ordering	**Technical Support**
Telephone: 800.662.2566 (toll-free)	Telephone: 800.662.2566 (toll-free)
Fax: 800.424.1350 (toll-free)	Fax: 650.424.1064
Web: www.clontech.com	Web: www.clontech.com
E-mail: orders@clontech.com	E-mail: tech@clontech.com

Notice to Purchaser