Takara Bio USA, Inc.

iDimerize™ Inducible Heterodimer System User Manual

Cat. No. 635067
PT5179-1 (013117)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: techUS@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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 Takara Bio USA, Inc. (TBUSA), as the iDimerize Inducible Dimerization Systems. This manual describes the **iDimerize Inducible Heterodimer System** (Cat. No. 635067), 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 Heterodimer System consists of components identical to those previously supplied in the ARGENT Regulated Heterodimerization Kit from ARIAD. The names of the plasmids, dimerization domains, and dimerization ligands have been changed 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:

- **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. The iDimerize Inducible Heterodimer System provides the heterodimerizer ligand—as well as DNA vectors that enable each dimerization domain to be fused to a different protein of interest. The two different dimerization domains (DmrA & DmrC) are each able to bind to the A/C Heterodimerizer ligand.
- **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.
- **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.

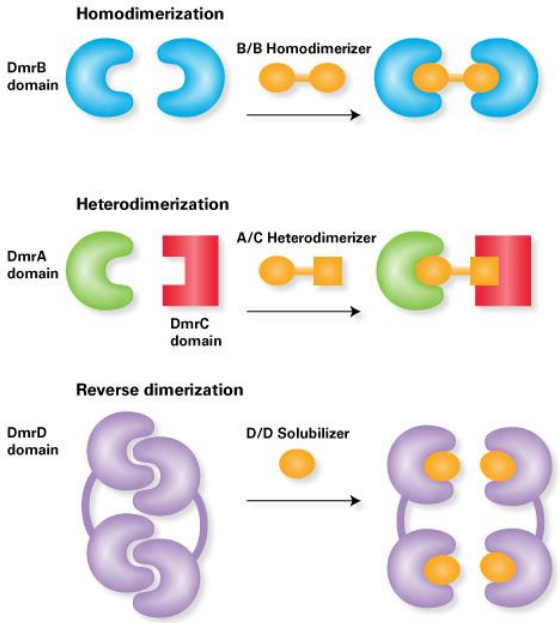

Figure 1. Different iDimerize kits use different dimerization strategies: homodimerization, heterodimerization, and reverse dimerization. Separate kits are available.

C. iDimerize Inducible Heterodimer System

The iDimerize Inducible Heterodimer System contains reagents to induce the heterodimerization of two proteins of interest by the addition of a small molecule, the "A/C Heterodimerizer". The system consists of three vectors that are used to create two fusion proteins, one containing a DmrC dimerization domain (pHet-1) and the other containing a Dmr A dimerization domain (pHet-Nuc1 or pHet-Mem1), each of which bind A/C Heterodimerizer, also included in the kit. The resulting fusion proteins, which contain different localization tags, are localized to the cytoplasm (pHet-1), nucleus (pHet-Nuc1), or plasma membrane (pHet-Mem1). The addition of A/C Heterodimerizer to live cells expressing fusion proteins containing DmrA and DmrC domains induce heterodimerization of the fusion proteins by promoting the interaction of these dimerization domains (Graef *et al.*, 1997, Castellano *et al.*, 1999). The plasmids in this kit provide an assortment of components (i.e., dimerization domains, an HA epitope tag, and localization sequences) that can easily be customized and exchanged to generate protein fusions whose activity and localization can be controlled by the heterodimerizer (Figure 2).

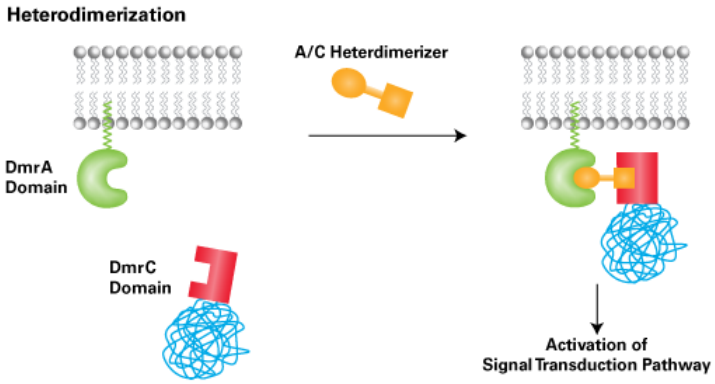

Figure 2. Controlling signal transduction using regulated heterodimerization.

II. List of Components

Store all components at -20°C.

- 1 each iDimerize Inducible Heterodimer Vector Set 1
  - 20 µl pHet-1 Vector (500 ng/µl)
  - 20 µl pHet-Mem1 Vector (500 ng/µl)
  - 20 µl pHet-Nuc1 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 A/C Heterodimerizer (0.5 mM)
  (also sold separately as Cat. Nos. 635056, 635055 & 635095—see Section III.A)

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

Table 1. ARGENT and TBUSA Nomenclature for iDimerize Inducible Heterodimer System Components

Ligand Name in ARIAD Kit	**Ligand Name in TBUSA Kit**
AP21967	A/C Heterodimerizer
Plasmid Name in ARIAD Kit	**Plasmid Name in TBUSA Kit**
pC_4-R_HE pC_4M-F2E pC_4EN-F1	pHet-1 pHet-Mem1 pHet-Nuc1
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in TBUSA Kit**
F F' FRB	DmrA DmrA DmrC

NOTES:

- The A/C Heterodimerizer is so named because it induces dimerization of a protein possessing the DmrA domain and a second protein containing the DmrC domain.
- The amino acid sequences for the F and F', 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-F2E plasmid (pHet-Mem1).

III. Additional Materials Required

A. A/C Heterodimerizer

Each iDimerize Inducible Heterodimer System includes 500 µl A/C Heterodimerizer (0.5 mM; see Section II). Additional A/C Heterodimerizer can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
635056	A/C Heterodimerizer (0.5 mM)	5 x 500 µl
635055	A/C Heterodimerizer*	5 mg
635095	A/C Heterodimerizer*	5 x 5 mg

* Designed for *in vivo* use; supplied in a dry-down format (see Section VI).

B. DmrA & DmrC Antibodies

The DmrA and DmrC Antibodies recognize the respective DmrA and DmrC dimerization domains expressed using any iDimerize Inducible Heterodimer System, and are recommended for Western blot analysis.

Cat. No.	Product Name	Size
635089	DmrA Monoclonal Antibody (0.5 µg/µl)	100 µg
635091	DmrC Polyclonal Antibody (0.5 µg/µl)	100 µg

C. 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)

D. Antibiotics for Selecting Stable Cell Lines

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

E. 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)

F. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that greatly simplifies cloning.
For more information, visit takarabio.com/in-fusion

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 pHet-1, pHet-Nuc1 and pHet-Mem-1 vectors (Figure 3). Cloning into the XbaI site places the DmrA or DmrC domain(s) at the C-terminus and cloning into the SpeI site places the DmrA or DmrC domain(s) at the N terminus of your protein of interest.
- If the sequence encoding your protein is cloned into the Xba1 site of the pHet-1 vector, it must be in-frame with the start codon (ATG) located upstream of the Xba I site, and be in-frame with the downstream DmrC domain. If your protein of interest is cloned into the Xba I site of the pHet-Nuc1 or pHet-Mem-1 vector, it must be in-frame with the N-terminal nuclear localization signal of pHet-Nuc1 or the N-terminal myristoylation signal of pHet-Mem-1. The coding sequence of a protein that is cloned into the Xba1 site of either vector must not contain a stop codon, and must be in-frame with the downstream DmrA or DmrC domain(s).
- Fusion proteins localize to the cytoplasm when created using pHet-1 (which contains no targeting signal), to the nucleus when created using pHet-Nuc1 (which contains an N-terminal nuclear localization signal between the EcoRI and XbaI sites), and to the inner leaflet of the plasma membrane when created using pHet-Mem1 (which contains an N-terminal myristoylation signal between the EcoRI and XbaI sites).
- A hemagglutinin (HA) epitope tag located between the SpeI and BamHI sites in the pHet-1 and pHet-Mem1 vectors is expressed at the C-terminus of fusion proteins created using any of the three vectors. 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 DmrA and DmrC domains should I use?

The number of DmrA and DmrC domains best suited for each application varies. We have generally found that fusing one DmrA domain or one DmrC domain to each signaling protein works well, although in some cases multiple tandem DmrC domains are required (e.g., when the event studied requires the formation of higher order oligomers). Often the optimal configuration is best determined empirically.

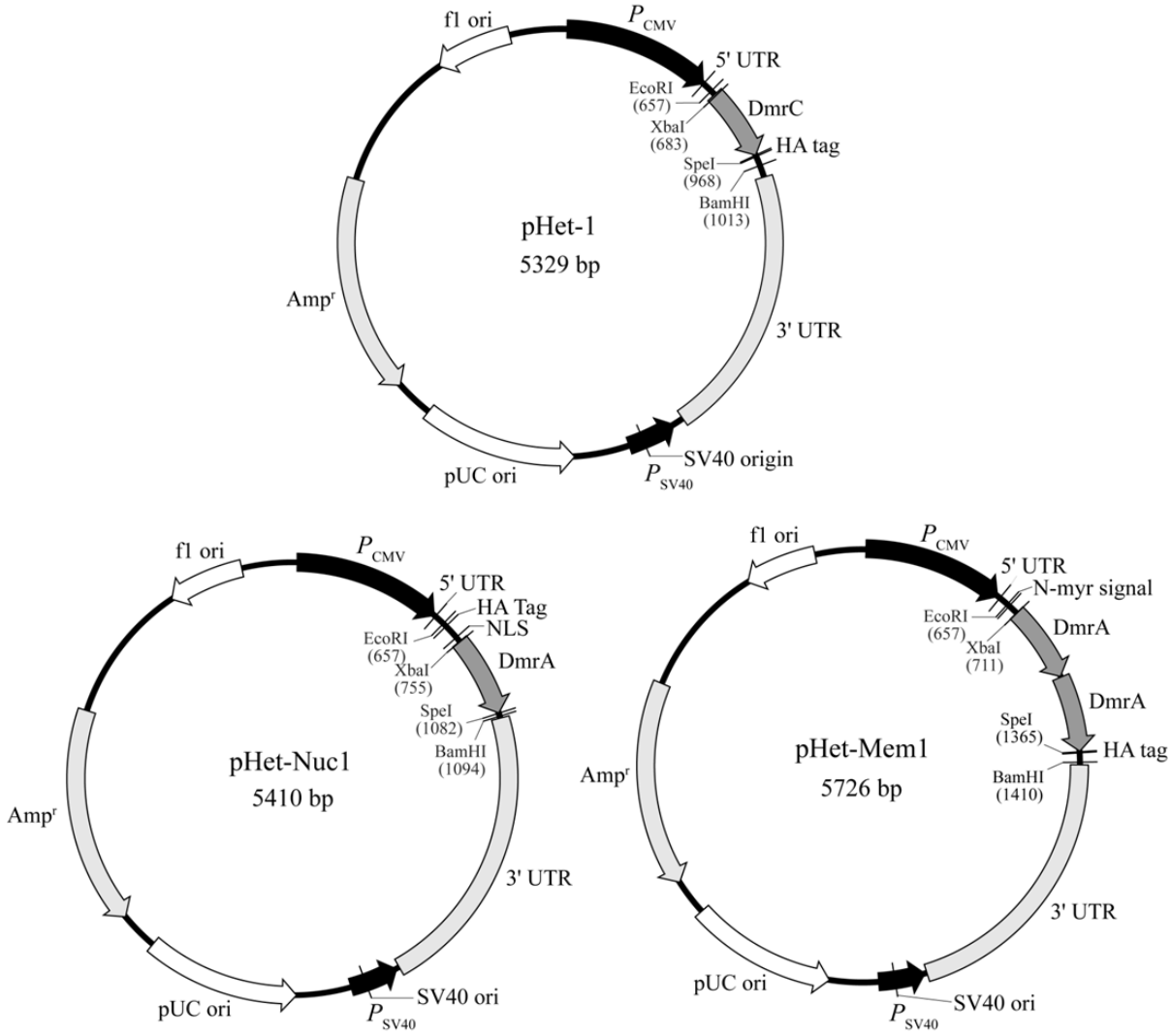

Figure 3. pHet-1, pHet-Nuc1, and pHet-Mem1 Vector Maps: Cloning Strategy. Use the XbaI site to clone your gene of interest upstream of the dimerizing domain(s), and the SpeI site to clone your gene of interest downstream of the domain(s) as described in Section IV.B.

B. Protocol: Cloning Strategy for Creating Fusion Proteins

Create fusion proteins containing the DmrA/DmrC domains and your protein of interest in the supplied 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.F.

1. Cloning options for pHet-1

- **XbaI Site**
  - Clone here to place the DmrC 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 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
- **SpeI Site**
  - Clone here to place the DmrC domain at the N-terminus of your protein.
  - Linearize the vector at the SpeI site and directionally clone using our 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 DmrC sequence (nucleotides 964–966). 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 pHet-Mem1

- **XbaI Site**
  - Clone here to place two DmrA 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 a 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 DmrA domains and the N-myr signal at the N-terminus of your protein.
  - Linearize the vector at the SpeI site and directionally clone using our 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 DmrA sequence (nucleotides 1361–1363). 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. Cloning options for pHet-Nuc1

- **XbaI Site**
  - Clone here to place a DmrA domain at the C-terminus and an HA-Tag and nuclear localization signal (NLS) 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.
  - The first ATG start codon will be supplied by the HA-Tag at nucleotides 673-675, so there is no need to include a start codon at the start of your gene of interest.
  - 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 sequence is in frame with the XbaI site in your primers, it will automatically be in the correct reading frame when cloned.
- **SpeI Site**
  - Clone here to place a DmrA domain, HA-tag, and NLS at the N-terminus of your protein.
  - Linearize the vector at the SpeI site and directionally clone using our 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 DmrA sequence (nucleotides 1084–1086). 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.
  - Include a stop codon at the end of your gene of interest.

4. Creating Fusion Proteins with Multiple Dimerization Domains

You may choose to add additional dimerization domains to your protein to allow for higher order oligomerization. Additional DmrA or DmrC 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 domains can be fused if desired.

V. *In Vitro* Inducible Heterodimerization

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

A. Protocol: Transient Transfection of Heterodimerizer

Prior to establishing a stable cell line that expresses the pHet-1 and pHet-Nuc1 (or pHet-Mem1) constructs containing your genes of interest, your constructs should be transiently transfected and tested for dimerization in response to A/C Heterodimerizer. For your initial *in vitro* experiments, we recommend testing medium containing different concentrations of A/C Heterodimerizer 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.E) to transfect your target cell line with 5 µl of the pHet-1 and (pHet-Nuc or pHet-Mem1) constructs containing your gene of interest. Follow the Xfect Protocol (Type PT5003-2 in the keyword field at takarabio.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 A/C Heterodimerizer at specific time intervals and concentrations, replace the medium in the plates containing the transfected cells with medium containing the appropriate amount of A/C Heterodimerizer, diluted as described below. Maintain at least one culture in medium containing no A/C Heterodimerizer as a negative control.

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

   a. Recommended A/C Heterodimerizer Concentrations and Time Points

   - Try A/C Heterodimerizer 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 A/C Heterodimerizer

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

   - Working concentrations of A/C Heterodimerizer 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 A/C Heterodimerizer 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 A/C Heterodimerizer 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 Heterodimerizer Constructs

To select for stable clones that express the pHet-1 and pHet-Nuc1 (or pHet-Mem1) constructs containing your gene of interest (and have been shown to be responsive to A/C Heterodimerizer 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 pHet-1 and pHet-Nuc1 (or pHet-Mem1) constructs into your target cells using Xfect Transfection Reagent. Follow the Xfect Protocol (PT5003-2 from takarabio.com/manuals), except use 2 µg of your pHet-1 and pHet-Nuc1 (or pHet-Mem1) 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 A/C Heterodimerizer according to the concentrations and incubation conditions determined in Section V.A.

C. Protocol: Washout Experiment—Removing A/C Heterodimerizer from Cells

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

1. Adherent cells

a. Remove the medium 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 A/C Heterodimerizer in medium at a concentration of your choice (positive control) and culture the second plate without A/C Heterodimerizer (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 A/C Heterodimerizer and another portion of the cells in medium without A/C Heterodimerizer.

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

D. Results Obtained Using *In Vitro* Heterodimerization

Peak effects of induced heterodimerization of two proteins using A/C Heterodimerizer are generally seen at concentrations of 500 nM (Muthuswamy, *et al.*, 1999; Castellano *et al.*, 1999; unpublished data). In initial experiments we recommend that A/C Heterodimerizer be tested across a broad range of concentrations (e.g. 0.5 nM to 500 nM) to provide a complete dose-response profile.

VI. *In Vivo* Inducible Heterodimerization

A. General Guidelines

For *in vivo* use, reconstitute the 5 mg format of A/C Heterodimerizer (Cat. No. 635055) following your established injection protocol (for 5 mg format—see Section III.A).

B. Results Obtained Using *In Vivo* Heterodimerization

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.

VII. References

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Muthuswamy, S. K., Gilman, M. & Brugge, J. S. (1999) Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol. Cell. Biol.* **19**(10): 6845–6857.

VIII. Troubleshooting

Description of Problem	Possible Explanation	Solution
Dimerization is observed in the absence of the A/C Heterodimerizer	The expression level of the protein(s) of interest fused to the DmrA and/or DmrC domain(s) is too high, especially in the case of a DmrA-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 A/C Heterodimerizer does not result in any of the expected effects	• The A/C Heterodimerizer concentration is too low. • The monitoring assay is not sensitive enough. • The volume of A/C Heterodimerizer used causes cells to die due to high solvent concentration. • Expression level too low	• Increase the amount of A/C Heterodimerizer 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-DmrA and anti-DmrC antibodies (Section III.B).

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