Clontech Laboratories, Inc.

iDimerize™ Inducible Expression System

PT5181-1 (PR143850)
Cat. No(s). 635065, 635055, 635056 & 635057
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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. This manual describes the **iDimerize Inducible Expression System** (Cat. No. 635065), which contains reagents for placing the transcription of a target gene under the control of a small molecule "dimerizer". The kit can be used to achieve tightly regulated conditional expression of genes of interest, allowing protein function to be investigated *in vitro* or *in vivo*. The iDimerize Inducible Expression System consists of components identical to those previously supplied in the ARGENT Regulated Transcription Plasmid Kit from ARIAD. The plasmid names, 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.

B. Using Dimerization to Regulate Gene Expression

Activation of gene expression in eukaryotes is controlled by the binding of transcription factors to the promoters of target genes. Transcription factors are bifunctional proteins that recognize specific DNA sequences near target genes and then recruit the transcriptional machinery of the cell to activate transcription. The two domains responsible for these activities--the DNA-binding domain and the transcriptional activation domain--are functionally separable and can reconstitute a sequence-specific transcriptional activator even when expressed as individual proteins that are brought together via a noncovalent interaction.

This modular architecture enables regulation of gene transcription using small molecule "dimerizers" that can induce dimerization (Spencer *et al.,* 1993). A dimerizer is a cell-permeant organic molecule with two separate motifs that each bind with high affinity to a specific protein module (Figure 1). By fusing such modules to a DNA-binding domain and a transcriptional activation domain, the reconstitution of a functional transcription factor, and therefore the expression of a target gene, can be made absolutely dependent on the presence of the dimerizer (Figure 2). In principle, the expression of any cloned gene can be brought under dimerizer control by equipping the gene with upstream sequences that are recognized by the engineered DNA-binding domain. Introducing the modified gene into cells that also express the engineered transcription factor proteins and adding dimerizer will lead to dose-dependent activation of target gene expression.

Different types of dimerizer (Figures 1 & 2) are available. **Homo**dimerizers incorporate two identical binding motifs, and can therefore be used to induce self-association of a single protein of interest, while **hetero**dimerizers contain two different binding motifs, allowing the specific dimerization of two different proteins when they are fused to two appropriate ligand binding domains.

- The iDimerize Inducible Expression System uses a heterodimerizer to provide regulated transcription (Figure 2) since this leads to efficient reconstitution of an active transcription factor. This system provides a heterodimerizer ligand, vectors encoding the engineered transcription factor fusion proteins, and an expression vector into which genes of interest can be inserted.
- For applications requiring homodimerization, we provide a separate kit, the **iDimerize Inducible Homodimer System** (Cat. No. 635068), that includes the homodimerizer ligand and vectors for fusing the ligand-binding domain to a single protein of interest.
- For applications requiring heterodimerization, we provide a separate kit, the **iDimerize Inducible Heterodimer System** (Cat. No. 635067), that includes the heterodimerizer ligand and DNA vectors that enable each dimerization domain to be fused to a different protein of interest.
- Another type of dimerizer, reverse dimerizers, promote the disaggregation of self-associating fusion proteins created by fusing a protein of interest to a "conditional aggregation domain" (DmrD), as in our **iDimerize Reverse Dimerization System** (Cat. No. 635066).

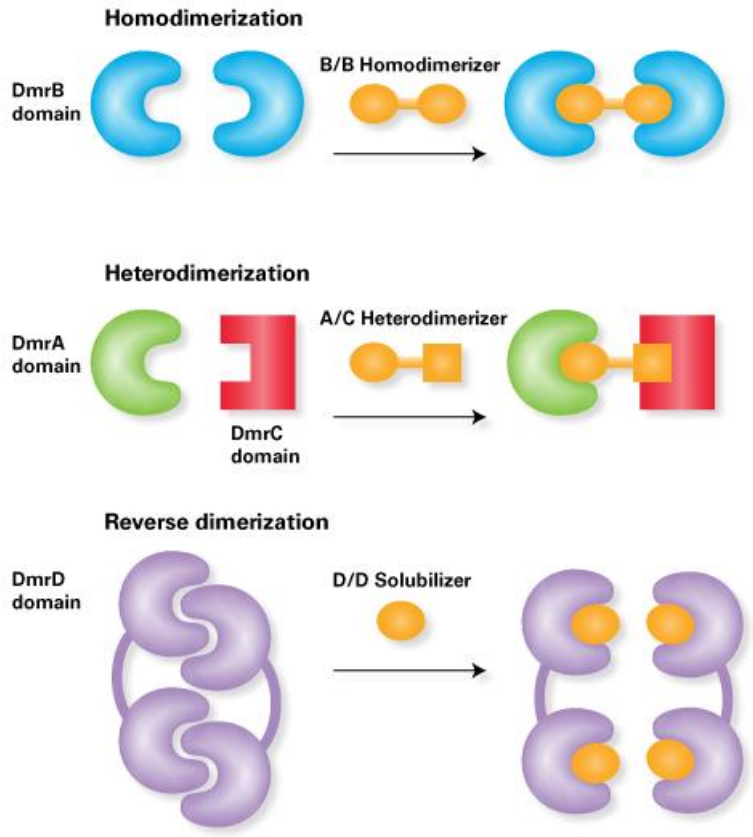


Figure 1. The different types of dimerization include homodimerization, heterodimerization, and reverse dimerization. Separate kits are available from Clontech.

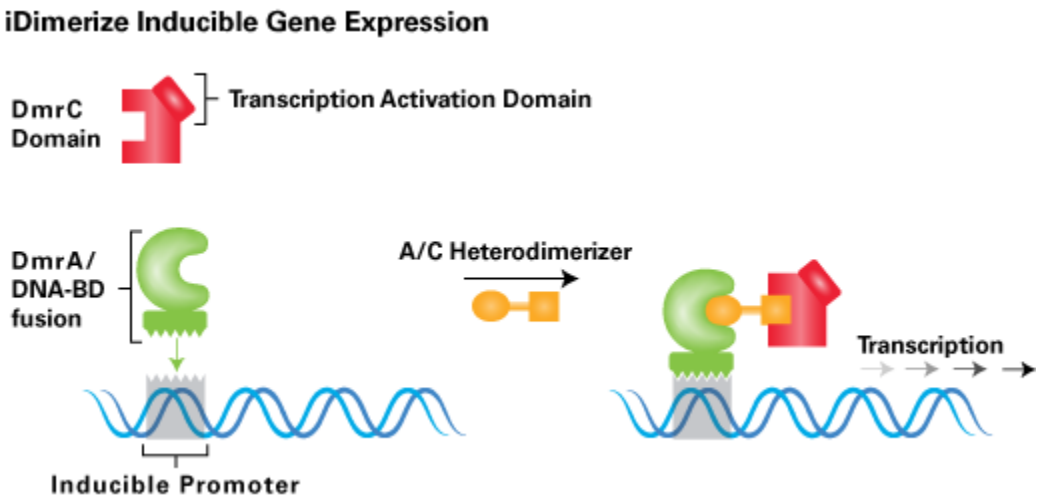


Figure 2. Using regulated heterodimerization to control transcription of a target gene.

C. iDimerize Inducible Expression System

The iDimerize Inducible Expression System contains reagents for placing transcription of a target gene under the control of a small molecule, the "A/C Heterodimerizer" (Figure 2). The system includes three vectors, pHet-Act1-1, pHet-Act2-1, and pZFHD-1 (Figures 3 & 4), as well as the heterodimerizer itself.

Two of these vectors (pHet-Act1-1 and pHet-Act2-1) express different combinations of transcription factor activation domains (AD) and DNA-binding domains (DBD) that are fused to single DmrC and multiple DmrA dimerization domains, respectively. When the dimerization domains on each of these fusion proteins bind A/C Heterodimerizer (or AP21967—see Section II, Table 1), they form heterodimers, reconstituting a functional transcription factor that only forms in the presence of the heterodimerizer (Figure 2). The fusion proteins expressed from pHet-Act1-1 and pHet-Act2-1 are both localized to the nucleus.

The third vector (pZFHD1-1) is an inducible mammalian expression vector designed to express a gene of interest under the control of the P_{ZI-1} promoter, which consists of 12 tandem ZFHD1 binding sites fused to a minimal IL-2 promoter. In the presence of A/C Heterodimerizer the reconstituted transcription factor binds P_{ZI-1} when pZFHD-1 is present in a double stable cell line that also contains the pHet-Act1-1 or pHet-Act2-1 vector.

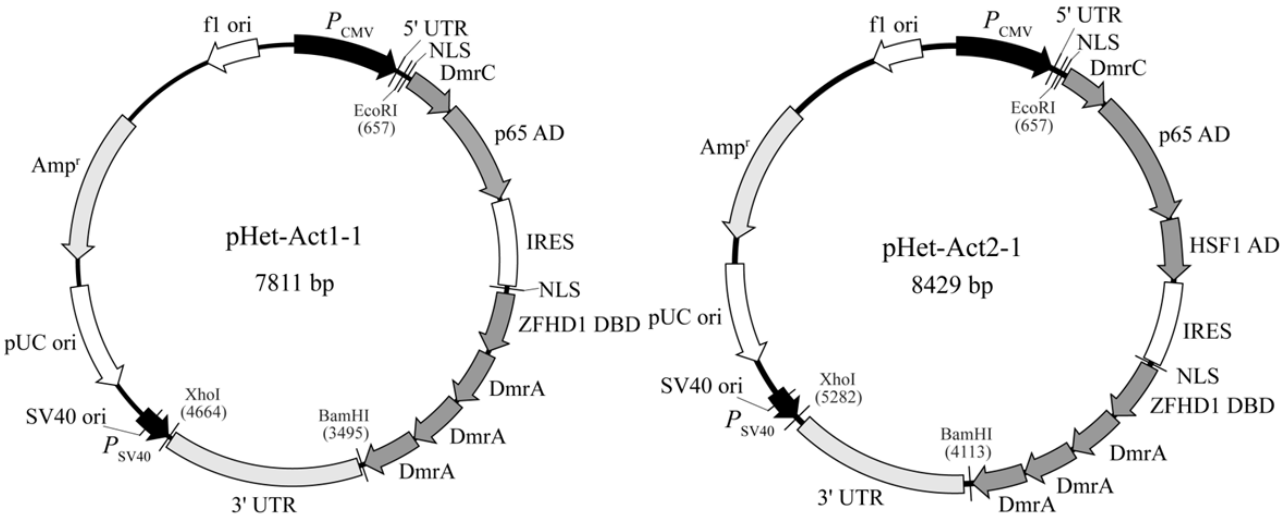
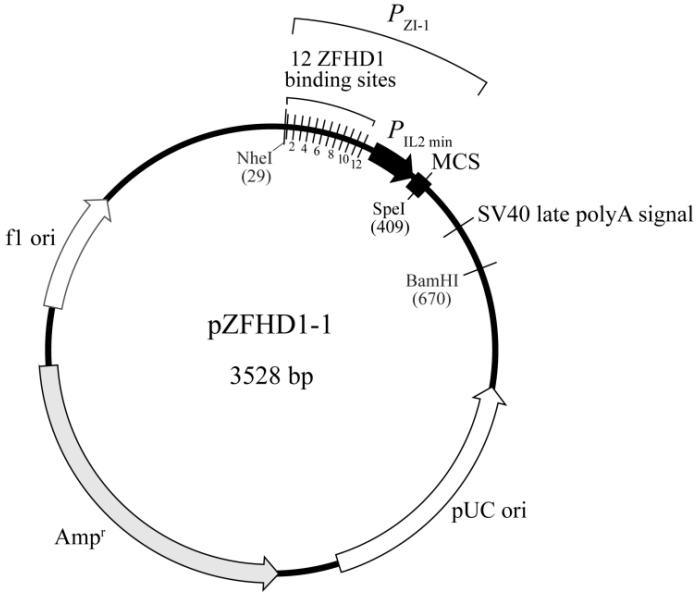


Figure 3. pHet-Act1-1 and pHet-Act2-1 Vector Maps.



	HindIII	PstI	EcoRI	SpeI	BglII	ClaI
381	ACAAGCTTGC	CCTGCAGCGG	GAATTCCACT	AGTCGAGATC	TCCATCGATG	
	TGTTCGAACG	GGACGTCGCC	CTTAAGGTGA	TCAGCTCTAG	AGGTAGCTAC	

Figure 4. pZFHD1-1Vector Map and Multiple Cloning Site (MCS).

II. List of Components

Store all components at -20°C.

- 1 each iDimerize Inducible Expression Vector Set 1
  - 20 µl pZFHD1-1 Vector (500 ng/µl)
  - 20 µl pHet-Act1-1 Vector (500 ng/µl)
  - 20 µl pHet-Act2-1 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. 635057, 635056 & 635055—see Section III.A)

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

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

Ligand Name in ARIAD Kit	Ligand Name in Clontech Kit
AP21967	A/C Heterodimerizer
Plasmid Name in ARIAD Kit	**Plasmid Name in Clontech Kit**
pC_4N_2-R_HS/ZF3 pC_4N_2-R_HS3H/ZF3 pZ_{12}I-PL-2	pHet-Act1-1 pHet-Act2-1 pZFHD1-1
Dimerization Domain Name in ARIAD Kit	**Dimerization Domain Name in Clontech 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.

III. Additional Materials Required

A. A/C Heterodimerizer

Each iDimerize Inducible Expression 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
635057	A/C Heterodimerizer (0.5 mM)	500 µl
635056	A/C Heterodimerizer (0.5 mM)	5 x 500 µl
635055	A/C Heterodimerizer*	5 mg

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

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 Your Target Gene Construct

We recommend using In-Fusion HD (Section III.E) for cloning your gene of interest into pZFHD1-1 (Figure 4). Follow the protocol outlined in the In-Fusion HD user manual (Type PT5162-1 in the keyword field at www.clontech.com/manuals).

V. Constructing a Stable Cell Line Providing Heterodimerizer-Mediated Transcription

To generate stable cell lines containing both the transcription factor and target gene plasmids, we always recommend creating stable clones via a two step process:

- First create a cell line that expresses the transcription factor components (single stable clone),
- Use the best single stable clone as a host to transfect the pZFHD1-1 plasmid containing your gene of interest (double stable clone).

NOTE: Creating stable clones using simultaneous cotransfection of both plasmids will result in high background as result of their tandem cointegration, and a high chance of the juxtaposition of a constitutive promoter from the transcription factor plasmid (CMV) and your gene of interest.

Sequentially transfect pZFHD1-1 and either pHet-Act1 or pHet-Act2 into your host cell line according to the following strategy:

- We recommend using Xfect Transfection Reagent (Section III.D) for all transfections. Follow the Xfect Protocol (Type PT5003-2 in the keyword field at www.clontech.com/manuals).
- Assays for the induction of target gene expression in the presence of A/C Heterodimerizer should be performed as described in Section V.B.

A. Protocol: Strategy for Creating a Double Stable Cell Line Expressing Transcription Factor & Target Gene Plasmids

1. Before making stable clones, test the function of your constructs using cotransient transfection:
   a. Transiently transfect your transcription factor plasmid of choice (pHet-Act1-1 or pHet-Act2-1) and your pZFHD1-1 target gene construct (from Section IV) into your host cell line, in a 2:1 ratio.
   b. Assay for induction of target gene expression in the presence of A/C Heterodimerizer to confirm the functionality of the two plasmids.

NOTES:

- Note that the background is always higher in transient transfection experiments than in properly screened stable clones, due to the high copy number of each plasmid in transiently transfected cells.
- If the level of expression of the transactivator component (DmrC\p65AD fusion) is too high following a transient transfection, you may see reduced expression of your gene of interest (see Section X).

2. Stably integrate your transcription factor plasmid (pHet-Act1-1 or pHet-Act2-1) and select a functional clone as follows:

    a. Cotransfect this plasmid into your cell line along with the puromycin linear selection marker (Pur^r), using Xfect Transfection Reagent, and select stable transfectants by screening for hygromycin or puromycin resistance. Guidelines describing how to select for independent stable clones are outlined in Appendix A.

    b. Select stable transfectants by adding puromycin (see Table 2 in Appendix A).

    c. Screen several independent clones (e.g., 20 clones):

    d. To test for the best clone, transiently transfect your target gene construct (from Section IV) into each positive clone from Step 3.c and compare target gene expression in the presence and absence of A/C Heterodimerizer (e.g. using using qRT-PCR ,Western analysis, or an assay specific to your protein. Recommended dimerizer concentrations are provided in Section V.B.

3. Stably integrate the second plasmid (i.e., the pZFHD-1 target gene construct) into the chosen clone. Select a double stable clone with the lowest background and the highest A/C Heterodimerizer-dependent induction of target gene expression as follows:

    a. Cotransfect the target gene plasmid with the hygromycin linear selection marker provided in the kit and select several clones for resistance to both hygromycin and puromycin (see Appendix A).

    b. Screen individual double stable clones for the lowest background and highest levels of A/C Heterodimerizer-dependent induction of target gene expression (Section V.B).

    c. Expand the best clone and freeze aliquots for long-term storage.

B. Protocol: Induce Target Gene Expression with A/C Heterodimerizer

1. Incubate double-stable clones (or a transient cotransfected population) with A/C Heterodimerizer using the concentration range described below. The optimal concentration for your experiments must be determined empirically. Always maintain at least one culture in medium containing no A/C Heterodimerizer as a negative control.

    a. Recommended A/C Heterodimerizer Concentration

    - Working concentration = 0.1 nM to 500 nM .

    b. General Guidelines for Preparing Media 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 media 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.

2. Assay for induced expression of your target gene using an assay that is appropriate for your experiment. Expression may be first detected after as little as 30 min and reach a maximum after 24–48 hr.

NOTE: The amount of time required for detection depends on many factors, such as sensitivity of the assay, the specific protein expressed, and the host cell line.

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 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 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.

VI. *In Vitro* Heterodimerizer-Inducible Transcription

In initial experiments we recommend that A/C Heterodimerizer be tested across a broad range of concentrations (e.g., .01 to 1,000 nM) to provide a complete dose response profile.

VII. *In Vivo* Heterodimerizer-Inducible Transcription

For *in vivo* use, reconstitute the 5 mg dry-down format of A/C Heterodimerizer following your established injection protocol (5 mg format—see Section III.A).

VIII. References

Natesan, S., Rivera, V. M., Molinari, E. & Gilman, M. (1997) Transcriptional squelching reexamined. *Nature* **390**(6658): 349–350.

Spencer, D. M., Wandless, T. J., Schreiber, S. L. & Crabtree, G. R. (1993) Controlling signal transduction with synthetic ligands. Science **262**(5136): 1019–1024.

IX. Troubleshooting

Description of Problem	Possible Explanation	Solution
Transcription is observed in the absence of the A/C Heterodimerizer	The expression level of the transcription factor domains fused to the DmrA and/or DmrC domain(s) is too high.	Transfect cells with a lower amount of plasmid (in the case of transient transfection) or screen additional stable clones 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.	• 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.
Low levels of induced expression in cotransient transfection	• "Transcriptional squelching": Expression of the transactivator component (DmrC\p65AD fusion) is too high following a transient transfection, resulting in sequestration of endogenous transcription machinery by excess unbound transactivator (Natesan et al., 1997).	• Further optimize the plasmid amounts and ratios to limit the amount of pHet-Act1-1 or pHet-Act2-1 introduced. • If this results in insufficient plasmid for efficient transfection, you can introduce an additional irrelevant plasmid (e.g.. pUC18) as carrier DNA.
High background in stable clones	• Integration of pZFHD1-1 vector into a suboptimal host DNA locus (e.g. adjacent to an endogenous enhancer). • Mixed population of cells, not independent clones	• Screen at least 20 independent clones to find an integrant with low background (Section V.A.)

Appendix A: Selection of Independent Stable Clones Using Hygromycin or Puromycin

To select for stable clones that contain stable integrants of iDimerize plasmids, cotransfect the plasmid 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 iDimerize plasmid construct 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 plasmid 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.

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