Takara Bio USA, Inc.

iDimerize™ Regulated Transcription System User Manual

Cat. No. 635081
(013117)

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I. Introduction

The iDimerize Regulated Transcription System (Cat. No. 635081) is an optimized expression-control system based on ARGENT dimerization control technology which was formerly available from ARIAD Pharmaceuticals, Inc. This manual explains how to use the iDimerize Regulated Transcription System to control the transcription of a target gene via a small molecule "dimerizer." Use this kit to achieve tightly regulated, conditional expression of genes of interest in order to investigate protein function *in vitro* or *in vivo*.

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 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 they are expressed as individual proteins that are brought together via a noncovalent interaction. The iDimerize Regulated Transcription System utilizes this modular architecture to achieve tightly regulated conditional gene expression (Figure 1).

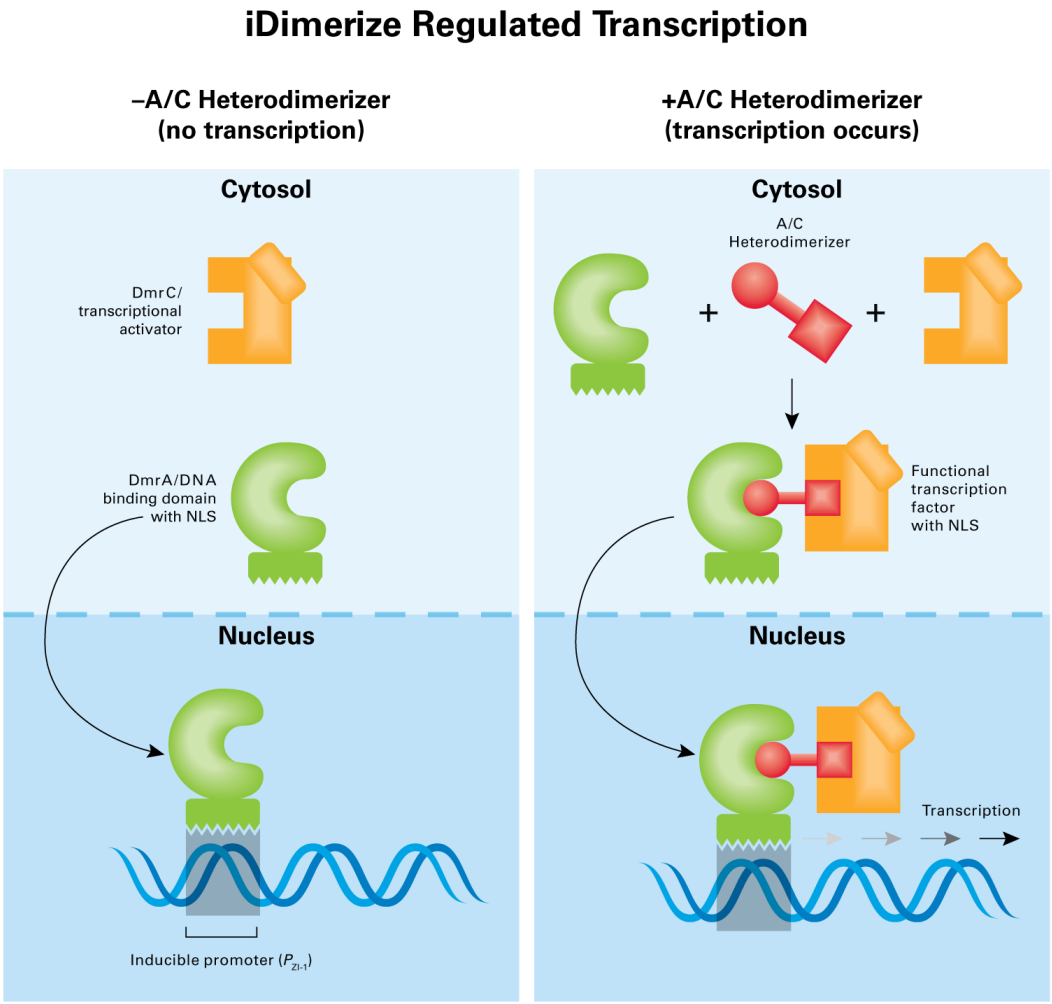

Figure 1. The iDimerize Regulated Transcription System uses the tight P_{ZI-1} promoter, which exhibits very low background in the absence of induction because it is not recognized by host transcription factors. A further level of control is provided separating the DNA binding component (DmrA/DB; green) and transcription activation component (DmrC/TA; yellow) into separate compartments in the cell. DmrC/TA does not possess a nuclear localization signal (NLS), so it resides predominantly in the cytoplasm; however, it is brought to the nucleus by the addition of A/C heterodimerizer which causes its interaction with DmrA/DB. DmrA/DB does possess an NLS, so when A/C heterodimerizer is added, the transcription factor is completed through dimerization. The complete transcription factor is translocated into the nucleus, where it binds the P_{ZI-1} promoter and induces expression of the gene of interest.

Dimerizers are cell-permeant organic molecules with two separate motifs that each bind with high affinity to a specific protein module. Heterodimerizers contain two different binding motifs, which allow the specific dimerization of two *different* proteins. The iDimerize Regulated Transcription System uses the A/C heterodimerizer to reconstitute an active transcription factor and trigger regulated transcription.

iDimerize Regulated Transcription System

The iDimerize Regulated Transcription System includes three vectors, plus the A/C heterodimerizer:

- pHet-Act1-2 (Figure 2) encodes both the transcription factor activation domain and the DNA-binding domain. The activation domain is fused to a single DmrC domain, and the DNA-binding domain is fused to multiple DmrA dimerization domains. When DmrC and DmrA bind the A/C Heterodimerizer, they form heterodimers that reconstitute the functional transcription factor (Figure 1, bottom right). The nuclear localization signal on the DNA-binding domain causes the newly formed complex between the DNA-binding domain and the transcription factor activation domain to enter the nucleus, inducing expression of the target gene cloned into the pZFDH1-2 vector. This vector contains a puromycin resistance cassette.
- pZFHD1-2 (Figure 3, left) is an inducible mammalian expression vector designed to express your gene of interest under the control of the $P_{ZI\text{-}1}$ 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 $P_{ZI\text{-}1}$ in a double-stable cell line that also contains the pHet-Act1-2 vector. This vector contains a neomycin resistance cassette.
- pZFHD1-2 Control (Figure 3, right) is the same vector as pZFHD1-2, with the gene for firefly luciferase cloned into the MCS. When pZFHD1-2 Control is cotransfected with pHet-Act1-2, you should detect only minimal expression in the absence of the A/C heterodimerizer. Adding the A/C Heterodimerizer results in fast, tunable, luciferase gene expression.

Since pHet-Act1-2 and pZFHD1-2 contain different resistance cassettes, you can establish double-stable cell lines containing both vectors (see Appendix A for more information).

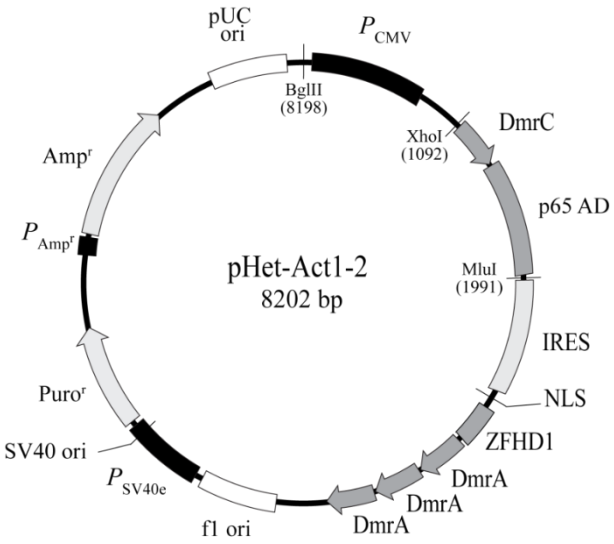

Figure 2. pHet-Act1-2 vector map.

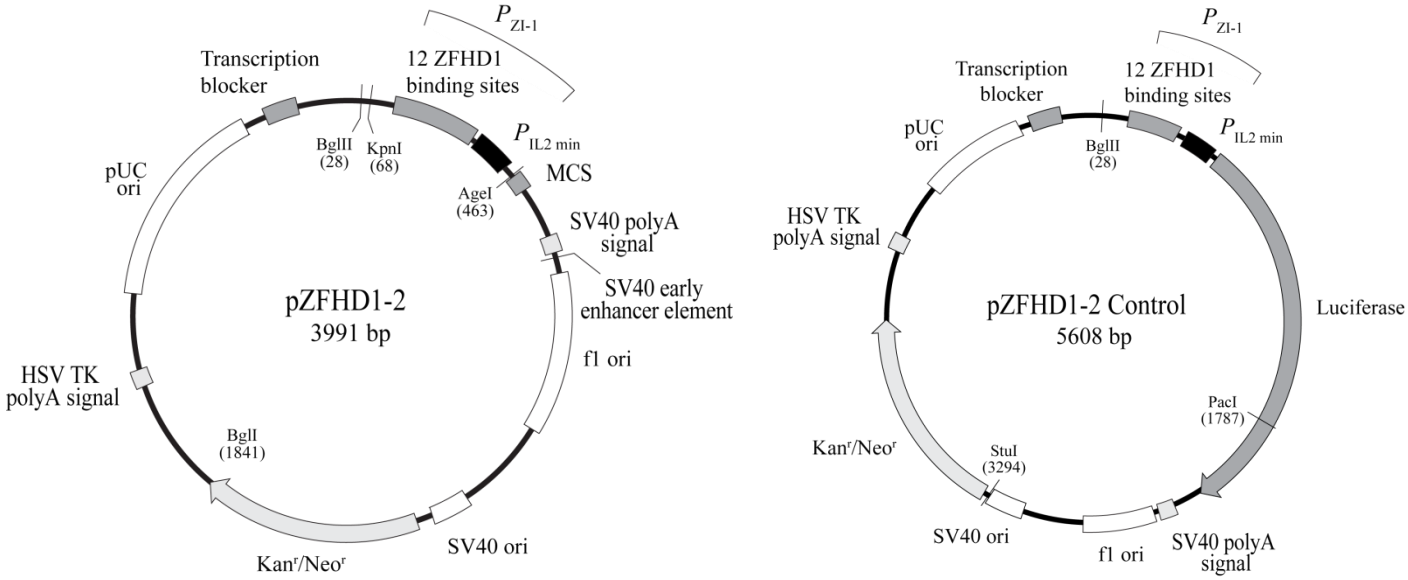

Figure 3. pZFHD1-2 and pZFHD1-2 Control vector maps. Clone your gene of interest into the MCS of pZFHD1-2.

II. List of Components

Store all components at –20°C.

- 1 each iDimerize Regulated Transcription Vector Set 2 (Cat. No. 635082; not sold separately)
  - 20 µl pHet-Act1-2 Vector (500 ng/µl)
  - 20 µl pZFHD1-2 Vector (500 ng/µl)
  - 20 µl pZFHD1-2 Control Vector (500 ng/µl)
- 500 µl A/C Heterodimerizer (0.5 mM)
  (also sold separately as Cat. Nos. 635056, 635055 & 635095—see Section III.A)

III. Additional Materials Required

A. A/C Heterodimerizer

Each iDimerize Regulated Transcription System includes 500 µl A/C Heterodimerizer (0.5 mM; see Section II). Additional A/C Heterodimerizer can 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.E).

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	Size
631306	Puromycin	100 mg
631305		25 mg
631307	G418	1 g
631308		5 g

E. Xfect™ Transfection Reagent

Xfect Transfection Reagents provide high transfection efficiency and low cytotoxicity.

Cat. No.	Transfection Reagent	Size
631317	Xfect Transfection Reagent	100 rxns
631318		300 rxns

F. In-Fusion® HD Cloning System

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

Cat. No.	Cloning Kit	Size
639645	In-Fusion HD Cloning System	10 rxns
639646		50 rxns
639647		100 rxns

IV. Creating Your Target Gene Construct

We recommend using the In-Fusion HD Cloning System to clone your gene of interest into pZFHD1-2. Follow the protocol outlined in the In-Fusion HD user manual. (To find the manual, go to takarabio.com/manuals and type "In-Fusion HD" in the search box.)

V. Performing a Transient Double Transfection of pHet-Act1-2 & pZFHD1-2

1. Transiently transfect pHet-Act1-2 and your pZFHD1-2 target gene construct (from Section IV) into your host cell line, in a 2:1 ratio.
2. Assay for induction of target gene expression in the presence of A/C Heterodimerizer to confirm the functionality of the two plasmids.

NOTES:

- The background in transient transfection experiments can be higher than in properly screened stable clones, due to the high copy number of each plasmid in transiently transfected cells.
- You may be able to reduce the background in transiently transfected cells by decreasing the amount of the two co-transfected vectors; however, the ratio of the two vectors should remain 2:1.

VI. Constructing a Stable Cell Line That Provides Heterodimerizer-Mediated Transcription

We recommend using a two-step process to generate a double-stable cell line containing both the transcription factor and target gene plasmids. First, create a cell line that expresses the transcription factor components from pHet-Act1-2 (the single-stable clone), using puromycin for selection. Then use the best single-stable clone as a host to transfect the pZFHD1-2 plasmid containing your gene of interest, followed by G418 treatment to obtain the double-stable clone.

NOTES:

- Use the Xfect Transfection Reagent for all transfections.
  Follow the Xfect Protocol (to find the protocol, type "Xfect" in the keyword field at takarabio.com/manuals).
- Do not create stable clones by simultaneous cotransfection of both plasmids. Cotransfection results in high background as result of tandem cointegration, and a significant chance of juxtaposing the constitutive promoter from the pHet-Act1-2 transcription factor plasmid with your gene of interest.

A. Protocol: Construct a Double Stable Cell Line

1. Stably integrate the transcription factor plasmid (pHet-Act1-2) and select a functional clone:
   a. Transfect pHet-Act1-2 into your cell line using Xfect Transfection Reagent.
   b. Select stable transfectants by screening for puromycin resistance (see Appendix A for guidelines).
   c. Screen several independent clones (e.g., 20 clones).
   d. To select the best pHet-Act1-2 clone, transiently transfect your pZFHD1-2 target gene construct (from Section IV) into each positive clone from Step 1.c and compare target gene expression in the presence and absence of A/C Heterodimerizer using qRT-PCR, Western analysis, or an assay specific to your protein.

2. Create a double-stable clone by transfecting your chosen pHet-Act1-2 clone with the pZFHD1-2 vector containing your gene of interest. Select the double-stable clone with the lowest background and the highest A/C Heterodimerizer-dependent induction of target gene expression:
   a. Transfect your pZFHD1-2 target gene construct (from Section IV) into your chosen pHet-Act1-2 clone (from step IV.A.1.d) using Xfect Transfection Reagent.
   b. Use puromycin and G418 (see Appendix A) to establish double-stable clones.
   c. Screen individual double-stable clones for the lowest background and highest levels of A/C Heterodimerizer-dependent induction of target gene expression (Section VI.C).
   d. Expand the best clone and freeze aliquots for long-term storage.

B. Prepare A/C Heterodimerizer

- Recommended working concentration of A/C Heterodimerizer: 0.1 nM to 500 nM.
- 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. *You must determine the optimal concentration for your experiments empirically.*

EXAMPLE: To prepare 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 A/C Heterodimerizer directly from ethanol stocks, or by diluting A/C Heterodimerizer 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.
- The final concentration of ethanol in the medium should be kept below 0.5% (a 200-fold dilution of a 100% ethanol solution) to prevent the ethanol from having a detrimental effect on the cells.

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

1. Incubate double-stable clones (or a transiently cotransfected population) with A/C Heterodimerizer.

NOTES:
- Recommended working concentration of A/C Heterodimerizer = 0.1 nM to 500 nM.
- You must determine the optimal concentration for your experiments empirically.
- Always maintain at least one culture in medium containing no A/C Heterodimerizer as a negative control.

2. Assay for induced expression of your target gene using an appropriate assay. Expression may be detected after as little as 30 min and will reach its maximum level after 24–48 hr.

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

D. *In Vitro* Heterodimerizer-Inducible Transcription

In initial experiments we recommend that you test A/C Heterodimerizer across a broad range of concentrations (e.g., 0.01 to 1,000 nM) to provide a complete dose response profile.

E. *In Vivo* Heterodimerizer-Inducible Transcription

Reconstitute the 5 mg dry-down format of A/C Heterodimerizer following your established injection protocol (Cat. No. 635055; see Section III.A).

VII. Protocol: Removing A/C Heterodimerizer from Cells (Washout Experiment)

Perform this experiment to compare target cells before and after A/C Heterodimerizer treatment.

A. Adherent cells

1. Remove the media from transfected and A/C Heterodimerizer-treated cells (from Protocol VI.C).
2. Rinse cells with warm PBS containing Ca^{2+} and Mg^{2+}.
3. Detach cells by your method of choice (trypsin, cell dissociation buffer, etc.).
4. Split cells into at least two new cell culture plates.
5. Culture one plate with media containing A/C Heterodimerizer at a concentration of your choice (positive control) and culture the second plate in media without A/C Heterodimerizer (negative control).
6. Collect cells at a specific time after splitting that is defined by your needs. Analyze and compare cells cultured with or without A/C Heterodimerizer.

B. Suspension cells

1. Collect the transfected and A/C Heterodimerizer-treated cells (from Protocol VI.C) via centrifugation.
2. Resuspend one portion of the cells with media containing A/C Heterodimerizer at a concentration of your choice (positive control). Resuspend the other portion of the cells in media without A/C Heterodimerizer (negative control).
3. Collect cells at a specific time after splitting that is defined by your needs. Analyze and compare cells cultured with or without A/C Heterodimerizer.

VIII. 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.	• Transient transfections: Transfect cells with a lower amount of plasmid • Stable transfections: screen additional stable clones for the lowest background. • Monitor protein expression in your cells by Western blot using the anti-DmrA and anti-DmrC antibodies (Section III.B).
Addition of A/C Heterodimerizer does not result in any of the expected effects	• 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. • Include the pZFHD1-2 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) is too high following a transient transfection, resulting in sequestration of endogenous transcription machinery by excess unbound transactivator.*	• Optimize the plasmid amounts and ratios to limit the amount of pHet-Act1-2 that is 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 pZFHD1-2 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 VI.A.)

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

Appendix A: Selecting Independent Stable Clones Using G418 or Puromycin

To select for stable clones that contain stable integrants of iDimerize plasmids, transfect each plasmid into your target cell line as described in Section V, and screen for G418 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 the Xfect Transfection Reagent*. Follow the Xfect Protocol, except use 2 µg of your plasmid per well.
   * To find the Xfect protocol, type "Xfect" in the keyword field at takarabio.com/manuals.

   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 G418 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 G418 at the selection concentration that is optimal for your cell line (Table 1).

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

Cat. No.	Antibiotic	Recommended Concentration (µg/ml)	
		Selection[1]	Maintenance
631306	Puromycin (100 mg)	0.25–10	0.25
631305	Puromycin (25 mg)		
631307	G418 (1 g)	100–800	200
631308	G418 (5 g)		

[1] When selecting for single colonies, you must determine the appropriate dose for your specific cell line empirically. 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 G418 and/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 1). Expand and test clones using your preferred assay.

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