

ARGENT™
Regulated Homodimerization Kit

Version 2.0

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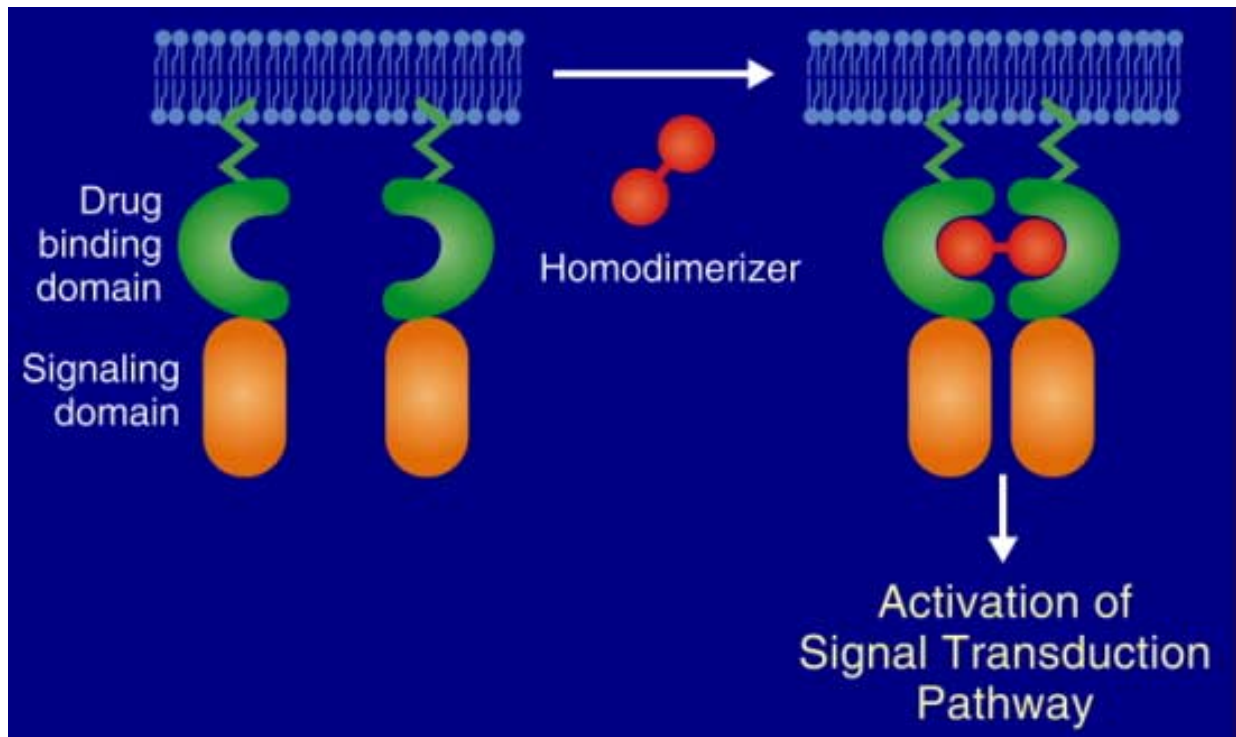
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ARGENT™ Regulated Homodimerization Kit

The ARGENT™ Regulated Homodimerization Kit 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.



[Controlling Signal Transduction Using Regulated Homodimerization](#)

Overview

Many cellular processes are triggered by the induced interaction, or "dimerization", of signaling proteins (1). 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. 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 protein recognized by the dimerizer. Addition of the dimerizer then crosslinks the chimeric signaling protein, activating the cellular event that it controls (see the figure).

There are two classes of dimerizers. **Homodimerizers** (as shown in the figure) incorporate two identical binding motifs, and can therefore be used to induce self-association of a single signaling domain. The ARGENT™ Regulated Homodimerization Kit described here provides a homodimerizer and DNA vectors for making appropriate fusion proteins. **Heterodimerizers** have two different binding

motifs, allowing the dimerization of two different signaling domains when they are fused to the two appropriate ligand binding domains. For applications requiring heterodimerization, we offer a separate regulation kit that includes a heterodimerizer (see [ARGENT Regulated Heterodimerization Kit](#)).

Applications of the ARGENT Homodimerization Kit

Induced protein homodimerization has proved to be broadly applicable, and a large number of signaling proteins have been brought under homodimerizer control. Published examples include:

- Transmembrane signaling receptors such as Fas, gp130, and the receptors for Epo, Tpo, insulin, TGF- β PDGF, EGF and HGF;
- Intracellular signaling molecules such as Src, Sos, Vav, ZAP70, Raf, Bax, FADD, CED3, caspases 1, 3, 8 and 9, RIP, IKKs, and T cell receptor zeta chain; and
- Other cellular proteins with dimerization-based mechanisms, including integrins, cadherins and transcription factors.

These studies have allowed a wide variety of cellular processes to be chemically controlled, including proliferation, differentiation, adhesion, transformation and apoptosis. A complete list of publications describing the use of homodimerizing reagents can be found in the [Regulation Kit Bibliography](#).

Many of these applications have progressed from *in vitro* work to analysis in transgenic mice, or in mice implanted with cells expressing dimerizer constructs. The dimerizer provided with the kit, AP20187, is suitable for *in vivo* work and has been used in several mouse studies. In addition, the plasmid reagents provided with the kit have been adapted for delivery using a variety of vector systems, including retroviral and adenoviral vectors. A product based on the regulated homodimerization of Fas is in clinical development for the treatment of graft-versus-host disease (2).

Regulated dimerization has applications in many areas of functional genomics research and drug discovery. Inducible alleles of orphan receptors or other signaling proteins can be created with no knowledge of the natural ligand. These systems can be used for functional analysis of the signaling pathway in multiple cell types, potentially identifying downstream target proteins, or genes whose expression is modulated by the signaling event. Inducible animal models can be established of disease states associated with an activated signaling protein. In addition, cell lines in which a specific signal can be chemically induced may be useful in the configuration of targeted cell-based assays for small molecule drugs.

Design of the kit components

The reagents in the ARGENT Regulated Homodimerization Kit are based on the human protein FKBP12 (FKBP, for FK506 binding protein) and its small molecule ligands. FKBP is an abundant cytoplasmic protein that serves as the initial intracellular target for the natural product immunosuppressive drugs FK506 and rapamycin. In the original homodimerizer system developed by the Schreiber and Crabtree laboratories (3), a dimerizer was created by chemically linking two molecules of FK506 in a manner that eliminated immunosuppressive activity. The resulting molecule, called FK1012, was able to crosslink fusion proteins containing wild-type FKBP domains. A second generation FKBP homodimerizer, AP1510, was subsequently developed by scientists at ARIAD (4). AP1510 has the advantages of being completely synthetic, as well as being smaller and simpler than FK1012 and more potent in many applications.

More recently, we have improved the affinity and specificity of these molecules further by eliminating their ability to bind to endogenous FKBP. This was achieved by remodelling the FKBP-ligand interface using protein engineering (5). The resulting third generation homodimerizers, AP1903 and

AP20187, bind with subnanomolar affinity to FKBP with a single amino acid substitution, Phe36Val (F_V), while binding with 1000-fold lower affinity to the wild-type protein. The new system invariably provides more potent activation of homodimerization, and the third-generation ligands have pharmacologic properties suitable for *in vivo* use. AP20187 and F_V form the basis of the reagents provided in the ARGENT Regulated Homodimerization Kit.

Notes on the use of this kit

Use of the Homodimerization Kit to induce heterodimers

It is also possible to use the Homodimerization Kit to induce heterodimerization, by fusing two different signaling proteins to the same ligand binding domain. Addition of the homodimerizer creates a mixture of homodimeric and heterodimeric complexes. However, to induce heterodimers exclusively, the [ARGENT™ Regulated Heterodimerization Kit](#) should be used

Use to control transcription

We have found that when AP20187 is used to dimerize individual components of a transcription factor, the resulting complexes are typically inactive, perhaps due to the very high affinity of the ligand or inappropriate geometry of the complexes. Therefore this kit should **not** be used for transcriptional applications. If you are attempting to regulate a transcriptional event by inducing homodimerization of a single protein we can provide an alternate F_V ligand for this purpose (please request the F_V homodimerizer AP1889 (6)). However, if (as in most applications) your aim is to activate transcription by inducing heterodimerization of two separate components of a transcription factor, then we recommend that you use either the [ARGENT™ Regulated Heterodimerization Kit](#) or one of the [ARGENT™ Regulated Transcription Kits](#).

Use of the previous kit based on AP1510

This kit replaces our original Homodimerization Kit that used the dimerizer AP1510 and wild-type FKBP fusion proteins (4). The AP20187-based system has the advantages of working at lower concentrations, and AP20187 has better pharmacokinetic properties than AP1510, allowing it to be used *in vivo*. However, we will continue to provide AP1510 to those investigators who have already made wild-type FKBP fusion proteins and wish to complete their experiments.

Kit contents

To control the activity of a signaling domain, the domain of interest is fused to one or more copies of an F_V domain and the dimerization state controlled by administration of the dimerizer AP20187.

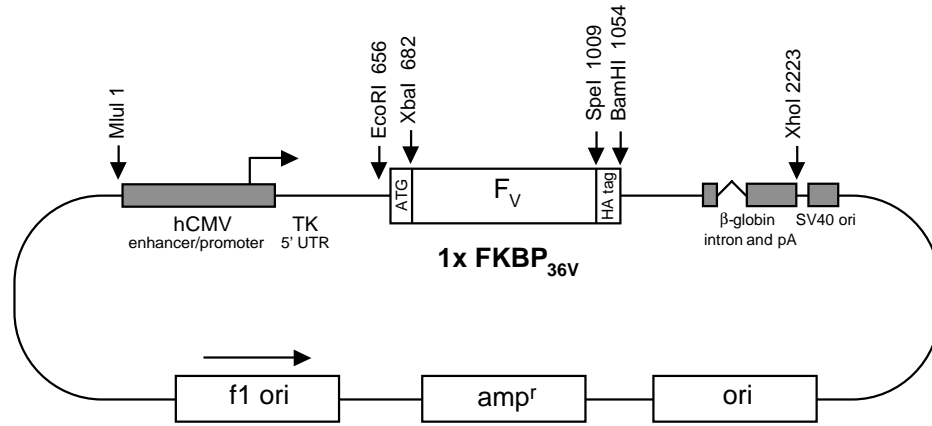
The ARGENT™ Regulated Homodimerization Kit contains two plasmids, pC₄-F_V1E and pC₄M-F_V2E, and an aliquot of AP20187. As described below, the plasmids in this kit provide an assortment of components (i.e. multiple F_V domains, an epitope tag, localization sequences) that can be easily manipulated to generate protein fusions whose activity and localization can be controlled by dimerizer.

Expression plasmids

pC₄-F_V1E

Description

pC₄-F_V1E (5371 bp)



Not drawn to scale

- In pC₄-F_V1E, a chimeric fusion protein containing a single copy of F_V (F_V1) followed by a carboxy-terminal epitope tag (E, from the influenza hemagglutinin [HA] gene) is expressed under control of the human CMV enhancer/promoter (C).
- The F_V domain is flanked by XbaI and SpeI sites. To fuse the protein of interest to a single F_V domain clone it into the adjacent XbaI or SpeI sites as described below.
- Unless the domain fused to FKBP contains a signal that targets it to another location, fusion proteins should be localized to the cytoplasm by default as there is no targeting signal in this vector (the amino terminus of this fusion protein, upstream of the XbaI site, consists only of a methionine).

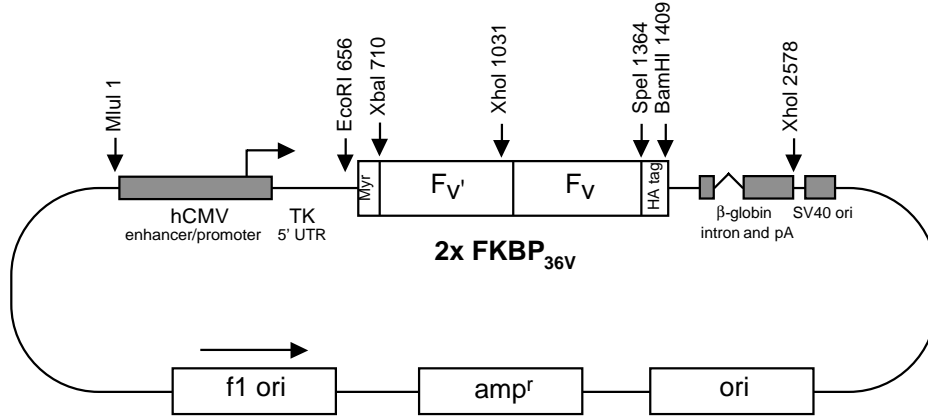
[Annotated Sequence](#)

[Raw sequence](#)

pC₄M-F_v2E

Description

pC₄M-F_v2E (5726 bp)



Not drawn to scale

- In pC₄M-F_v2E, a chimeric fusion protein containing an amino-terminal myristoylation signal (M), two copies of F_v (F_v2), followed by a carboxy-terminal epitope tag (E, from the influenza hemagglutinin [HA] gene) is expressed under control of the human CMV enhancer/promoter (C).
- The two F_v domains are flanked by XbaI and SpeI sites. To fuse the protein of interest to two F_v domains clone it into the adjacent XbaI or SpeI sites as described below.
- One of the F_v domains has changes in the codons used that do not change the amino acid sequence, but which significantly reduce the match between the F_v domains at the nucleotide level. We have found that this “wobble” reduces potential for recombination (i.e. making these constructs fit for use in retroviral vectors).
- Due to the presence of the amino-terminal myristoylation signal, fusion proteins will be targeted to the cytoplasmic face of membranes.

[Annotated Sequence](#)

[Raw sequence](#)

General vector information

Creating fusion proteins

The creation of fusion proteins using vectors in any of the ARIAD Regulation Kits is based on a standard cloning strategy involving the stepwise addition of compatible XbaI-SpeI fragments. To do this, amplify the coding sequence of interest by PCR so that it contains the six nucleotides specifying an XbaI site immediately 5' to the first codon (take care not to create an overlapping Dam methylation sequence, GATC, on either strand), and the six nucleotides specifying a SpeI site immediately 3' to the last codon. Then, for example, to fuse the protein of interest amino terminal to 2 F_v domains, clone the XbaI-SpeI fragment into the XbaI site of pC₄M-F_v2E (XbaI and SpeI have compatible cohesive ends). If inserted in the proper orientation, the XbaI and SpeI sites, now flanking the new fusion protein, will be maintained, with the junction of the two peptides consisting of the two amino acids specified by the SpeI and XbaI sites that were fused. Alternatively, to fuse the XbaI-SpeI

fragment carboxy-terminal to 2 F_V domains, insert it into the SpeI site of pC₄M-F_V2E. In both cases, since the flanking XbaI and SpeI sites are maintained, additional fragments can still be fused at the amino- and carboxy- terminal ends if desired.

This strategy can also be applied to create 3 tandem F_V domains. For example, the XbaI-SpeI fragment of pC₄-F_V1E can be inserted into the SpeI site of pC₄M-F_V2E (or vice versa).

If the sequence to be fused contains internal XbaI or SpeI sites, fusions can still be made either by using XbaI or SpeI at both ends, or by using NheI or AvrII which also generate ends that are compatible with XbaI and SpeI. Note, though, that in these cases unique flanking XbaI and SpeI sites will not be regenerated.

The sequence between the SpeI and BamHI sites of pC₄-F_V1E and pC₄M-F_V2E encodes a carboxy-terminal HA epitope tag followed by a stop codon. Therefore, stop codons should not be included in the fused sequences.

Controlling the localization of fusion proteins

The region between the EcoRI and XbaI sites in all pC₄- vectors contains a leader sequence which determines the default localization of fusion proteins. In pC₄-F_V1E, pC₄M-F_V2E and pC₄EN-F1 (from the ARGENT™ Regulated Heterodimerization Kit) the default localization is to the cytoplasm, inner face of plasma membranes and nucleus, respectively. Fusion proteins can be cloned as XbaI-SpeI or XbaI-BamHI fragments (note the location of the epitope tag) into the appropriate vector to target them to the desired location.

How many FKBP domains should I use?

The number of F_V domains best suited for each application varies. Fusion to a single F_V domain is generally preferred if formation dimers is sufficient to induce the desired signaling event. Fusion to 2 or more F_V domains may be preferred when induction of a signaling event requires the formation of higher order oligomers. Often the optimal configuration is best determined empirically (7, 8).

Antibodies to detect fusion proteins

Anti-HA (Babco #MMS101R-500) and anti-FKBP12 (Affinity Bioreagents #PA1-026) antibodies are available commercially. The anti-FKBP12 reagent binds equally well to wild-type FKBP and F_V. Each F_V domain is ~12 kDa.

Addition of a selectable marker

Stable integration of plasmid vectors into cells is greatly facilitated by co-expression of the fusion protein of interest and the selectable marker gene on the same mRNA transcript. Such bicistronic mRNAs can be created by inserting the coding region of the fusion protein (as an EcoRI-BamHI fragment) downstream of an enhancer/promoter and upstream of an internal ribosome entry sequence (IRES) which drives expression of a selectable marker gene. Several such vectors are available from Clontech (e.g. pIRESneo2, pIRESHyg2, pRESpuro2, pRESbleo).

The advantage of using this configuration is that essentially all drug resistant cells will express the protein of interest since any mRNA that expresses the selectable marker as its second cistron should also express the protein of interest as its first cistron.

Additional pC₄ expression vector information

Origin of vector

pC₄ expression vectors are derived from the vector pCGNN (9). To create pC₄ several existing restriction sites were eliminated and several others added in order to have all functional regions of the plasmid be flanked by unique restriction sites (i.e. MluI, EcoRI, XbaI, SpeI, BamHI and XhoI).

Configuration of vectors

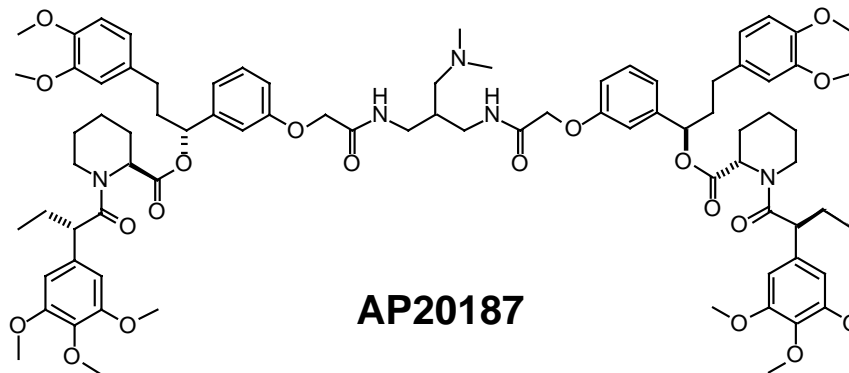
- **MluI-EcoRI:** Contains the enhancer/promoter from human CMV and the 5' UTR from the Herpes simplex virus TK gene. This fragment can be replaced with an alternate enhancer/promoter of choice (i.e. to promote tissue-specific expression).
- **EcoRI-XbaI-SpeI-BamHI:** Contains the coding region subdivided as follows:
 - *EcoRI-XbaI:* leader sequence (e.g. a myristoylation sequence or simply an ATG).
 - *XbaI-SpeI:* ligand binding domains and signaling domain
 - *SpeI-BamHI:* Carboxy-terminal sequence. In addition to the stop codon may contain an epitope tag.
- **BamHI to XhoI:** contains an 830-bp portion of the 3'UTR of the rabbit β -globin gene that includes its final intron and poly(A) signal. Note that in pC₄M-F_v2E the XhoI site is not unique due to the presence of a second site within the first FKBP domain.

Production of single stranded DNA for mutagenesis

All pC₄ plasmids contain f1 origins for rescue of single stranded DNA. The strand generated upon rescue is indicated by the arrow in plasmid maps. For example, in pC₄-F_v1E the antisense strand is generated, therefore oligonucleotides used for mutagenesis should correspond to the sense stand of the vector (the strand shown in the vector sequences).

AP20187

Description



AP20187 is a synthetic dimerizer that can be used to induce homodimerization of F_v domain-containing fusion proteins. AP20187 has no immunosuppressive activity and is non-toxic to cells.

To date, AP20187 has only been tested *in vitro* and in mice. We do not yet know whether it crosses the blood-brain barrier in mice or whether it works in yeast or any other model organisms.

AP20187 cannot be used to dimerize wild type FKBP domains. If you have already made constructs using wild type FKBP domains you must use the dimerizer AP1510 (4). We will continue to supply AP1510 for use *in vitro* upon request.

Reconstituting AP20187

AP20187 (molecular mass 1428.8 Da) is provided in lyophilized form which should be reconstituted as a concentrated stock in an organic solvent. We recommend dissolving the lyophilized material in absolute ethanol to make a 1 mM solution (e.g. dissolve 250 μ g AP20187 in 175 μ l ethanol). After adding the appropriate volume of ice-cold ethanol, seal and vortex periodically over a period of a few minutes to dissolve the compound. Keep on ice during dissolution to minimize evaporation.

Storage and handling of AP20187

Once dissolved, the stock solution can be kept at -20°C indefinitely, in a glass vial or a microfuge tube. Further dilutions in ethanol can be similarly stored. At the bench, solutions in ethanol should always be kept on ice, and opened for as short a time as possible, to prevent evaporation and consequent changes in concentration.

Using AP20187 *in vitro*

Working concentrations of dimerizer can be obtained by adding compound directly from ethanol stocks, or by diluting serially in culture medium just before use. In the latter case we recommend that the highest concentration does not exceed 5 µM, to ensure complete solubility in the (aqueous) 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 detrimental effects of the solvent on the cells.

Use of AP20187 in animals

AP20187 has been successfully used in mice with maximal effects seen at doses in the range of 0.5-10 mg/kg delivered intravenously (10; see below).

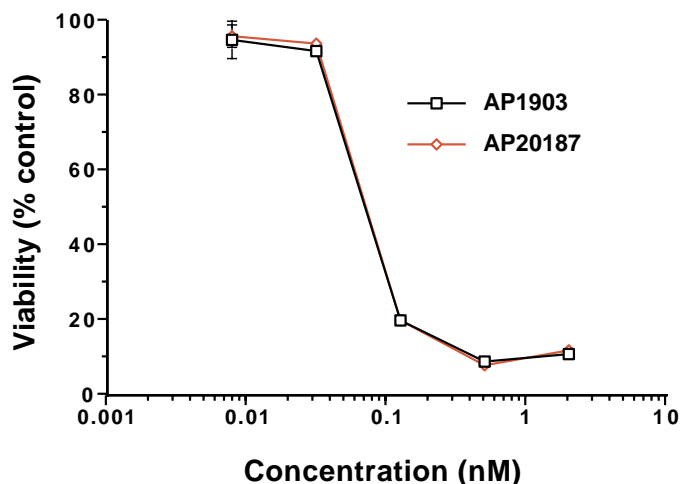
Once preliminary *in vitro* experiments have been carried out successfully we will be happy to provide quantities of AP20187 necessary for use in mice. The protocol for formulating AP20187 for *in vivo* use can be found [here](#).

Expected results

AP20187 is chemically virtually identical to AP1903 (5) and has the same binding properties. In all applications tested to date, AP20187 has identical activity to AP1903 *in vitro* and *in vivo* (as shown in the plot below).

In vitro usage

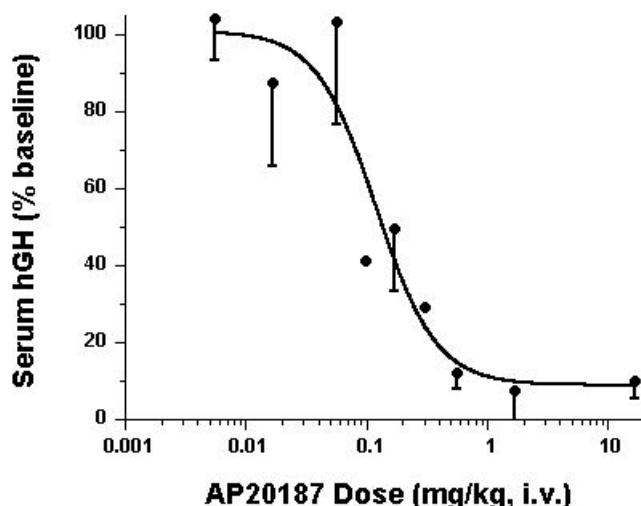
The example below shows the activation of Fas signaling by AP20187 in cells transduced with a construct containing the death domain of Fas cloned into pC₄M-F_v2E (5). In these experiments, maximal Fas-induced cell death upon overnight treatment with dimerizer was seen with concentrations below 1 nM of AP20187. In other applications, concentrations of dimerizer as high as 10 to 100 nM are required to see the maximal effect. In initial experiments we recommend that AP20187 be tested across a broad range of concentrations (e.g. 0.01 nM to 100 nM) to provide a complete dose-response profile.



In vivo usage

The example below shows the activation of Fas signaling in mice after intravenous (i.v.) administration of AP20187. Cells were transduced with two constructs, one containing the death domain of Fas cloned into a vector similar to pC₄M-F_v2E (5) and the other containing a human growth hormone (hGH) cDNA expressed constitutively from the CMV enhancer. These cells were then implanted intramuscularly into mice. Twenty-four hours later the mice were administered increasing doses of AP20187. 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 (10). In initial experiments we recommend that AP20187 be tested across a broad range of concentrations (e.g. 0.005 to 10 mg/kg) to provide a complete dose-response profile and to allow determination of the minimal effective dose.



Effect of protein expression level

For some proteins, low level signaling can be observed in the absence of dimerizer if the expression level of the fusion protein is too high. In such cases it is possible to eliminate basal signaling by reducing protein expression levels. For example, some cell death is observed in the absence of dimerizer in cells transiently transfected with the FKBP-Fas fusion protein, but not in cells stably transduced with a retroviral vector in which the expression level of the fusion protein is lower.

Conditions of use

Please bear in mind that these materials will be provided to you pursuant to a Material Transfer Agreement (MTA). Our MTA contains, among other provisions, certain restrictions on the transfer to others of our materials and any derivatives you create using or incorporating our materials. If you wish to share the materials or derivatives with colleagues or collaborators, they must first complete our MTA. Please also be aware that our Kits are not to be used in research funded by, or conducted on behalf of, a commercial or for-profit entity. Those situations require a [commercial agreement](#).

We certainly hope that you obtain interesting results and that they are presented and published without delay. But please note that under the terms of the MTA, you need to give us advance notice of any such presentations or publications, including talks, posters, and submissions of abstracts or manuscripts for publication. Also, in the event of a patent filing, a copy of the patent application must be provided to ARIAD. Advance notice is usually 4 weeks prior to submission, but please check your MTA for specific details.

Please also be aware that the use of intellectual property or materials of others, in conjunction with the Regulation Kit, may have additional ramifications. For example, if you plan to use a Regulation Kit together with human embryonic stem cells from WiCell (WARF), we and you are required to execute an additional MTA which will be provided to you.

We appreciate your cooperation in this regard.

References

References cited here are listed below. A complete list of articles that have used homodimerizers can be found in the [Regulation Kit Bibliography](#).

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Appendix

pC₄M-F₂E Annotated Sequence

MluI <--
1 acgcgttcgcagctcgcgccgttacataacttacggtaaatggccgcctggctgaccgcccaacgacccccgccattgacgtcaataatgacgtatggt 100
101 ccctagtaaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaaactgccacttggcagtacatcaagtgtatcatatgccaa 200
201 gtacgccccctattgacgtcaatgacggtaaatggccgcctggcattatgccagtacatgaccttatgggactttcctacttggcagtacatctcagct 300
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601 atccagcctccgggggatcttgggtggcgtgaaactcccgcacctcttcggccagcgaattcgcgcgct ATG GGG AGT AGC AAG AGC AAG CCT 691
1 M G S S K S K P 8
692 AAG GAC CCC AGC CAG CGC TCT AGA GGC GTC CAA GTC GAA ACC ATT AGT CCC GGC GAT GGC AGA ACA TTT CCT AAA 766
9 K D P S Q R S R G V Q V E T I S P G D G R T F P K 33
767 AGG GGA CAA ACA TGT GTC GTC CAT TAT ACA GGC ATG TTG GAG GAC GGC AAA AAG GTG GAC AGT AGT AGA GAT CGC 841
34 R G Q T C V V H Y T G M L E D G K K V D S S R D R 58
842 AAT AAA CCT TTC AAA TTC ATG TTG GGA AAA CAA GAA GTC ATT AGG GGA TGG GAG GAG GGC GTG GCT CAA ATG TCC 916
59 N K P F K F M L G K Q E V I R G W E E G V A Q M S 83
917 GTC GGC CAA CGC GCT AAG CTC ACC ATC AGC CCC GAC TAC GCA TAC GGC GCT ACC GGA CAT CCC GGA ATT ATT CCC 991
84 V G Q R A K L T I S P D Y A Y G A T G H P I I P 108
992 CCT CAC GCT ACC TTG GTG TTT GAC GTC GAA CTG TTG AAG CTC GAG ACT AGA GGA GTG CAG GTG GAG ACT ATC TCC 1066
109 P H A T L V F D V E L L K L E T R G V Q V E T I S 133
1067 CCA GGA GAC GGG CGC ACC TTC CCC AAG CGC GGC CAG ACC TGC GTG GTG CAC TAC ACC GGG ATG CTT GAA GAT GGA 1141
134 P G D G R T F P K R G Q T C V V H Y T G M L E D G 158
1142 AAG AAA GTT GAT TCC TCC CGG GAC AGA AAC AAG CCC TTT AAG TTT ATG CTA GGC AAG CAG GAG GTG ATC CGA GGC 1216
159 K K V D S S R D R N K P F K F M L G K Q E V I R G 183
1217 TGG GAA GAA GGG GTT GCC CAG ATG AGT GTG GGT CAG AGA GCC AAA CTG ACT ATA TCT CCA GAT TAT GCC TAT GGT 1291
184 W E E G V A Q M S V G Q R A K L T I S P D Y A Y G 208
1292 GCC ACT GGG CAC CCA GGC ATC ATC CCA CCA CAT GCC ACT CTC GTC TTC GAT GTG GAG CTT CTA AAA CTG GAA ACT 1366
209 A T G H P G I I P P H A T L V F D V E L L K L E T 233
1367 AGT TAT CCG TAC GAC GTA CCA GAC TAC GCA TAA gaaaagtgaggaatcctgagaactcaggggtgagtttggggacccttgattgttctt 1455
234 S Y P Y D V P D Y A * 244
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