

RPD™
Regulated Secretion/Aggregation Kit

Version 2.0

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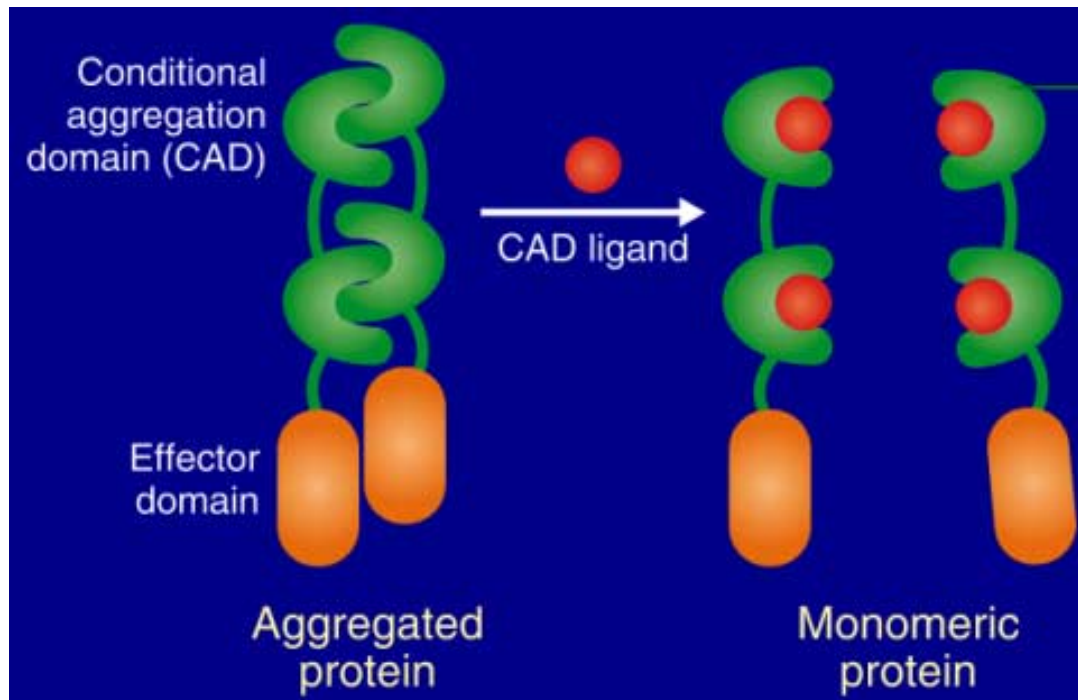
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ARGENT™ Regulated Secretion/Aggregation Kit

The RPD™ Regulated Secretion/Aggregation Kit contains reagents for regulating the properties of proteins by controlling their aggregation state with a small molecule. In contrast to ARGENT, RPD is a “reverse dimerization” system— aggregation is the resting state and the ligand breaks up protein-protein interactions. The Kit can be used to achieve rapid, reversible changes in the subcellular location, aggregation state and/or biological activity of engineered proteins, *in vitro* or *in vivo*. A particular application is the rapid induction of protein secretion following storage of engineered proteins in the endoplasmic reticulum.



[Controlling Protein Activity Using Regulated Aggregation](#)

Overview

Many cellular processes are mediated through protein-protein interactions (1). Examples include the stepwise recruitment and activation of intracellular signaling molecules, and the subsequent activation of gene expression. Methods that allow such processes to be manipulated at will using small molecules are powerful tools for investigating and controlling cellular activities. The use of chemical inducers of dimerization, or “dimerizers”, has proved to be a particularly versatile approach (2). Cells are engineered to express a protein of interest fused to a drug-binding domain; treatment with the bivalent dimerizer crosslinks the proteins and initiates signaling. This approach has been used to control numerous cellular activities and forms the basis of our four [ARGENT Regulation Kits](#).

Recently, we devised a new technology for controlling the activities of proteins that functions instead as a “reverse dimerization” system (3). In this system, called RPD™, the protein of interest is fused to a module called a “conditional aggregation domain” (CAD). A CAD is a multivalent protein module that self-aggregates, through interactions that can be blocked by a small molecule. Expression of this fusion protein in cells will therefore lead to the constitutive formation of aggregates, which can be broken up rapidly and reversibly by addition of the small molecule ligand. Any property of the fusion protein that is dependent on its aggregation

state— such as its subcellular localization or catalytic activity— can therefore be brought under real-time small molecule control.

Because aggregation is the resting state and addition of ligand breaks up the protein-protein interactions, the RPD system represents a complementary technology to ARGENT for controlling protein-protein interactions, and can be used in analogous ways to create inducible alleles. In addition, the ability to create large aggregates (rather than discrete dimers) has unique applications. For example, we found that adding a secretory signal sequence to fusion proteins allows them to be reversibly stored as aggregates in the endoplasmic reticulum of engineered cells. This approach provides a means to induce rapid pulses of protein secretion from cells in response to ligand (4). The RPD Regulated Secretion/Aggregation Kit provides vectors encoding conditional aggregation domains, as well as a specific ligand, for achieving regulated aggregation and/or secretion of any protein of interest.

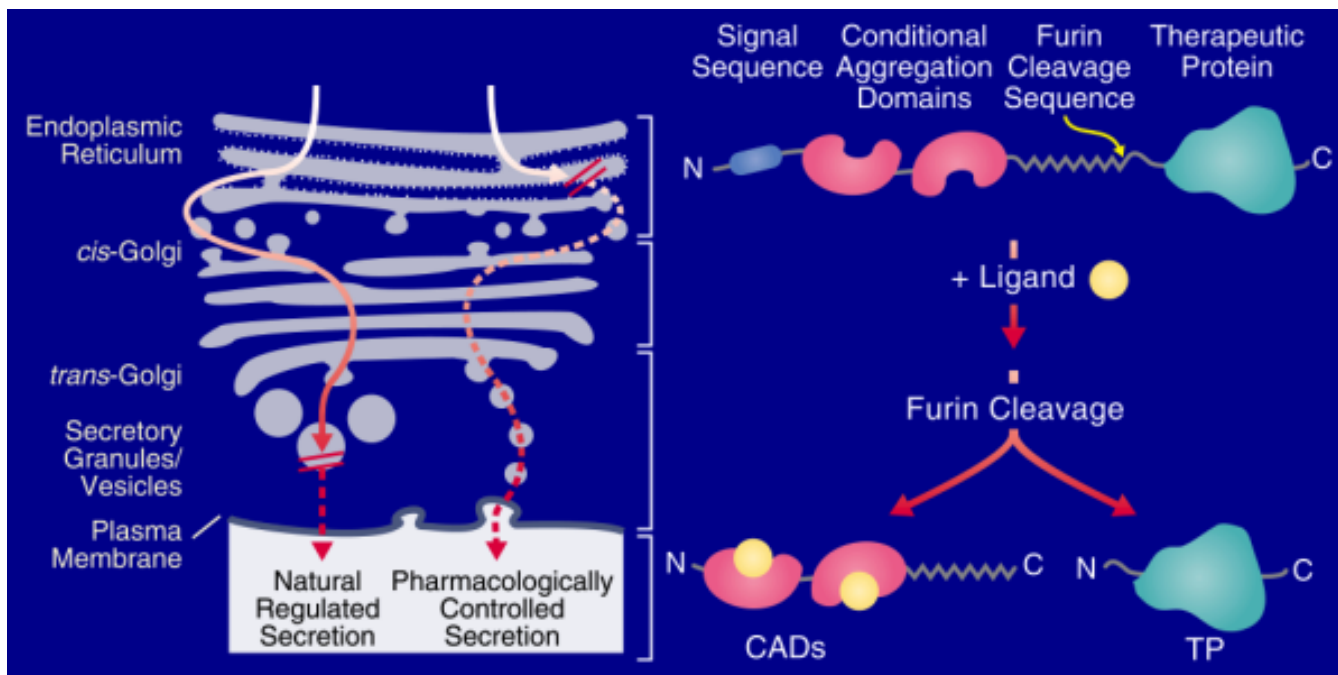
Applications of the RPD Kit

The development of RPD was stimulated by the discovery of an unusual self-dimerizing mutant of the protein FKBP, called F_M (see next section) (3). By constructing GFP fusion proteins, we found that multimerization of this domain results in the formation of large aggregates that can be completely dissociated within minutes by exposure to specific, monovalent F_M ligands. Thus reiterated F_M domains can be used as CADs. The extent of aggregation could be controlled by varying the number of F_M domains, with four typically providing maximal aggregation. In addition, the subcellular localization of the aggregates could be controlled by appending the appropriate targeting signal.

RPD technology is generally useful for regulating the function of proteins that can be activated or inactivated by self-association. In principle, most processes that can be brought under dimerizer control using the ARGENT system can also be controlled in the reverse manner using RPD technology. RPD can therefore be used to turn *off* a process that is *activated* by oligomerization. For example, we have fused F_M domains separately to the DNA-binding and activation domains of a transcription factor, providing an “off-switch” for transcription (in which gene expression is abolished by addition of ligand) (3). Similarly, F_M domains could be used to create inhibitable alleles of signaling proteins that are activated by protein-protein interactions, or vice versa. Although F_M domains homodimerize, it is possible to use the RPD system to regulate heterodimerization between two different fusion proteins (as illustrated by the example of transcription factors). In these applications, each protein is equipped with reiterated F_M domains, and the resulting aggregates contain a mixture of homodimeric and heterodimeric complexes.

Controlling secretion

The second general use of RPD is to turn *on* a process that is *inactivated* by oligomerization. A key example is the regulation of protein secretion through controlled aggregation in the endoplasmic reticulum. The use of RPD to control secretion (see figure below) involves the insertion of F_M domains into a secreted protein of interest, between the signal sequence and the mature protein (4). The resulting fusion proteins localize in the endoplasmic reticulum (ER) as massive aggregates. Addition of a monovalent F_M ligand dissolves the aggregates and allows the protein to be exported through the secretory apparatus. To ensure secretion of the authentic protein, a cleavage site for the specific endo-peptidase furin is interposed between the F_M domains and the protein of interest. Since furin is exclusively expressed in the *trans* Golgi, the fusion protein will be processed as it traverses this compartment, resulting in the secretion of the correctly processed protein (as well as the separate F_M moiety).



Pharmacologic Control of Protein Secretion

We have shown that rapid, transient and tightly-regulated secretion of human growth hormone (hGH) or insulin can be achieved in this manner, with protein secretion initiating within 15 minutes of compound addition and terminating within one hour of compound withdrawal (4). The level of basal secretion could be controlled by varying the number of F_M domains. By implanting engineered cells into mice, we also demonstrated the utility of the RPD system *in vivo*. Brief pulses of protein could be induced in a dose-dependent manner using a RPD ligand, and the system could be used to control insulin secretion and glucose levels in a mouse model of insulin-dependent diabetes.

The ability rapidly to create and destroy large protein aggregates in discrete locations in the secretory pathway has particular utility in protein trafficking research. For example this approach has been used to discover the existence of “mega-vesicles” transporting cargo across the Golgi stack (5). In this study, specific temperature blocks were used in conjunction with the RPD system to localize F_M aggregates to the ER or the *cis* or *trans* Golgi, and to control the movement of aggregates between these compartments.

The ability to rapidly activate or inactivate the activity or secretion of proteins also has many applications in functional genomics research and drug discovery. Analogously to the use of ARGENT, inducible alleles of signaling proteins can provide tools for functional analysis of signaling pathways, and the basis for animal models of associated diseases. Rapid induction of protein secretion offers a more physiologically accurate means to induce protein production than regulated transcription for several important classes of proteins, such as cytokines, for which inducible models of associated diseases are highly valuable. In addition, cell lines in which the activity of a single protein can be rapidly activated or inactivated may be useful in the configuration of targeted cell-based assays for small molecule drugs.

Design of the kit components

The reagents in the RPDTM Regulated Aggregation/Secretion Kit, like those in the ARGENT-based regulation kits, 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. The original ARGENT system used dimers of FK506 (2), or synthetic equivalents (6), to bring together fusion proteins containing wild-type FKBP domains. 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 (7). 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. These components form the basis of the [ARGENT Regulated Homodimerization Kit](#).

In the course of this protein engineering work, we discovered by chance that a different mutation at the Phe36 position, Phe36Met, converts the normally monomeric FKBP protein into a ligand-reversible dimer (3). Two-hybrid, gel filtration, analytical ultracentrifugation and x-ray crystallographic studies showed that the mutant (F_M) forms discrete homodimers that can be completely dissociated within minutes by adding FKBP ligands. Fusion proteins containing reiterated F_M domains formed ligand-reversible aggregates when expressed in cells. We found that monomeric versions of AP1903 and AP20187, modified to enhance their ability to permeate cells, were the most potent ligands for dissociating aggregates (3). The F_M mutant of FKBP, and one of these monomeric ligands (AP21998), form the basis of the reagents provided in the RPD Regulated Aggregation/Secretion Kit.

Notes on the use of this kit

Use of FK506 and rapamycin to induce disaggregation

Both FK506 and rapamycin retain the ability to bind to the F_M mutant of FKBP, albeit with reduced affinity compared to the wild-type protein (3). Therefore these compounds can be used to break up aggregates of F_M -containing fusion proteins as an alternative to the AP21998 provided in the Kit. However, because AP21998 is designed to bind specifically to the F_M mutant, this compound is typically 3-10 fold more potent than the natural product ligands (3). In addition, the synthetic ligand is devoid of the inhibitory effects of the natural ligands on cell growth and signaling, which may compromise some experimental uses.

Can AP21998 be used in vivo?

Our studies have shown that AP21998 cannot easily be formulated and administered to experimental mice at doses suitable for induction of the RPD system. We have identified other, related ligands that are more appropriate for use in mice. Therefore, when you are ready to initiate *in vivo* studies, please visit our website to request a larger aliquot of a suitable compound.

Kit contents

To control the aggregation state of a protein, and thereby control its secretion or activity, the protein of interest is fused to 2 or more F_M domains.

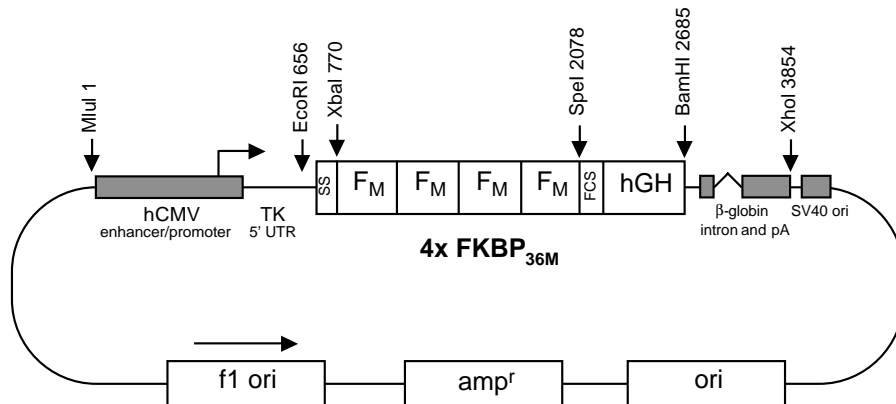
The RPD™ Regulated Secretion/Aggregation Kit contains 3 plasmids, pC₄S₁-F_M4-FCS-hGH, pC₄EN-F_M3, and pC₄-F_M2E, and an aliquot of the F_M ligand AP21998. As described below, the three plasmids provide an assortment of modular components— F_M domains with different numbers of repeats, an epitope tag, localization sequences, and a furin cleavage site. These modules can be easily manipulated and exchanged to generate the fusion protein whose aggregation state you wish to control.

Expression plasmids

pC₄S₁-F_M4-FCS-hGH

Description

pC₄S₁-F_M4-FCS-hGH (7002 bp)



Not drawn to scale

- In pC₄S₁-F_M4-FCS-hGH a chimeric fusion protein containing a signal sequence (S₁; amino acids -26 to -1 of human growth hormone), 4 tandem repeats of F_M (F_M4), a furin cleavage signal (FCS; from human stromelysin-3 [SARNRQKR]) and the entire mature coding sequence for human growth hormone (hGH) is expressed under control of the human CMV enhancer/promoter (C).
- The principal application of this vector is to control the secretion of hGH, as described (4), or of any other protein of interest. hGH can be easily substituted with another protein by replacing the FCS-hGH portion of the vector (SpeI-BamHI fragment) as described below.
- In some instances it may be desirable to have less efficient retention of the fusion protein in the ER to increase the rate of secretion in the absence of ligand. This can be achieved by constructing a fusion protein that contains fewer than 4 F_M domains (see figure 4 in reference 4). To construct such proteins, the XbaI-SpeI fragment of pC₄S₁-F_M4-FCS-hGH is replaced with an XbaI-SpeI fragment from pC₄EN-F_M3 or pC₄-F_M2E to create a fusion protein that contains 3 or 2 F_M domains, respectively.
- This vector may also be used as a source of a fragment encoding 4 F_M domains, which can be subcloned into the desired vector as an XbaI-SpeI fragment.

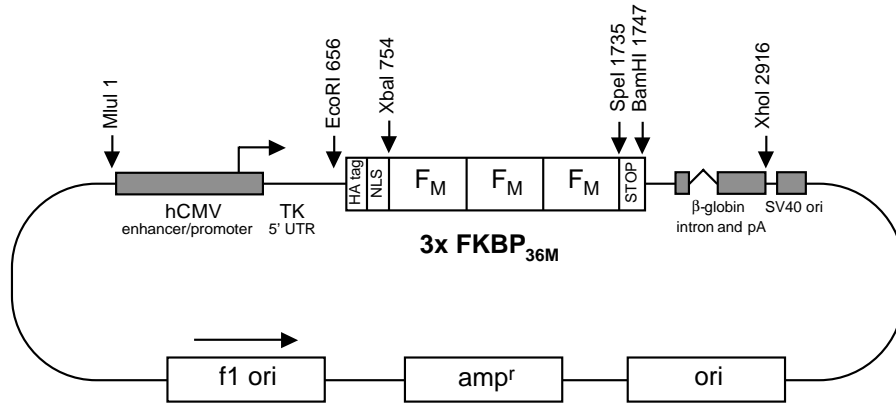
[Annotated Sequence](#)

[Raw sequence](#)

pC₄EN-F_M3

Description

pC₄EN-F_M3 (6064 bp)



Not drawn to scale

- In pC₄EN-F_M3 a chimeric fusion protein containing an amino-terminal epitope tag (E, from the influenza hemagglutinin [HA] gene), a nuclear localization sequence (N; from the SV40 large T antigen) and 3 tandem repeats of F_M (F_M3) is expressed under control of the human CMV enhancer/promoter (C).
- To make a protein fusion that contains 3 F_M domains that will form aggregates in the nucleus, the gene for the protein of interest should be cloned into either the XbaI or SpeI sites as described below.
- The XbaI-SpeI fragment of pC₄EN-F_M3 can be replaced with the XbaI-SpeI fragment from pC₄-F_M2E or pC₄S₁-F_M4-FCS-hGH to create fusion proteins that contain 2 or 4 F_M domains, respectively.
- This vector may also be used as a source of a fragment encoding 3 F_M domains, which can be subcloned into the desired vector as an XbaI-SpeI fragment.

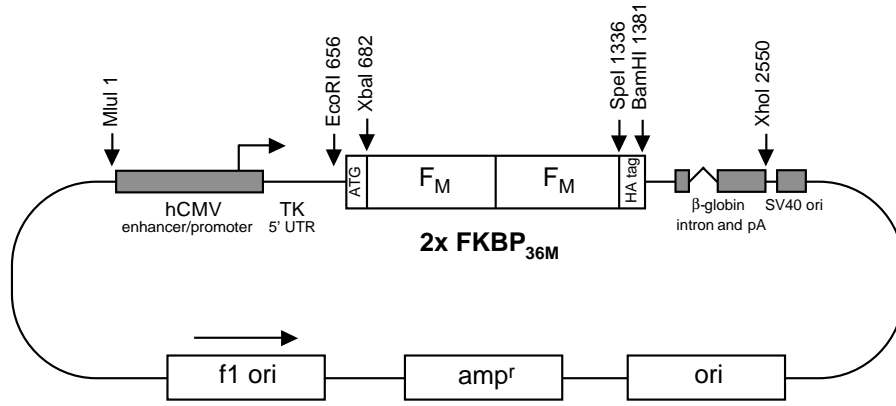
[Annotated Sequence](#)

[Raw sequence](#)

pC₄-F_M2E

Description

pC₄-F_M2E (5698 bp)



Not drawn to scale

- In pC₄-F_M2E a chimeric fusion protein containing 2 tandem repeats of F_M (F_M2) and a carboxy-terminal epitope tag (E, from the influenza hemagglutinin [HA] gene) is expressed under control of the human CMV enhancer/promoter (C). This vector contains no targeting sequence (the amino terminus of the protein consists only of a methionine), so that the default localization of the fusion protein will be to the cytoplasm.
- To make a protein fusion that contains 2 F_M domains, the gene for the protein of interest should be cloned into either the XbaI or SpeI sites as described below.
- This vector may also be used as a source of a fragment encoding 2 F_M domains, which can be subcloned into the desired vector as an XbaI-SpeI fragment.
- Also, an XbaI-SpeI or XbaI-BamHI fragment assembled in one of the other 2 vectors in this kit can be inserted into this vector to target fusion proteins containing 3 or 4 F_M domains to the cytoplasm.

[Annotated Sequence](#)

[Raw sequence](#)

General vector information

Controlling secretion of a protein other than hGH

To control the secretion of a protein other than hGH, replace the SpeI-BamHI fragment of pC₄S₁-F_M4-FCS-hGH with an SpeI-BamHI fragment that contains a furin cleavage sequence followed by the mature coding sequence of the protein of interest. This is most easily accomplished by designing two PCR primers to amplify the gene for the protein of interest to append a SpeI site and the coding sequence for a FCS at the 5' end, and a BamHI site after the stop codon at the 3' end. For example, to incorporate hGH into this vector the following upstream primer was used:

5'- act agt gct aga aac cgt cag aag aga ttc cca acc att ccc tta agc agg -3' where

- act agt: encodes the SpeI site,
- agt gct aga aac cgt cag aag aga: encodes the FCS (the first 3 nucleotides overlap with the SpeI site) and

- ttc cca acc att ccc tta agc agg: encodes the first 8 amino acids of mature hGH with the first amino acid (ttc=Phe) being the one left after natural removal of the hGH signal sequence.
- Replace the portion of the primer underlined above with the corresponding portion for your protein of interest.

The downstream primer used was:

5' gga tcc cggg cta gaa gcc aca gct gcc -3' where

- gga tcc: encodes the BamHI site and
- cggg cta gaa gcc aca gct gcc (= ggc agc tgt ggc ttc tag cccg on the coding strand): encodes the last 5 amino acids of hGH, its stop codon and 4 nucleotides from the 3' UTR.
- Replace the portion of the primer underlined above with the corresponding portion for your protein of interest.

Making F_M protein fusions

To fuse a protein of interest directly to F_M domains to control its aggregation state, the coding sequence of interest should be amplified 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 upstream of 3 F_M domains, clone the XbaI-SpeI fragment into the XbaI site of pC₄EN-F_M3 (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 downstream of 2 F_M domains, insert it into the SpeI site of pC₄-F_M2E. 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.

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 all three expression vectors includes an in-frame stop codon. Therefore stop codons should not be included in the fused sequences.

Finally, XbaI-SpeI or XbaI-BamHI fragments can be cloned into either the pC₄S₁-, pC₄EN- or pC₄- vector backbones to create fusion proteins targeted to the endoplasmic reticulum, nucleus or cytoplasm, respectively.

Incorporating EGFP into fusion proteins

Enhanced green fluorescent protein (EGFP; 8) must be obtained from commercial sources (i.e. Clontech) due to licensing restrictions. To make protein fusions containing EGFP follow the scheme described above in which EGFP coding sequence is amplified with an upstream XbaI site and a downstream SpeI site. For example, we used the following primers to amplify an XbaI-SpeI fragment containing EGFP (amino acids 2-239) for insertion into the XbaI site of pC₄S₁-F_M4-FCS-hGH:

5'- gg TCT AGA gtg agc aag ggc gag gag ctg -3'

5'- gg ACT AGT ctt gta cag ctc gtc cat gcc -3'

Addition of a selectable marker

Stable integration of plasmid vectors into cells is greatly facilitated by co-expression of the gene for the fusion protein of interest and the selectable marker gene on the same mRNA transcript. Such bicistronic mRNAs can be created by expressing the coding region of the fusion protein downstream of an enhancer/promoter and upstream of an internal ribosome entry sequence (IRES) which drives expression of a selectable marker

gene. Using the “pIRES-” vectors available from Clontech (e.g. pIRESneo2, pIREShyg2, pRESpuro2, pRESbleo), two alternative approaches can be used to generate such a vector.

- The BamHI-XhoI fragment from one of the vectors in this Kit can be replaced by a BamHI-XhoI fragment from a pIRES vector. This replaces the intron and polyA sequence from the vector in the Kit with an intron, an IRES element, the selectable marker gene and a polyA sequence.
- Alternatively, an EcoRI-BamHI fragment containing the protein fusion of interest can be cloned into the MCS of a pIRES vector.

The advantage of using this approach to introduce a selectable marker 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 signal sequence or simply an ATG)
 - *XbaI-SpeI*: ligand binding domains
 - *SpeI-BamHI*: Carboxy-terminal sequence. In addition to the stop codon may contain an epitope tag. In vectors designed for protein secretion, contains a furin cleavage sequence and the secreted protein of interest.
- 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.

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 pC₄S₁ 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).

Antibodies to detect fusion proteins

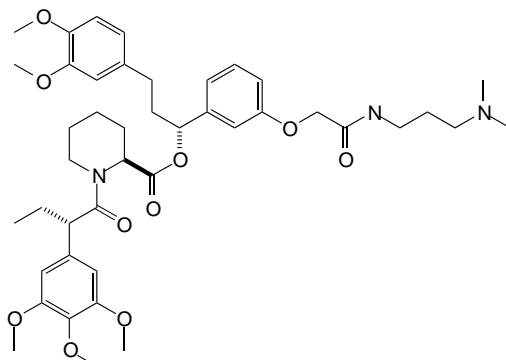
Anti-HA (Babco #MMS101R-500) and anti-FKBP12 (Affinity Bioreagents #PA1-026) antibodies are available commercially. The anti-FKBP12 antiserum recognizes F_M as efficiently as it does the wild-type protein. Each F_M domain is ~12 kDa.

hGH assays

Several kits are available for the quantitation of hGH levels, including an RIA from Nichols Diagnostic (# 40-2155) and an ELISA from Roche (# 1 585 878).

AP21998

Description



AP21998

AP21998 (3) is a monovalent synthetic compound that is designed to bind with high affinity and specificity to F_M , and other FKBP mutants with “hole” mutations at position 36. AP21998 binds to a single F_M domain with a K_d of approximately 1 nM, and about 1000-fold less tightly to the wild-type FKBP protein. The compound is a monovalent derivative of the synthetic dimerizers AP1903 and AP20187 (7), modified to enhance cellular permeancy.

AP21998 can be used to induce disaggregation of F_M -containing fusion proteins *in vitro* or in cultured mammalian cells. AP21998 has no immunosuppressive activity and is non-toxic to cells. To date, AP21998 has only been tested on mammalian cells. We do not yet know whether it functions in yeast or any other model organisms.

Reconstituting AP21998

AP21998 (molecular mass 778 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 500 μ g AP21998 in 643 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 AP21998

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 AP21998 *in vitro*

Working concentrations of ligand 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 μ M, 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.

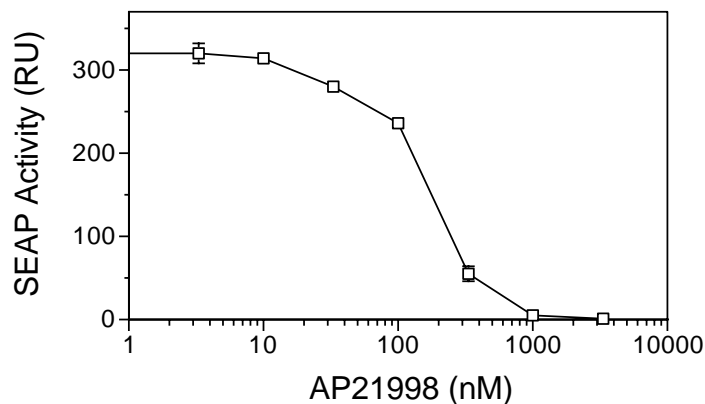
Use of AP21998 in animals

Our studies have shown that AP21998 cannot easily be formulated and administered to experimental mice at doses suitable for induction of the RPD system. We have identified other, related ligands that are more appropriate for use in mice, including AP22542 (4). Therefore, when you are ready to initiate *in vivo* studies, please visit our website to request a larger aliquot of a suitable compound.

Expected results

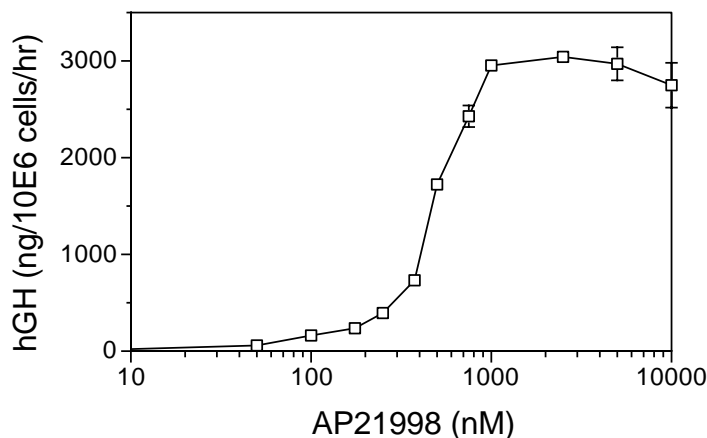
Turning off a process that is activated by oligomerization — regulated transcription

An example of the use of this Kit to turn off a process that is *activated* by oligomerization — activation of transcription — is shown below. Vectors expressing fusion proteins containing three F_M domains fused to a DNA binding domain and three F_M domains fused to a transcriptional activation domain were transiently transfected into cells containing a target gene (secreted alkaline phosphatase (SEAP)) with upstream binding sites for the DNA binding domain (3). Interactions between the F_M domains in the absence of ligand result in constitutive activation of SEAP expression. By disrupting this interaction, AP21998 turns gene expression off in a dose-dependent manner.



Turning on a process that is inactivated by oligomerization — regulated secretion

An example of the use of this Kit to turn on a process that is *inactivated* by oligomerization is shown in the figure below. In this case, the process that is being regulated is the secretion of a human growth hormone (hGH) fusion protein introduced into cells using the vector $pC_4S_1-F_M4-FCS-hGH$. In the absence of ligand, the F_M4-hGH fusion protein forms large aggregates and is retained in the endoplasmic reticulum with little or no secreted protein detected. Upon addition of AP21998, protein disaggregation occurs rapidly, leading to the detection of secreted protein within 20 minutes (4). Peak levels of disaggregation and secretion occur in response to 1-2 μM AP21998 as shown in the figure. Disaggregation of fusion proteins containing fewer than 4 F_M domains occurs at somewhat lower doses of AP21998.



In HT1080 fibrosarcoma cells, hGH fusion protein stored in the endoplasmic reticulum in the absence of ligand is fully depleted within 2 hours (4). Note that we have found that the extent of depletion may be lower for other fusion proteins in other cell types.

In our studies to date, we have seen no evidence for the toxicity of proteins stored as aggregates in the endoplasmic reticulum. Cells stably expressing stored aggregates have been passaged for greater than 1 year with no changes in morphology, protein retention, or protein release in response to ligand. In addition, no activation of the ER stress sensing kinase PERK has been observed (4).

Finally, we have recently found that long-term regulated secretion of hGH can be achieved in mice upon delivery of the F_M4-hGH gene into skeletal muscle using AAV vectors (data not shown).

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 reagents provided in this kit can be found in the [Regulation Kits Bibliography](#).

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Appendix


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--> BamHI <--
2648 TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC TAG cccggatcctgagaacttcagggtagtttggggacccttgattgttcttcttt 2736
653 C R S V E G S C G F * 663

2737 ttcgctattgtaaaattcatgttatatggagggggcaagtttccaggggtgtgtttagaatgggaagatgtcccttgatccaccatggaccctcatgat 2836
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rabbit β-globin intron
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and 3' UTR
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Poly A signal
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XhoI
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pC₄EN-F_M3 Annotated Sequence

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MluI <--
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201 gtacgccccctattgacgtcaatgacggtaaatggccccgctggcattatgccagtcacatgaccttatgggactttcctacttggcagtcacatctacgt 300
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401 gacgtcaatgggagttgtttttggcaccacaaatcaacgggactttccaaaatgctgtaacaaactccgccccattgacgcaaatggcggttagcggtgac 500
501 ggtgggaggtctatataagcagagctcggttagtgaaccgctcagatcgctggagacgccatccacgctgttttgacctccatagaagacaccgggaccg 600
601 atccagcctccgggggactcttgggtggcgtgaaactcccgcacctcttcggccagcgaattccagaagcgcgt  ATG GCT TCT AGC TAT CCT TAT 693
1  --> <-- HSV TK 5' UTR --> EcoRI <--
1  HA epitope tag --> <-- SV40 NLS --> XbaI <--
694 GAC GTG CCT GAC TAT GCC AGC CTG GGA GGA CCT TCT AGT CCT AAG AAG AAG AGA AAG GTG TCT AGA GGA GTG CAG 768
8  D V P D Y A S L G G P S S P K K K R K V S R G V Q 32
769 GTG GAA ACC ATC TCC CCG GGA GAC GGG CGC ACC TTC CCC AAG CGC GGC CAG ACC TGC GTG GTG CAC TAC ACC GGG 843
33 V E T I S P G D G R T F P K R Q T C V V H Y T G 57
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58 M L E D G K K M D S S R D R N K P F K F M L G K Q 82
919 GAG GTG ATC CGA GGC TGG GAA GAA GGG GTT GCC CAG ATG AGT GTG GGT CAG AGA GCC AAA CTG ACT ATA TCT CCA 993
83 E V I R G W E E G V A Q M S V G Q R A K L T I S P 107
994 GAT TAT GCC TAT GGT GCC ACT GGG CAC CCA GGC ATC ATC CCA CCA CAT GCC ACT CTC GTC TTC GAT GTG GAG CTT 1068
108 D Y A Y G A T G H P G I I P P H A T L V F D V E L 132
1069 CTA AAA CTG GAA GTC GAG GGC GTG CAG GTG GAA ACC ATC TCC CCA GGA GAC GGG CGC ACC TTC CCC AAG CGC GGC 1143
133 L K L E V E G V Q V E T I S P G D G R T F P K R G 157
1144 CAG ACC TGC GTG GTG CAC TAC ACC GGG ATG CTT GAA GAT GGA AAG AAA ATG GAT TCC TCC CGG GAC AGA AAC AAG 1218
158 Q T C V V H Y T G M L E D G K K M D S S R D R N K 182
1219 CCC TTT AAG TTT ATG CTA GGC AAG CAG GAG GTG ATC CGA GGC TGG GAA GAA GGG GTT GCC CAG ATG AGT GTG GGT 1293
183 P F K F M L G K R G E V A Q M S V A Q 207
1294 CAG AGA GCC AAA CTG ACT ATA TCT CCA GAT TAT GCC TAT GGT GCC ACT GGG CAC CCA GGC ATC ATC CCA CCA CAT 1368
208 Q R A K L T I S P D Y A Y G A T G H P G I I P P H 232
1369 GCC ACT CTC GTC TTC GAT GTG GAG CTT CTA AAA CTG GAA ACT AGA GGA GTG CAG GTG GAA ACC ATC TCC CCA GGA 1443
233 A T L V F D V E L L K L E T R G V Q V E T I S P G 257
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258 D G R T F M L G K R G E V T G M L E D G A K K 282
1519 ATG GAT TCC TCC CGG GAC AGA AAC AAG CCC TTT AAG TTT ATG CTA GGC AAG CAG GAG GTG ATC CGA GGC TGG GAA 1593
283 M D S S R D R N K P F K F M L G K Q E V I R G W E 307
1594 GAA GGG GTT GCC CAG ATG AGT GTG GGT CAG AGA GCC AAA CTG ACT ATA TCT CCA GAT TAT GCC TAT GGT GCC ACT 1668
308 E G V A Q M S V G Q R A K L T I S P D Y A Y G A T 332
1669 GGG CAC CCA GGC ATC ATC CCA CCA CAT GCC ACT CTC GTC TTC GAT GTG GAG CTT CTA AAA CTG GAA ACT AGT TAT 1743
333 H P G I I P P H A T L V F D V E L L K L E T S Y 355
1744 TAA ggatcctgagaacttcagggtgagtttggggacccttgattgttctctcttttttcgctattgtaaaatcatgttatatggagggggcaagtttt 1842
356 * <--
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