Frequently Asked Questions about the Destabilizing Domains (DDs)

1) How do I propagate the plasmids?
All plasmids are pBMN retroviral (MMLV-derived) plasmids containing the ampicillin resistance gene. We typically propagate and clone in TOP10 cells at 30 ˚C to prevent recombination, which we have seen when cloning at 37 ˚C. You should be able to propagate these plasmids in any garden-variety *E. coli* "cloning strain" such as DH5α, but be aware that recombination can be a problem. These plasmids can be used to make both ecotropic and amphotropic virus, depending on the packaging cell line or helper plasmid used to make virus.

2) What sites are available for cloning?
The full sequences of the plasmids that we distribute are available as PDF files on our website at: http://wandless.stanford.edu/Plasmidseq.htm. You will likely need to design your own constructs to fit with the experiments that you are planning.

3) Can you recommend a good anti-FKBP12 antibody?
Yes, at least we can recommend two not-so-bad antibodies. Affinity Bioreagents (Golden, Colorado, bioreagents.com, 800-527-4535) catalog PA1-026A. Product info claims that this antibody is good for IHC, IP and Western. It's a rabbit polyclonal against a 13-residue N-terminal FKBP epitope. More recently we have used a monoclonal from BD Pharmingen (http://www.bdbiosciences.com/) catalog 610808. It has also been pretty good for Westerns of the L106P DD.

4) Can you recommend a good anti-ecDHFR antibody?
Nope – we don't know of one. Remember to include an epitope tag in your construct.

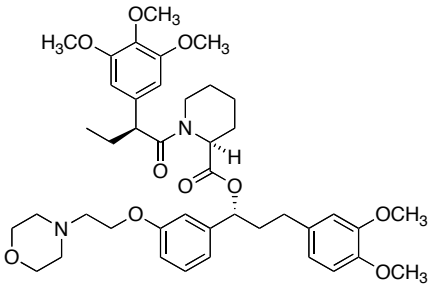
5) Where can I get Shield-1 and/or related plasmids?
We have shipped more than 500 samples to over 300 different labs. As you might imagine, this has taken quite a bit of effort. There are two commerical suppliers of the DDs as of December 2008. Clontech sells DD-related reagents under the "ProteoTuner" name. They offer both the FKBP-derived and the ecDHFR-derived DDs. A contract synthesis company called Cheminpharma (New Haven, CT) also sells Shield-1.

6) How do I formulate Shield-1 (Shld1)?
The molecular weight of Shield-1 is 748.91 ($C_{42}H_{56}N_2O_{10}$). We typically make a 1 mM stock in absolute (200 proof) ethanol, which we store at –20 ˚C. This stock is good for at least 6 months, probably longer. We use this as a 1000× stock, which we dilute directly into cell culture media for each experiment, giving a final concentration of 1 μM Shld1 (or whatever is required). You may be able to use less Shld1, based on the functional threshold of your protein of interest, which can be determined experimentally.

7) Can I just make a dilution of Shield-1 into media and store at 4 ˚C?
We don't know. We've never tried this before, and we're not sure how stable the ligand will be for extended periods of time in aqueous solution. If you decide to give it a try, please let us know how it works.

8) How do I formulate Trimethoprim (TMP)?
We typically make a 10 mM stock in DMSO and use this as a 1000× stock.

9) Help!!! My vial of Shield-1 is empty – the FedEx guy must have taken it!
Relax. Shield-1 is a clear, colorless oil. Go back to step (4) to make a stock and then test it using one of the plasmids encoding DD-YFP. You should see Shld1-dependent YFP by immunofluorescence, Western blot, or flow cytometry.



Shield-1

10) How soluble is Shield-1 in aqueous solutions?
Don't expect this compound to be very soluble in water. The highest concentration we've worked with in aqueous solution is 250 μM (1% EtOH). The solution looked a bit cloudy, but we still observed Shld1-depedent activity for our protein of interest. Other than that, the highest concentration we've used in a cell-based experiment at final dilution is 3 μM, with a 0.3% final EtOH concentration (a 1:300 dilution of a 1 mM stock). Keep in mind that the presence of EtOH may affect the outcome of your experiment, so plan your controls accordingly.

11) Do the DD systems work in mice?
We have done some experiments demonstrating that Shield-1 is active in mice when delivered intravenously or i.p. See Banaszynski et al. *Nature Medicine* (2008) **14**, 1123-1127. TMP crosses the blood-brain barrier in rodents as demonstrated in the 2010 *Chem. & Biol.* paper.

12) Can I give the plasmids and Shield-1 to my family, friends, casual acquaintances?
Go crazy – we're easy! Share these reagents with anyone you care to. We do not require MTAs for academic labs. We will send the Shield-1 stabilizing ligand to anyone who requests it as long as they pay for shipping.

13) Does this DD system work in other cell types or organisms?
Yes. Two papers in the December 2007 issue of *Nature Methods* demonstrated that the FKBP-derived DD system regulates protein stability in the apicomplexan parasites, *Toxoplasma gondii* and *Plasmodium falciparum*. We have received anecdotal evidence from other users that the system works in zebrafish, *Xenopus*, *Ostreococcus*, and *C. elegans*, but no papers have been published in these organisms to our knowledge. Yeast is still a bit tricky, but we are working on a DD system in yeast. You should be able to find a list of publications that have used one or more DD systems at: http://wandless.stanford.edu/links.html

14) What is the best way to do "washout" experiments?
You might have to experiment a bit to find the best way for your system. The best protocol for doing a washout in 10-cm dishes might be a bit different for doing a washout from cells on coverslips. However, we can say with confidence that using cell culture media doped with a stable form of the DD-protein (i.e., FKBP-F36V or ecDHFR) is very important. When you've got ligand stabilizing a DD-fusion in cells, you want to give that ligand an attractive alternative to the DD that it's stabilizing. Putting 1-5 μM of unliganded protein outside of the cell works very well for us.

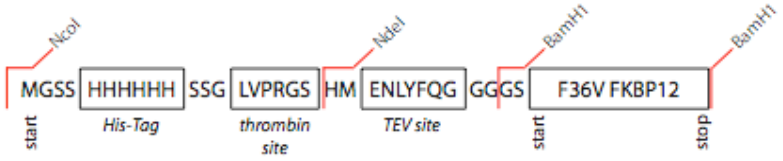
FAQ sheet last updated: 3/3/11

Preparing F36V for Conditioned Media

Use the following protocol to purify His_6-TEV-GGGS-F36V-FKBP12 for use in conditioned media. Protein yield after purification and dialysis is ~70 mg/L culture; enough for over 850 mL conditioned media when used at 5 μM.

A. Expression Plasmid: pET 15b His_6-TEV-GGGS-F36V [DAY 1]

F36V FKBP12 is cloned into a pET 15b expression vector with a hexa-histidine purification tag. There are thrombin and TEV protease recognition sequences between the tag and the FKBP domain if it becomes necessary to remove the His_6-tag, though we typically leave the His_6 tag intact. The full construct description is as follows:



full length protein = 15307 Da

Transform plasmid into BL21(DE3) bacteria; plasmid has ampicillin resistance. Grow @ 37 °C.

B. Protein Expression [DAY 2-3]

1. Inoculate 1 colony into LB-amp overnight culture. Grow @ 37 °C, 8-12 h.
2. Inoculate LB-amp growth culture with 1/50 dilution from overnight (i.e. 20 mL from overnight into 1 L growth culture.)
3. Grow @ 37 °C to OD_{600} = 0.5-0.8 (log phase).
    - BL21(DE3) double in ~20 min in LB.
4. Induce protein expression with 2 mM IPTG. Grow @ 37 °C for 3-5 h.
    - Take 1 mL sample at beginning and end of induction for gel analysis. Pellet bacteria sample in 1.5 mL tubes @ 4000 rpm, 10 min. Pour off supernatant and freeze at -20 °C.
5. Harvest bacteria in centrifuge bottles @ 5000 rpm, 4 °C, 20 min. Freeze pellet @ -80 °C.
    - If desired, can use multiple spins to concentrate pellet into fewer bottles.

C. Protein Purification (taken from Qiagen handbook) [DAY 4]

Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0
Wash Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0
Elution Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8.0

1. While thawing pellets on ice, add between 1/30 to 1/50 culture volume of lysis buffer + [1 mg/mL] lysozyme + 1 mM PMSF (ex. for 1L culture: 30 mL buffer + 0.030 g lysozyme + 0.3 mL PMSF [100mM stock]).
    - If 1/50 volume is difficult to resuspend the bacteria in, increase the lysis volume a little to allow better resuspension and sonication.
2. Resuspend bacteria and transfer to an Oakridge tube (can combine pellets if multiple bottles used in previous step). Incubate on ice, 30 min.
3. Sonicate bacteria on ice until sound changes, usually 2x 2 min(10s/10s pulse on/off; 50% amplitude), with a rest between rounds.
4. Add 20% TritonX-100 to final concentration of 1%. Incubate on ice, 30 min.

5. Pellet debris in Oakridge tube @19,000 rpm (50K g), 4 °C, 1 h.
6. Pour supernatant into a clean tube → SOLUBLE fraction
    - reserve 50 μL for gel analysis, store @ -20 °C.

D. Purification with NiNTA resin [ALSO DAY 4]

We use gravity-flow Poly-Prep columns from Bio-Rad, but this can also be done in batch format in Falcon tubes with a centrifuge instead.

1. Pipette 50% NiNTA slurry into a column and let empty by gravity flow. Use 2 columns with 2 mL 50% slurry each for 1 L culture.
    - volume of 50% slurry = "column volume" **(CV)**
    - volume of resin = "bed volume" **(BV)**; 1 BV = 1/2 CV
2. Equilibrate resin with 4 CV lysis buffer. Allow column to empty by gravity.
3. Cap end of column and add soluble protein fraction to the resin. Cap top.
4. Incubate @ 4 °C, 1h with constant mixing.
5. Remove caps and let unbound fraction flow through.
    - reserve 50 μL for gel analysis, store @ -20 °C.
6. Wash column 3 times with 4 CV wash buffer each time.
    - reserve 50 μL for gel analysis, each wash, store @ -20 °C.
7. Elute protein 3-5 times with 1 BV elution buffer each time.
    - reserve 50 μL for gel analysis, each elution, store @ -20 °C.
8. Combine elution fractions and dilute with an equal volume of wash buffer {to deter concentration-dependent protein precipitation}. Store @ 4 °C.

E. Analyze Protein [DAY 5-6]

1. Assay protein purity by SDS-PAGE, using samples collected over purification.
2. Dialyze protein into PBS + 4% glycerol. {The glycerol is not necessary, but seems to prevent some protein precipitation during dialysis. Can be omitted if cells do not tolerate glycerol.}
    - If protein precipitates during dialysis, centrifuge @ 3000 rpm, 4 °C, 5 min to pellet debris. Transfer the supernatent to a clean tube.
3. Read protein concentration by absorbance at 280 nm.
    - $\varepsilon \sim 9860\ M^{-1}cm^{-1}$
    - MW = 15307 Da
4. Dilute protein to 500 μM (100X stock for conditioned media). Aliquot into small fractions and flash freeze in liquid nitrogen. Store at -80 °C. These stocks aren't sterile, but usually conditioned media is an acute treatment before analysis, so contamination is not a large concern.

- Yield to this step: 68 mg H-T-G-F36V / 1 L culture.

F. Conditioned Media

Aspirate Shield^{+} media from cells and add back media "conditioned" with 5 μM F36V-FKBP12. Incubate cells @ 37 °C, 10 min. Cells can either be left in conditioned media, or replaced with regular media. We have not found intermediate washing steps to be necessary, but you may need to increase the concentration of F36V or add media washes if using more than 1 μM Shield. We do not currently have data about how long F36V is stable in conditioned media.