

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 DH5 α , but be aware that recombination can be a problem. These plasmids can be used to make both ecotropic and amphitropic 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 commercial 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 (C₄₂H₅₆N₂O₁₀). 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 \times stock, which we dilute directly into cell culture media for each experiment, giving a final concentration of 1 μ M 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)?

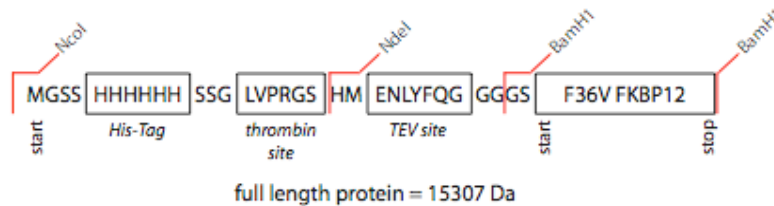
We typically make a 10 mM stock in DMSO and use this as a 1000 \times stock.

Preparing F36V for Conditioned Media

Use the following protocol to purify His₆-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 μ M.

A. Expression Plasmid: pET 15b His₆-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 His₆-tag, though we typically leave the His₆ tag intact. The full construct description is as follows:



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 OD₆₀₀ = 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 NaH₂PO₄, 300 mM NaCl, 10 mM imidazole; pH 8.0

Wash Buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole; pH 8.0

Elution Buffer: 50 mM NaH₂PO₄, 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 supernatant to a clean tube.
 3. Read protein concentration by absorbance at 280 nm.
 - $\epsilon \sim 9860 \text{ M}^{-1}\text{cm}^{-1}$
 - 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.