USP5 Zf-UBD Expression & Purification

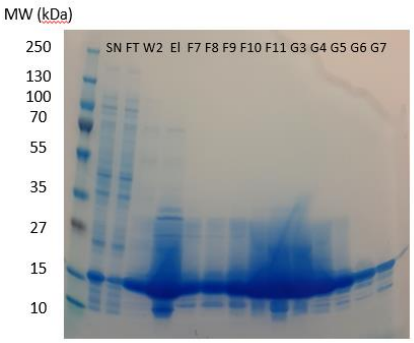
1. Growth

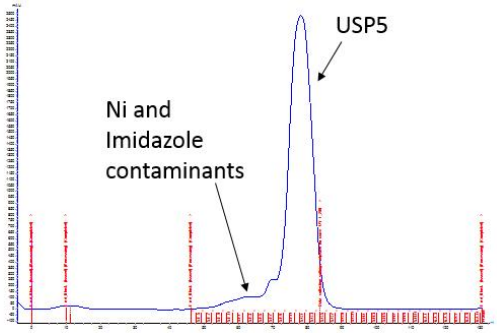
6x1 L M9 minimal media culture in BL21 Codon Plus RIL grown at 37˚C in shaker, induced at OD_{600} ~0.6 with 0.5 mM IPTG. Cultures grown overnight at 15˚C.

Construct ID	Vector	Cloned AA Sequence	MW	pI
T0C011-B06	p28BIOH-LIC	MSGLNDIFEAQKIEWHEGSAGGSG GEVRQVSKHAFSLKQLDNPARIPPCGWKCSK CDMRENLWLNLTDGSILCGRRYFDGSGGNN HAVEHYRETGYPLAVKLGTITPDGADVYSYDE DDMVLDPSLAEHLSHFGIDMLKMQKTD GGSGHHHHHH	17043.91	5.77

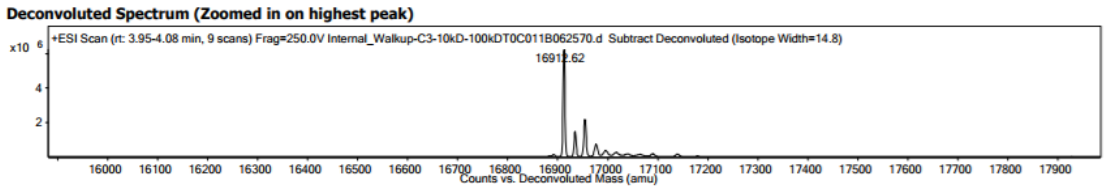
2. Purification

Cells were harvested by centrifugation. Cells were re-suspended in 400 mL of resuspension buffer 50 mM Tris pH 8, 150 mM NaCl, 2 mM TCEP supplemented with benzonase and 1x protease inhibitors and lysed by sonication. Clarified lysate (supernatant-SN) rocked with 5 mL Ni-NTA resin for 1 hour at 4˚C (flow through-FT). Beads washed with 100 mL resuspension buffer, then 200 mL resuspension buffer + 15 mM imidazole (Wash 2-W2) before elution with 2x15 mL resuspension buffer + 300 mM imidazole (elution-E1). E1 of USP5 concentrated to 5 mL and run on S75 16/60. Fractions from Ni-NTA column and S75 16/60 analyzed with SDS-PAGE.





Fractions were concentrated to 16.8 mg/mL and aliquoted (50 µL x 76), frozen in liquid N2 and stored at -80˚C. The final yield was 63.8 mg. Mass spectrometry revealed a non-biotinylated protein with a Met residue cleaved. The purified protein was not biotinylated in vivo, as B21 cells were used. Will be purifying construct in BirA cells in the future.



USP5 Zf-UBD Fluorescence Polarization (FP) Displacement Assay Development

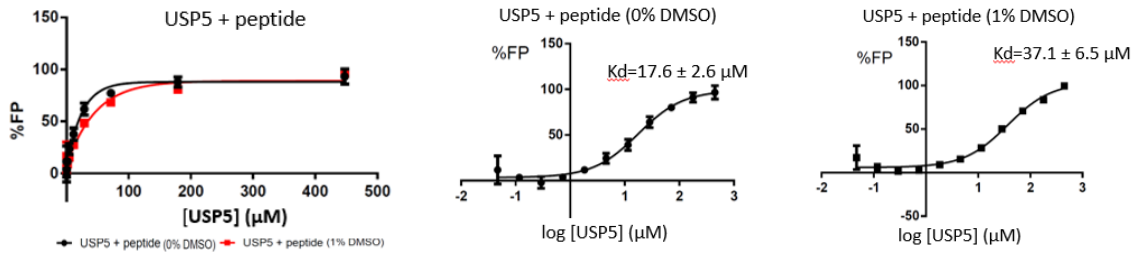
1. Buffer Screen

The buffer screen was performed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using BioTek Synergy 4. The excitation and emission wavelengths were 485 nm and 528 nm respectively. FP was measured for 50 nM FITC-RLRGG peptide incubated ± 75 µM USP5 Zf-UBD (SGC ID T0C011-B06) at the conditions reported in the table below (N=2). Following a 1 minute centrifugation step at 800 rpm, FP was measured. FP was calculated by subtracting the peptide-only FP from the protein+peptide FP measurement.

Buffer (50 mM)	pH	[NaCl]=50 mM	[NaCl]=100 mM	[NaCl]=150 mM
Tri-Na Citrate	6.5	23.5	34.5	36.5
Na-acetate	5.6	24.5	0	1.5
Glycine	9.0	44.5	34	66
Na-cacodylate	6.8	0	7	16.5
Hepes	7.4	2	0	29.5
Na-Hepes	7.0	0.5	19	23.5
Bis-Tris-Propane	7.0	48.5	83.5	77
Tris	8.5	50	29.5	30.5
Bis-Tris	6.0	0	0	0.5

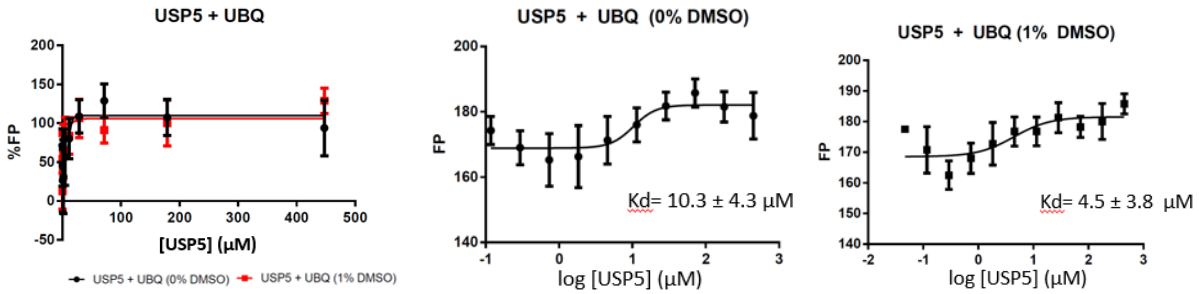
2. Optimization:

The experiment was performed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using BioTek Synergy 4. The excitation and emission wavelengths were 485 nm and 528 nm respectively. Reactions were completed with USP5 (T0C011-B06), 50 nM FITC FITC-RLRGG/FITC-UBQ in 50 mM Bis-Tris Propane pH 7.0, 100 mM NaCl. Following a 1 minute centrifugation step at 800 rpm, FP was measured. Experiments completed in replicate n=4 in a 1:2.5 12-step dilution series. Data was processed in GraphPad Prism using Sigmoidal, 4PL, X is log(Concentration) fit.



The final FP conditions for the FITC-RLRGG peptide are: 50 mM Bis Tris-Propane, pH 7.0, 100 mM NaCl, 50 nM peptide, 75 µM USP5 Zf-UBD. The protein concentration is quite high, so for future

inhibitor screening, inhibitor concentrations will also have to be high. This may present a problem for sensitivity when screening small molecule inhibitors.



The signal to noise ratio of USP5 with ubiquitin is poor. Need to explore better conditions which optimize the signal to noise ratio of FITC-ubiquitin.