USP Zf-UBD Crystallography Pipeline

Objective: To design, clone, purify and crystallize protein constructs of USP Zf-UBD's for X-ray structure determination

Experiment & Results:

A) Clone Design

Clone constructs were designed by analyzing available solution structures (**Table 1**) and/or using secondary structure prediction tools including: J Pred, SOPMA and Protein Predict. Amino acid start and end positions (based on reference sequences from Uniprot) were chosen such that structured regions of the Zf-UBD were included and the start or end position was varied by a couple of amino acids. Please see the attached excel file for all the designed clone constructs.

Table 1. USP Zf-UBD & Available PDB Structures

Target Zf-UBD	UniProt ID	Structure (PDB ID)
USP3	Q9Y6I4	N/A
USP5	P45974	2G43 (X-ray) 2G45 (X-ray) 3IHP (X-ray) 6DXH (X-ray) 6DXT (X-ray)
USP13	Q92995	2L80 (NMR)
USP16	Q9Y5T5	2I50 (NMR)
USP20	Q9Y2K6	5Z4I (NMR)
USP22	Q9UPT9	N/A
USP33	Q8TEY7	2UZG (NMR)
USP39	Q53GS9	N/A
USP44	Q9H0E7	N/A
USP45	Q70EL2	N/A
USP49	Q70CQ1	N/A
USP51	Q70EK9	N/A

B) Cloning

Based on the construct amino acid and DNA sequence, primers were ordered for each protein. Ligation-independent cloning was completed using Clontech's In-Fusion enzyme via LIC-infusion into expression vector pET28-MHL with an N-terminal His tag to allow expression and purification from bacterial production. Please see the attached cloning files for more details.

C) Test Expression

E.coli BL21(DE3)-RIL cells were transformed with the plasmid clones and grown in 3 mL of TB media and M9 media with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Harvested cells were lysed and the clarified lysates subject to Ni-affinity chromatography. TestX gels show the

lysate and Ni elution fraction for each clone construct. Please see the attached TOC013_testX.ppt file for more details. Clones that expressed well were selected for large scale growth and purification.

D) Growth & Purification

6-12 L TB or M9 minimal media culture in *E.coli* BL21 Codon Plus RIL grown at 37°C in a LEX system, induced at OD600 ~ 0.6 with 0.5 mM IPTG. Cultures grown overnight at 15°C. Cells were harvested by centrifugation, and the pellet was collected and stored at -80°C. Pellets were thawed and re-suspended in 400 mL of resuspension buffer 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP and supplemented with benzonase and 1x protease inhibitors and then lysed by sonication for 10 min at 5 second intervals, 7 second pause. Clarified lysate (supernatant-SN) was then rocked with 5 mL Ni-NTA resin for 1 hour at 4^{o}C (flow through-FT). Beads were washed with 100 mL resuspension buffer (wash 1-W1), then 200 mL resuspension buffer + 15 mM imidazole (wash 2-W2) before elution with 30 mL resuspension buffer + 300 mM imidazole (elution-El). Eluent supplemented with a molar ratio of 1:1000 TEV protease and dialyzed with snakeskin MWCO 3500 against 2 L re-suspension buffer (no imidazole) overnight (cleaved protein-CP). Cleavage of protein was verified by SDS-PAGE analysis. Cleaved protein was incubated with 5 mL Ni-NTA resin and rocked for 1 hour at 4^{o}C (FT-2). The beads were eluted with dialysis buffer + 300 mM imidazole (El-2). FT2 of the protein was concentrated to 5 mL and run on S75 1660 column. Protein was concentrated, aliquoted, flash frozen and stored at -80^{o}C. Please see attached TOC013_purification.ppt for specific construct purification details.

E) X-ray Crystallography

Purified proteins were used to set up crystal trays using SGC and RW screens (attached) in 96-well intelli plates (Art Robbins Instruments). 70 µL of each condition was dispensed into the well of the plate and 0.5 µL well solution was dispensed into the drop by a liquid handling robot (PHOENIX) followed by 0.5 µL of 5 mg/mL protein in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP. Crystal plates were sealed and stored at 18^{o}C. Crystals were cryo-protected with 25% ethylene glycol (v/v) and mounted using a nylon loop and cryo-cooled in liquid nitrogen. The crytals were screened using our in house diffractometer collecting 2 images at 90 degrees with a 0.5 deg oscillation, 20 s exposure and 100 mm crystal-detector distance at a wavelength of 1.54178 Å.

The results to date are summarized in **Table 2**.

Table 2. Summary of USP Zf-UBD Crystallization Pipeline To Date

Clone	Target	AA	Vector	Media	Amount Grown (L)	Purification Yield (mg)	Crystal Plate Setup (Y/N)	Crystals? (Y/N)	Diffraction (Y/N)
TOC013E09	USP44	27-110	pET28-MHL	M9	12	No/low expression			
TOC013F02	USP49	15-105	pET28-MHL	M9	12	0.25 mg	Y	N	

TOC013D10	USP33	37-124	pET28-MHL	M9	6	15.7 mg	Y	Y	N
TOC013D11	USP33	38-124	pET28-MHL	M9	6	0.35 mg	Y	N	
TOC013E02	USP39	97-197	pET28-MHL	M9	6	1.6 mg	Y		
TOC013E05	USP39	97-205	pET28-MHL	M9	6				
TOC013C02	USP13	188-292	pET28-MHL	M9	6	Insoluble			
TOC013C03	USP13	208-292	pET28-MHL	M9	6	Insoluble			
TOC013C04	USP13	208-308	pET28-MHL	TB	6				
TOC013B11	USP03	10-152	pET28-MHL	TB	6				
TOC013C08	USP16	26-146	pET28-MHL	M9	6	0.7 mg	Y	N	
TOC013C09	USP16	32-146	pET28-MHL	TB	6				
TOC013C11	USP20	1-100	pET28-MHL	M9	6	4.8 mg	Y	N	
TOC013D03	USP20	1-110	pET28-MHL	M9	6	13.2 mg	Y	Y: optimizing	
TOC013D04	USP20	8-110	pET28-MHL	M9	6				
TOC013-D07	USP22	43-142	pET28-MHL	M9	6	No/low expression			
TOC013D08	USP22	49-142	pET28-MHL	M9	6				
Constructs previously available in SGC database that have high expression:									
SDC093D04	USP13	183-307	pET28-MHL	TB	6				
SDC093D05	USP13	187-301	pET28-MHL	TB	6				
SDC024F07	USP16	34-185	pET28-LIC	TB	6				
SDC024F09	USP16	16-185	pET28-LIC	TB	6				
SDC024F10	USP16	8-185	pET28-LIC	TB					
SDC036B06	USP20	1-141	pET28-LIC	TB	6				
SDC042G05	USP33	7-139	pET28-LIC	TB	6				
SDC042G06	USP33	7-167	pET28-LIC	TB	6				
SDC042G07	USP33	7-183	pET28-LIC	TB					
SDC168E10	USP44	1-167	pET28-MHL	TB					
SDC231F11	USP51	160-350	pET28-LIC	TB					
SDC231-G03	USP51	176-350	pET28-LIC	TB	6				

Conclusions & Future Directions:

At this time, I've been able to crystallize the zinc finger ubiquitin binding domain of USP33 and USP20. Unfortunately, there was no diffraction of the USP33 crystals which grew in the following conditions:

RW-D01, RW-E01 and SGC-F03. I tried different cryo-protectant conditions: 25% ethylene glycol (v/v), 25% glycerol (v/v), and 100% paratone (v/v), but there was still no diffraction. Next, I'll be using seeding to set up some more USP33 Zf-UBD crystals for the TOC013D10 construct. For USP20, there was some very small microcrystals for TOC013D03 construct in the initial crystal screen with several ammonium sulfate RW/SGC conditions. I've set up some more crystal plates at a lower concentration (3 mg/mL). Hopefully, this will allow for the growth of larger crystals, which can be mounted to determine if they diffract. I am working through growing and purifying the rest of the proteins in my list and will continue to set up crystal trays.