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5-fluoro-tryptophan USP5 Zf-UBD Growth & Purification for ^{19}F NMR Screening

Objective: To express and purify USP5 Zf-UBD$^{171-290}$ in media supplemented with 5-fluoro-tryptophan (5F-W). Verification of label incorporation, protein folding and ubiquitin peptide binding done by mass spectrometry, DSF and FP respectively. Preliminary ^{19}F NMR of purified protein to determine if there are perturbations in one of the ^{19}F resonances with a ubiquitin peptide, LRLRGG.

Experiment & Results:

The USP5 Zf-UBD construct (Table 1) has two tryptophan residues, where W209 is an important residue in the binding pocket. For this reason, this protein is an ideal candidate for ^{19}F NMR spectroscopy screening. Protocol for fluorinated protein expression and purification adapted from *Nat Prot*. 2016. (11) 1414-1427.

Table 1. USP5 Construct Summary

Cloned AA sequence (171-290)	**pI**	**MW (Da)**	**Expected MW with F-Trp labels (Da)**
MHHHHHHSSGRENLYFQGGEVRQVSKHAFSLKQLDNPARIP PCGWKCSKCDMRENLWLNLTDGSILCGRRYFDGSGGNNHA VEHYRETGYPLAVKLGTITPDGADVYSYDEDDMVLDP SLAEHLSHFGIDMLKMQKTD	6.1	15653	15689

A. Growth:

1 L of LB culture of USP5 Zf-UBD$^{171-290}$ construct in BL21 Codon Plus RIL grown at 37˚C in shaker until OD_{600} ~0.8. Culture was centrifuged at 5000 RPM for 20 min. The supernatant was discarded and the pellet was re-suspended in defined media containing 5F-W (Table 2). Media was shaken at 37˚C in shaker at 170 RPM for 90 minutes as recovery time before decreasing temperature to 15˚C. Culture was induced with 0.5 mM IPTG and shaken for 18 h.

Table 2. Defined media preparation

Component for Solution A	**5x stock (g)**
Alanine	2.5
Arginine	2
Asparagine	2
Aspartic acid	2
Cysteine	0.25
Glutamine	2
Glutamic acid	3.25
Glycine	2.75
Histidine	0.5
Isoleucine	1.15
Leucine	1.15
Lysine	2.1
Methionine	1.25
Phenylalanine	0.65
Proline	0.5

Serine	10.5
Threonine	1.15
Tyrosine	0.85
Valine	1.15
Sodium acetate	7.5
Succinic acid	7.5
Ammonium chloride	2.5
Sodium hydroxide	4.25
Potassium phosphate dibasic	52.5
Adenine	2.5
Guanosine	3.25
Thymine	1.0
Uracil	2.5
Cytosine	1.0

NB: autoclave solution A

NB: add the following sequentially to sterilized 1xSolution A	**Amount for 1-L expression**
40% (w/v) glucose solution	50 mL
0.01 M FeCl3	1 mL
1 M CaCl2	0.02 mL
1 M ZnSO4	0.01 mL
MnSO4	2 mg
Thiamine	50 mg
Niacin	50 mg
Biotin	1 mg
1 M MgSO4	4 mL

NB: adjust pH to 7.2	
5-fluoro-tryptophan	60 mg

B. Purification

Cells were harvested by centrifugation and re-suspended in 400 mL resuspension buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP) supplemented with benzonase and 1x protease inhibitors and lysed by sonfication. Clarified lysate (supernatant-SN) was rocked with 5 mL Ni-NTA resin for 1 hour at 4˚C (flow through-FT). Beads were washed with 100 mL resuspension buffer (Wash 1-W1), then 200 mL of resuspension buffer + 15 mM imidazole (Wash 2-W2) before elution with 45 mL resuspension buffer + 300 mM imidazole (elution-El). Fractions were assessed by SDS-PAGE (Figure 1a). Elution was concentrated to 5 mL and loaded on gel filtration column Superdex 75 16 60 (Figure 1b).

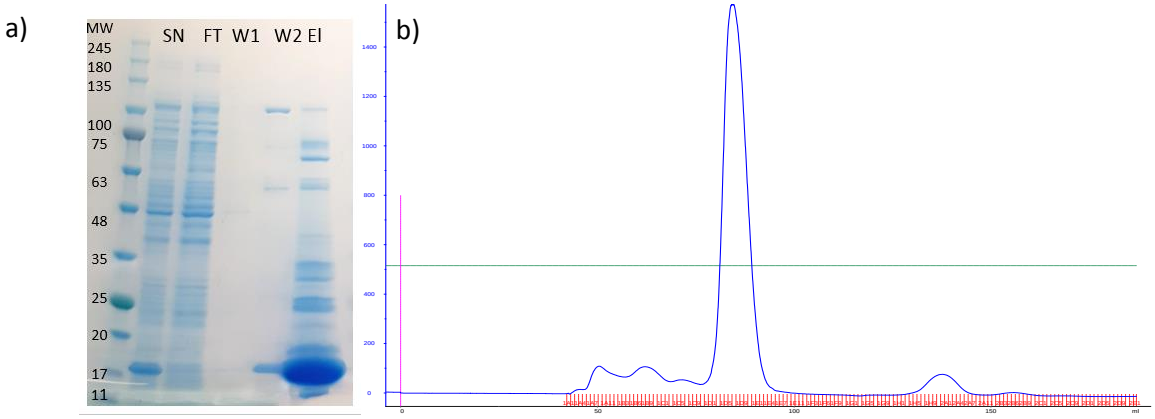

Figure 1. a) SDS-PAGE of Ni-NTA purification of USP5$^{171-290}$ b) S75 16 60 chromatogram

SDS-PAGE was used to analyze the fractions from the GF column (Figure 2). Fractions D9-E2 were pooled and concentrated to 5.7 mg/mL and aliquoted (24 x 50 µL), frozen in liquid N_2 and stored at -80˚C. The purity of the pooled sample was verified by mass spectrometry, with a m/z of 15689.74, indicating both tryptophan residues in the construct are fluorinated (Figure 3).

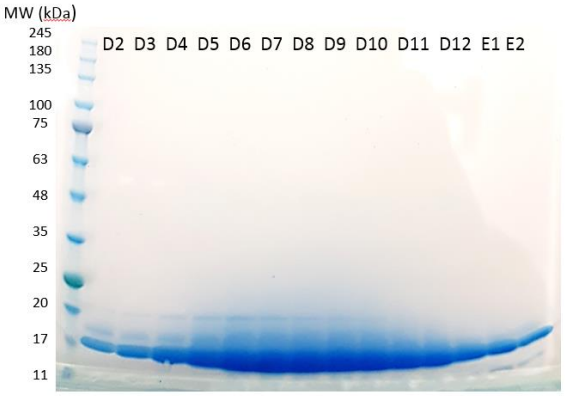

Figure 2. SDS-PAGE of S75 1660 fractions

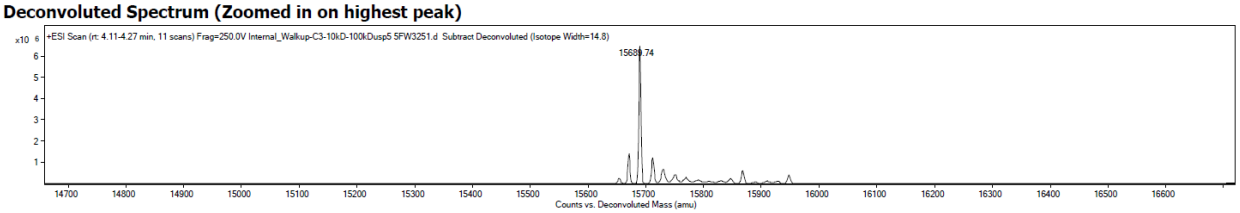

Figure 3. Mass spectrometry spectrum of fractions D9-E2

Fractions D2-D8 were pooled and diluted 1:2 in 20 mM Tris pH 8, 1 mM TCEP and run on an ion exchange Hi-Load Q column (Figure 4).

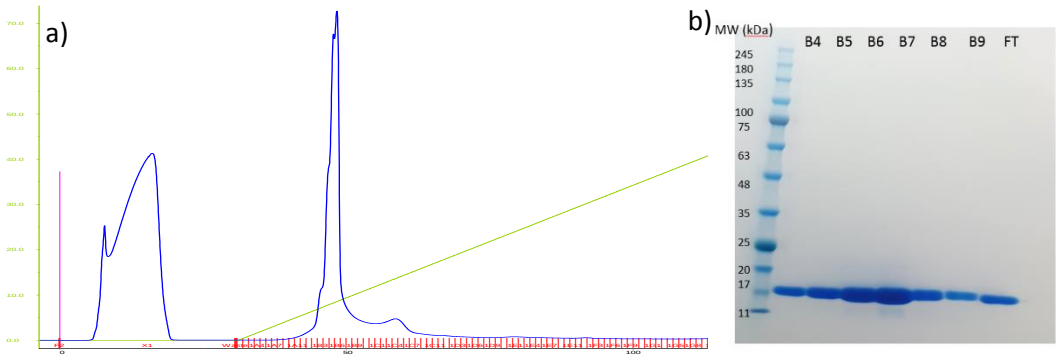

Figure 4. a) Q-column chromatogram b) SDS-PAGE of Q-column run

B4-B9 and FT fractions were concentrated to 6.7 mg/mL and aliquoted (18 x 50 µL), frozen in liquid N_2 and stored at -80˚C. The purity of the pooled sample was verified by mass spectrometry, with a m/z of 15689.88, indicating both tryptophan residues in the construct are fluorinated (Figure 5).

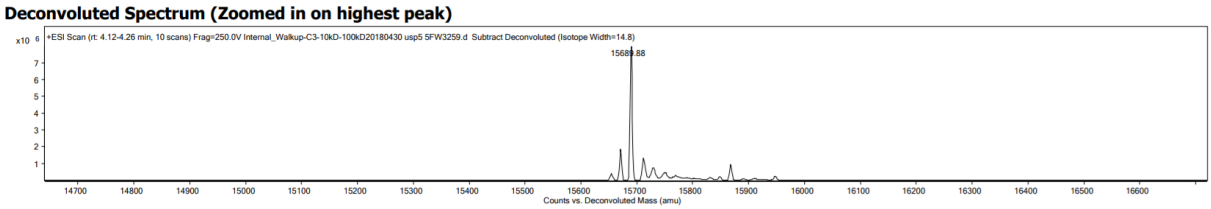

Figure 5. Mass spectrometry spectrum of Q-column fractions

C. Protein Folding

The experiment was performed in a total volume of 20 µL in a white, Roche 384-well PCR plate. Fluorescence was measured using a LightCycler 480 PCR system with excitation and emission spectrum of 465 nm and 580 nm from 20-95˚C. The ramp rate was 4.8; acquisition 6 for 4˚C/min. 0.4 mg/mL of 5F-W USP5$^{171-290}$ (~26 µM) and 5x SYPRO orange prepared in 100 mM HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.01% Triton X-100 (v/v) was prepared in a 384-well plate (n=3). The 384-well plate was sealed with an optical seal, centrifuged at 1000 RPM for 1 minute before fluorescence was measured (Figure 6).

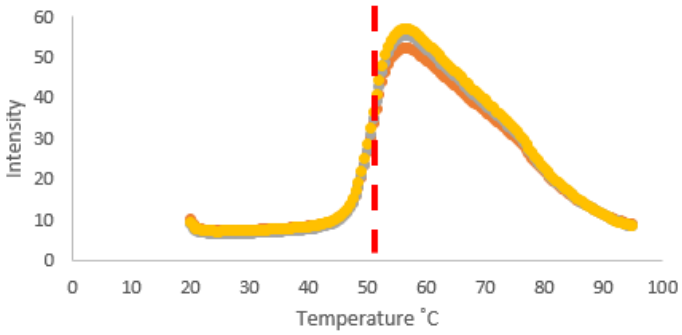

Figure 4. Melting curves of 5F-W USP5$^{171-290}$ (n=3)

The melting temperature of the 5F-W USP5$^{171-290}$ is ~52˚C. Previous protein constructs of USP5$^{171-290}$ had a melting temperature of ~54-55˚C. The slight difference in melting temperature could be attributed to the extra mass of the fluorine and the presence of the N-terminal tag (MHHHHHHSSGRENLYFQG) which was not cleaved for 5F-W USP5$^{171-290}$.

D. Ubiquitin Peptide Binding

Reactions were completed with 5F-W USP5$^{171-290}$ and 50 nM FITC-RLRGG peptide in 50 mM bis-tris propane, 150 mM NaCl, 1 mM TCEP, 0.01% TX-100 in replicate n=2 (1:2.5 12-step dilution series). Data was processed in GraphPad Prism using non-linear regression, one site binding (Figure 7).

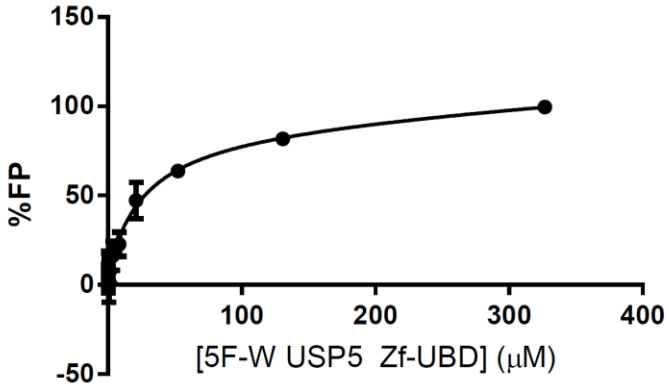

Figure 7. Binding curve of 5F-W USP5$^{171-290}$ and FITC-RLRGG

The K_d was calculated to be 26.2 ± 8.3 µM. This is similar to the K_d of ~25 µM seen previously with the non-fluorinated USP5$^{171-290}$ construct.

E. ^{19}F NMR

Before compound screening with ^{19}F NMR I first had to check if there were perturbations in at least one of the ^{19}F resonances using a LRLRGG ubiquitin peptide that binds to the USP5 Zf-UBD. The following samples (Table 3) were prepared in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20, 2.5% DMSO (v/v), 5% D_2O (v/v):

Table 3. Control and peptide samples prepared for initial ^{19}F NMR screen

	5F-W USP5$^{171-290}$ (µM)	**LRLRGG peptide (µM)**
Control	50	0
1:1	50	50
1:3	50	150
1:6	50	300

^{19}F measurements were done with Bruker 600 MHz NMR. Raw data was processed using TopSpin (Bruker), LB=5. The control spectra showed 2 well-resolved peaks of equal intensity at approximately 118 ppm and 125 ppm (Figure 8).

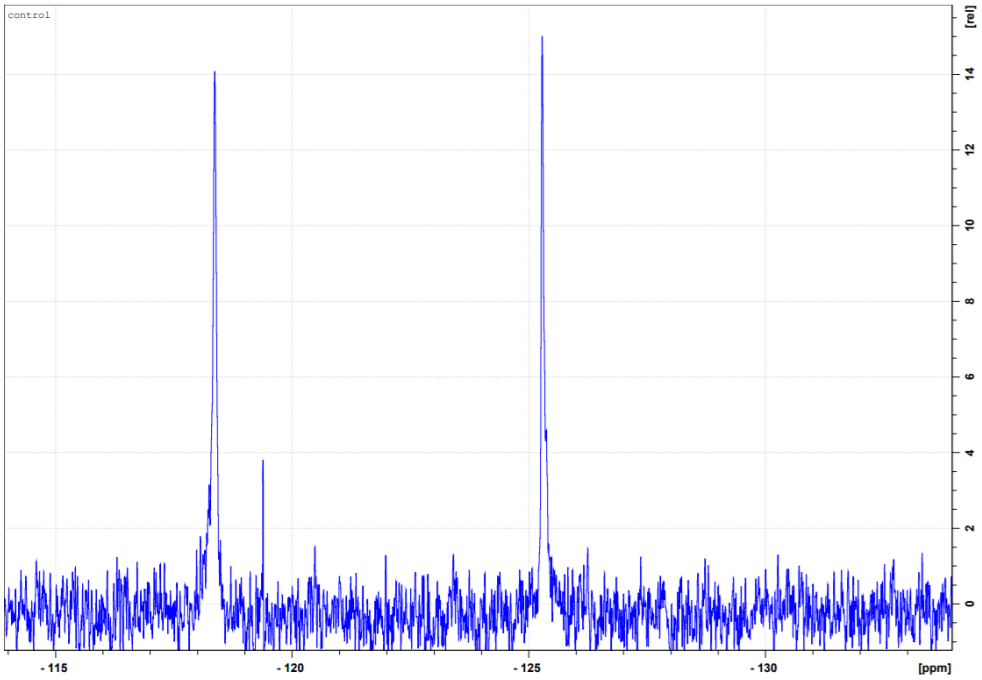

Figure 8. 50 µM 5F-W USP5$^{171-290}$ ^{19}F NMR spectra

The addition of the LRLRGG ubiquitin peptide resulted in a chemical shift to the right, significant broadening of the peak and the area under the peak was knocked down by ~50% (Figure 9 & 10).

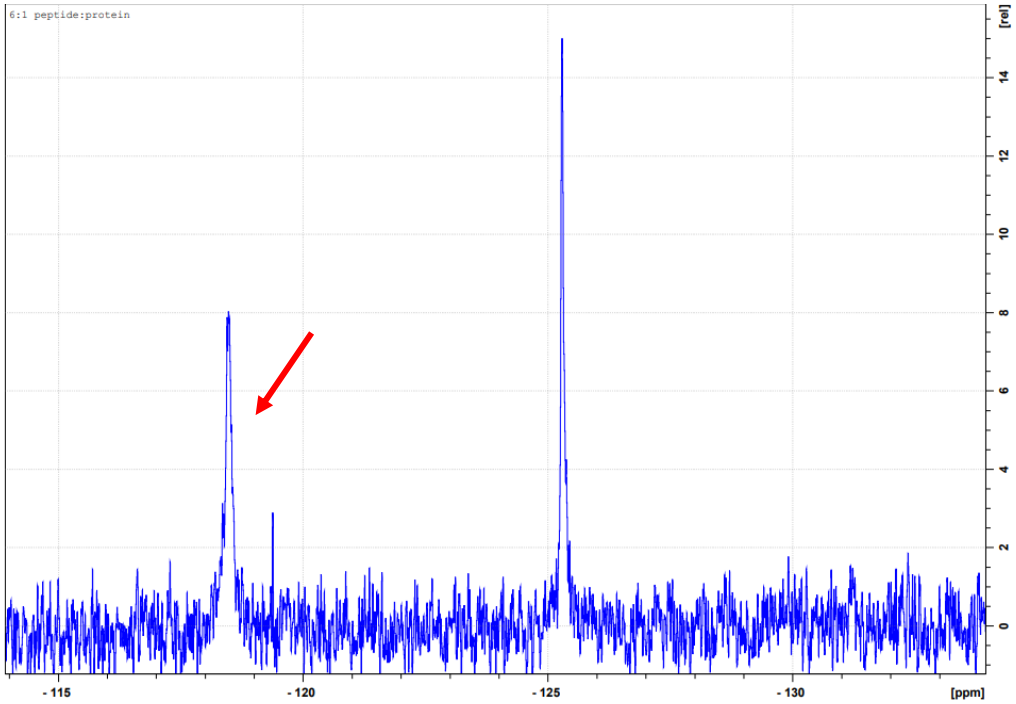

Figure 9. 1:6 5F-W USP5$^{171\text{-}290}$: LRLRGG ^{19}F NMR spectra

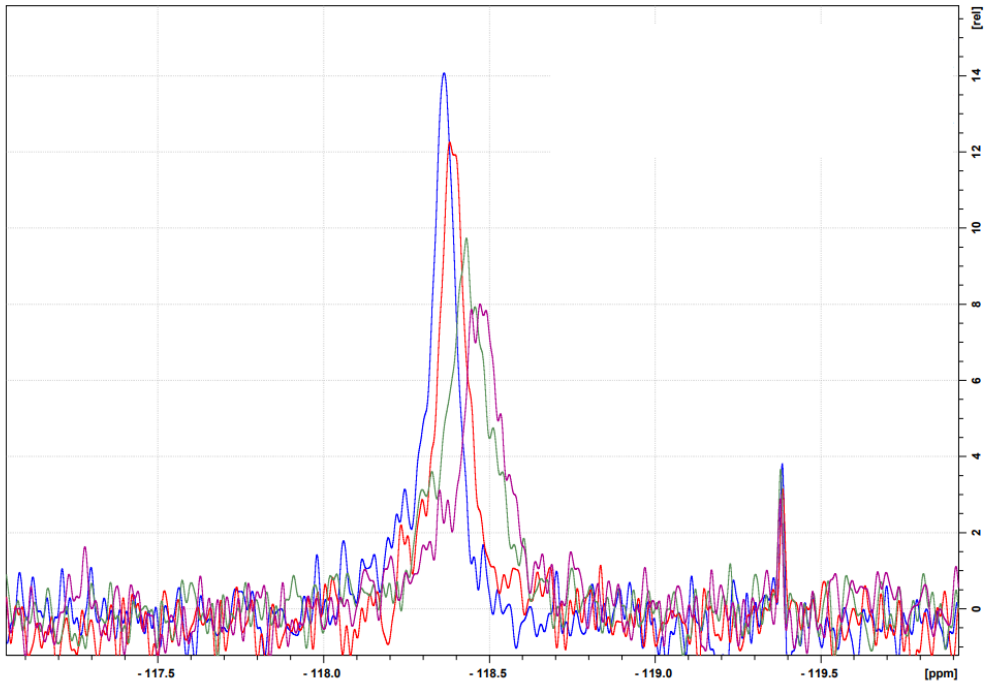

Figure 10. Overlay of spectra (blue=control, red=1:1, green=1:3, purple=1:6 [protein:peptide])

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Conclusions & Future Directions:

The total yield for 1 L growth in defined media for $USP5^{171-290}$ with 5F-Trp was 12.8 mg. Mass spectrometry analysis verifies 5F-W incorporation at both tryptophan sites in the $USP5^{171-290}$ construct. Analysis with DSF demonstrates the purified protein is folded, as we see a similar melting temperature to the previous protein construct. Analysis with FP reveals the ubiquitin RLRGG peptide binds to the 5F-W $USP5^{171-290}$. Preliminary ^{19}F NMR of the purified protein, showed two well-resolved peaks of equal intensity. There is a significant chemical shift in one of the two ^{19}F-W peaks which decreases in a dose-dependent manner with the addition of a LRLRGG ubiquitin peptide. Next, I will be using ^{19}F NMR to screen compounds against 5F-W $USP5^{171-290}$.