Mandeep Mann
2018/03/07

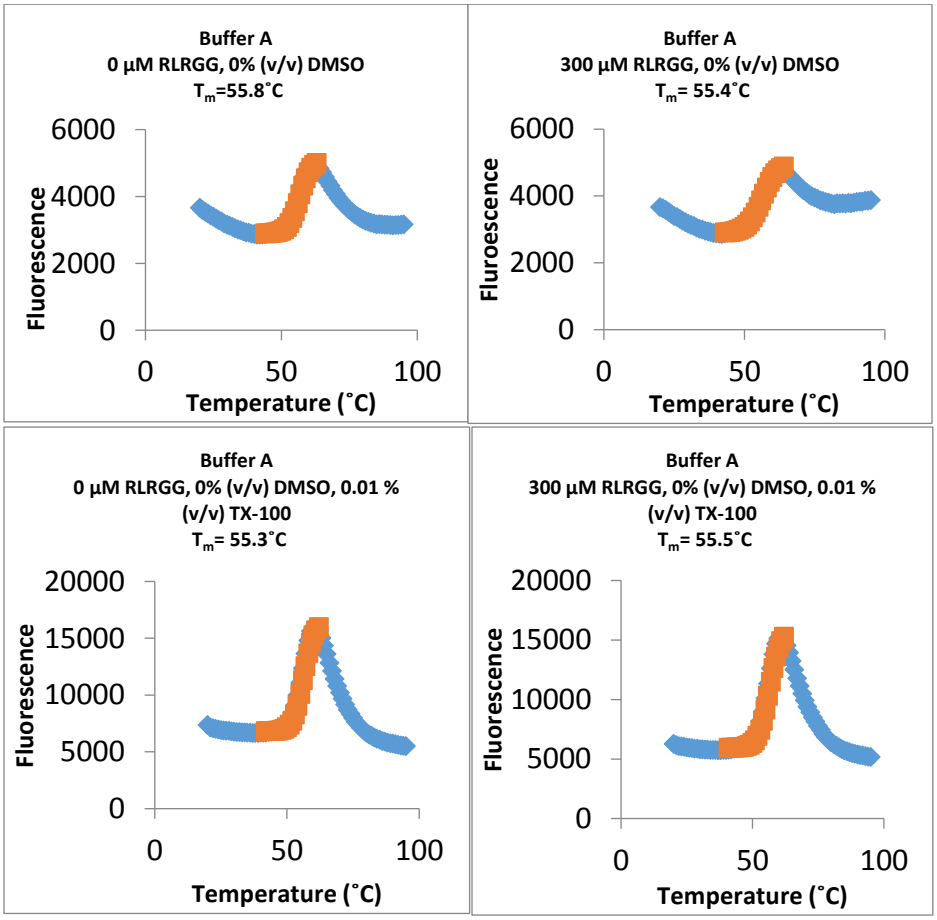
USP5 Zf-UBD Differential Scanning Fluorimetry Assay Development

Objectives: To determine if the presence of detergent in buffer conditions results in significant difference in melting temperatures of USP5 Zf-UBD and if the addition of increasing concentrations of ubiquitin RLRGG peptide results in thermal shift of USP5 Zf-UBD. Previous experiments on optimizing protein concentrations for DSF can be found here.

Experiments & Results:

1. **RLRGG-peptide titration in Buffer A** (100 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP) ± 1% (v/v) DMSO ± 0.01% (v/v) TX-100

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. 2.5 µL of USP5 Zf-UBD$^{171-290}$at 3.6 mg/mL (270 µM) and 45x Sypro Orange buffer solution was added to 20 µL of 1.11x[RLRGG-peptide] in a 96-well plate (1:2 11 pt-dilution series, n=3). 20 µL of the reaction mixture was then transferred to a 384-well plate. The 384-well plate was sealed with an optical seal, centrifuged at 1000 RPM for 1 minute, and incubated at room temperature for 20 minutes before fluorescence was measured. The data was processed using Bafcon 6 & BioActive. Representative regression charts of highest and lowest RLRGG-peptide concentrations for various Buffer A conditions are shown. See attached Excel files for all raw T_m data.



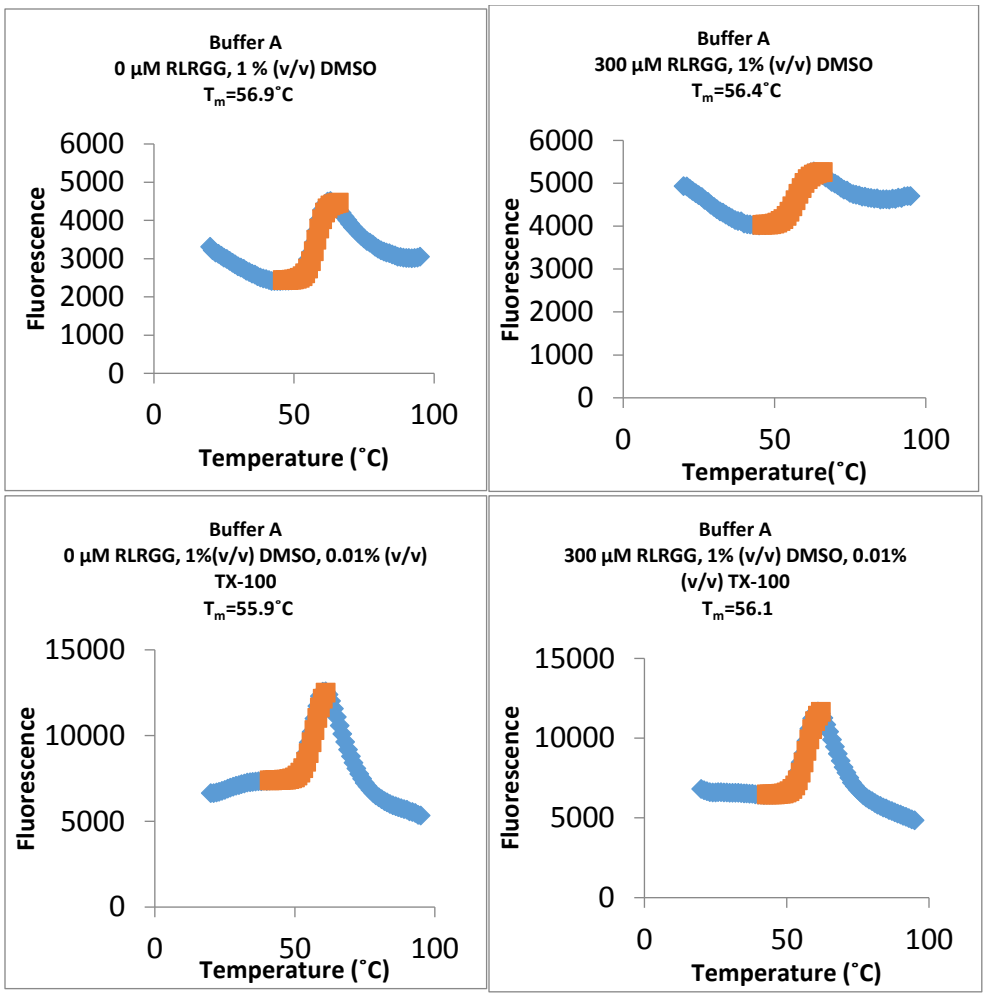


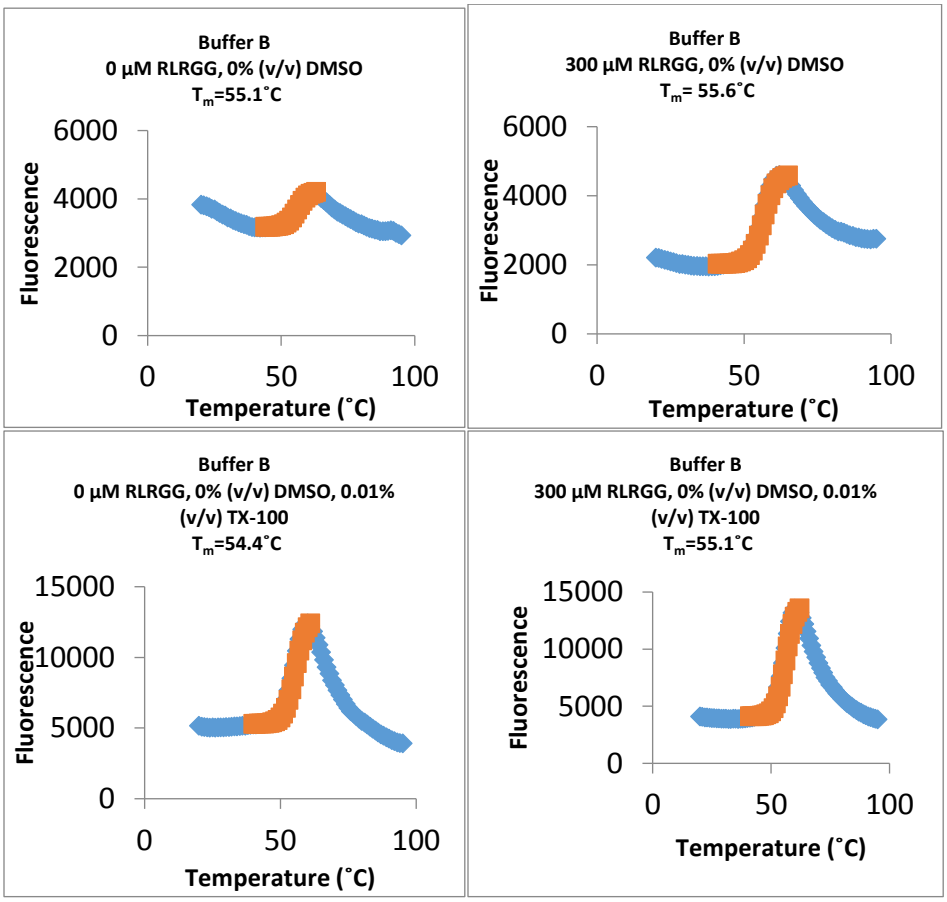
Table 1. Summary of average T_m of USP5 Zf-UBD in Buffer A ± 1% (v/v) DMSO ± 0.01% (v/v) TX-100

[RLRGG] µM	Buffer A 0% (v/v) DMSO Average T_m ± SD	Buffer A 0% (v/v) DMSO, 0.01% (v/v) TX-100 Average T_m ± SD	Buffer A 1% (v/v) DMSO Average T_m ± SD	Buffer A 1% (v/v) DMSO, 0.01% (v/v) TX-100 Average T_m ± SD
0	56.11 ± 0.29	55.25 ± 0.04	56.84 ± 0.36	60.03 ± 6.79
0.29	55.92 ± 0.60	55.06 ± 0.25	56.34 ± 0.45	63.06 ± 5.89
0.59	55.88 ± 2.3	55.09 ± 0.16	56.49 ± 0.52	64.09 ± 11.72
1.2	55.70 ± 0.22	55.29 ± 0.18	57.33 ± 2.41	56.21 ± 0.56
2.3	55.72 ± 0.21	55.01 ± 0.45	55.34 ± 0.37	55.94 ± 0.46
4.7	55.59 ± 0.08	54.95 ± 0.22	55.07 ± 0.33	58.33 ± 2.14
9.4	56.06 ± 0.11	55.13 ± 0.24	56.03 ± 0.60	56.10 ± 0.25
18.75	56.44 ± 0.60	55.18 ± 0.14	55.65 ± 0.72	55.89 ± 0.02
37.5	55.89 ± 0.22	55.33 ± 0.26	55.99 ± 0.42	56.24 ± 0.53
75	56.26 ± 1.36	55.20 ± 0.02	56.19 ± 1.92	56.15 ± 0.16
150	56.30 ± 0.12	55.47 ± 0.32	55.20 ± 2.29	56.10 ± 0.17
300	55.65 ± 0.49	55.77 ± 0.46	56.69 ± 0.55	56.40 ± 0.36

There is no significant difference in the melting temperature of USP5 Zf-UBD in Buffer A in the presence of detergent, both at 0% and 1% (v/v) DMSO. In the presence of detergent, the melting curves show a higher dynamic range of fluorescence, suggesting the presence of 0.01% (v/v) helps in preventing non-specific interactions. The standard deviation of lower RLRGG-peptide concentration at 1% (v/v) DMSO and 0.01% TX-100 is quite high; nonetheless, for all Buffer A conditions it is shown that USP5 Zf-UBD is not stabilized in the presence of increasing concentrations of RLRGG-peptide. If RLRGG-peptide was binding and stabilizing the USP5 Zf-UBD we would expect to see increasing melting temperatures as the molar ratio of the RLRGG peptide increased.

2. **RLRGG-peptide titration in Buffer B** (50 mM bis-tris propane pH 7.0, 100 mM NaCl, 1 mM TCEP) ± 1% (v/v) DMSO ± 0.01% (v/v) TX-100

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. 2.5 µL of USP5 Zf-UBD$^{171-290}$at 3.6 mg/mL (270 µM) and 45x Sypro Orange buffer solution was added to 20 µL of 1.11x[RLRGG-peptide] in a 96-well plate (1:2 11 pt-dilution series, n=3). 20 µL of the reaction mixture was then transferred to a 384-well plate. The 384-well plate was sealed with an optical seal, centrifuged at 1000 RPM for 1 minute and fluorescence was measured. The data was processed using Bafcon 6 & BioActive. Representative regression charts of highest and lowest RLRGG-peptide concentrations for various Buffer B conditions are shown. See attached Excel files for all raw T_m data.



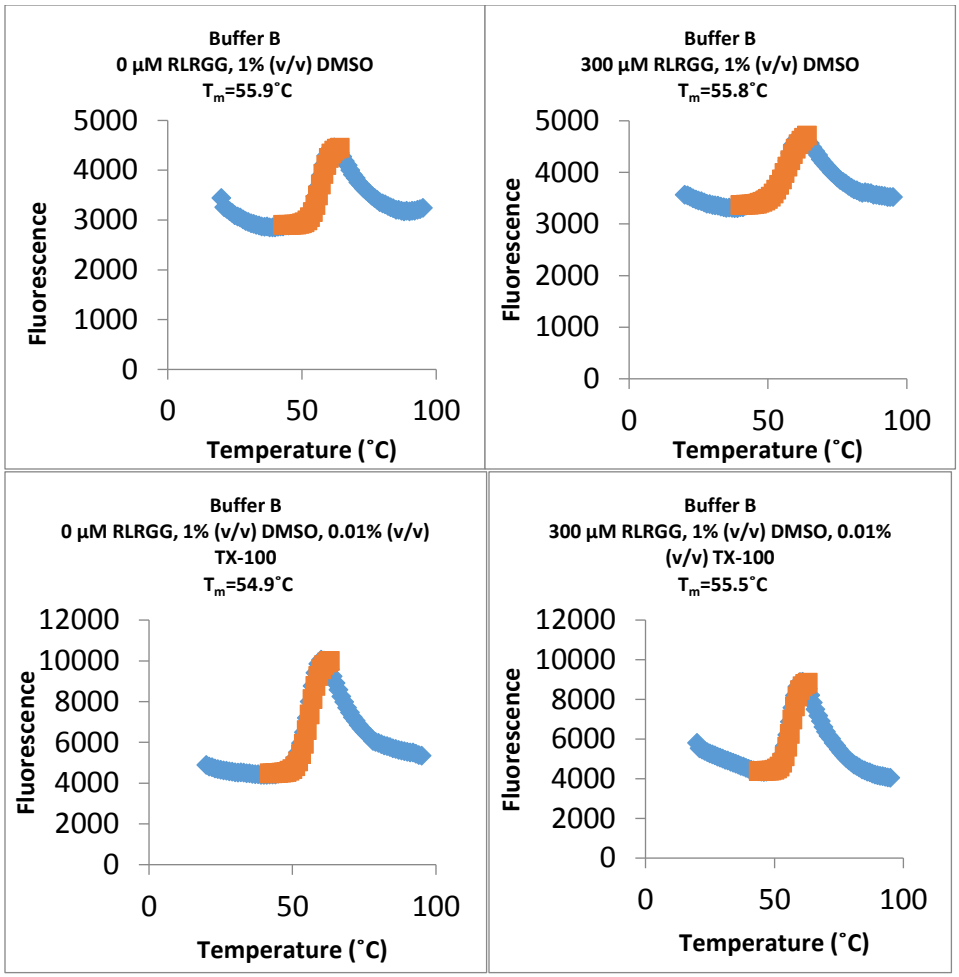


Table 2. Summary of average T_m of USP5 Zf-UBD in Buffer B ± 1% (v/v) DMSO ± 0.01% (v/v) TX-100

[RLRGG] µM	Buffer B 0% (v/v) DMSO Average T_m ± SD	Buffer B 0% (v/v) DMSO, 0.01% (v/v) TX-100 Average T_m ± SD	Buffer B 1% (v/v) DMSO Average T_m ± SD	Buffer B 1% (v/v) DMSO, 0.01% (v/v) TX-100 Average T_m ± SD
0	55.23 ± 0.14	54.44 ± 0.07	56.04 ± 0.33	54.92 ± 0.05
0.29	54.81 ± 0.40	54.47 ± 0.10	55.72 ± 0.21	55.00 ± 0.18
0.59	54.81 ± 0.12	54.42 ± 0.17	55.83 ± 0.15	54.81 ± 0.07
1.2	55.02 ± 0.46	51.27 ± 5.58	55.20 ± 1.08	54.90 ± 0.22
2.3	55.11 ± 0.37	54.42 ± 0.11	55.76 ± 0.11	54.94 ± 0.07
4.7	55.19 ± 0.11	54.41 ± 0.12	56.21 ± 1.35	54.77 ± 0.04
9.4	55.39 ± 0.71	54.48 ± 0.13	55.83 ± 0.03	54.78 ± 0.08
18.75	55.23 ± 0.66	54.38 ± 0.32	55.80 ± 0.05	54.88 ± 0.24
37.5	55.56 ± 1.07	54.44 ± 0.08	55.66 ± 0.10	54.96 ± 0.27
75	55.14 ± 0.95	55.12 ± 0.78	55.26 ± 0.86	55.20 ± 0.18
150	55.48 ± 0.38	54.86 ± 0.11	57.73 ± 2.12	55.18 ± 0.02
300	55.28 ± 0.31	55.03 ± 0.04	54.63 ± 1.72	55.68 ± 0.25

Similar to Buffer A, there is no significant difference in the melting temperature of USP5 Zf-UBD in Buffer B in the presence of detergent at 0% and 1% (v/v) DMSO. In the presence of detergent, the melting curves show a higher dynamic range of fluorescence, suggesting the presence of 0.01% (v/v) helps in preventing non-specific interactions of the protein. For all Buffer B conditions it is shown that USP5 Zf-UBD is not stabilized in the presence of increasing concentrations of RLRGG-peptide, as an increase in melting temperature is not seen.

Conclusions & Future Directions:

In conclusion, for both Buffer A and B, the presence of 0.01% TX-100, a non-ionic detergent, does not significantly affect the melting temperature of USP5 Zf-UBD but does increase the dynamic range of fluorescence measurements, likely because the detergent prevents non-specific interactions and aggregation of the protein. There is no apparent stabilization of USP5 Zf-UBD in the presence of ubiquitin RLRGG-peptide for both Buffer A and B, most likely due to low affinity of the peptide to the USP5 Zf-UBD as shown in previous fluorescence polarization experiments (K_{disp} ~ 55 µM).

For future experiments pertaining to DSF assay development, I will be testing different lengths of ubiquitin peptide with USP5 Zf-UBD. Hopefully, I will see one of these peptides have a higher affinity to the protein and show stabilization as well as thermal shift with increasing peptide concentrations. I will then begin to screen inhibitor compounds against USP5 Zf-UBD using the DSF assay.