Mandeep Mann
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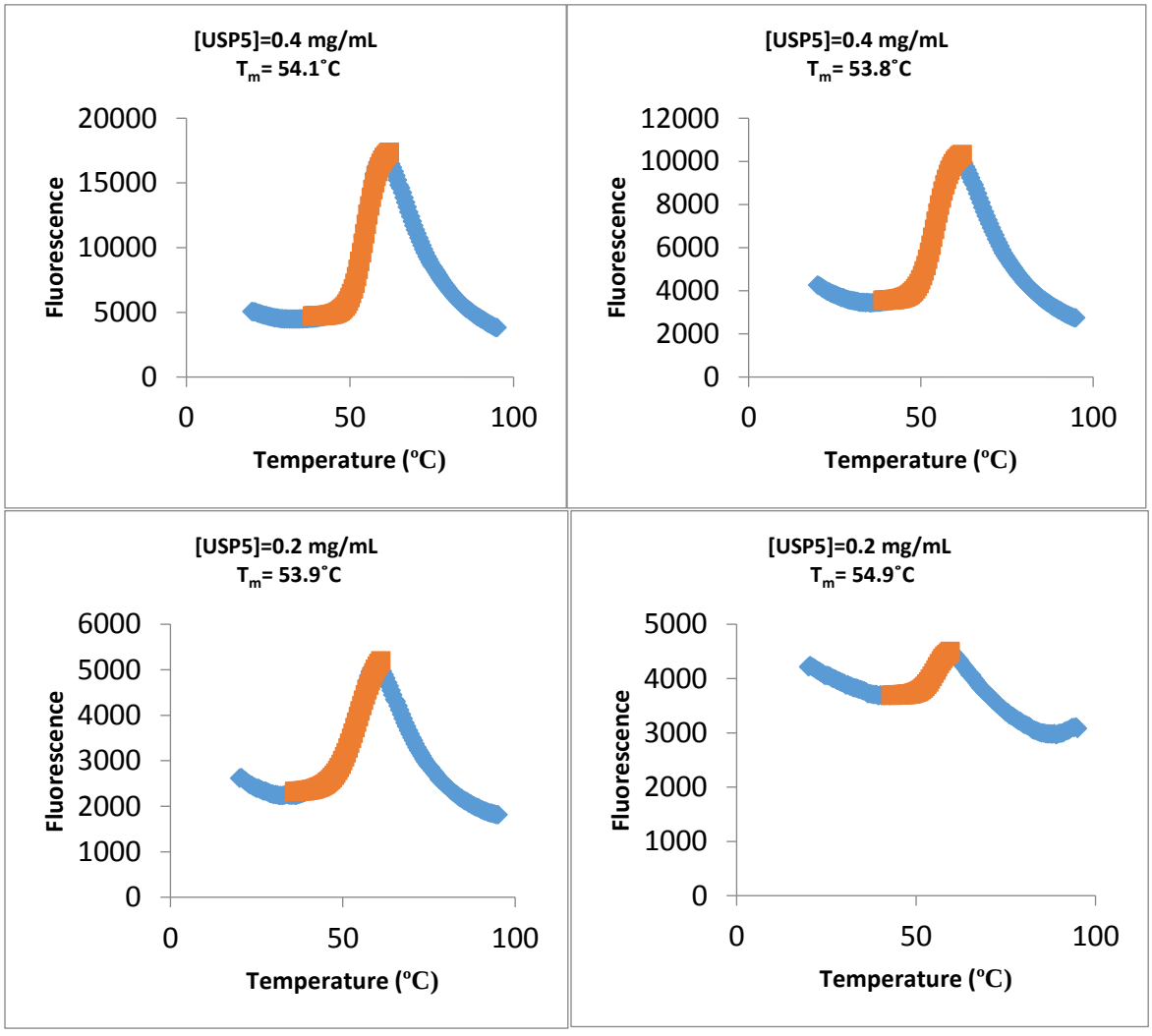
USP5 Zf-UBD Differential Scanning Fluorimetry Assay Development

Objective: Development of DSF assay for USP5 Zf-UBD to determine optimal protein screening conditions and significance of thermal shift of USP5 Zf-UBD in the presence of a ubiquitin RLRGG peptide

Experiments & Results:

1. **[USP5 Zf-UBD] Screen:**

The protein concentration screen 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. USP5 Zf-UBD$^{171-290}$ (Zenodo) of 0.4, 0.2, 0.1, 0.05 mg/mL were prepared in 5x Sypro Orange buffer solution (100 mM Hepes pH 7.4, 150 mM NaCl-a standard screening buffer). 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.



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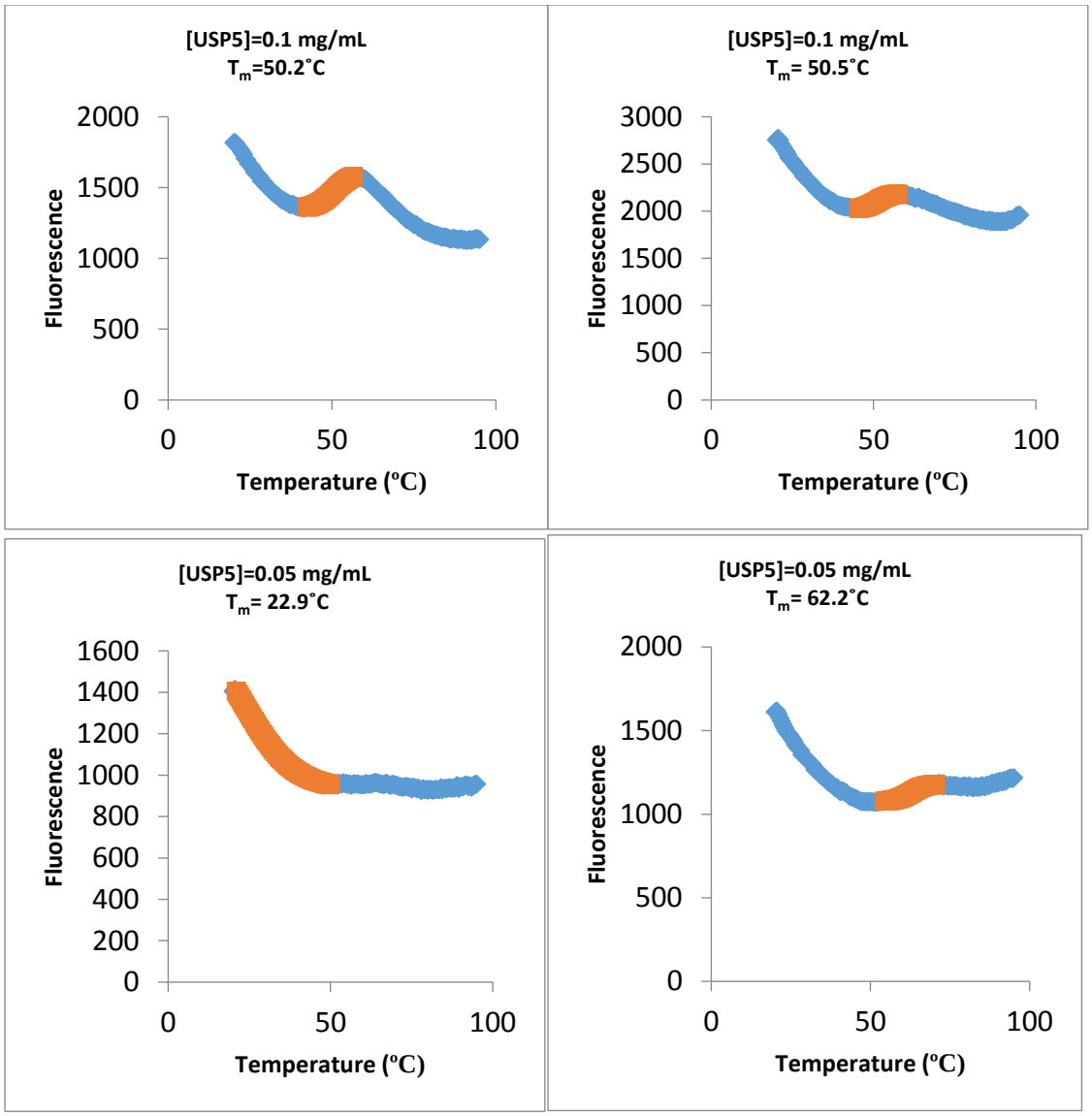
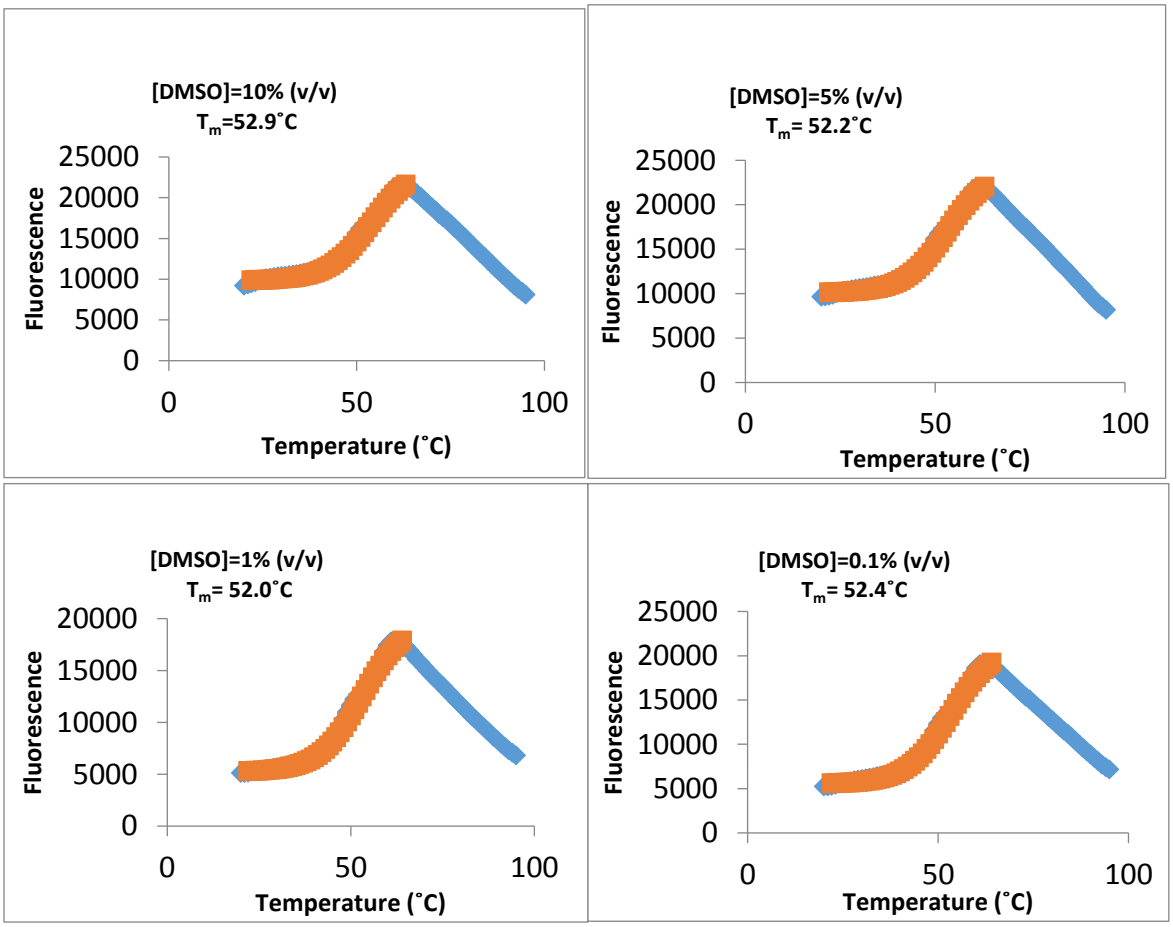


[USP5 Zf-UBD]$^{171-290}$	**Average T_m (n=2)**
0.4 mg/mL	53.95 ± 0.25
0.2 mg/mL	54.40 ± 0.65
0.1 mg/mL	50.3 ± 0.21
0.05 mg/mL	42.58 ± 27.8

The best protein concentration tested is 0.4 mg/mL, which is ~30 µM.

2. [DMSO] Screen:

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. USP5 Zf-UBD$^{171-290}$ of 0.4 mg/mL (30 µM) was prepared in 5x Sypro Orange buffer solution (100 mM Hepes pH 7.4, 150 mM NaCl), and [DMSO] (v/v). 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 are shown.



% [DMSO] (v/v)	Average T_m (n=2)
10	54.9 ± 2.88
5	54.7 ± 3.44
1	52.5 ± 0.34
0.1	52.4 ± 0.00

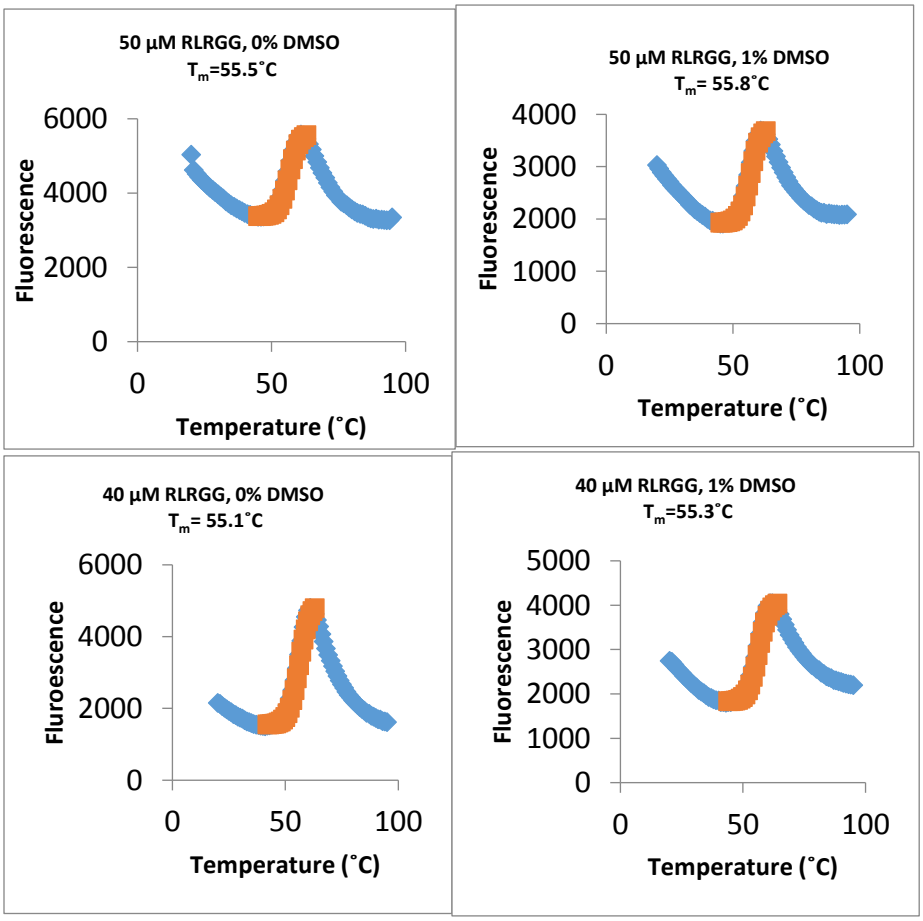
A DMSO screen was done because this assay will be used to screen small molecule inhibitors against USP5 Zf-UBD. These compounds are prepared in DMSO.

The melting temperature of USP5 Zf-UBD decreases from 54˚C to ~52˚C when DMSO is added to the reaction mixture, likely because DMSO destabilizes the protein.

3. USP5 Zf-UBD & Ubiquitin Peptide:

Next, I tested how the addition of a RLRGG ubiquitin peptide, changed the melting temperature of USP5 Zf-UBD.

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 (100 mM Hepes pH 7.4, 150 mM NaCl ± 1% (v/v) DMSO) was added to 20 µL of 1.11x[RLRGG-peptide] in a 96-well plate. 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 are shown.



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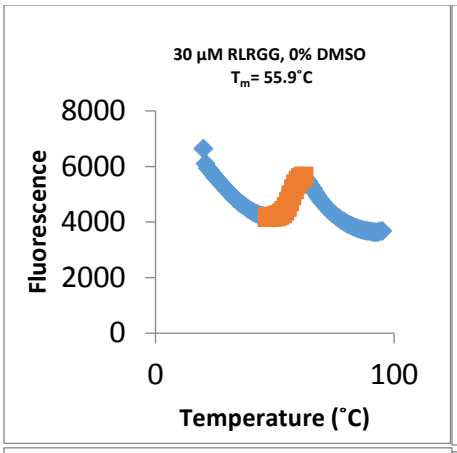

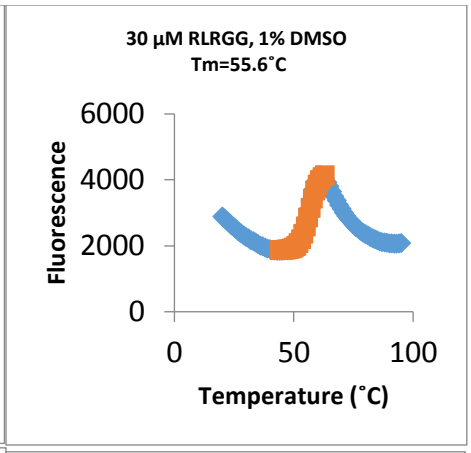

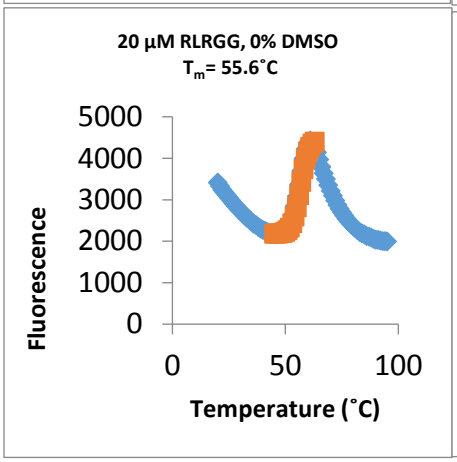

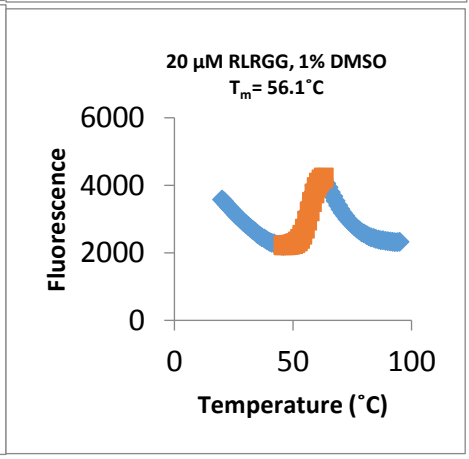

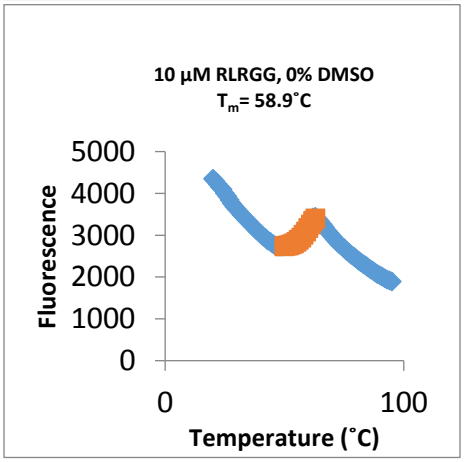

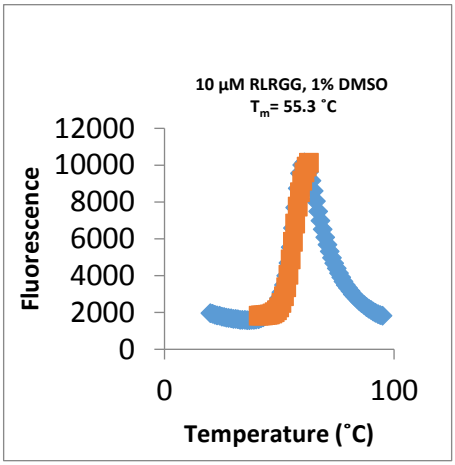

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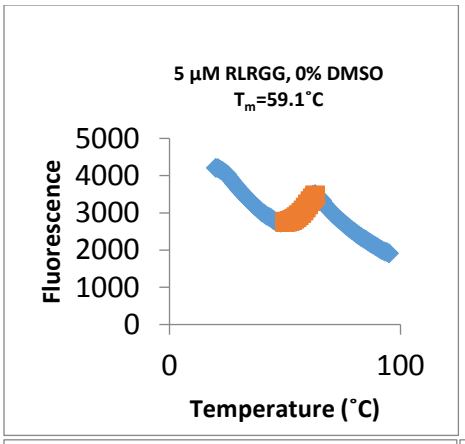

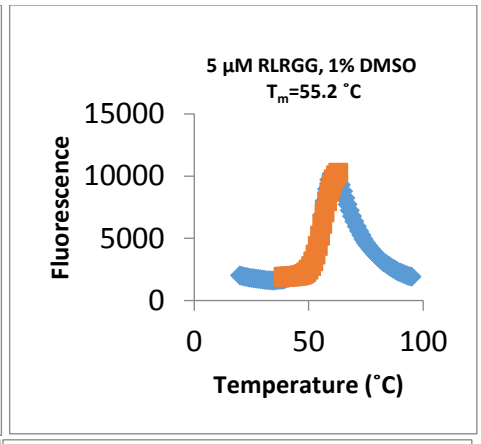

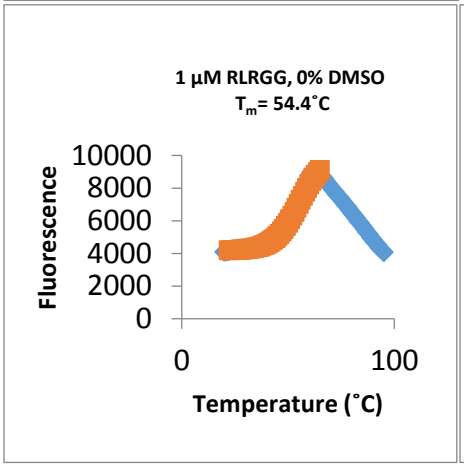

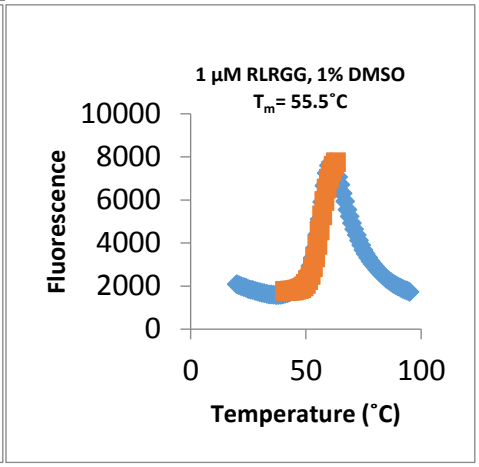

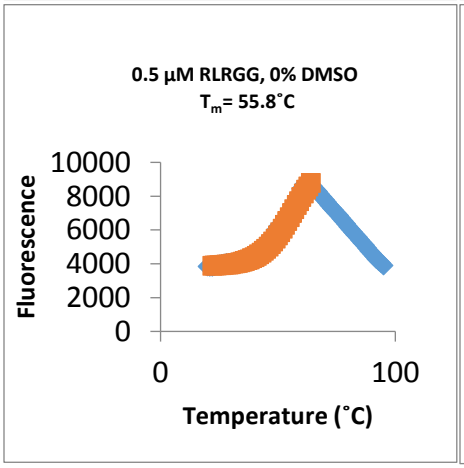

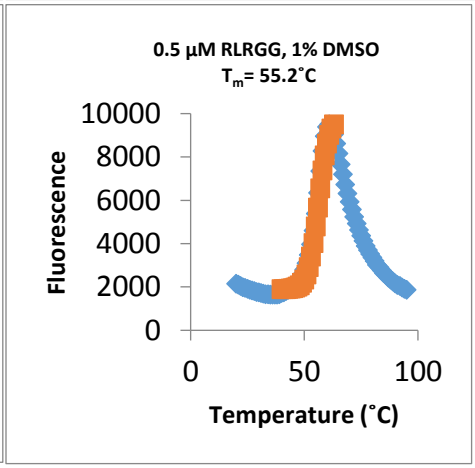

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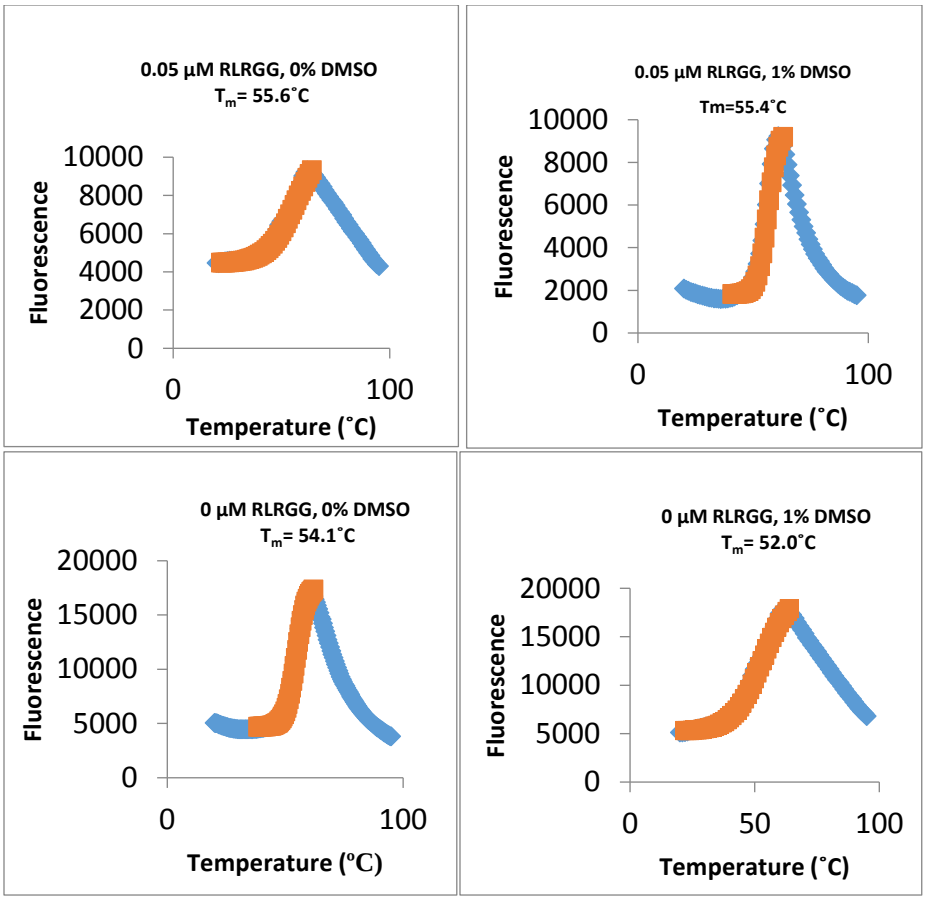


[RLRGG] (µM)	Average T_m (n=2) 0% DMSO (v/v)	Average T_m (n=2) 1% DMSO (v/v)
50	55.5 ± 0.10	55.6 ± 0.32
40	55.1 ± 0.06	55.4 ± 0.13
30	56.0 ± 0.004	55.9 ± 0.32
20	55.8 ± 0.14	56.1 ± 0.37
10	58.9	55.4 ± 0.03
5	59.1	55.2 ± 0.008
1	54.4	55.4 ± 0.10
0.5	53.7 ± 1.8	55.4 ± 0.22
0.05	55.5 ± 0.37	55.3 ± 0.14
0	54.0 ± 0.20	52.4 ± 0.24

The melting curves for higher peptide concentrations at 0% DMSO are not ideal. This may be caused by a number of reasons (i.e. Pipetting errors, non-specific interactions etc) but it's difficult to say exactly why these curves look a little odd. I will have to repeat this experiment, and optimize the buffer condition, perhaps by adding detergent to avoid non-specific interactions.

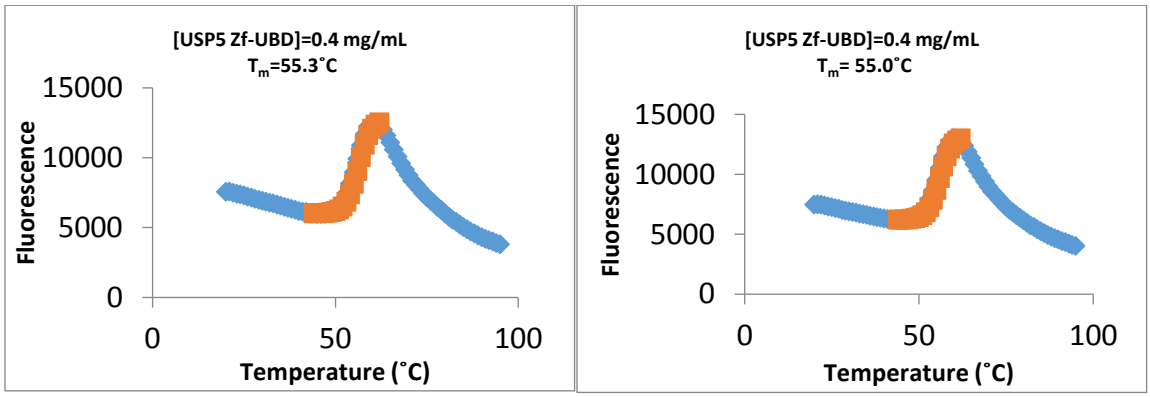
The melting temperature of the RLRGG peptide and the protein stays about the same despite the peptide titration. This is not what I expected as the molar ratio of the peptide decreases, I expect less stabilization of the protein. This may be due to weak affinity with the peptide. Perhaps incubating the plates for some time before DSF analysis will give the RLRGG-peptide enough time to bind to the protein

and I will see a shift in the RLRGG-peptide titration. Further experiments are required to confirm the thermal shift of the protein with the RLRGG-peptide. In future experiments I will use higher concentrations of the RLRGG peptide and incubate plates for a longer period of time before analysis to confirm if the RLRGG peptide stabilizes the USP5 Zf-UBD.

4. USP5 Zf-UBD Screen-Alternate buffer

In a previous post (Zenodo), in which I did a buffer screen for a fluorescence polarization assay, I optimized the assay using the buffer condition: 50 mM bis-tris propane pH 7.0, 100 mM NaCl. I was interested in seeing how this buffer condition would effect the melting temperature of USP5 Zf-UBD in the DSF assay compared to the Tm measured with the standard screening buffer.

The protein screen 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. USP5 Zf-UBD$^{171-290}$ of 0.4 mg/mL (30 µM) were prepared in 5x Sypro Orange buffer solution (50 mM bis-tris propane pH 7.0, 100 mM NaCl). 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.



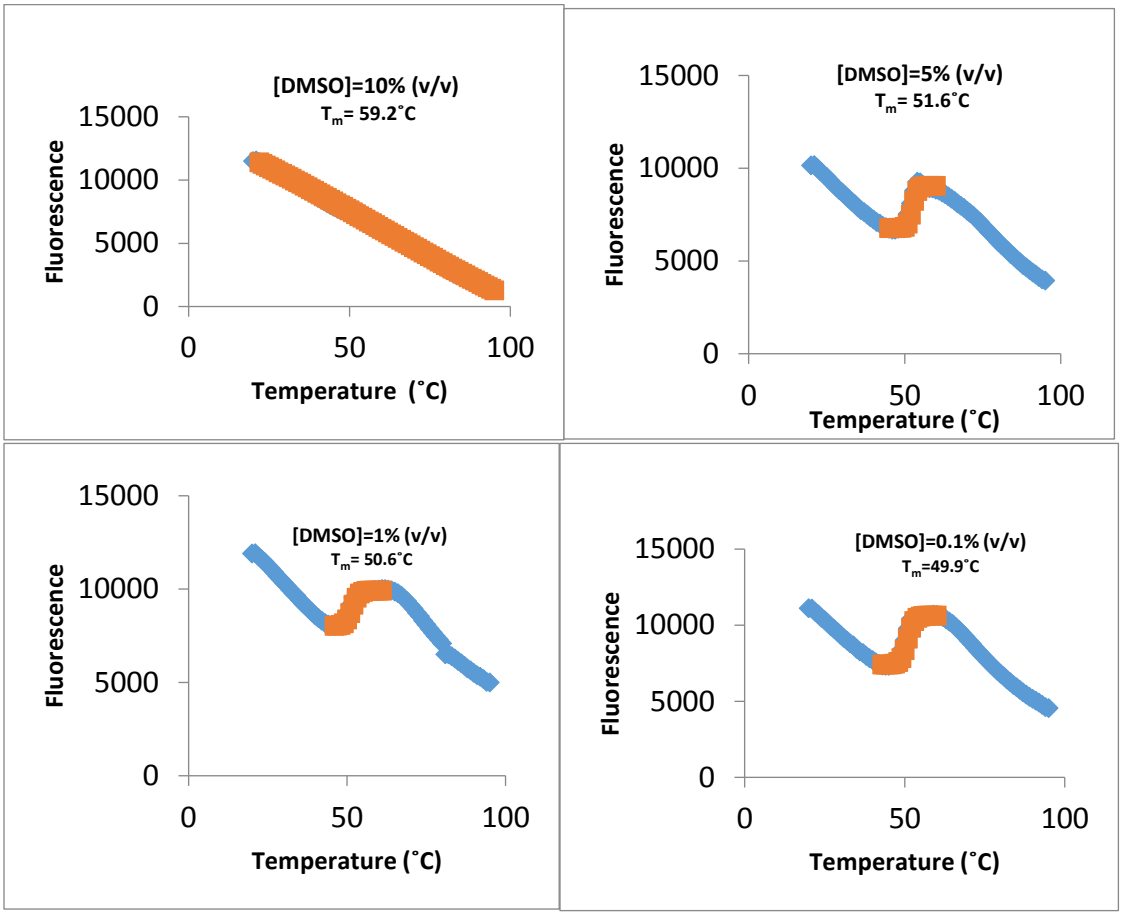
[USP5 Zf-UBD]$^{171-290}$	Average T_m (n=2)
0.4 mg/mL	55.2 ± 0.21

The melting temperature of USP5 Zf-UBD in the buffer 50 mM bis-tris propane pH 7.0, 100 mM NaCl is ~55˚C, which is 1˚ higher than the Tm of USP5 Zf-UBD in the standard screening buffer.

5. [DMSO] Screen with alternate buffer

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. USP5 Zf-UBD$^{171-290}$ of 0.4 mg/mL (30 µM) was prepared in 5x Sypro Orange buffer solution (50 mM bis-tris propane pH 7.0, 100 mM NaCl), and [DMSO] (v/v). 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 are shown.



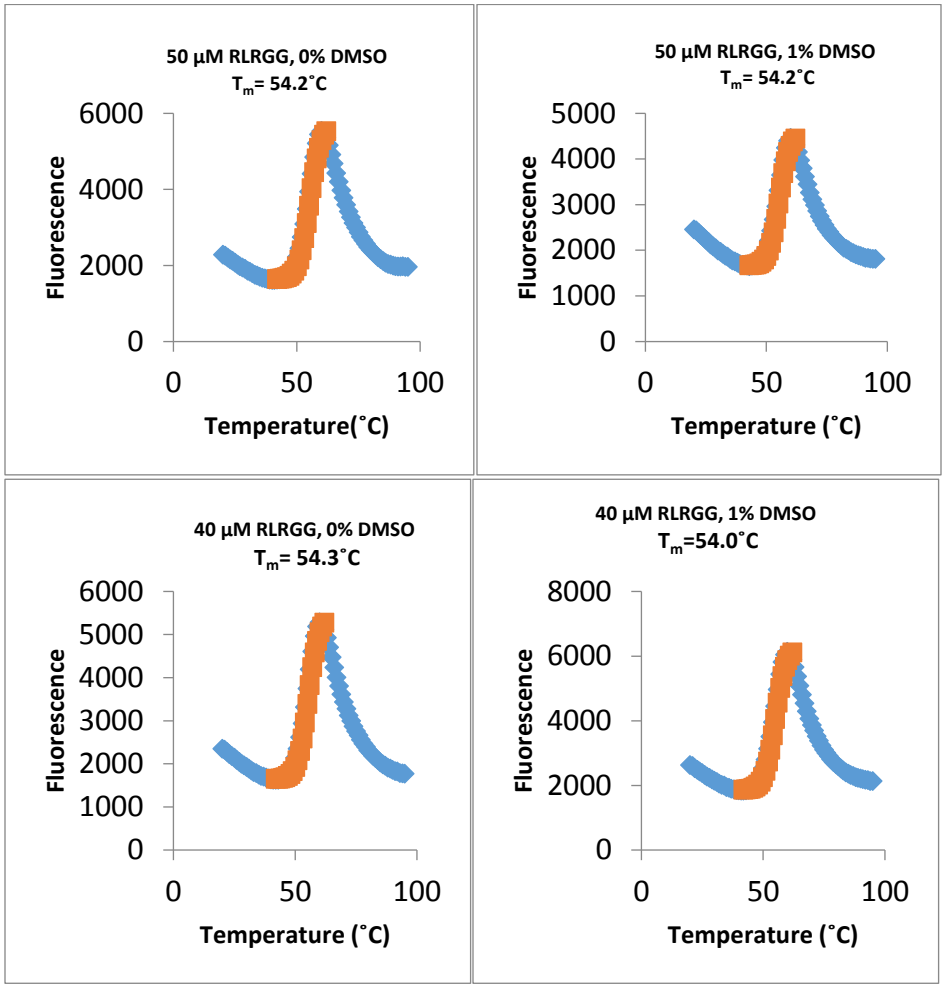
% [DMSO] (v/v)	Average T_m (n=2)
10	41.1 ± 25.6
5	51.5 ± 0.01
1	50.6 ± 0.09
0.1	49.6 ± 0.13

A DMSO screen was done because this assay will be used to screen small molecule inhibitors against USP5 Zf-UBD. These compounds are prepared in DMSO.

At 10% DMSO (v/v) there is no melting curve, suggesting this data should be ignored. The melting curves for all DMSO concentrations are not ideal, again leading to the conclusion that the buffer conditions need to be optimized. The melting temperature of USP5 Zf-UBD decreases from 55˚C to ~51˚C when DMSO is added to the reaction mixture, likely because DMSO destabilizes the protein.

6. USP5 Zf-UBD, Ubiquitin Peptide & DMSO with alternate buffer conditions

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$^{170-290}$ at 3.6 mg/mL (270 µM) and 45x Sypro Orange prepared in 50 mM bis-tris propane pH 7.0, 100 mM NaCl ± 1% (v/v) DMSO was added to 20 µL of 1.11x[RLRGG-peptide] in a 96-well plate. 20 µL was transferred to a 384-well plate and 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 are shown.



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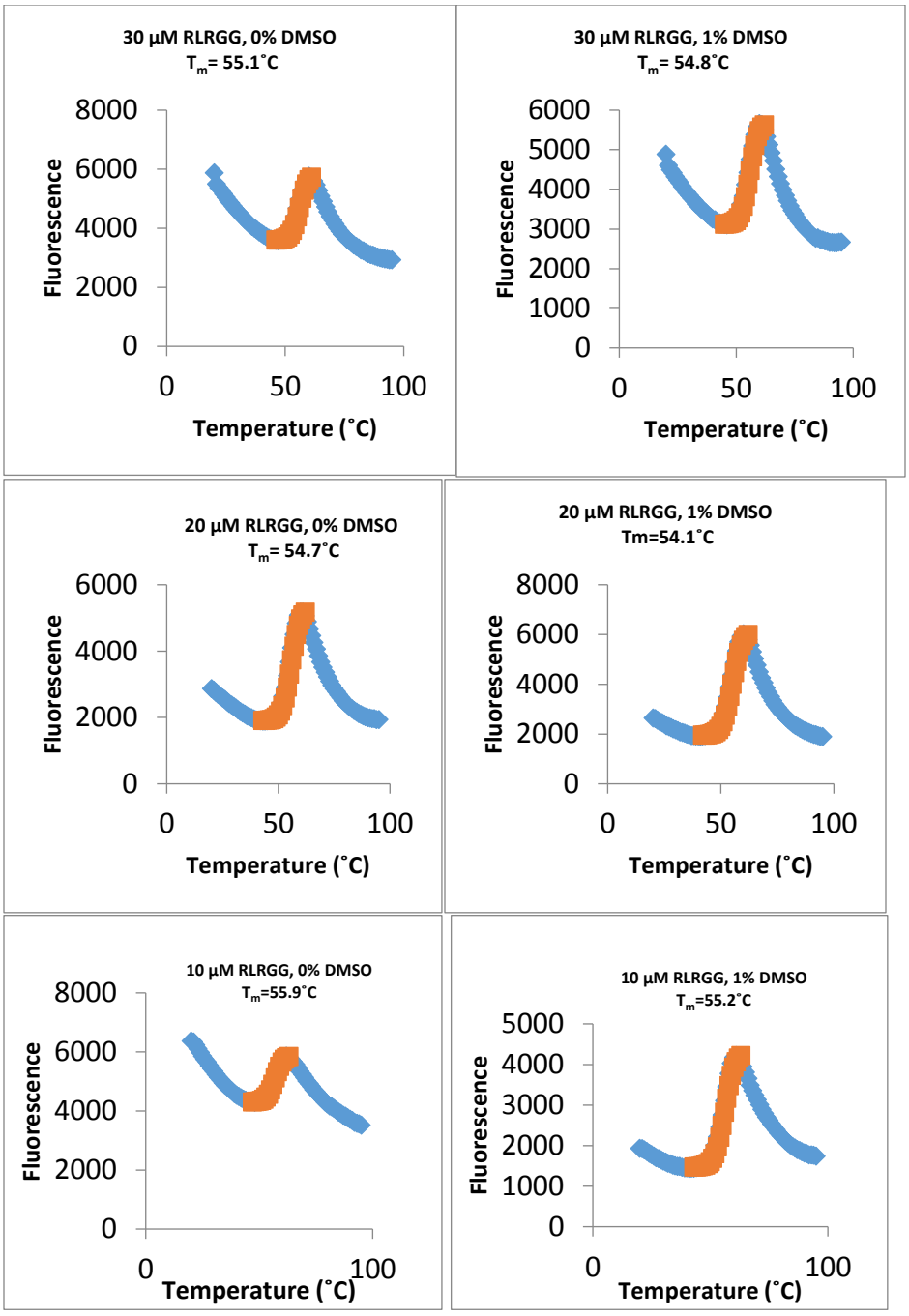

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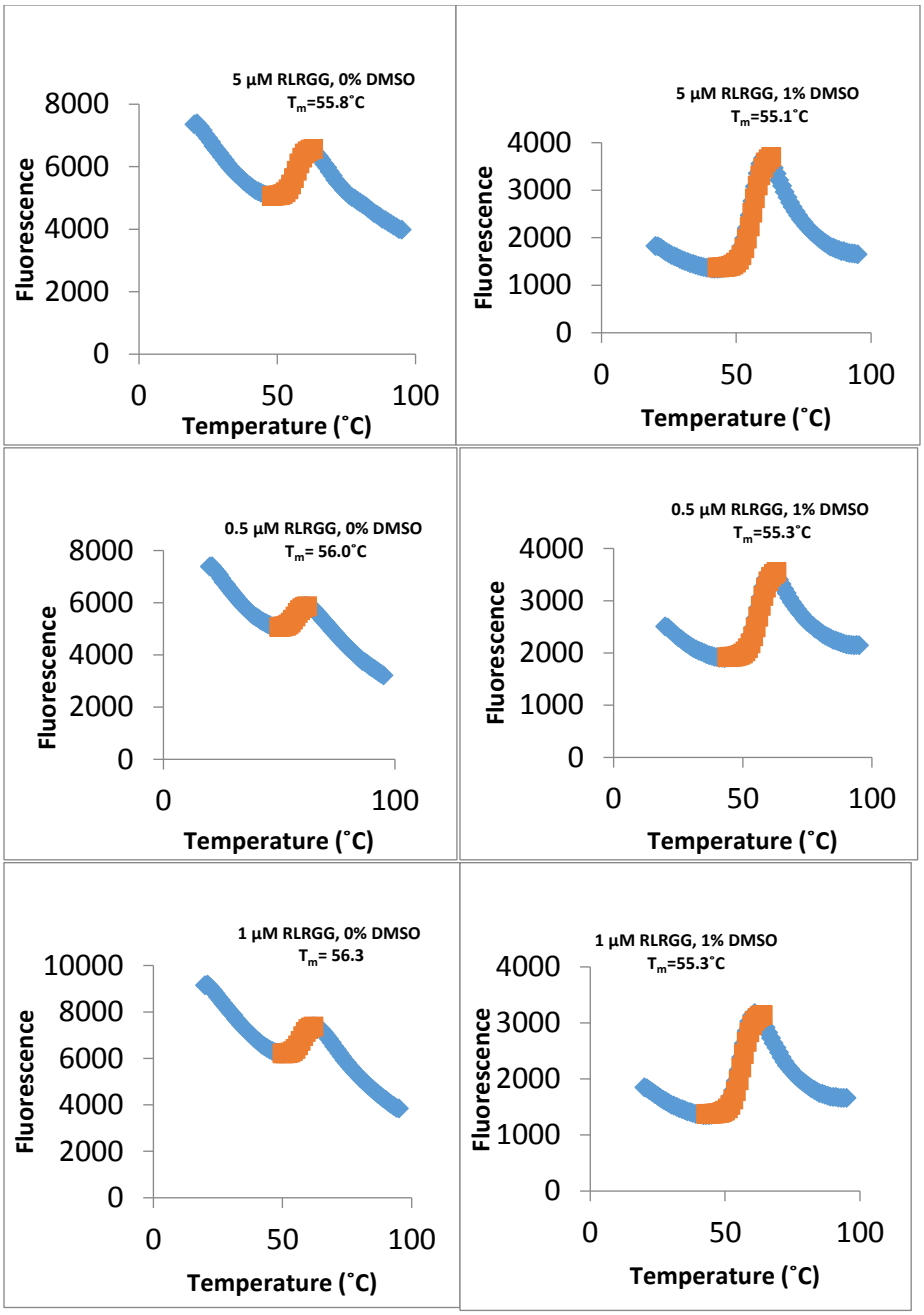

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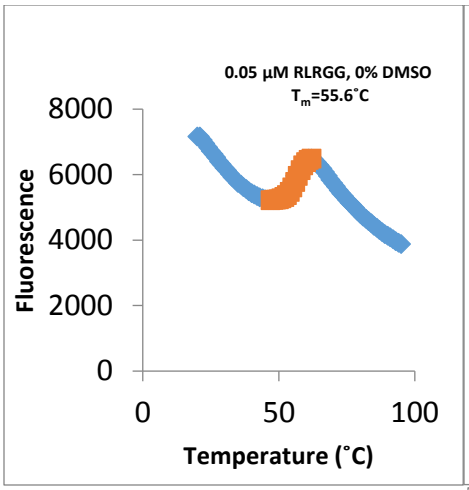

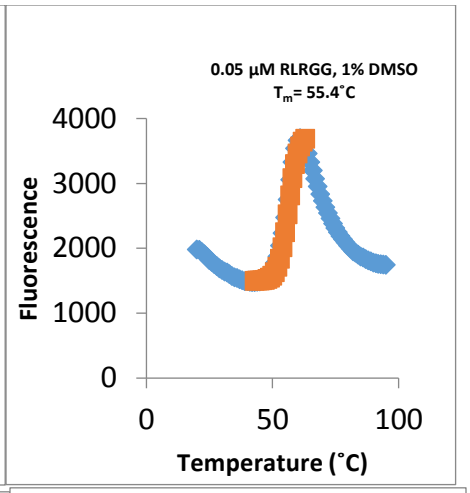

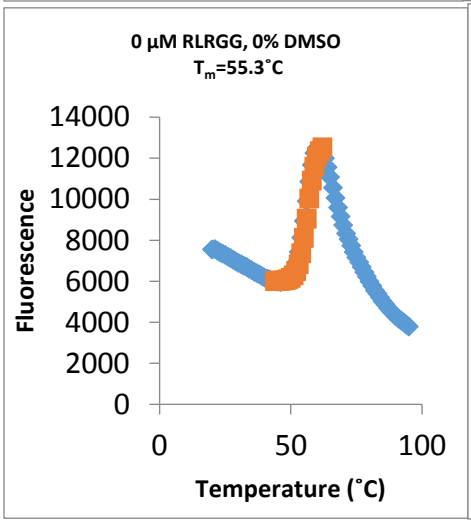

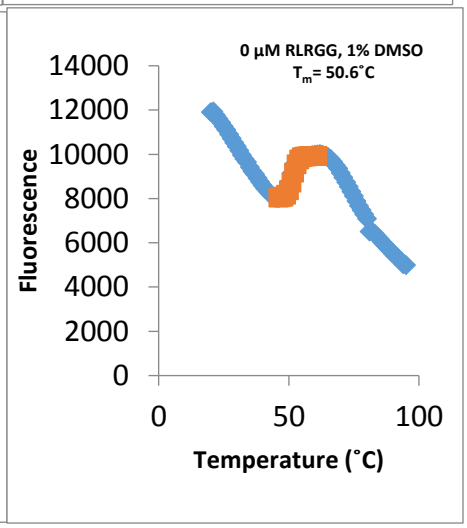

[RLRGG] (µM)	Average T_m (n=2) 0% DMSO (v/v)	Average T_m (n=2) 1% DMSO (v/v)
50	54.3 ± 0.05	53.9 ± 0.02
40	53.3 ± 0.04	54.1 ± 0.11
30	55.0 ± 0.14	54.8 ± 0.06
20	54.7 ± 0.04	54.4 ± 0.4
10	55.8 ± 0.08	55.3 ± 0.08
5	55.9 ± 0.14	55.2 ± 0.13
1	56.2	52.6 ± 0.49
0.5	56.0	55.4 ± 0.009
0.05	55.6	55.4 ± 0.07
0	55.2 ±0.21	49.7 ± 0.11

The melting temperature of USP Zf-UBD with the peptide is ~56˚C and ~55˚C in 0% (v/v) and 1% (v/v) DMSO respectively. The melting temperature of the RLRGG-peptide and the protein stays about the same despite the peptide titration in both 0% and 1% DMSO conditions. As mentioned before, this is not expected as the decrease in molar ratio of the RLRGG-peptide should not stabilize the protein to the same extent as higher molar ratios of the RLRGG-peptide. This may be attributed to weak affinity of the

RLRGG-peptide to the protein and incubation time before DSF analysis but further experiments will have to be conducted to answer these questions.

In the standard screening buffer (100 mM Hepes pH 7.4, 150 mM NaCl), the addition of a ubiquitin peptide increases the melting temperature by 2-3 ˚C versus the 50 mM bis-tris propane pH 7.0, 100 mM NaCl condition which has an increase of 1-4˚C. A thermal shift of 2˚C is considered significant; however, because no temperature shift is seen from 50 µM RLRGG-peptide to 0.05 µM RLRGG-peptide I cannot conclude that this shift is significant. Further experiments are required to confirm these results for USP5 Zf-UBD with the RLRGG-peptide.

Next, I will repeat these experiments with a trace amount of detergent in the buffer. On the one hand, detergent in the buffer helps to prevent non-specific interactions and aggregation. On the other hand, in DSF, detergents have also been shown to interfere with the hydrophobic binding of the dye to the protein. I will then be able to compare melting temperatures with and out without detergent to see if there is a significant difference. If there is no significant difference, the presence of detergent will deter non-specific interactions and I will hopefully see melting curves with a sharp transition. I will also increase the starting concentration of the RLRGG-peptide for the titration and increase the incubation time of the plates after the protein is added to ensure sufficient binding time of the RLRGG-peptide to the protein. I hope to see a significant change in melting temperatures for the RLRGG-peptide titration.