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USP5 Zf-UBD Differential Scanning Fluorimetry Assay Development #3

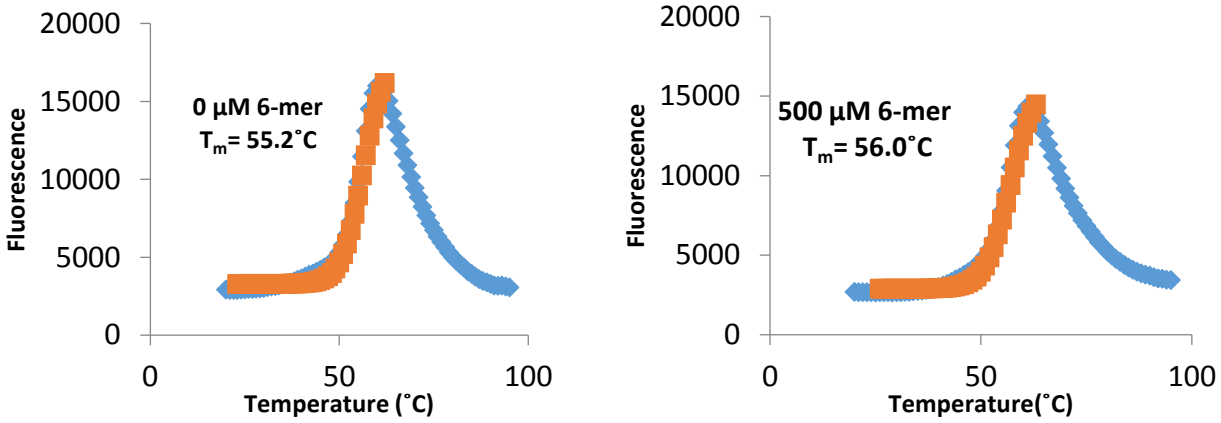
Objective: To determine if ubiquitin peptides of different length results in a thermal shift of the USP5 zinc finger ubiquitin binding domain (Zf-UBD). Previous experiments for DSF assay development for USP5 Zf-UBD can be found here.

Experiment & Results:

In previous experiments, there was no apparent stabilization of USP5 Zf-UBD in the presence of a RLRGG ubiquitin peptide. Different lengths of ubiquitin peptide were designed to see if increasing the peptide length resulted in a thermal shift for USP5 Zf-UBD in the DSF assay. The rational behind the design of the peptides can be found here.

1. 6-mer peptide: LRLRGG titration

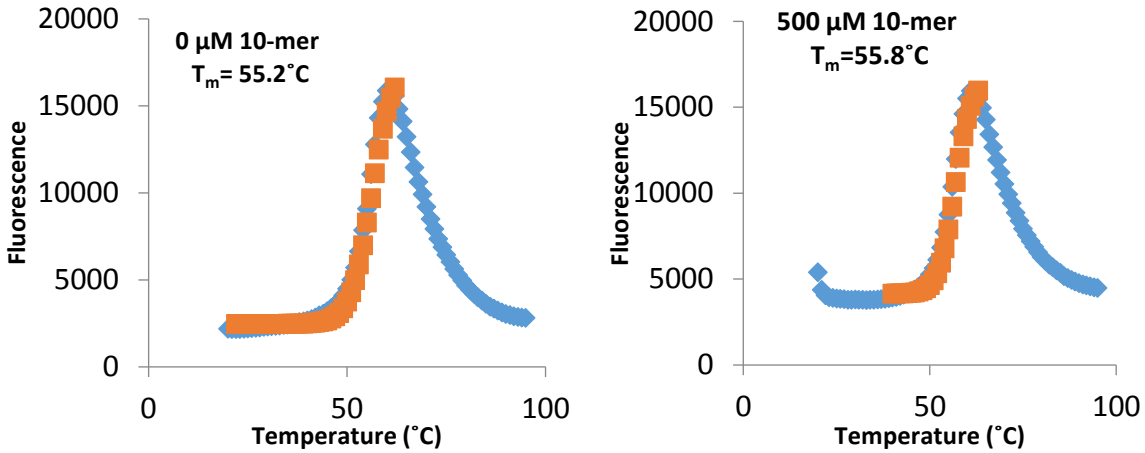
The experiment was performed in a total volume of 20 µL in a white, Roche 384-well PCR plate. Fluorescence was measured using a LightCycler 480 PCR system with excitation and emission spectrum of 465 nm and 580 nm from 20-95˚C. The ramp rate was 4.8; acquisition 6 for 4˚C/min. 2.5 µL of USP5 Zf-UBD171-290 at 3.6 mg/mL (270 µM) and 45x SYPRO orange prepared in 100 mM HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.01% Triton X-100 (v/v) was added to 20 µL of 1.11xLRLRGG 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 30 minutes before fluorescence was measured. The data was processed using Bafcon 6 & Bioactive. Representative regression charts of the highest and lowest LRLRGG peptide concentration are shown. See attached Excel files for raw T_m data.



2. 10-mer peptide: RAHGLRLRGG titration

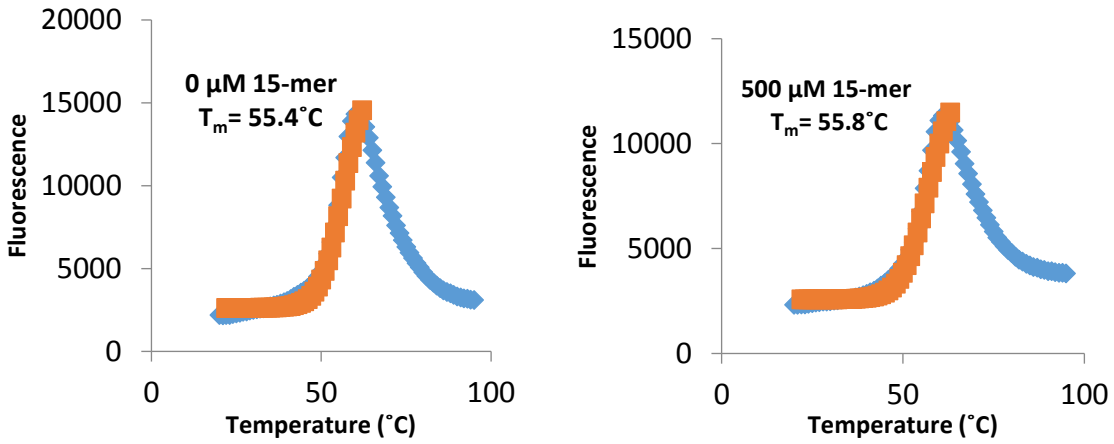
The experiment was performed in a total volume of 20 µL in a white, Roche 384-well PCR plate. Fluorescence was measured using a LightCycler 480 PCR system with excitation and emission spectrum of 465 nm and 580 nm from 20-95˚C. The ramp rate was 4.8; acquisition 6 for 4˚C/min. 2.5 µL of USP5 Zf-UBD171-290 at 3.6 mg/mL (270 µM) and 45x SYPRO orange prepared in 100 mM

HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.01% Triton X-100 (v/v) was added to 20 µL of 1.11xRAHGLRLRGG 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 30 minutes before fluorescence was measured. The data was processed using Bafcon 6 & Bioactive. Representative regression charts of the highest and lowest RAHGLRLRGG peptide concentration are shown. See attached Excel files for raw T_m data.



3. 15-mer peptide: RAHGRAKHGLRLRGG titration

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-UBD171-290 at 3.6 mg/mL (270 µM) and 45x SYPRO orange prepared in 100 mM HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.01% Triton X-100 (v/v) was added to 20 µL of 1.11xRAHGRAKHGLRLRGG 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 30 minutes before fluorescence was measured. The data was processed using Bafcon 6 & Bioactive. Representative regression charts of the highest and lowest RAHGRAKHGLRLRGG peptide concentration are shown. See attached Excel files for raw T_m data.



The T_m data for the peptide titrations is summarized in Table 1.

Table 1. Summary of average Tm of USP5 Zf-UBD with different lengths of ubiquitin peptide

Concentration (µM)	Average Tm ± SD (˚C); n=3 6-mer LRLRGG	Average Tm ± SD (˚C); n=3 10-mer RAHGLRLGG	Average Tm ± SD (˚C); n=3 15-mer RAHGRAKHGLRLRGG
0	55.2 ± 0.0	55.2 ± 0.0	55.3 ± 0.2
0.5	55.2 ± 0.0	55.2 ± 0.0	55.1 ± 0.1
1.0	55.2 ± 0.0	55.1 ± 0.1	55.3 ± 0.1
2.0	55.2 ± 0.0	55.1 ± 0.1	55.3 ± 0.1
3.9	55.3 ± 0.1	55.1 ± 0.1	55.4 ± 0.1
7.8	55.2 ± 0.1	55.1 ± 0.1	55.3 ± 0.0
15.6	55.3 ± 0.1	55.2 ± 0.1	55.4 ± 0.0
31.2	55.2 ± 0.2	55.1 ± 0.1	55.3 ± 0.2
62.5	55.3 ± 0.2	55.3 ± 0.1	55.5 ± 0.2
125.0	55.3 ± 0.1	55.2 ± 0.1	55.3 ± 0.4
250.0	55.5 ± 0.1	55.3 ± 0.1	55.3 ± 0.0
500.0	56.0 ± 0.2	55.9 ± 0.1	55.8 ± 0.1

Conclusions & Future Directions:

Increasing the length of the previously tested RLRGG ubiquitin peptide did not improve the stabilization of the peptide to the USP5 Zf-UBD as no thermal shift is seen with high concentrations (500 µM) of the 6-mer, 10-mer and 15-mer peptide. The DSF assay with the ubiquitin peptides and the Zf-UBD will not be used for screening libraries due to the weak affinity of the peptides against the protein domain. In the future, I'll be using SPR and NMR assays to screen libraries and identify compound hits.