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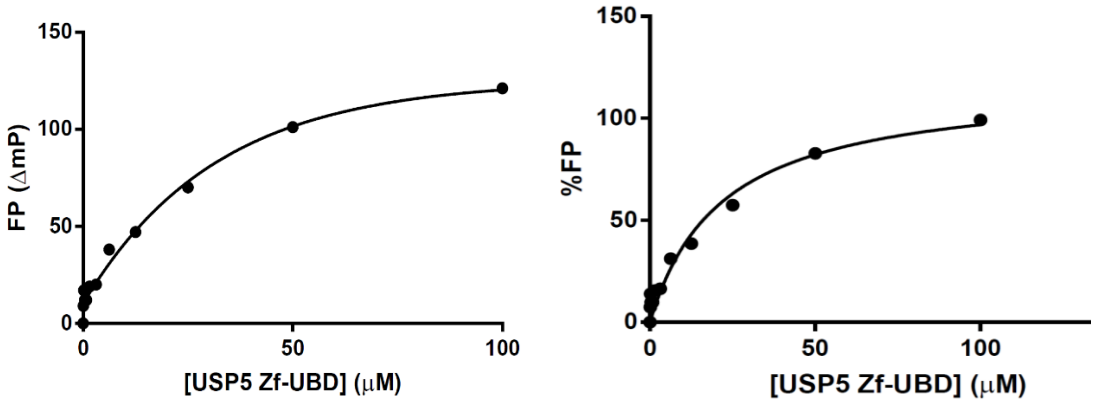
USP5 Zf-UBD Fluorescence Polarization Displacement Assay Optimization

Objectives: Optimizing FP assay conditions for USP5 Zf-UBD with an N-terminal FITC-labeled ubiquitin RLRGG peptide. See previous experiment on the buffer screen here.

Experiments & Results:

1. **[USP5 Zf-UBD] titration** with addition of detergent, 0.01% (v/v) Triton X-100 and reducing agent, 1 mM DTT in buffer to prevent non-specific interactions and protein aggregation

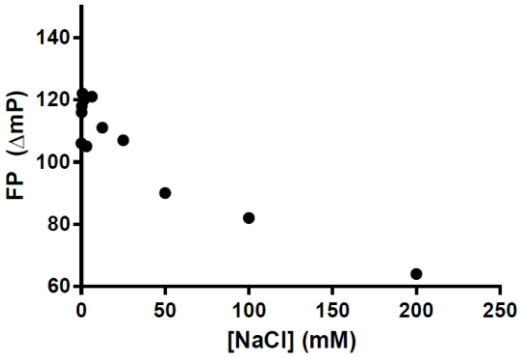
Experiments were completed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM DTT, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=1 in a 1:2 11-step dilution series. Data was processed in Graphpad Prism, non-linear regression with one site-specific binding.

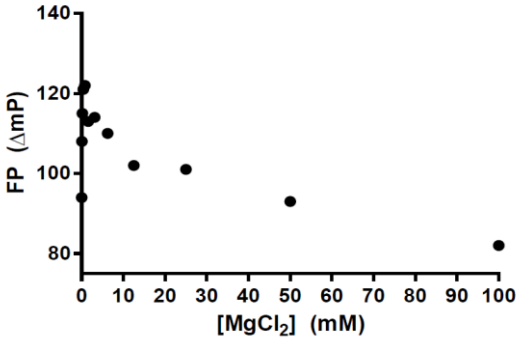


To determine the binding of the fluorescent probe with USP5 Zf-UBD, increasing concentrations of protein were mixed with a fixed concentration of FITC-RLRGG peptide. The K_d was calculated to be 22 ± 5.5 µM. A concentration of 25 µM of USP5 Zf-UBD was selected as it provides ~60% of maximum change in polarization of the FITC-RLRGG peptide. For competition assays, the protein is typically present at a concentration that allows 50-80% of the ligand (FITC-RLRGG) to be bound[1].

2. **NaCl vs. $MgCl_2$ titration** to determine maximal FP dynamic range

Experiments were completed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with 25 µM $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM DTT, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=1 in a 1:2 11-step dilution series. Data was processed in Graphpad Prism.





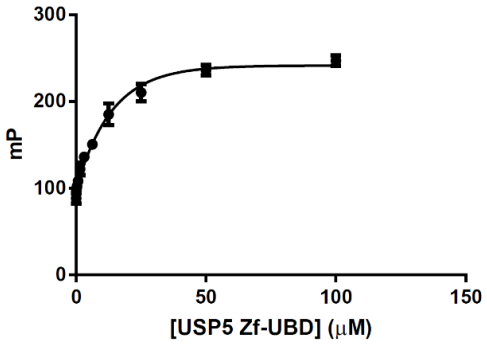
[NaCl] (mM)	mP
0	196
0.195313	206
0.390625	208
0.78125	**212**
1.5625	210
3.125	195
6.25	211
12.5	201
25	197
50	180
100	172
200	154

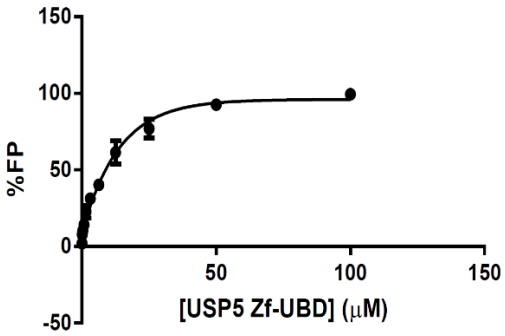
[$MgCl_2$] (mM)	mP
0	184
0.097656	198
0.195313	205
0.390625	211
0.78125	**212**
1.5625	203
3.125	204
6.25	200
12.5	192
25	191
50	183
100	172

Similar mP measurements were seen for both $MgCl_2$ and NaCl. 1 mM $MgCl_2$ was chosen as an initial additive to the buffer to see if it lowered the [USP5 Zf-UBD] required for maximum change in polarization.

3. **USP5 Zf-UBD titration** with buffer condition: 50 mM bis-tris propane pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.01% (v/v) Triton X-100

Experiments were completed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with 25 µM $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=3 in a 1:2 11-step dilution series. Data was processed with Graphpad Prism, non-linear regression with one site-specific binding.

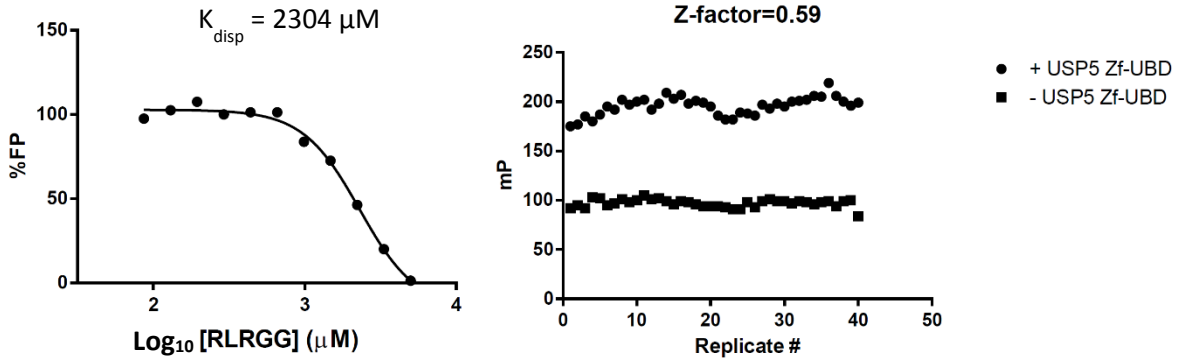




The K_d was calculated to be 8.1 ± 0.84 µM in the buffer condition: 50 mM bis-tris propane pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.01% (v/v) Triton X-100. The K_d is significantly lower with the addition of 1 mM $MgCl_2$. The concentration of the protein used in subsequent experiments was lowered from 25 to 20 µM for maximum change in polarization.

4. **RLRGG titration** in 50 mM bis-tris propane pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.01% (v/v) Triton X-100

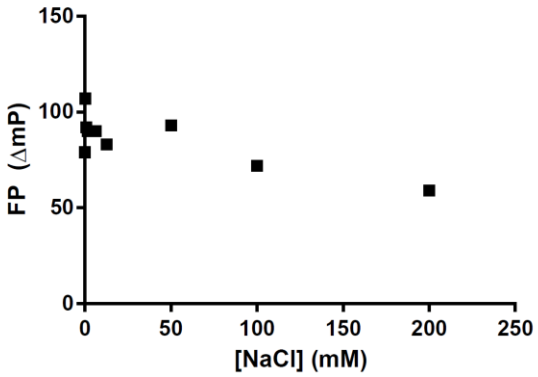
Experiments were completed in a total volume of 20 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with 20 µM $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=1 in a 1:1.5 11-step dilution series. Data was processed in Graphpad Prism using Sigmoidal, 4PL, X is log(Concentration) fit.



A titration of unlabeled RLRGG peptide resulted in a K_{disp} of approximately 2300 µM, showing a weak affinity of USP5 Zf-UBD to the peptide, despite a lower K_d with the addition of $MgCl_2$ to the buffer. This suggests that there may be non-specific binding outside of the ubiquitin binding domain and the calculated K_d may not be accurate. Considering there was no NaCl in the buffer condition, it was hypothesized that the lack of ionic strength in the buffer did now allow for sufficient binding of the peptide to the protein domain. A quick determination of the quality of the FP assay showed a Z-factor greater than 0.5 indicating the assay is okay to use for compound screening; however, since the RLRGG peptide has a weak K_{disp}, there may be non-specific binding outside of the ubiquitin binding pocket which is why this is likely not a good assay condition to be screening compounds. Next, experiments were repeated with the addition of NaCl to the buffer. DTT, due to its instability was replaced by TCEP for ease of use in experiments.

5. **NaCl titration** in 50 mM bis-tris propane pH 7.0, 1 mM TCEP, 0.01% (v/v) Triton X-100/

Experiments were completed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with 20 µM $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM TCEP, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=1 in a 1:2 11-step dilution series. Data was processed in Graphpad Prism.

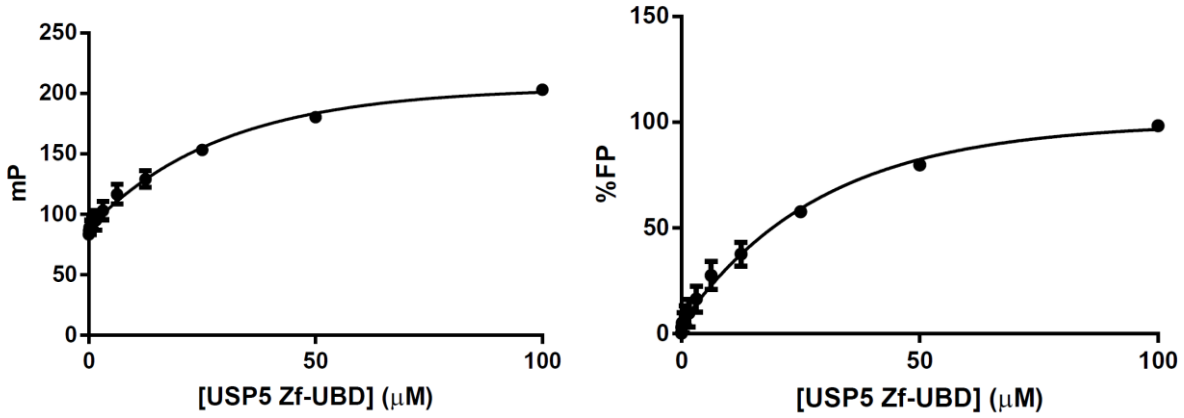


The [NaCl] titration showed a FP range of ~60-100. 50 mM NaCl gave a FP window of ~100 and was added to the buffer condition for further experiments.

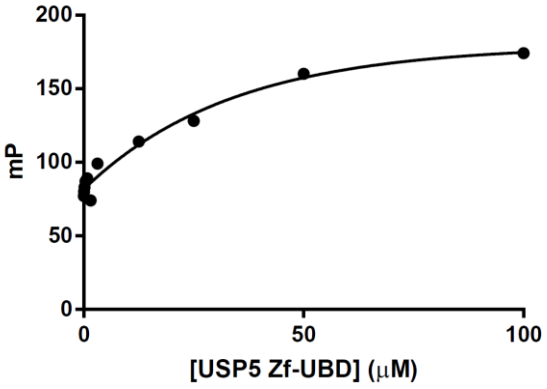
6. **USP5 Zf-UBD titration** in 50 mM bis-tris propane, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100 ± 1 mM $MgCl_2$

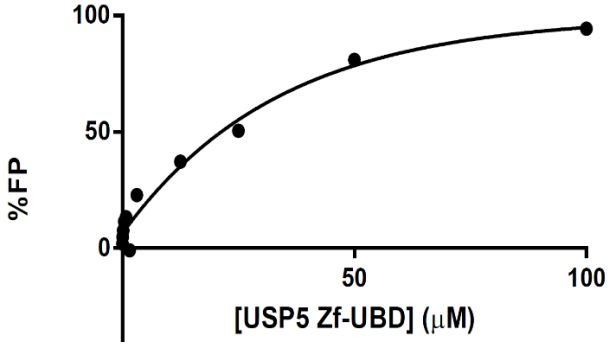
Experiments were completed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100 ± $MgCl_2$. Experiments completed in replicate n=3 in a 1:2 11-step dilution series. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Data was processed in Graphpad Prism, non-linear regression with one site-specific binding.

50 mM bis-tris propane, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100:



50 mM bis-tris propane, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100, 1 mM MgCl2:

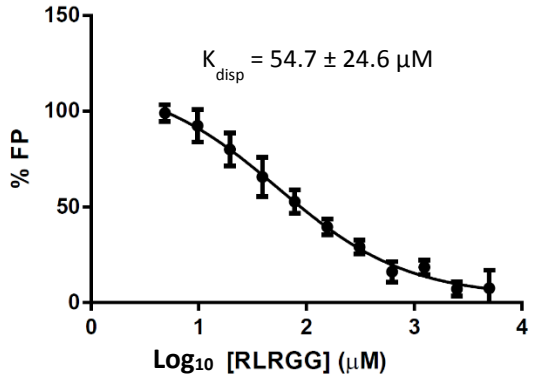


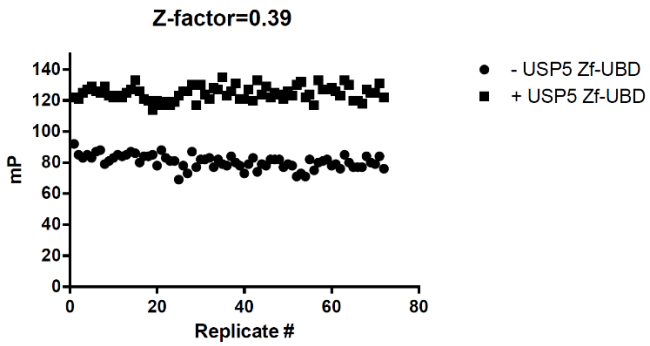


The K_d with no $MgCl_2$ in the buffer was 25 ± 2.6 µM and with 1 mM $MgCl_2$ in the buffer the K_d is 27 ± 9.0 µM. The addition of 1 mM $MgCl_2$ did not significantly change the [USP5 Zf-UBD] that gave ~60% change in polarization with FITC-RLRGG which is ~30 µM in the buffer condition 50 mM bis-tris propane pH 7.0, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100. For this reason, 1 mM $MgCl_2$ was not added to the final buffer condition.

7. **RLRGG peptide titration** in 50 mM bis-tris propane, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100

Experiments were completed in a total volume of 20 µL in 384-well black polypropylene PCR plates (Axygen). FP was measured using Biotek Synergy 4. The excitation and emission wavelengths were 485 and 528 nm respectively. Reactions were completed with 20 µM $USP5^{171-290}$, 40 nM FITC-RLRGG in 50 mM bis-tris propane pH 7.0, 1 mM MgCl2, 1 mM DTT, 0.01% (v/v) Triton X-100. Following a 1 minute centrifugation step at 800 RPM and incubation at room temperature for 30 minutes, FP was measured. Experiments completed in replicate n=3 in a 1:2 11-step dilution series. Data was processed in Graphpad Prism, Sigmoidal, 4PL, X is log(concentration) fit.





The K_{disp} of ~55 µM of the FITC-RLRGG to the USP5 Zf-UBD more accurately corresponds to the [USP5 Zf-UBD] that gives maximal change in polarization; however, the Z-factor of the assay is below 0.5 indicating this assay is not ideal for screening compounds.

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Conclusions & Future Directions:

At this time, the buffer condition: 50 mM bis-tris propane, 50 mM NaCl, 1 mM TCEP, 0.01% (v/v) Triton X-100 allows for sufficient RLRGG peptide binding to USP5 Zf-UBD (K_{disp} ~ 55 µM) but decreases the FP window (~60-70). NaCl was needed in the buffer condition for proper binding of the peptide to the protein domain. In the future, I will be testing different lengths of ubiquitin peptides. I hope one of these peptides will have a higher affinity for USP5 Zf-UBD as well as maintain a FP range of ~80-100.

References:

[1]Hall, M., Yasgar, A., Peryea, T., Braisted, J., Jadahv, A., Simeonav, A., & Coussens, N. Fluorescence polarization assays in high-throughput screening and drug discovery: a review. *Methods Appl Fluoresc.* 2016, **4**(2): 022001.