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2018/09/21

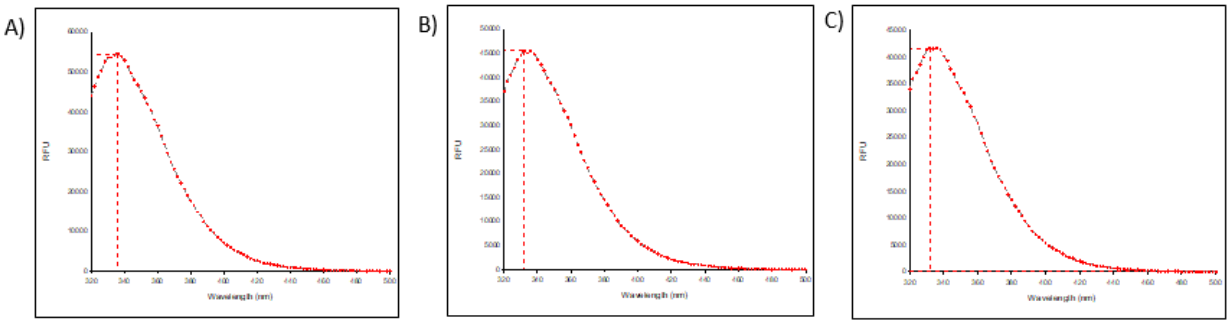
Developing a Tryptophan Fluorescence Assay for Screening Ligands against USP5 Zf-UBD

Objective: to develop a fluorescence based assay by measuring changes in UV tryptophan fluorescence of USP5 zinc finger ubiquitin binding domain (Zf-UBD) with addition of ligands.

Experiment & Results:

1. Emission Spectral Scan:

   Experiment was completed in a total volume of 10 µL in a 384-well black polypropylene PCR plate (Axygen). Fluorescence intensity was measured using Biotek Synergy H1 microplate reader (Biotek). 20 µM USP5 Zf-UBD$^{171-290}$ was prepared in three different buffers: A) 1xPBS, 0.005% Tween-20 (v/v), 1 mM TCEP B) 20 mM Hepes pH 7.4, 0.005% Tween-30 (v/v), 1 mM TCEP C) 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 1 mM TCEP. The following parameters were used for the emission spectral scan:
   - Start λ: 320 nm
   - End λ: 500 nm
   - Step: 2 nm
   - Gain: 100
   - Read speed: normal
   - Read height: 9.5 mm



	Maximum RFU	Emission Wavelength (nm)
Buffer A	54580	335
Buffer B	45560	332
Buffer C	41597	332

Figure 1. Emission spectral scan of USP5 Zf-UBD in buffers A) 1xPBS, 0.005% Tween-20, 1 mM TCEP B) 20 mM Hepes pH 7.4, 0.005% Tween-20, 1 mM TCEP C) 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 1 mM TCEP

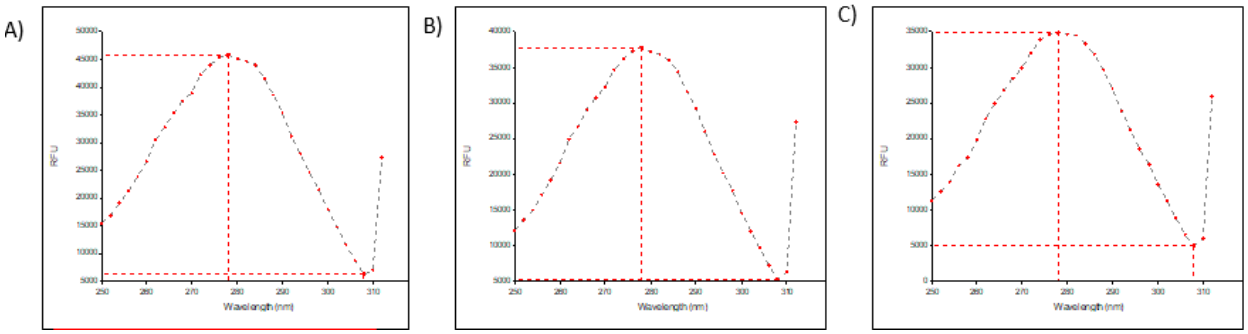
The maximum fluorescence intensity was seen with an emission wavelength of 335 nm with buffer A.

2. Excitation spectral scan:

   Experiment was completed in a total volume of 10 µL in a 384-well black polypropylene PCR plate (Axygen). Fluorescence intensity was measured using Biotek Synergy H1 microplate reader (Biotek). 20 µM USP5 Zf-UBD$^{171-290}$ was prepared in three different buffers: A) 1xPBS, 0.005%

Tween-20 (v/v), 1 mM TCEP B) 20 mM Hepes pH 7.4, 0.005% Tween-30 (v/v), 1 mM TCEP C) 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 1 mM TCEP. The following parameters were used for the excitation spectral scan:

- Emission λ=335 nm
- Excitation start λ=250 nm
- Excitation end λ=320 nm
- Step: 2 nm
- Read speed: normal
- Read height: 9.50



	Maximum RFU	Excitation Wavelength (nm)
Buffer A	45851	278
Buffer B	37772	278
Buffer C	34929	278

Figure 2. Excitation spectral scan of USP5 Zf-UBD in buffers A) 1xPBS, 0.005% Tween-20, 1 mM TCEP B) 20 mM Hepes pH 7.4, 0.005% Tween-20, 1 mM TCEP C) 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 1 mM TCEP

Buffer A had the highest maximum fluorescence intensity with an emission and excitation wavelength of 335 nm and 278 nm respectively.

3. USP5 Zf-UBD Concentration Curve

Experiment was completed in a total volume of 10 µL in a 384-well black polypropylene PCR plate (Axygen). Fluorescence intensity was measured using a Biotek Synergy H1 Multimode microplate reader with an excitation and emission wavelengths of 278 and 335 nm respectively. 100 µM USP5 Zf-UBD$^{171-290}$ was prepared in Buffer A with a 1:2.5 12-pt dilution series (n=2).

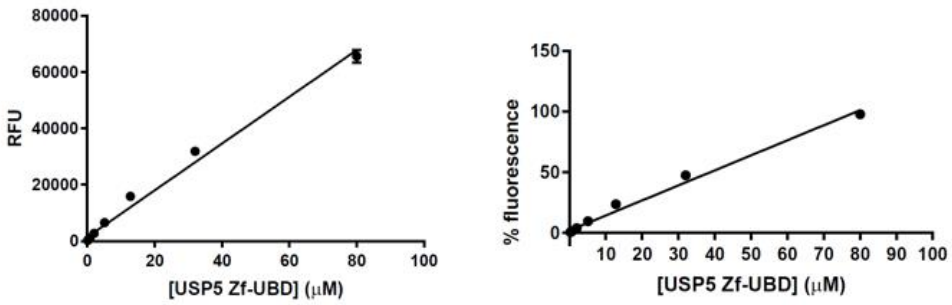


Figure 3. USP5 Zf-UBD fluorescence intensity measurements

As expected, as the concentration of USP5 Zf-UBD increases, the fluorescence intensity increases linearly. In other words, as the number of aromatic amino acids increases, the fluorescence intensity also increases.

4. LRLRGG titration

Experiment was completed in a total volume of 10 µL in a 384-well black polypropylene PCR plate (Axygen). Fluorescence intensity was measured using a Biotek Synergy H1 Multimode microplate reader with an excitation and emission wavelength of 278 and 335 nm respectively. A 1:2.5-12 point dilution series of a ubiquitin peptide, LRLRGG with 40 µM USP5 Zf-UBD$^{171-290}$ was prepared in Buffer A and fluorescence measured after 30 minute incubation at room temperature (n=2). (Figure 4).

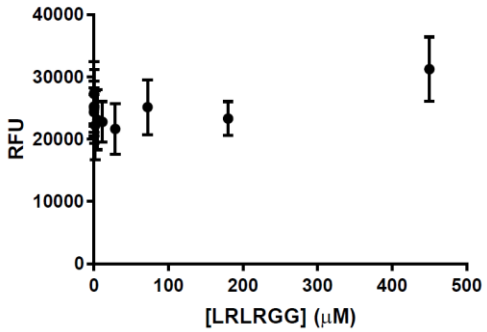


Figure 4. 1:2.5 12 point dilution series of a ubiquitin peptide, LRLRGG

A clear relationship between the ubiquitin peptide, LRLRGG and USP5 Zf-UBD fluorescence could not be discerned, most likely because the fluorescence signal is reaching saturation. For this reason, the experiment was repeated with 10 µM USP5$^{171-290}$ (Figure 5).

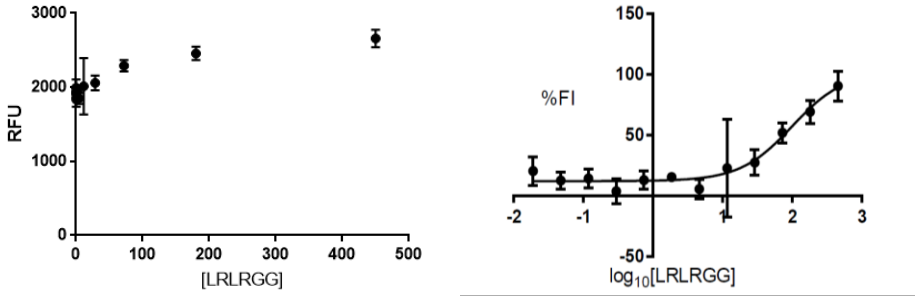


Figure 5. 1:2.5 12 pt dilution series LRLRGG and 10 µM USP5$^{171-290}$

As the concentration of LRLRGG increases, an increase in fluorescence is observed. The calculated K_D was 99 µM; however, the binding curve does not reach saturation and therefore it not an accurate K_D. Please note this was a preliminary experiment to determine whether a change in fluorescence could be observed. This data was not blank subtracted to account for the LRLRGG peptide fluorescence or inner filter effect phenomena. This experiment was repeated with the controls in experiment 5 below.

5. Compound Screening

Experiment was completed in a total volume of 10 µL in a 384-well black polypropylene PCR plate (Axygen). Fluorescence intensity was measured using a Biotek Synergy H1 Multimode microplate reader with an excitation and emission wavelength of 278 and 335 nm respectively. A 1:2.5-12 point dilution series of compound, with 10 µM USP5 Zf-UBD$^{171-290}$ was prepared in Buffer A + 1% DMSO (v/v) and fluorescence measured after 30 minute incubation at room temperature (n=2). Data was analyzed with GraphPad Prism.

I previously used ^{19}F-NMR spectroscopy for ligand screening, where ligand binding could be detected by structural perturbations in the resonance of a fluorinated tryptophan at the binding pocket. The ligands that showed binding in the ^{19}F NMR assay were used in the tryptophan fluorescence assay.

Compound titrations in buffer only were used for blank subtraction to eliminate the fluorescence of the compounds from measured fluorescence of the protein + compound.

Compound titrations with N-acetyl L-tryptophanamide (NATA) (Sigma Aldrich), a tryptophan analog was used as a control for the inner filter effect (IFE) (Figure 6).

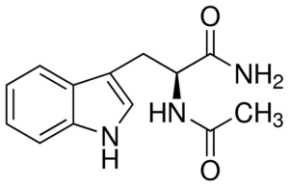


Figure 6. Structure of N-acetyl L-tryptophanamide

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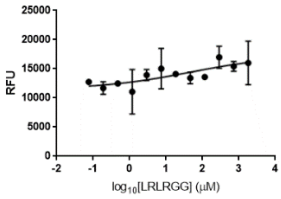
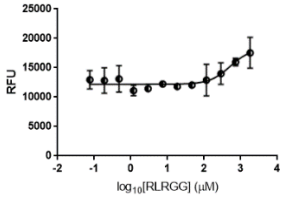
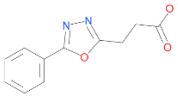
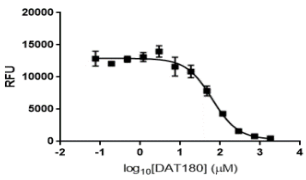
The inner filter effect is the dependence of the fluorescence intensity on the concentration of the substrate[1], where the primary IFE is caused by "fluorescence that is not uniformly distributed due to the excitation radiation which is absorbed by the ligand"[2]. In other words, the compound and protein both absorb at the excitation wavelength and the absorbance of the compound decreases emission of the protein. Theoretically, no change in fluorescence should be observed with compound titrations with the same molar concentration of NATA as $USP5^{171-290}$ (i.e. 10 µM) as the compounds are not expected to bind NATA. If a change in fluorescence is observed, this can be attributed to the inner filter effect.

There are several mathematical models used to correct for the IFE due to its complexity. For my purposes, I used the IFE correction factor: $W= F(\lambda_{ex})/F_o(\lambda_{ex})$[1] where F is the fluorescence intensity excited at λ_{ex}[1]. For NATA, $W=F(\lambda_{ex})_{NATA}/F_o(\lambda_{ex})_{NATA+ligand}$ was applied for each compound titration concentration. There may be better suited mathematical models to account for the IFE for testing ligands with $USP^{171-290}$ in this assay format, though due to my limited knowledge on this complex matter I cannot with absolute certainty state the method I used for IFE correction is the correct one. For those with more knowledge on this matter, please feel free to contact me with alternative suggestions.

The results of compound screening are summarized in Table 1. DAT192 and DAT199 were used as negative controls, as they showed no binding in a previous ^{19}F NMR assay. Please see the attached GraphPad Prism file to see fluorescence intensity data.

Compound Name	Compound Structure	KD IFE corrected (µM) (n=2)		IFE (Y/N)
LRLRGG		NC		N
RLRGG		~500		N
DAT180		62 ± 17		N

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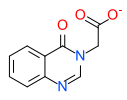
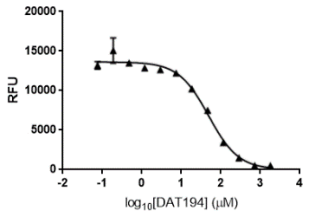
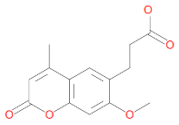
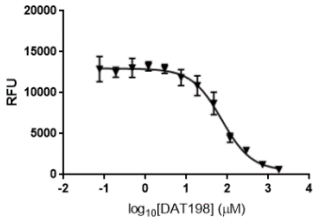
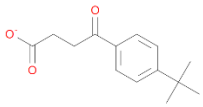
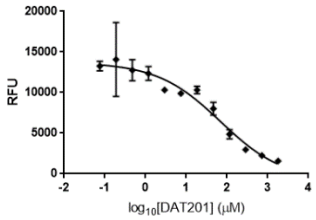
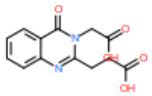
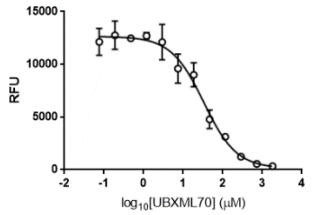
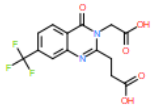
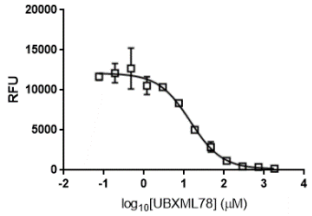
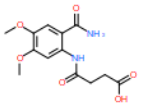
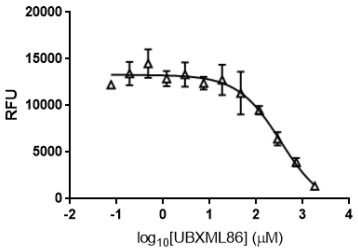
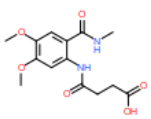
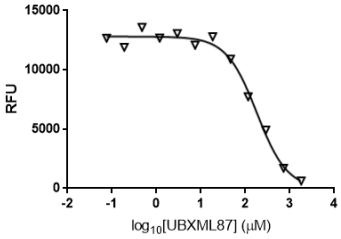
DAT194		49 ± 12		N
DAT198		72 ± 24		Y
DAT201		~ 70		Y
UBXML70		33 ± 13		N
UBXML78		14 ± 5		N
UBXML86		~ 332		Y
UBXML87		180 ± 93		N

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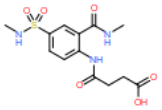
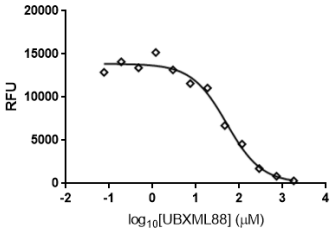
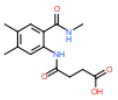
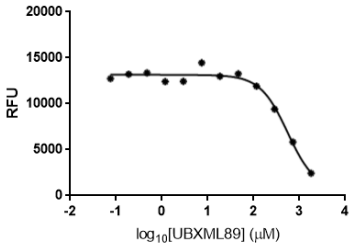
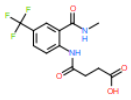
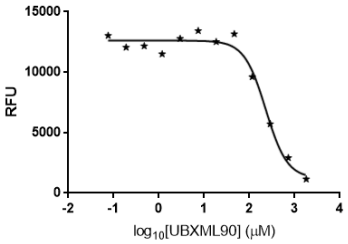
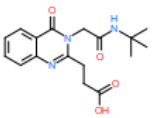
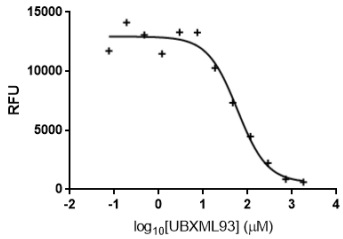
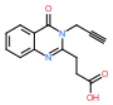
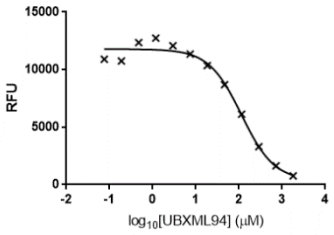
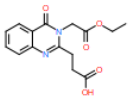
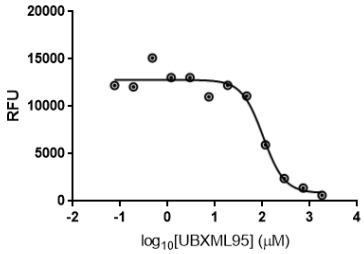
UBXML88		51 ± 29		N
UBXML89		~ 552		N
UBXML90		~ 229		N
UBXML93		~ 58		N
UBXML94		~ 114		N
UBXML95		~ 103		N

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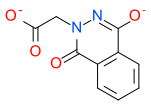
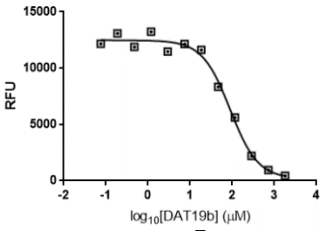
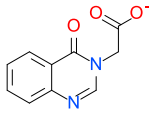
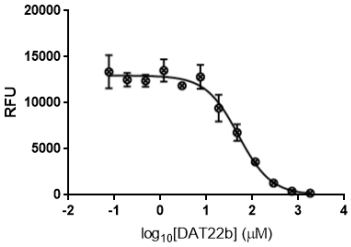
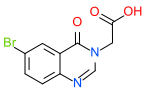
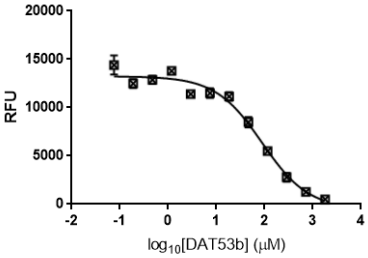
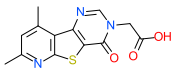
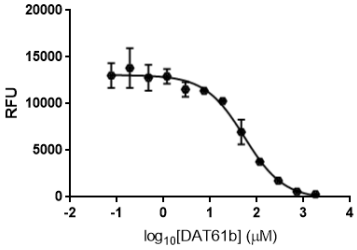
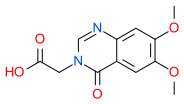
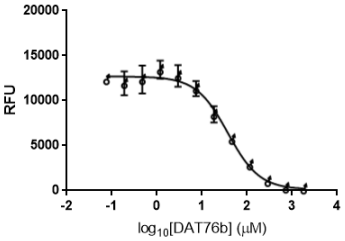
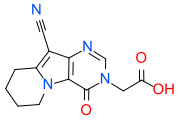
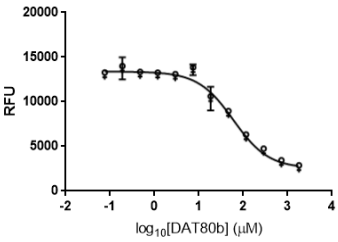
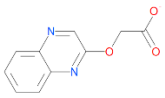
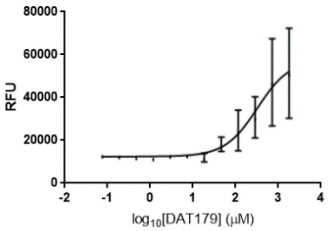
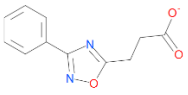
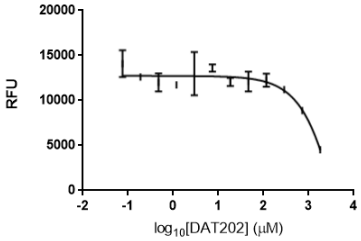
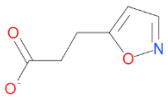
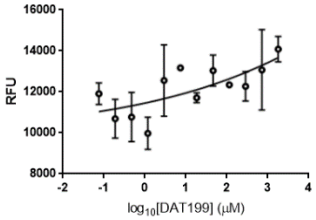
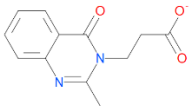
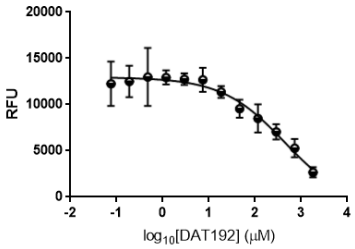
DAT19b		~ 89		N
DAT22b		49 ± 16		N
DAT53b		89 ± 51		Y
DAT61b		54 ± 24		N
DAT76b		37 ± 11		N
DAT80b		60 ± 22		Y

DAT179		~ 326		Y
DAT202		NC		N
DAT199		NC		N
DAT192		~ 436		N

Conclusions & Future Directions:

Using USP5$^{171-290}$ intrinsic fluorescence, I hoped to detect changes in fluorescence when ligands were bound to the ubiquitin binding pocket of USP5, due to structural perturbations of the aromatic amino acids within the pocket.

The ubiquitin peptides, RLRGG and LRLRGG were used as positive controls for USP5. Although there is a slight increase in fluorescence as the concentration of the ubiquitin peptide increases, binding affinity could not be measured. This could be due to the low affinity of the peptide to the protein domain. Interestingly, while the ubiquitin peptides showed an increase in fluorescence, all the compounds showed a decrease in fluorescence as the concentration of the compounds increased. I am not sure why there is difference in the change in the fluorescence of the peptides versus the compounds, as both the peptide and the compounds showed perturbations of the tryptophan in the binding pocket with the ^{19}F NMR assay.

The IFE was very evident for some of the compounds, as at high concentrations there was a marked decrease in NATA fluorescence (DAT198, DAT201, UBXML86, DAT53b, DAT80b, DAT179). The negative

controls, DAT199 and DAT 192 showed little to no binding which is encouraging that the tryptophan fluorescence assay can be used to detect ligand binding; however, it is difficult to conclude that the binding affinities of the ligands are accurate due to the number of questions that have arisen. **Anyone with expertise on this assay, could you please advise?**

1) Why is there a difference in the change of fluorescence with ubiquitin peptides vs. small molecule compounds?

2) Was the IFE correction method used the right mathematical model for this assay?

3) Are the binding affinities measured for the small molecule compounds accurate, especially for those compounds that display the IFE?

As of right now, it doesn't seem like I'll be using this assay to screen compounds as I am uncertain how to interpret the data. In the future, I will be using a surface plasmon resonance (SPR) to measure the binding affinities of all these compounds. It will be interesting to see if the binding affinities measured by tryptophan fluorescence can be correlated to SPR binding affinities; if so, perhaps the tryptophan fluorescence assay will be a viable screening assay. Nonetheless, for USP5 I have already developed a ^{19}F NMR spectroscopy assay for qualitative screening and SPR for quantitative validation, which seems to be working well.

References:

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[2] Wang, T., Zeng, L., & Li, D. *A review on the methods for correcting the fluorescence inner-filter effect of fluorescence spectrum.* Applied Spectroscopy Reviews, 2017: **52** (10).

[3]Epps, D., Raub, T., Caiolfa, V., Chiari, A., & Zamai, M. *Determination of the Affinity of Drugs toward Serum Albumin by Measuremnet of the Quenching of the Intrinsic Tryptophan Fluorescence of the Protein*. J. Pharm. Pharmacol, 1999. **51**: 41-48.