Purification
+
Assay Development of Janus Kinase 2

Elliot Dean 17 April 2019 Lab Meeting / Project Updates

High-Throughput Screening Core
Perelman School of Medicine, University of Pennsylvania

Why do we care about Janus Kinase 2?

Janus Kinase 2 (JAK2) is a non-receptor tyrosine kinase involved in a number of different signaling cascades (type II cytokine receptor family, gp130 receptor family, GM-CSF receptor family, and the single chain receptors)

Knock out of JAK2 in mice is lethal by day 12

Aberrant fusions of JAK2 with other genes can be seen in some Leukemia cases

Mutations in JAK2 (specifically V617F) are implicated in polycythemia vera, thrombocythemia, and other myeloproliferative disorders

Goals of the project

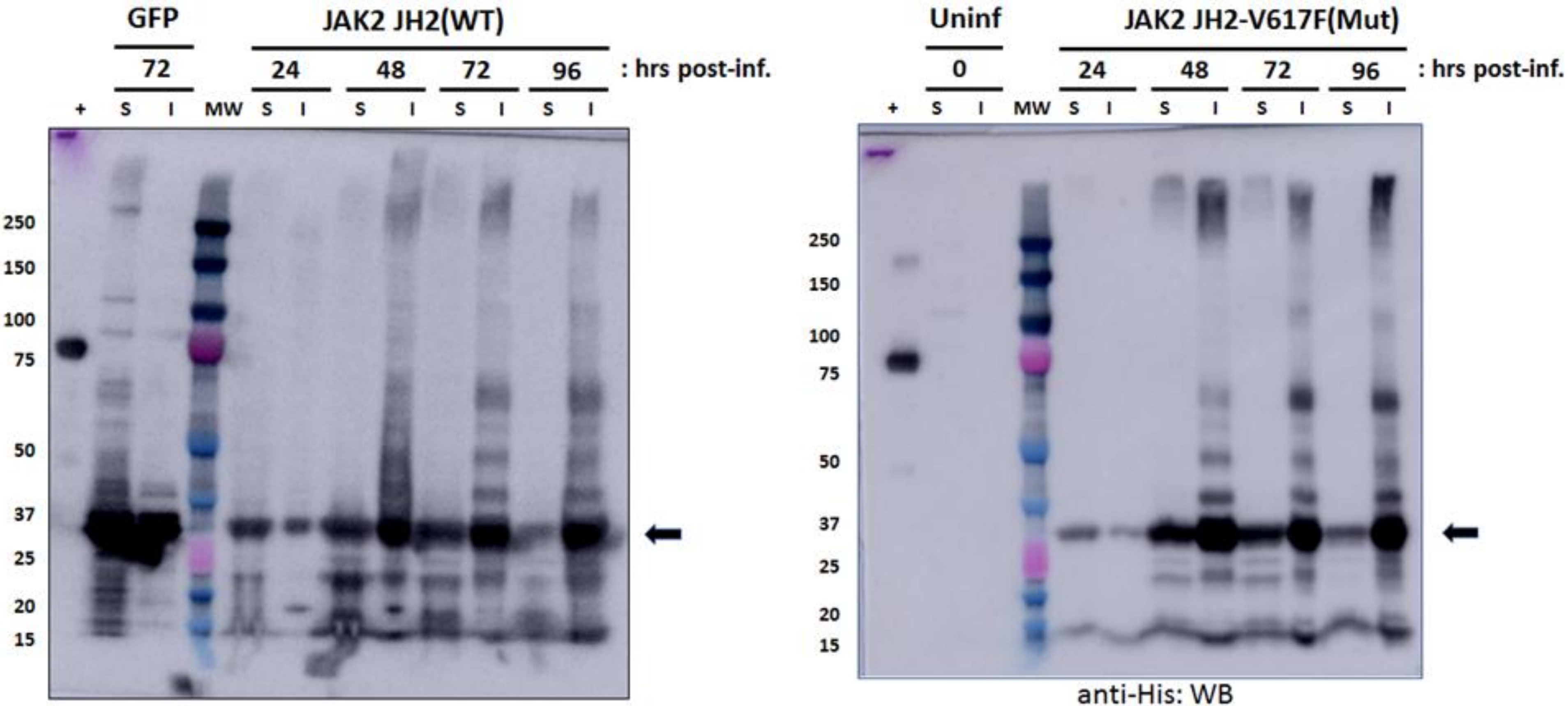
To successfully express JAK2_JH2 Wild-Type and Mutant (V617F) in Insect Cells

To purify both proteins, first by affinity column (Histidine affinity, TALON), followed by size exclusion chromatography

To obtain high purity, concentrated protein for assays (thermal shift / thermofluor)

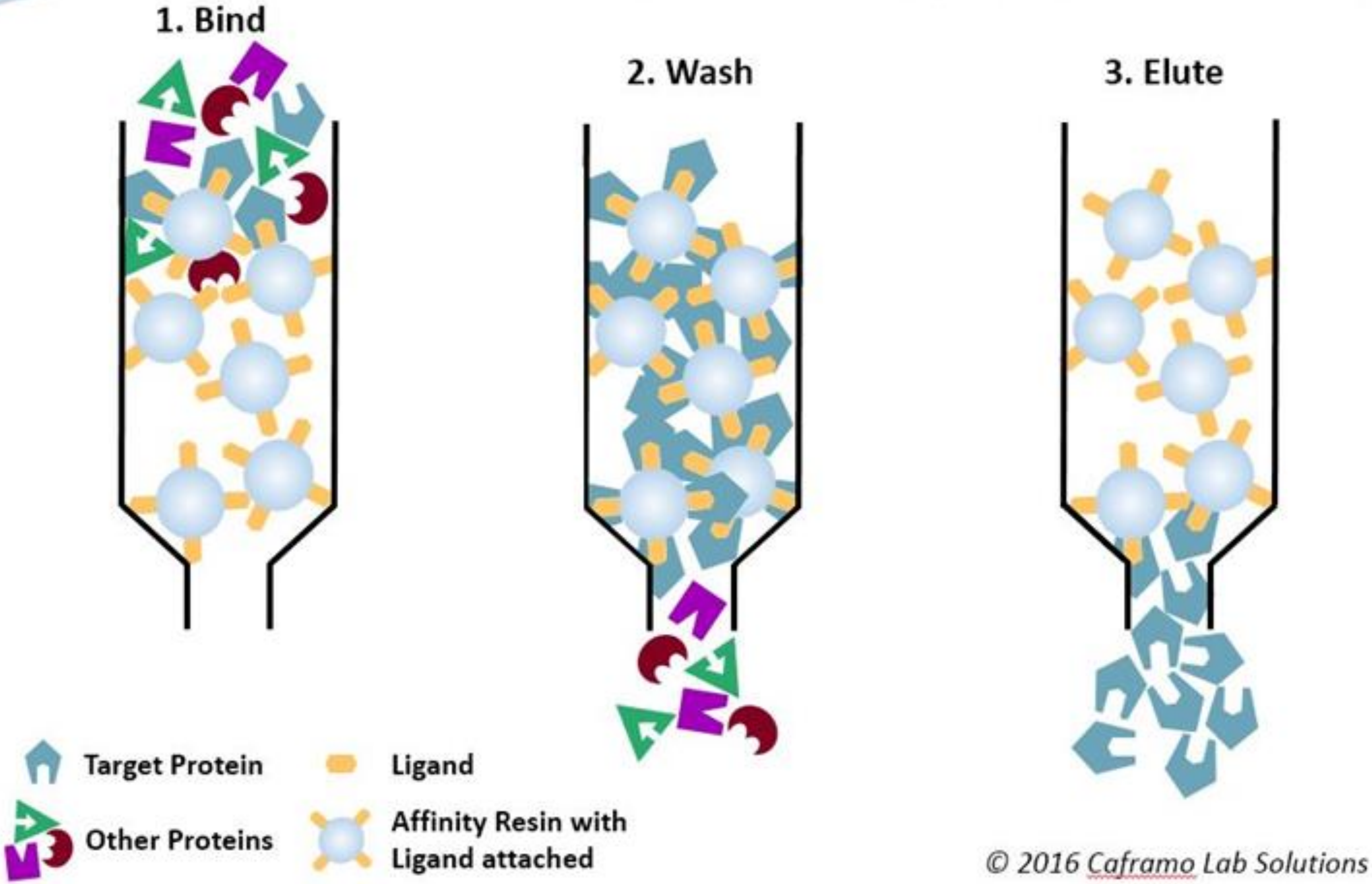
To screen against libraries of compounds that might inhibit the function of the kinase

Step One: Expression Confirmed

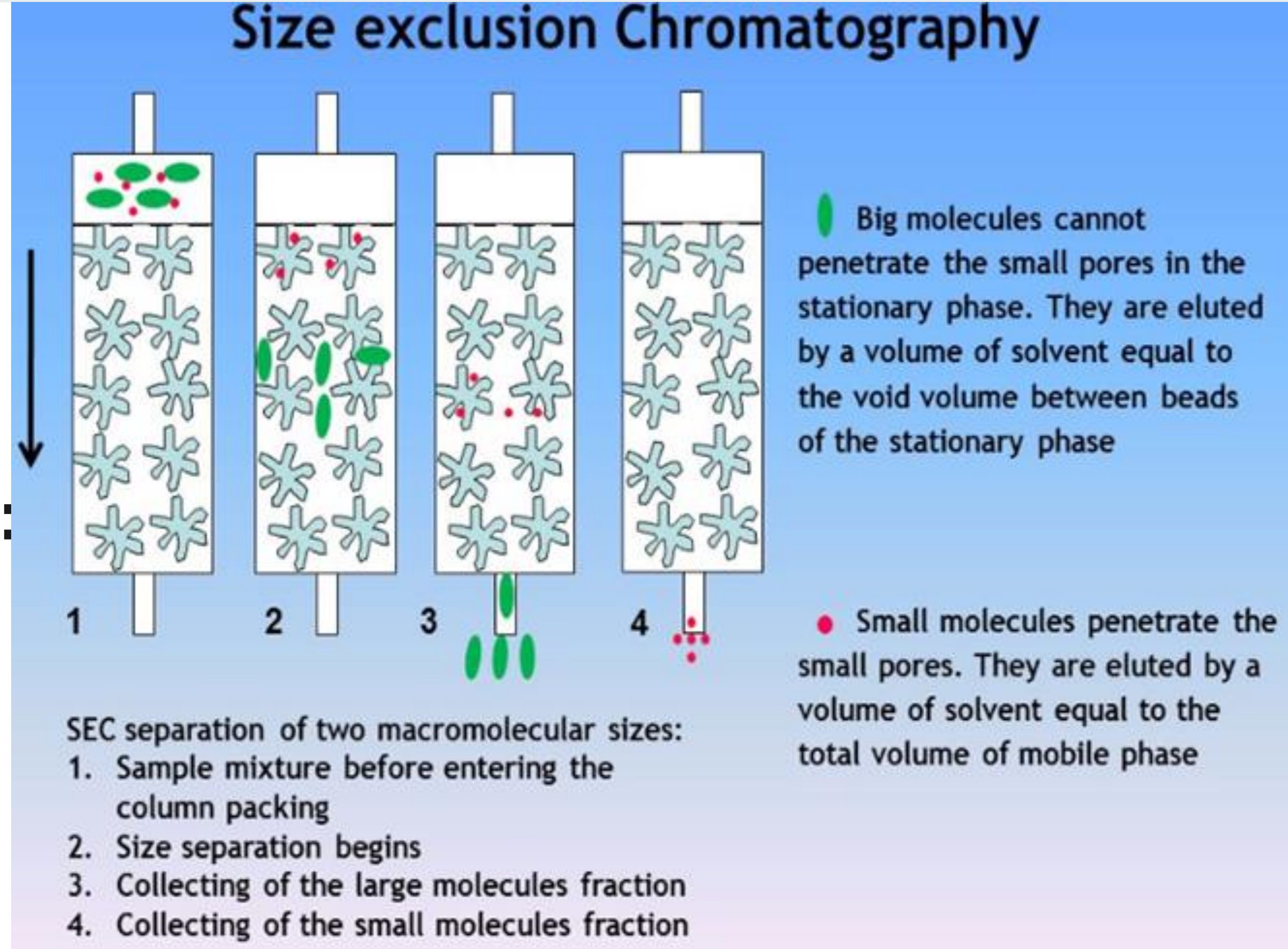


anti-His: WB

Protein Affinity Chromatography



Size Exclusion Chromatography: A Schematic



Size exclusion Chromatography

⬮ Big molecules cannot penetrate the small pores in the stationary phase. They are eluted by a volume of solvent equal to the void volume between beads of the stationary phase

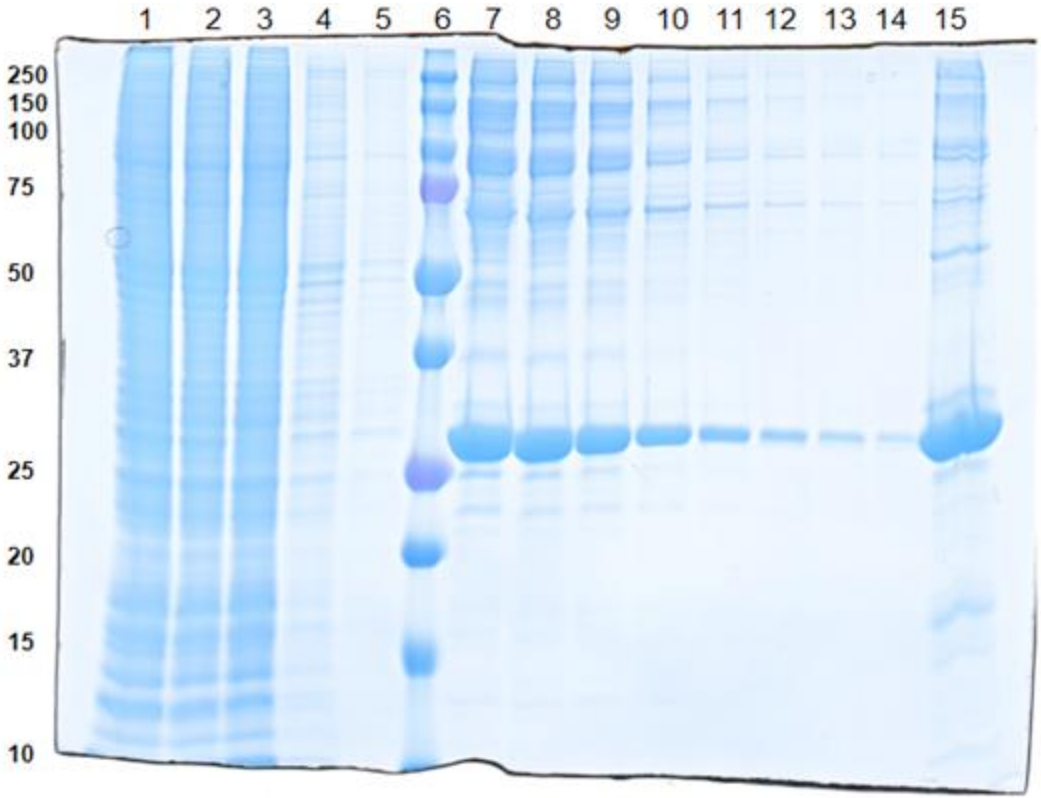
● Small molecules penetrate the small pores. They are eluted by a volume of solvent equal to the total volume of mobile phase

SEC separation of two macromolecular sizes:

1. Sample mixture before entering the column packing
2. Size separation begins
3. Collecting of the large molecules fraction
4. Collecting of the small molecules fraction

Large Scale Purification Yields Little Protein, but Viable for Assays

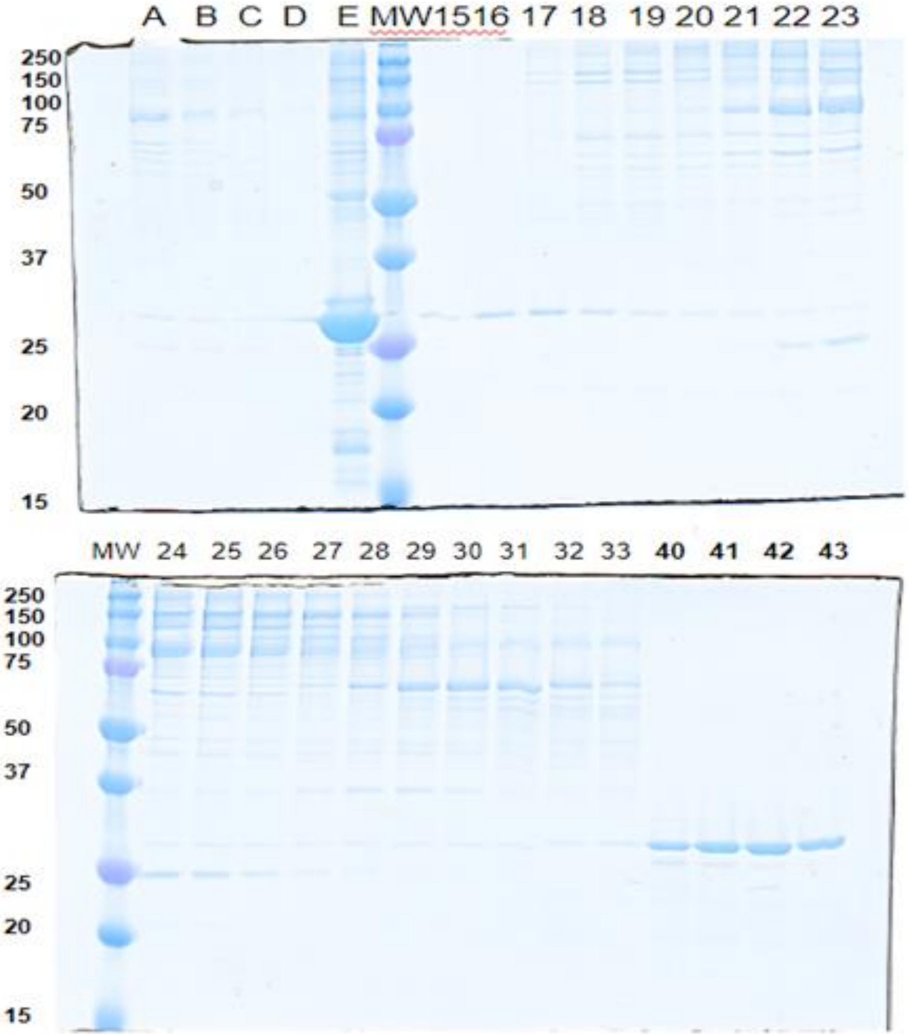
Human JAK2-JH2
Mutant V617F
Codon Optimized
(Epoch)
Purification 10
Start→
Talon nickel affinity
Coomassie gel
2/21/2019



1- Total cell SF9
2- Soluble
3- Flow Through
4- Wash 1 (5mM Imidazole)
5- Wash 2 (5mM Imidazole)
6- MW Marker
7- Elution 1 (250mM Imidazole)
8- Elution 2
9- Elution 3
10- Elution 4
11- Elution 5
12- Elution 6
13- Elution 7
14- Elution 8
15-TALON Beads

UNICORN 6.3
User: Default 2/22/2019 4:07:56 PM -08:00
Run By : Default 2/22/2019 11:04:51 AM -08:00
Result: 190222_jak2mutrun1_S200(125mL) Run ERD 001 001

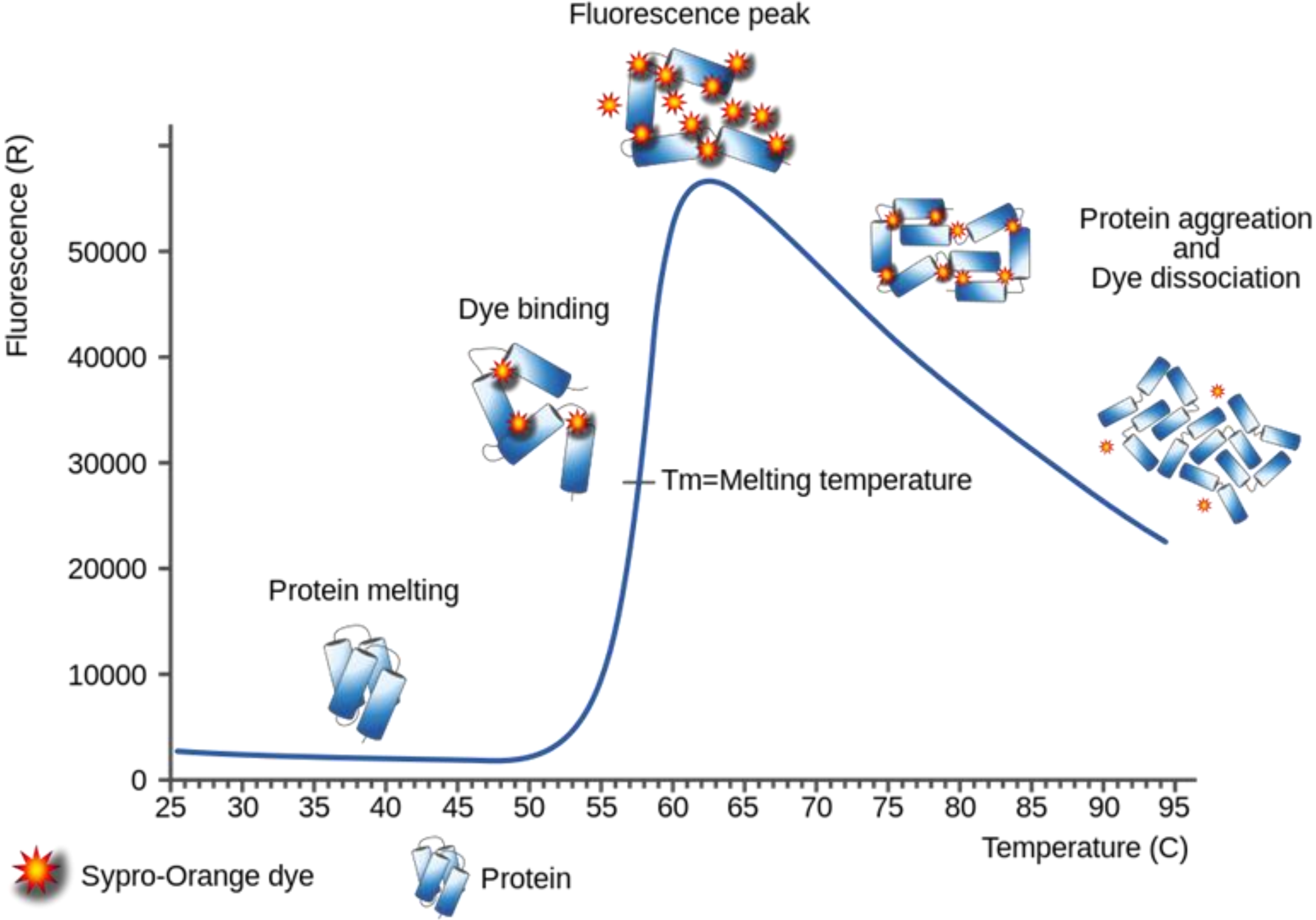
Human JAK2-JH2 V617F Codon Optimized (Epoch) Purification 10 S200 (120mL) Coomassie gel 2/21/2019

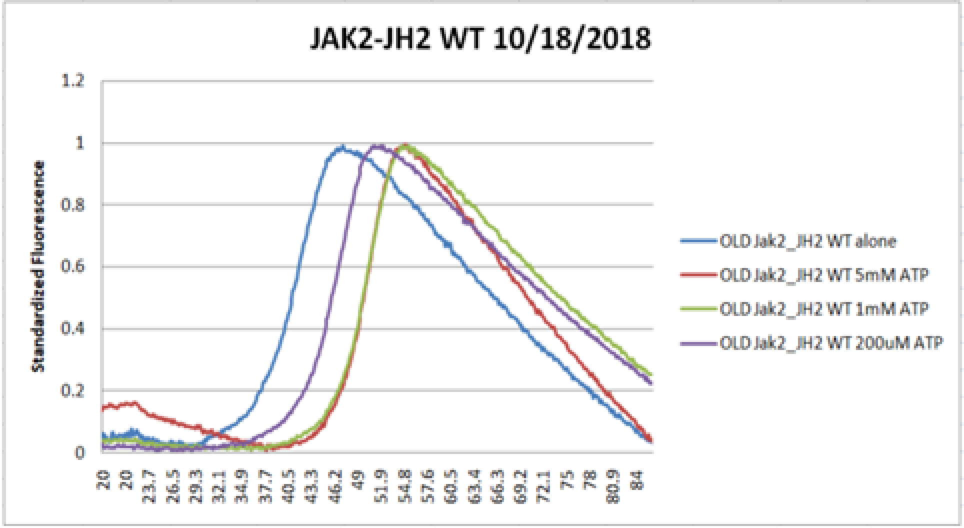


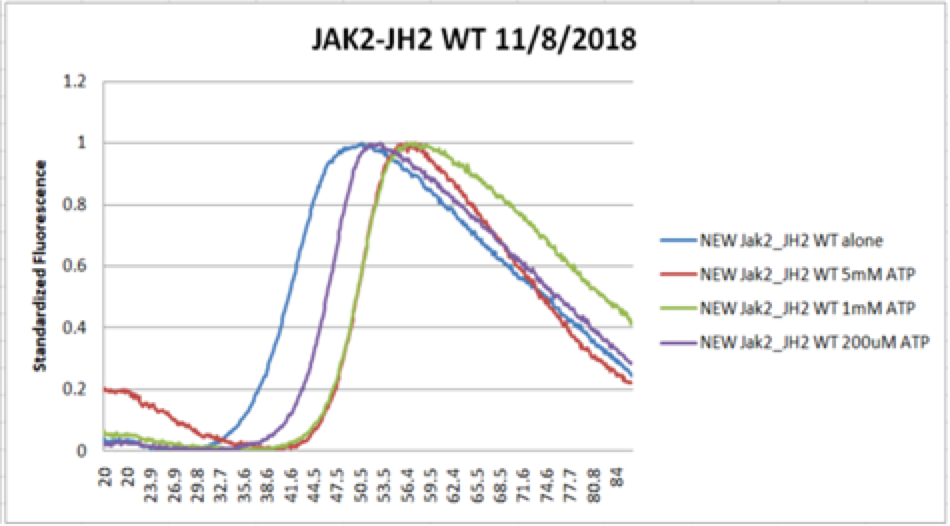
A-Elution 10 (400mM Imidazole)
B-Elution 11
C-Elution 12
D-Elution 13
E-Beads

Labeled Fraction #

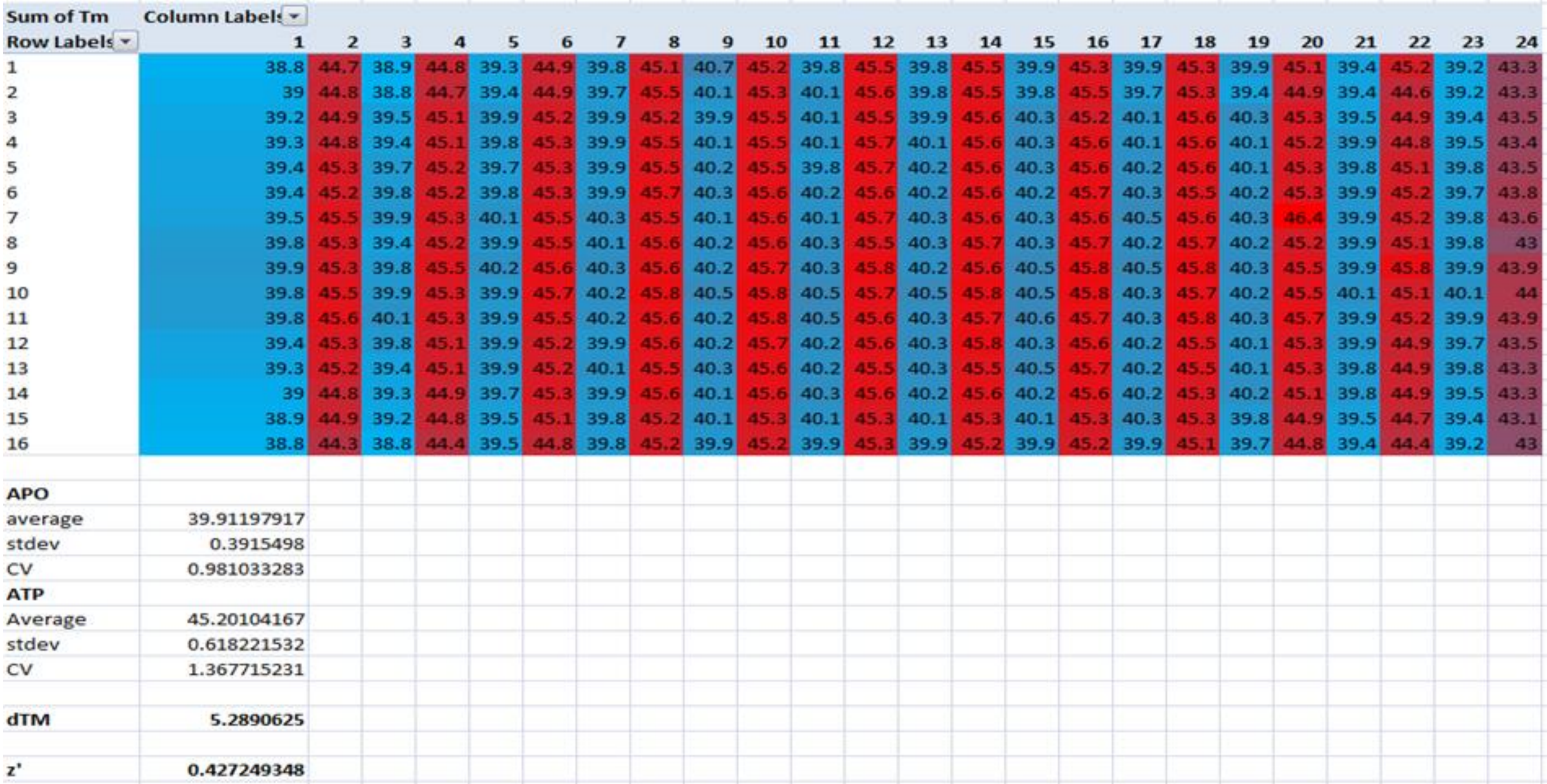
Thermal Shift Assays


Fluorescence peak
Fluorescence (R)
50000
40000
30000
20000
10000
0
Dye binding
Protein aggregation
and
Dye dissociation
Tm=Melting temperature
Protein melting
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
Temperature (C)
Sypro-Orange dye
Protein


JAK2-JH2 WT 10/18/2018
Standardized Fluorescence
1.2
1
0.8
0.6
0.4
0.2
0
20
20
23.7
26.5
29.3
32.1
34.9
37.7
40.5
43.3
46.2
49
51.9
54.8
57.6
60.5
63.4
66.3
69.2
72.1
75
78
80.9
84
OLD Jak2_JH2 WT alone
OLD Jak2_JH2 WT 5mM ATP
OLD Jak2_JH2 WT 1mM ATP
OLD Jak2_JH2 WT 200uM ATP


JAK2-JH2 WT 11/8/2018
Standardized Fluorescence
1.2
1
0.8
0.6
0.4
0.2
0
20
20
23.9
26.9
29.8
32.7
35.6
38.6
41.6
44.5
47.5
50.5
53.5
56.4
59.5
62.4
65.5
68.5
71.6
74.6
77.7
80.8
84
NEW Jak2_JH2 WT alone
NEW Jak2_JH2 WT 5mM ATP
NEW Jak2_JH2 WT 1mM ATP
NEW Jak2_JH2 WT 200uM ATP

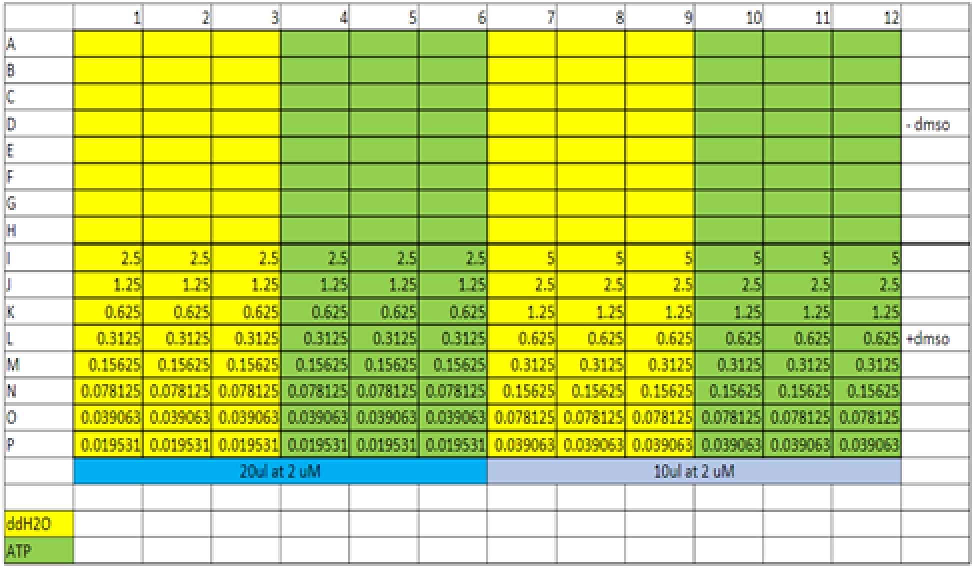
Optimizing Assay Conditions: Full Plate Testing



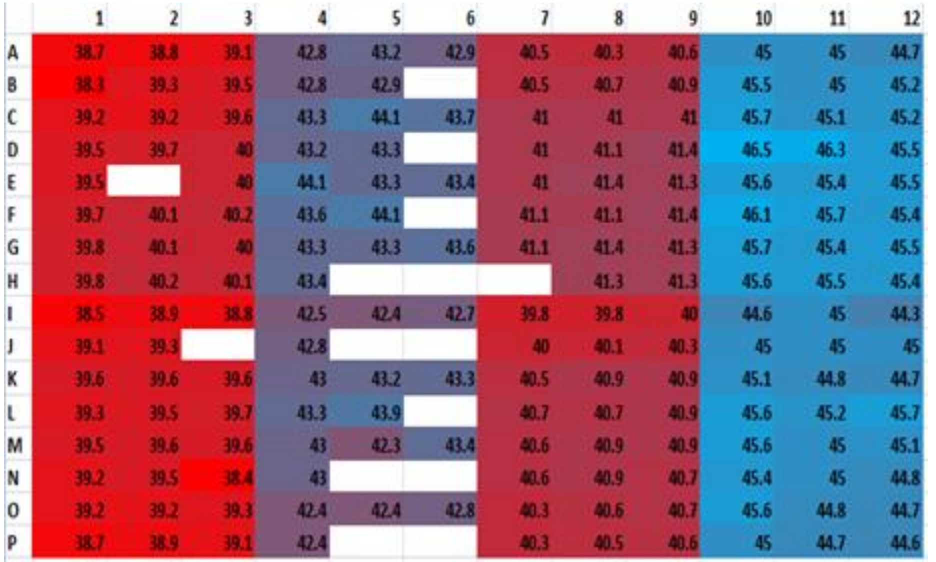
Sum of Tm	Column Labels																							
Row Labels	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	38.8	44.7	38.9	44.8	39.3	44.9	39.8	45.1	40.7	45.2	39.8	45.5	39.8	45.5	39.9	45.3	39.9	45.3	39.9	45.1	39.4	45.2	39.2	43.3
2	39	44.8	38.8	44.7	39.4	44.9	39.7	45.5	40.1	45.3	40.1	45.6	39.8	45.5	39.8	45.5	39.7	45.3	39.4	44.9	39.4	44.6	39.2	43.3
3	39.2	44.9	39.5	45.1	39.9	45.2	39.9	45.2	39.9	45.5	40.1	45.5	39.9	45.6	40.3	45.2	40.1	45.6	40.3	45.3	39.5	44.9	39.4	43.5
4	39.3	44.8	39.4	45.1	39.8	45.3	39.9	45.5	40.1	45.5	40.1	45.7	40.1	45.6	40.3	45.6	40.1	45.6	40.1	45.2	39.9	44.8	39.5	43.4
5	39.4	45.3	39.7	45.2	39.7	45.3	39.9	45.5	40.2	45.5	39.8	45.7	40.2	45.6	40.3	45.6	40.2	45.6	40.1	45.3	39.8	45.1	39.8	43.5
6	39.4	45.2	39.8	45.2	39.8	45.3	39.9	45.7	40.3	45.6	40.2	45.6	40.2	45.6	40.2	45.7	40.3	45.5	40.2	45.3	39.9	45.2	39.7	43.8
7	39.5	45.5	39.9	45.3	40.1	45.5	40.3	45.5	40.1	45.6	40.1	45.7	40.3	45.6	40.3	45.6	40.5	45.6	40.3	46.4	39.9	45.2	39.8	43.6
8	39.8	45.3	39.4	45.2	39.9	45.5	40.1	45.6	40.2	45.6	40.3	45.5	40.3	45.7	40.3	45.7	40.2	45.7	40.2	45.2	39.9	45.1	39.8	43
9	39.9	45.3	39.8	45.5	40.2	45.6	40.3	45.6	40.2	45.7	40.3	45.8	40.2	45.6	40.5	45.8	40.5	45.8	40.3	45.5	39.9	45.8	39.9	43.9
10	39.8	45.5	39.9	45.3	39.9	45.7	40.2	45.8	40.5	45.8	40.5	45.7	40.5	45.8	40.5	45.8	40.3	45.7	40.2	45.5	40.1	45.1	40.1	44
11	39.8	45.6	40.1	45.3	39.9	45.5	40.2	45.6	40.2	45.8	40.5	45.6	40.3	45.7	40.6	45.7	40.3	45.8	40.3	45.7	39.9	45.2	39.9	43.9
12	39.4	45.3	39.8	45.1	39.9	45.2	39.9	45.6	40.2	45.7	40.2	45.6	40.3	45.8	40.3	45.6	40.2	45.5	40.1	45.3	39.9	44.9	39.7	43.5
13	39.3	45.2	39.4	45.1	39.9	45.2	40.1	45.5	40.3	45.6	40.2	45.5	40.3	45.5	40.5	45.7	40.2	45.5	40.1	45.3	39.8	44.9	39.8	43.3
14	39	44.8	39.3	44.9	39.7	45.3	39.9	45.6	40.1	45.6	40.3	45.6	40.2	45.6	40.2	45.6	40.2	45.3	40.2	45.1	39.8	44.9	39.5	43.3
15	38.9	44.9	39.2	44.8	39.5	45.1	39.8	45.2	40.1	45.3	40.1	45.3	40.1	45.3	40.1	45.3	40.3	45.3	39.8	44.9	39.5	44.7	39.4	43.1
16	38.8	44.3	38.8	44.4	39.5	44.8	39.8	45.2	39.9	45.2	39.9	45.3	39.9	45.2	39.9	45.2	39.9	45.1	39.7	44.8	39.4	44.4	39.2	43

APO	
average	39.91197917
stdev	0.3915498
CV	0.981033283
ATP	
Average	45.20104167
stdev	0.618221532
CV	1.367715231
dTM	**5.2890625**
z'	**0.427249348**

Testing DMSO Sensitivity, Reaction Volume



	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B													
C													
D													- dmso
E													
F													
G													
H													
I	2.5	2.5	2.5	2.5	2.5	2.5	5	5	5	5	5	5	
J	1.25	1.25	1.25	1.25	1.25	1.25	2.5	2.5	2.5	2.5	2.5	2.5	
K	0.625	0.625	0.625	0.625	0.625	0.625	1.25	1.25	1.25	1.25	1.25	1.25	
L	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.625	0.625	0.625	0.625	0.625	0.625	+dmso
M	0.15625	0.15625	0.15625	0.15625	0.15625	0.15625	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	
N	0.078125	0.078125	0.078125	0.078125	0.078125	0.078125	0.15625	0.15625	0.15625	0.15625	0.15625	0.15625	
O	0.039063	0.039063	0.039063	0.039063	0.039063	0.039063	0.078125	0.078125	0.078125	0.078125	0.078125	0.078125	
P	0.019531	0.019531	0.019531	0.019531	0.019531	0.019531	0.039063	0.039063	0.039063	0.039063	0.039063	0.039063	
	20ul at 2 uM						10ul at 2 uM						
ddH2O													
ATP													



	1	2	3	4	5	6	7	8	9	10	11	12
A	38.7	38.8	39.1	42.8	43.2	42.9	40.5	40.3	40.6	45	45	44.7
B	38.3	39.3	39.5	42.8	42.9		40.5	40.7	40.9	45.5	45	45.2
C	39.2	39.2	39.6	43.3	44.1	43.7	41	41	41	45.7	45.1	45.2
D	39.5	39.7	40	43.2	43.3		41	41.1	41.4	46.5	46.3	45.5
E	39.5		40	44.1	43.3	43.4	41	41.4	41.3	45.6	45.4	45.5
F	39.7	40.1	40.2	43.6	44.1		41.1	41.1	41.4	46.1	45.7	45.4
G	39.8	40.1	40	43.3	43.3	43.6	41.1	41.4	41.3	45.7	45.4	45.5
H	39.8	40.2	40.1	43.4				41.3	41.3	45.6	45.5	45.4
I	38.5	38.9	38.8	42.5	42.4	42.7	39.8	39.8	40	44.6	45	44.3
J	39.1	39.3		42.8			40	40.1	40.3	45	45	45
K	39.6	39.6	39.6	43	43.2	43.3	40.5	40.9	40.9	45.1	44.8	44.7
L	39.3	39.5	39.7	43.3	43.9		40.7	40.7	40.9	45.6	45.2	45.7
M	39.5	39.6	39.6	43	42.3	43.4	40.6	40.9	40.9	45.6	45	45.1
N	39.2	39.5	38.4	43			40.6	40.9	40.7	45.4	45	44.8
O	39.2	39.2	39.3	42.4	42.4	42.8	40.3	40.6	40.7	45.6	44.8	44.7
P	38.7	38.9	39.1	42.4			40.3	40.5	40.6	45	44.7	44.6

ATP Titration and Protein Stability

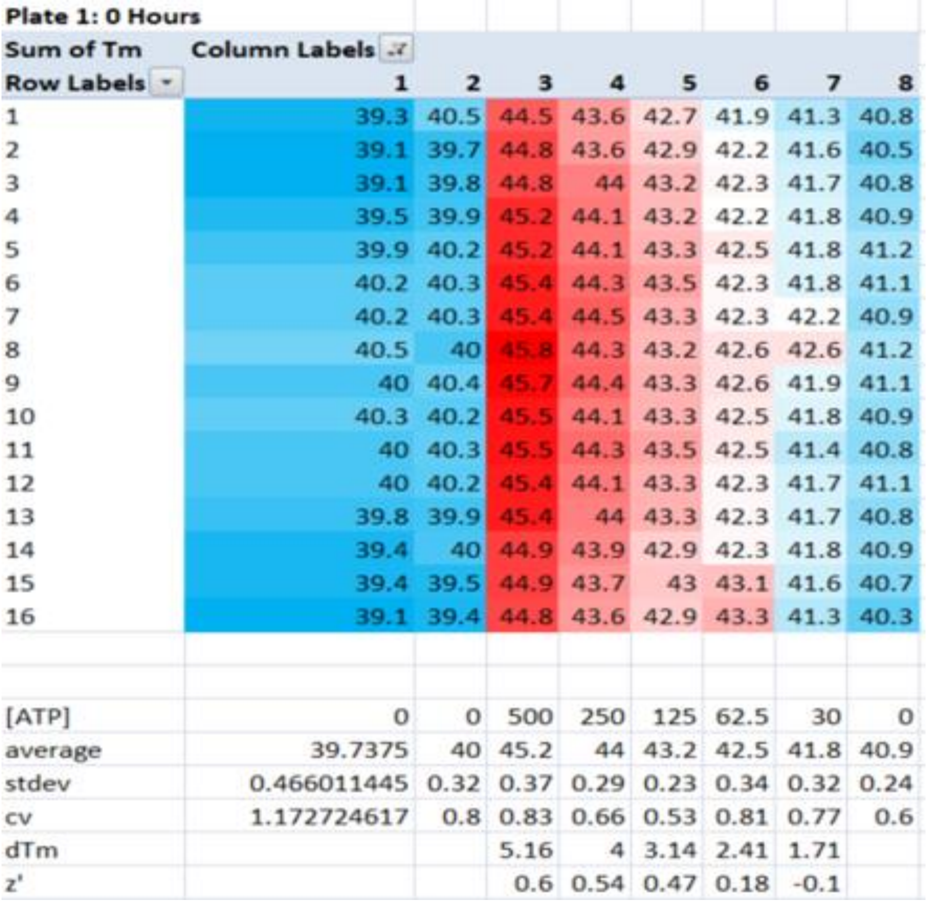


Plate 1: 0 Hours

Sum of Tm	Column Labels							
Row Labels	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	39.3	40.5	44.5	43.6	42.7	41.9	41.3	40.8
2	39.1	39.7	44.8	43.6	42.9	42.2	41.6	40.5
3	39.1	39.8	44.8	44	43.2	42.3	41.7	40.8
4	39.5	39.9	45.2	44.1	43.2	42.2	41.8	40.9
5	39.9	40.2	45.2	44.1	43.3	42.5	41.8	41.2
6	40.2	40.3	45.4	44.3	43.5	42.3	41.8	41.1
7	40.2	40.3	45.4	44.5	43.3	42.3	42.2	40.9
8	40.5	40	45.8	44.3	43.2	42.6	42.6	41.2
9	40	40.4	45.7	44.4	43.3	42.6	41.9	41.1
10	40.3	40.2	45.5	44.1	43.3	42.5	41.8	40.9
11	40	40.3	45.5	44.3	43.5	42.5	41.4	40.8
12	40	40.2	45.4	44.1	43.3	42.3	41.7	41.1
13	39.8	39.9	45.4	44	43.3	42.3	41.7	40.8
14	39.4	40	44.9	43.9	42.9	42.3	41.8	40.9
15	39.4	39.5	44.9	43.7	43	43.1	41.6	40.7
16	39.1	39.4	44.8	43.6	42.9	43.3	41.3	40.3
[ATP]	0	0	500	250	125	62.5	30	0
average	39.7375	40	45.2	44	43.2	42.5	41.8	40.9
stdev	0.466011445	0.32	0.37	0.29	0.23	0.34	0.32	0.24
cv	1.172724617	0.8	0.83	0.66	0.53	0.81	0.77	0.6
dTm			5.16	4	3.14	2.41	1.71	
z'			0.6	0.54	0.47	0.18	-0.1	

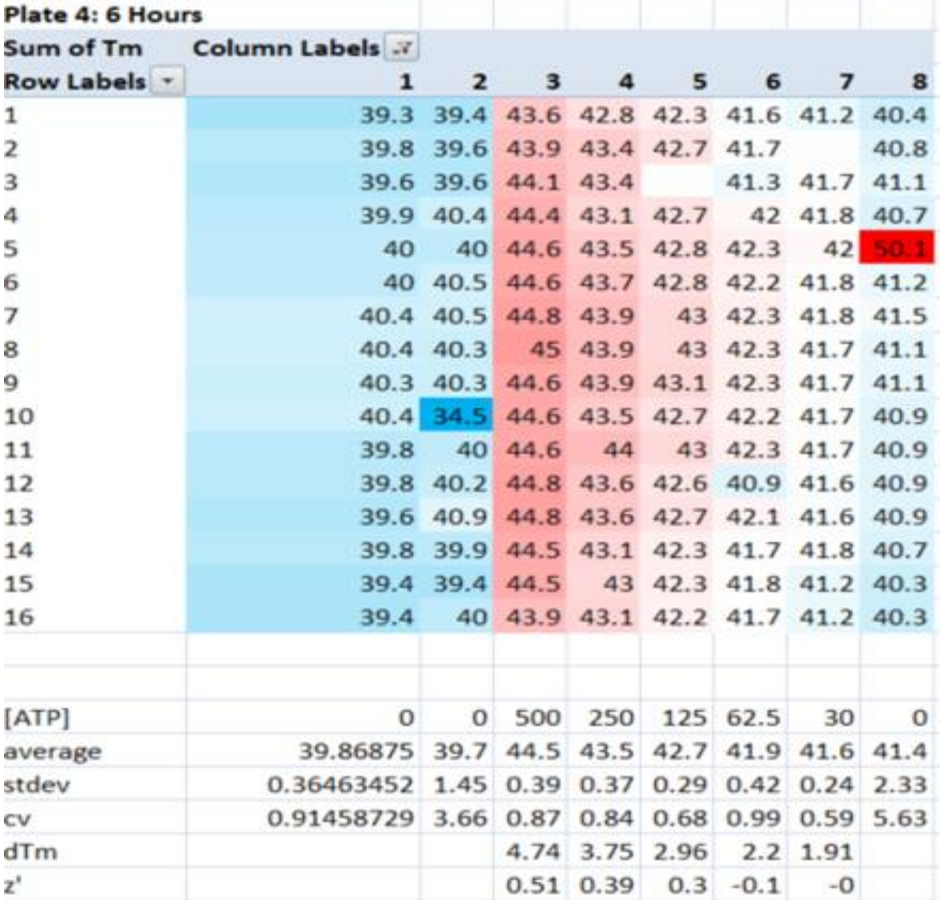


Plate 4: 6 Hours

Sum of Tm	Column Labels							
Row Labels	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	39.3	39.4	43.6	42.8	42.3	41.6	41.2	40.4
2	39.8	39.6	43.9	43.4	42.7	41.7		40.8
3	39.6	39.6	44.1	43.4		41.3	41.7	41.1
4	39.9	40.4	44.4	43.1	42.7	42	41.8	40.7
5	40	40	44.6	43.5	42.8	42.3	42	50.1
6	40	40.5	44.6	43.7	42.8	42.2	41.8	41.2
7	40.4	40.5	44.8	43.9	43	42.3	41.8	41.5
8	40.4	40.3	45	43.9	43	42.3	41.7	41.1
9	40.3	40.3	44.6	43.9	43.1	42.3	41.7	41.1
10	40.4	34.5	44.6	43.5	42.7	42.2	41.7	40.9
11	39.8	40	44.6	44	43	42.3	41.7	40.9
12	39.8	40.2	44.8	43.6	42.6	40.9	41.6	40.9
13	39.6	40.9	44.8	43.6	42.7	42.1	41.6	40.9
14	39.8	39.9	44.5	43.1	42.3	41.7	41.8	40.7
15	39.4	39.4	44.5	43	42.3	41.8	41.2	40.3
16	39.4	40	43.9	43.1	42.2	41.7	41.2	40.3
[ATP]	0	0	500	250	125	62.5	30	0
average	39.86875	39.7	44.5	43.5	42.7	41.9	41.6	41.4
stdev	0.36463452	1.45	0.39	0.37	0.29	0.42	0.24	2.33
cv	0.91458729	3.66	0.87	0.84	0.68	0.99	0.59	5.63
dTm			4.74	3.75	2.96	2.2	1.91	
z'			0.51	0.39	0.3	-0.1	-0	

“Pilot Screen” of Mutant Protein: TM Results

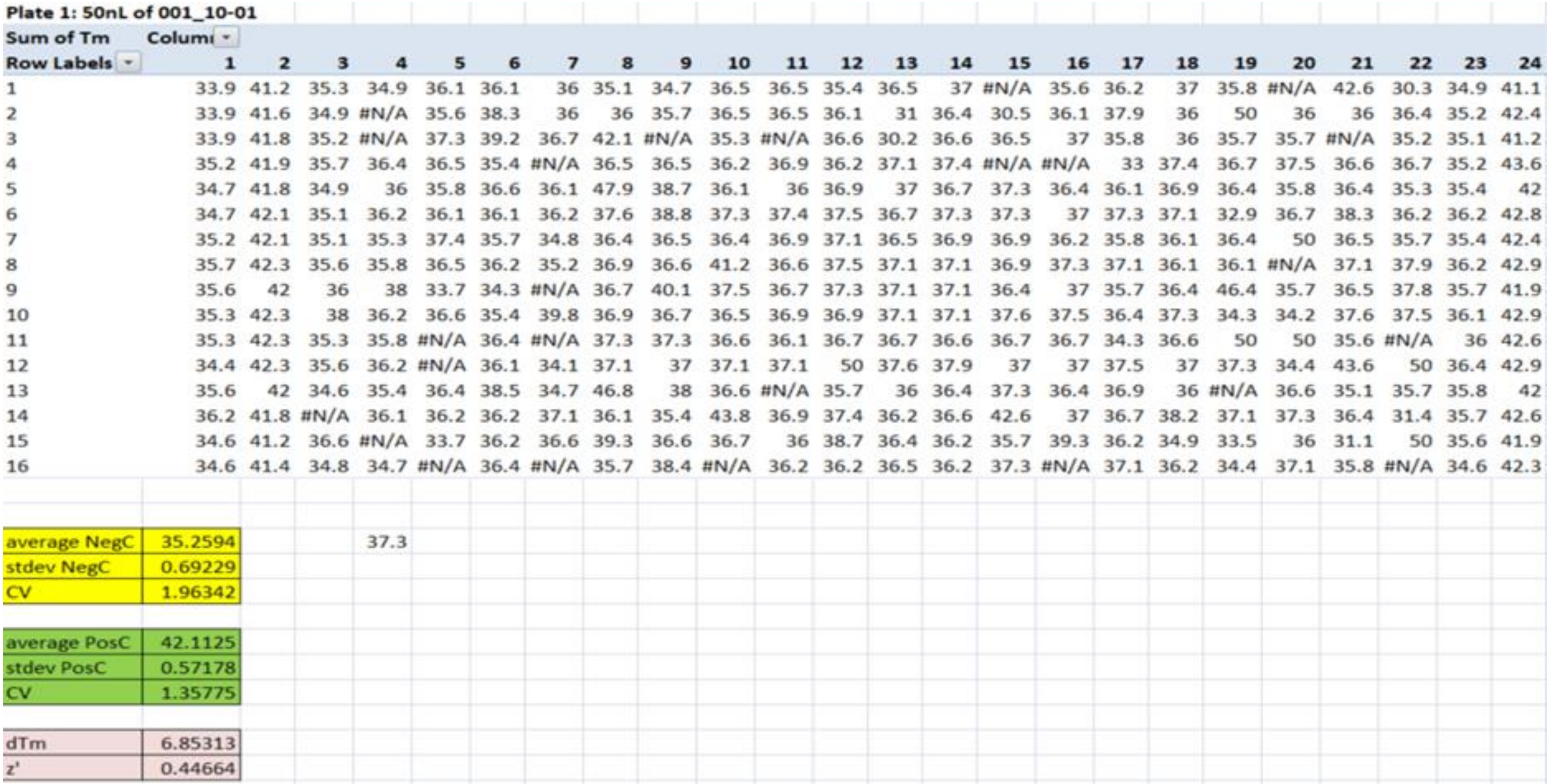


Plate 1: 50nL of 001_10-01

Sum of Tm **Columi**

Row Labels	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	33.9	41.2	35.3	34.9	36.1	36.1	36	35.1	34.7	36.5	36.5	35.4	36.5	37	#N/A	35.6	36.2	37	35.8	#N/A	42.6	30.3	34.9	41.1
2	33.9	41.6	34.9	#N/A	35.6	38.3	36	36	35.7	36.5	36.5	36.1	31	36.4	30.5	36.1	37.9	36	50	36	36	36.4	35.2	42.4
3	33.9	41.8	35.2	#N/A	37.3	39.2	36.7	42.1	#N/A	35.3	#N/A	36.6	30.2	36.6	36.5	37	35.8	36	35.7	35.7	#N/A	35.2	35.1	41.2
4	35.2	41.9	35.7	36.4	36.5	35.4	#N/A	36.5	36.5	36.2	36.9	36.2	37.1	37.4	#N/A	#N/A	33	37.4	36.7	37.5	36.6	36.7	35.2	43.6
5	34.7	41.8	34.9	36	35.8	36.6	36.1	47.9	38.7	36.1	36	36.9	37	36.7	37.3	36.4	36.1	36.9	36.4	35.8	36.4	35.3	35.4	42
6	34.7	42.1	35.1	36.2	36.1	36.1	36.2	37.6	38.8	37.3	37.4	37.5	36.7	37.3	37.3	37	37.3	37.1	32.9	36.7	38.3	36.2	36.2	42.8
7	35.2	42.1	35.1	35.3	37.4	35.7	34.8	36.4	36.5	36.4	36.9	37.1	36.5	36.9	36.9	36.2	35.8	36.1	36.4	50	36.5	35.7	35.4	42.4
8	35.7	42.3	35.6	35.8	36.5	36.2	35.2	36.9	36.6	41.2	36.6	37.5	37.1	37.1	36.9	37.3	37.1	36.1	36.1	#N/A	37.1	37.9	36.2	42.9
9	35.6	42	36	38	33.7	34.3	#N/A	36.7	40.1	37.5	36.7	37.3	37.1	37.1	36.4	37	35.7	36.4	46.4	35.7	36.5	37.8	35.7	41.9
10	35.3	42.3	38	36.2	36.6	35.4	39.8	36.9	36.7	36.5	36.9	36.9	37.1	37.1	37.6	37.5	36.4	37.3	34.3	34.2	37.6	37.5	36.1	42.9
11	35.3	42.3	35.3	35.8	#N/A	36.4	#N/A	37.3	37.3	36.6	36.1	36.7	36.7	36.6	36.7	36.7	34.3	36.6	50	50	35.6	#N/A	36	42.6
12	34.4	42.3	35.6	36.2	#N/A	36.1	34.1	37.1	37	37.1	37.1	50	37.6	37.9	37	37	37.5	37	37.3	34.4	43.6	50	36.4	42.9
13	35.6	42	34.6	35.4	36.4	38.5	34.7	46.8	38	36.6	#N/A	35.7	36	36.4	37.3	36.4	36.9	36	#N/A	36.6	35.1	35.7	35.8	42
14	36.2	41.8	#N/A	36.1	36.2	36.2	37.1	36.1	35.4	43.8	36.9	37.4	36.2	36.6	42.6	37	36.7	38.2	37.1	37.3	36.4	31.4	35.7	42.6
15	34.6	41.2	36.6	#N/A	33.7	36.2	36.6	39.3	36.6	36.7	36	38.7	36.4	36.2	35.7	39.3	36.2	34.9	33.5	36	31.1	50	35.6	41.9
16	34.6	41.4	34.8	34.7	#N/A	36.4	#N/A	35.7	38.4	#N/A	36.2	36.2	36.5	36.2	37.3	#N/A	37.1	36.2	34.4	37.1	35.8	#N/A	34.6	42.3

average NegC	35.2594			37.3
stdev NegC	0.69229			
CV	1.96342			

average PosC	42.1125
stdev PosC	0.57178
CV	1.35775

dTm	6.85313
z'	0.44664

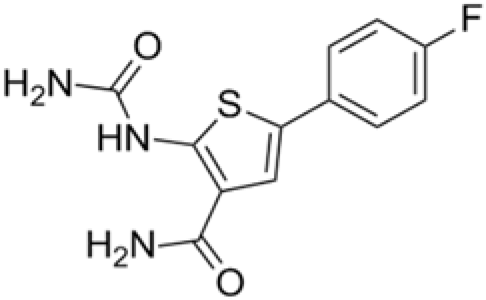
“Pilot Screen” of Mutant Protein: Fluorescence

Plate 1: 50nL of 001_10-01 Raw TAMRA Values

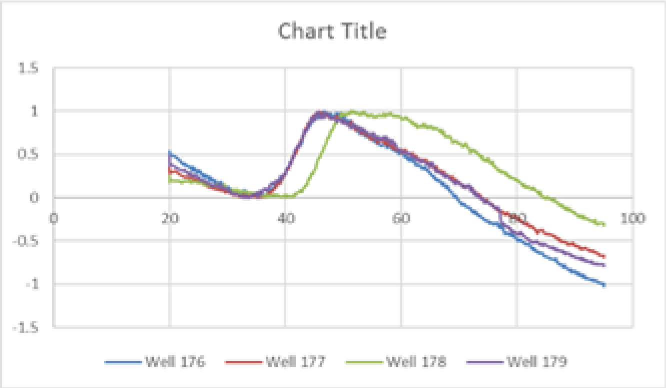
Sum of max	Column Labels																							
Row Labels	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	3034	2277	2215	4234	3960	2919	4427	1708	10296	4351	3292	4387	3261	3859	12557	3801	5105	5307	5165	8401	3381	6846	3959	3181
2	3716	3209	4880	6596	4091	3247	4893	5562	3335	4852	4721	5360	5351	2178	15696	5214	4950	4836	7338	4536	4449	4441	3486	1285
3	4387	3030	4205	8348	4152	4744	2360	9885	1557	4335	7778	8554	4645	4705	6302	3679	18422	6056	4175	5416	15094	6383	4899	4190
4	4650	3668	2928	4294	5243	6340	9091	5615	5393	4157	5093	3157	5266	4653	4832	6647	4688	4798	4593	3858	3672	4210	3537	2845
5	3977	3410	4687	5297	5326	5154	4550	3893	4186	4661	4863	5111	4616	5445	5564	5350	5953	5419	5889	5565	3650	5266	5211	3754
6	4359	3409	5309	4132	5207	4506	6313	5341	3209	4861	5371	4670	5849	4896	5068	5528	5136	4974	4368	4532	3485	3640	2950	2175
7	5005	3875	5541	4832	6935	3792	6713	6618	6237	6128	6483	5404	5664	6108	6373	5835	6546	9662	4869	13049	6146	6960	5893	4110
8	5275	4558	7351	7412	6775	7144	9555	8047	7670	5125	7285	6402	6503	5406	5495	6836	5593	6612	4421	13342	5019	4158	4125	2732
9	4774	4037	5314	5158	4788	13950	9154	6147	22493	5744	5223	5681	5301	6623	5250	6053	7684	8146	22708	11366	5605	6448	5339	4172
10	4438	3242	6143	5741	4333	5352	7704	6001	5818	6136	5554	3676	4838	5310	4129	4123	4260	4986	5985	5593	5138	3858	3369	2134
11	4217	3433	5180	4626	23636	4674	7714	4773	5125	4966	5178	5275	5310	3935	4119	4882	6544	5188	13188	6693	5084	2873	4585	3528
12	3873	2911	4634	4200	4264	4637	8028	5004	4703	4799	4932	10591	4550	3202	4607	4120	4470	3552	3759	2488	12171	11289	2825	1637
13	3953	3109	5170	5478	5763	10946	4475	10014	4528	5733	17858	5898	6480	4890	5377	5655	5478	7304	10628	4561	7149	5970	4994	3759
14	5468	3592	1403	5516	5364	5682	5829	6285	4269	3243	7135	5421	4612	2689	19118	5253	7140	4208	3860	3773	4833	4086	3357	1567
15	3991	3857	4663	3308	5236	5233	6482	5618	5444	5388	6436	3228	5541	4252	6190	5994	5624	4322	8676	5823	3081	13230	5222	4295
16	5015	3649	5862	6674	12863	3014	21373	4851	6440	10748	7782	6989	7094	6592	5450	17021	3964	5175	6378	2212	4837	5728	3457	2417

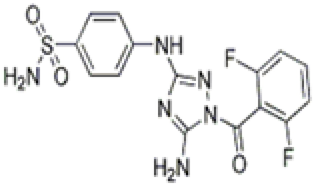
average NegC	4291.910403
stdev NegC	807.7896834
CV	18.82121497
average PosC	3220.228944
stdev PosC	836.8826813
CV	25.9882976
average sample	6014
stdev sample	3152.328019
CV	52.41396692

Hit me baby one more time!
(compounds that stabilize / bind the protein significantly)

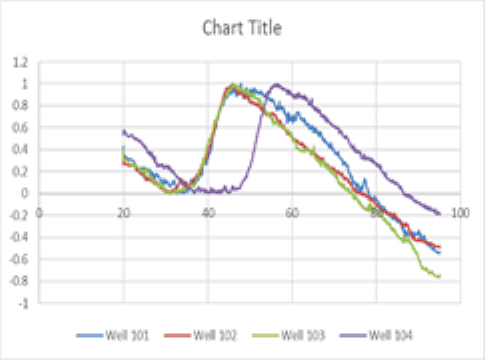


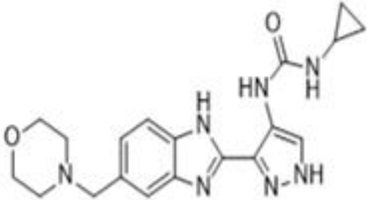
H10/178: TPCA1





E8/104: JNJ-7706621





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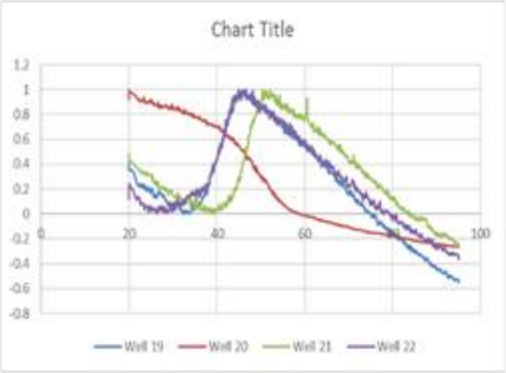

Future Directions

Purification: Continue working with codon-optimized constructs

Figure out how to get best recovery from TALON resin

(incubation time, temperature, elution volume, concentration of Imidazole, number of fractions)

Figure out best size exclusion protocol

Assays: Optimizing qPCR program, duration of thermal shift program, streamline analysis