Fragment Library Screening by SPR

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Biochemical Pharmacology

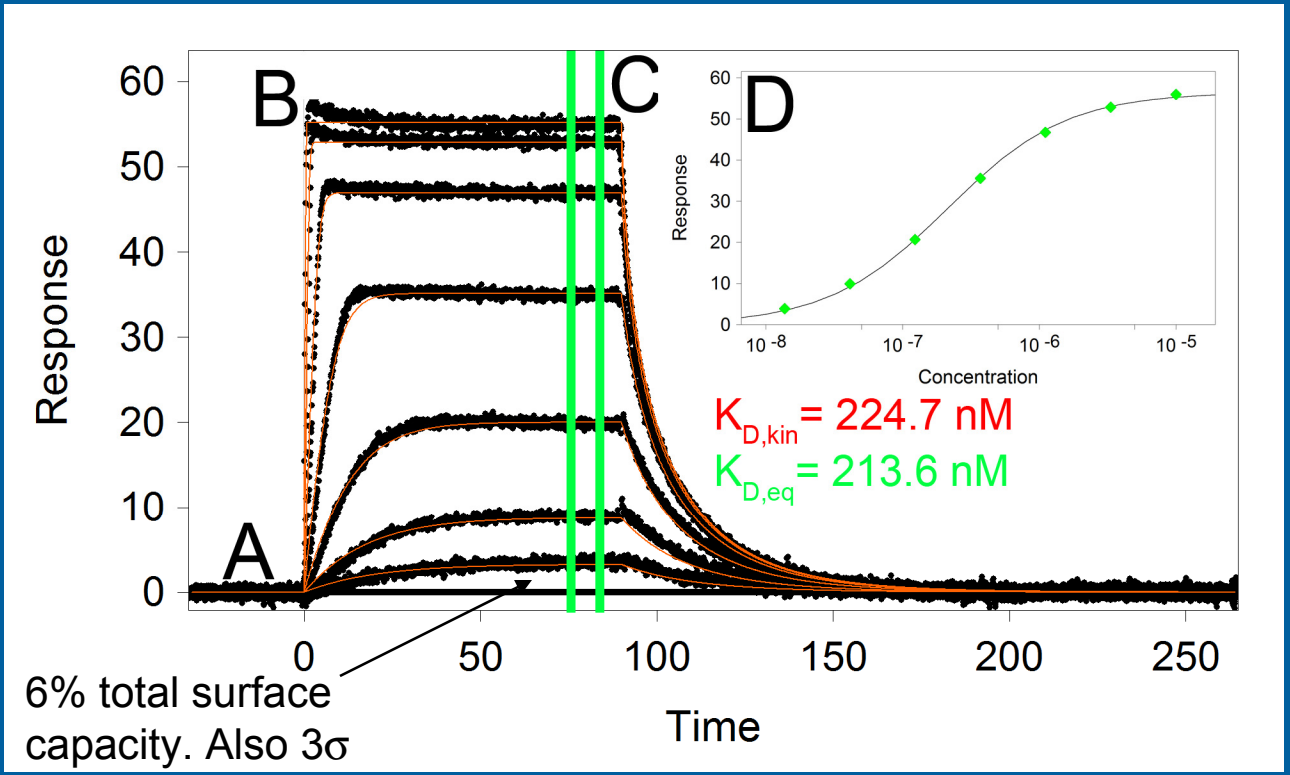
Genentech

Best data require best practices

- Methods in Enzymology: A Volume on Fragment-Based Drug Design – Tools, practical approaches, and examples.
  - Out in early 2011
  - SPR fragment screening covered in extensive detail based on ~34 screens on ~20 targets
- Today:
  - Review of the general screening procedure
  - Refinements to key steps
  - Case studies and examples at each step

SPR instrumentation capabilities

- Binding of matter detected in real time
- Capable of detecting as little as 5% surface occupancy
- Practical affinity range spans 9 orders of magnitude (50 pM – 20 mM)
  - Different methods and practices required for low vs. high affinity
- Usually limit low-affinity to 2-5 mM for compound solubility and chemical/crystallographic interest

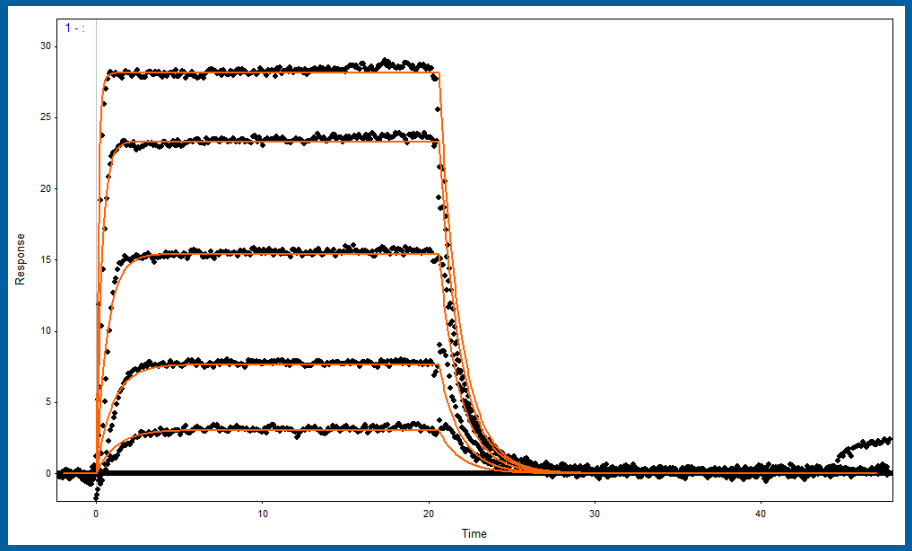


Protein Immobilization: A few strategies cover most targets

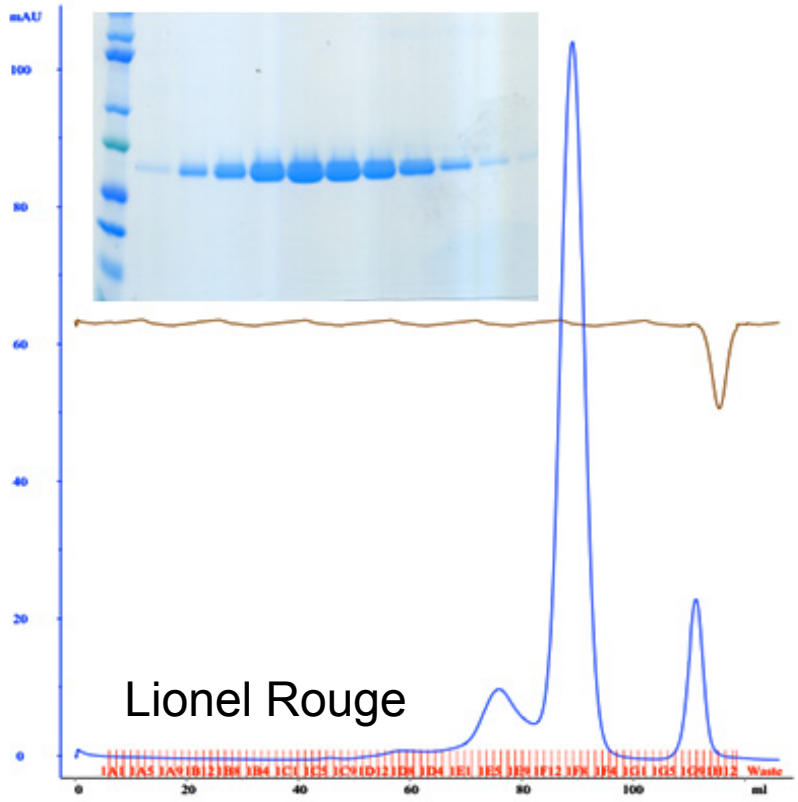
- SPR assays for 23 targets and off targets developed in the last 2 years at GNE and immobilization accomplished with one or more of:
  - Direct amine coupling to the hydrogel
  - Limiting biotinylation (~1 biotin/protein, mass spec confirmed)
  - Tagging with Avi-tag (Avi = GLNDIFEAQ**K**IEWHE)
  - Thiol modification for low pI proteins (e.g. Acidic alpha-glycoprotein)
- Surfaces validated during assay development with a starting set of control compounds, when available
  - Compare K_D to IC_{50} (ideally ~20-50 cmpds) over range from ~100 nM to 10 uM
- Assay development times range from 1 day to 2 weeks (assuming good quality protein and tool compounds)
- Need good quality protein!

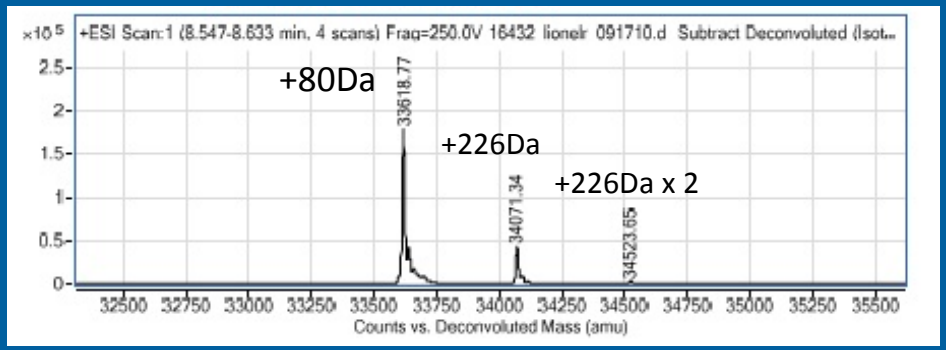
Example of limiting biotinylation for neutravidin-chip capture

- Purified protein incubated with 0.7:1 NHS-LC-LC-biotin:protein (Pierce)
- Mass-spec reveals most biotinylated protein has 1 tag with a very small population of 2-tagged
- K_D new protein lot = 1.44 uM
- Historic K_D = 1.39 +/- 0.22 uM



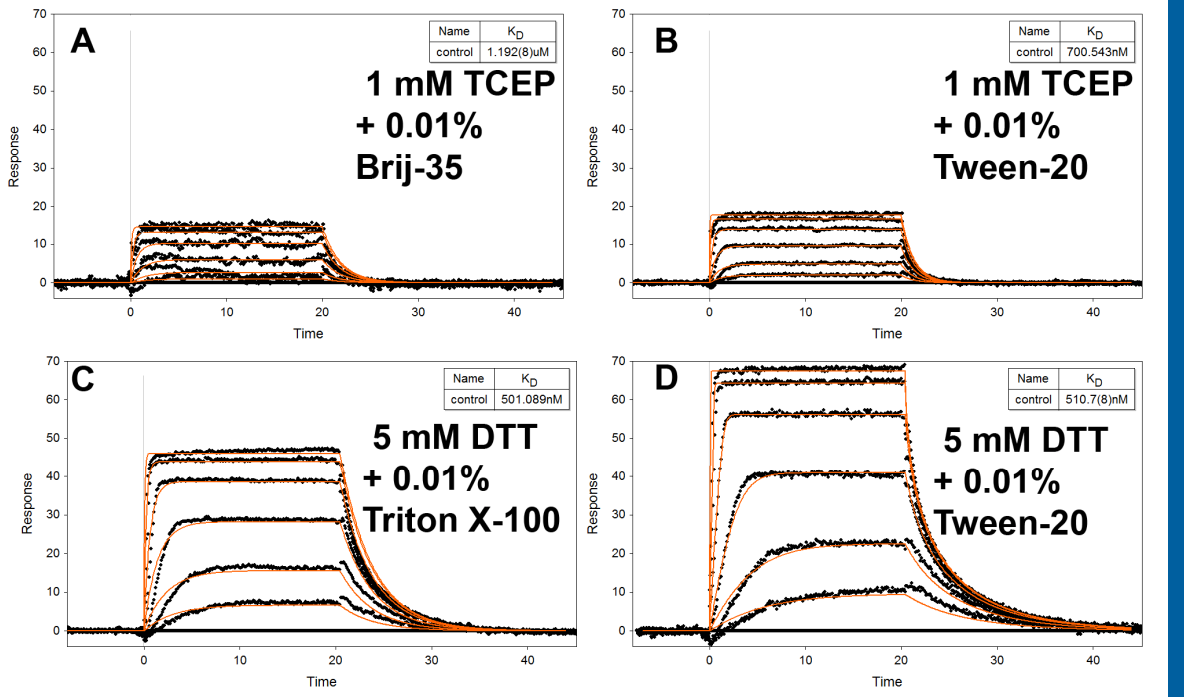
Kinase expressed in Tni (Ni-NTA, monoQ, S75)

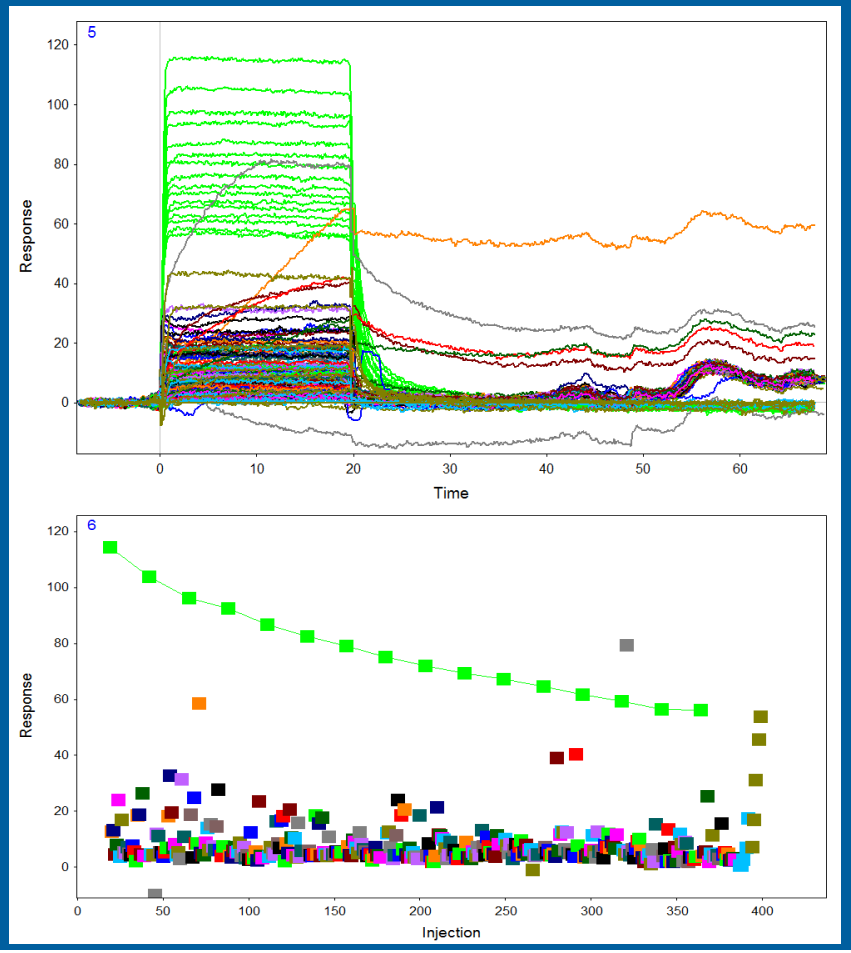




Buffer optimization cycle and pilot screen

- Chemically biotinylated kinase
- Controls (~20 cmpds) tested under several cycles of buffer optimization
- Evolved towards improvements in both quality of data and assay window (not all buffer mixtures are compatible with the instrument) and crystallographic compatibility (PEG, MPD, pH, salts)
- Best buffer tested for long-term stability with a pilot screen. Decay of ~50% is acceptable





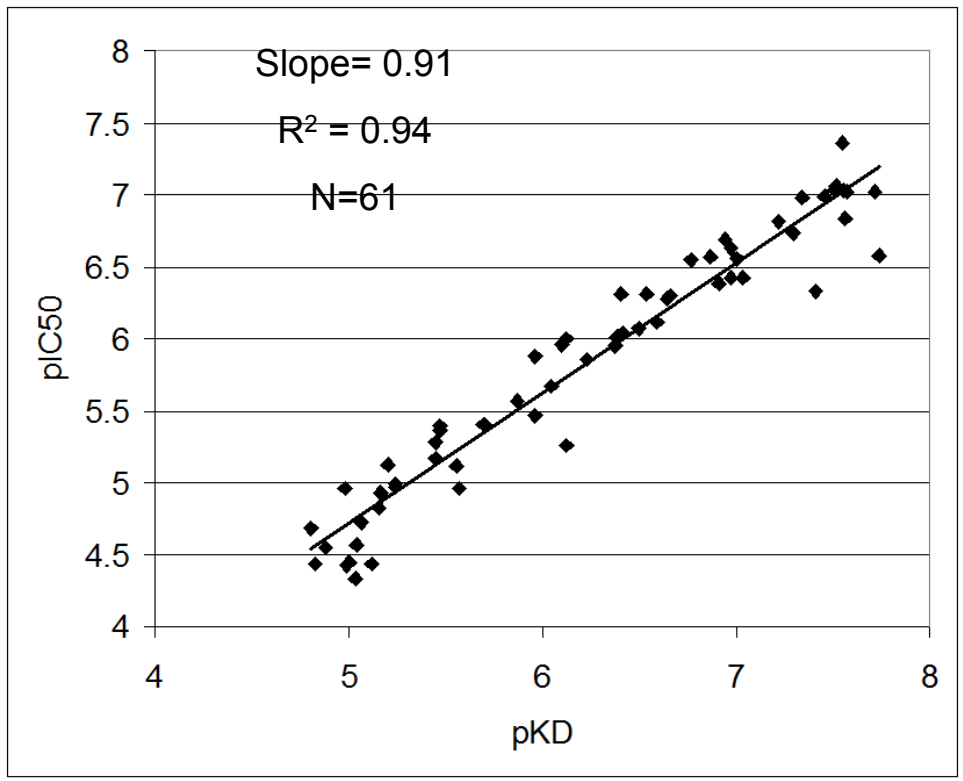
K_D and IC_{50} need to correlate and be reproducible

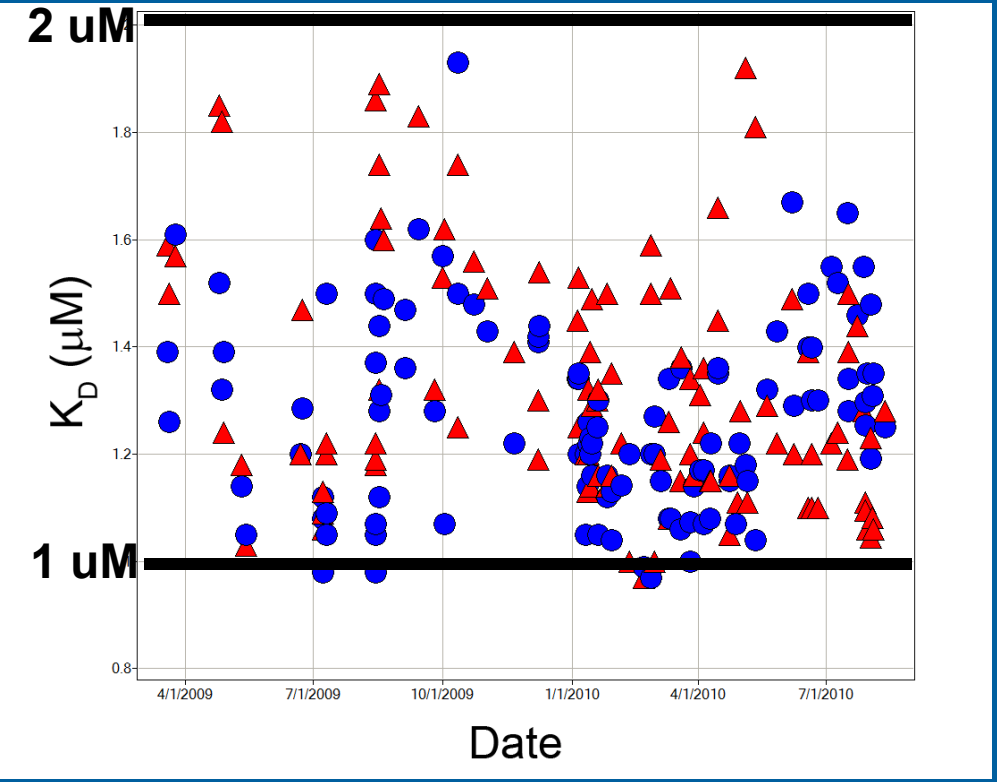
- 61 compounds chosen to validate SPR assay. High agreement over the full range of the enzyme assay
- Early and late controls from every run of the SPR assay for one project over an 18 month period shows very little variance despite different protein and compound lots, instruments, users, pipettes, etc.

Early: K_D = 1.27 +/- 0.18 uM

Late: K_D = 1.32 +/- 0.22 uM

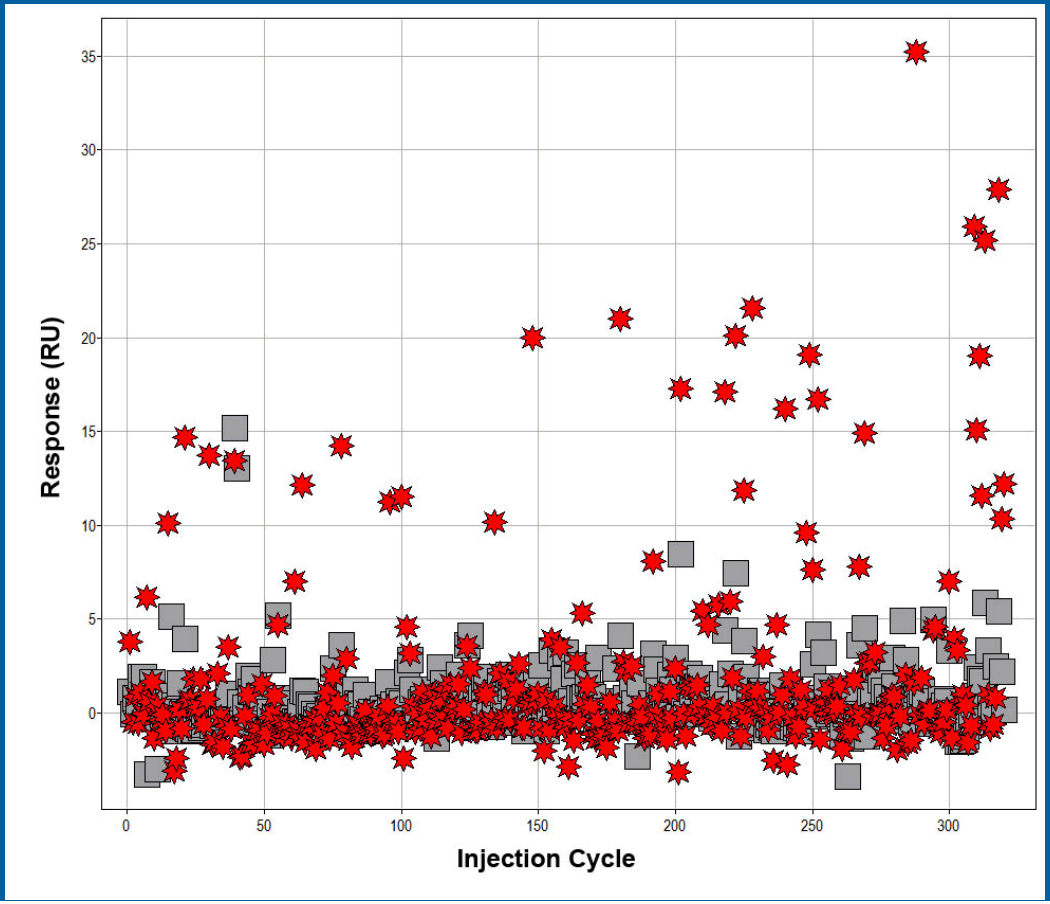
N = 112 in each set





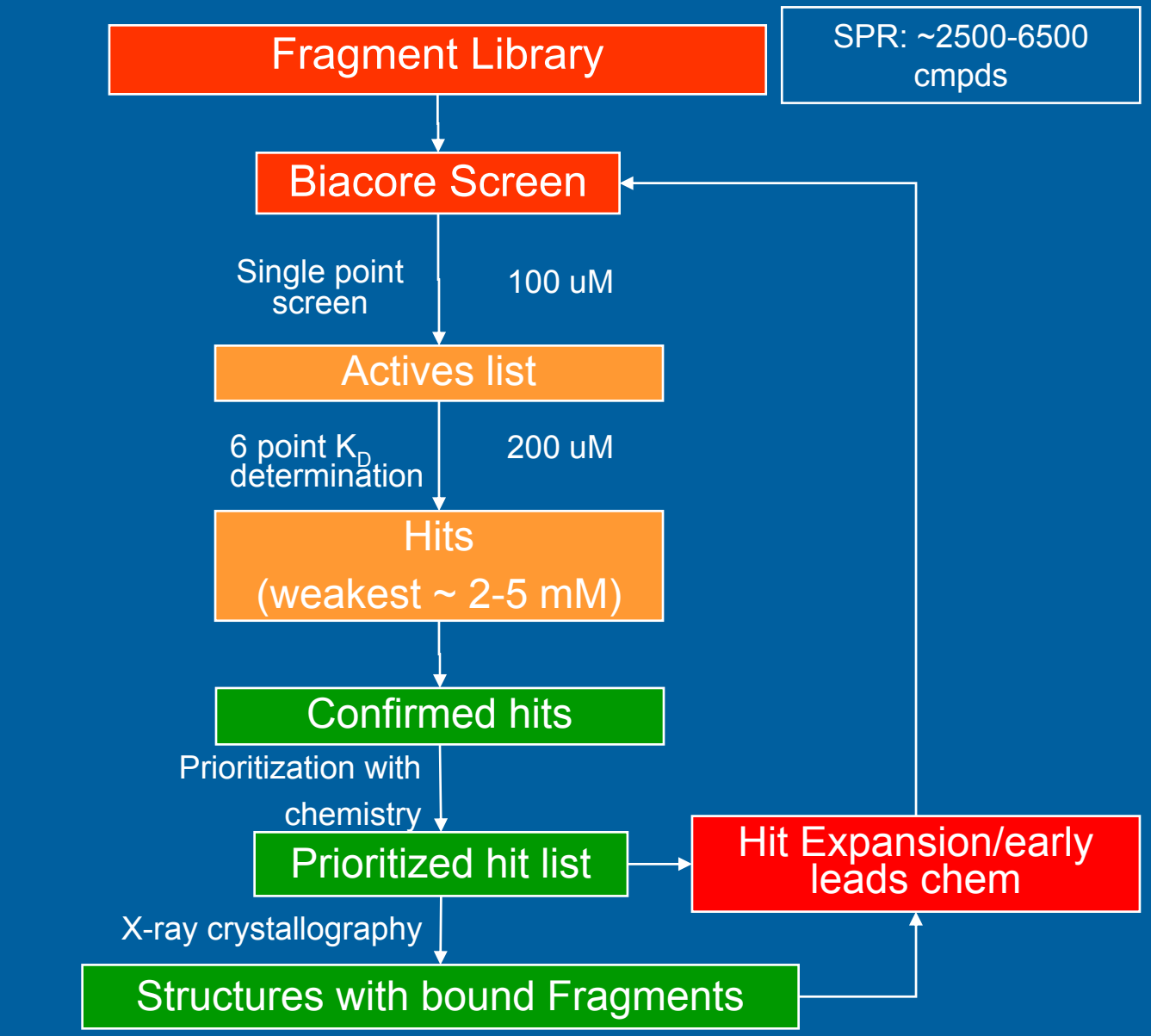
Pilot Screening can reveal protein quality issues

- No controls available for a new target
- Attempted blind fragment screen searching for controls
- Initial pilot screen hit rate was 16.4% (too high for a protease in general) (red points)
- Protein purification procedure refined and specific activity of the protease greatly improved
- Repeat screen hit rate was 6.4% owing to reduced non-specific binding



SPR-based Fragment Screening Workflow

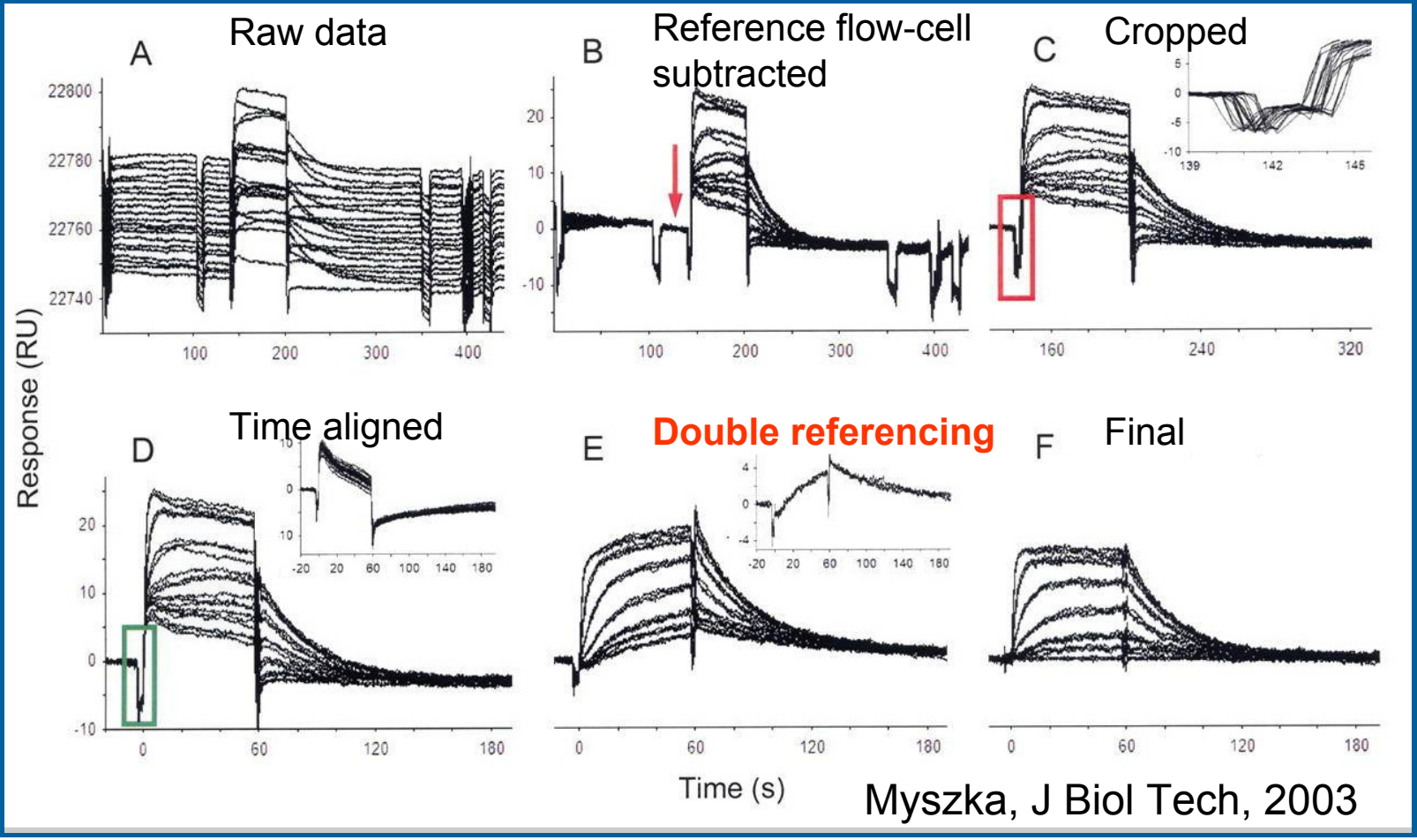
- Screening of low molecular weight (<300 Da) low affinity compounds (10 uM < K_D < 5000 uM)
- Screening cascade adapted from HTS methods
- Three targets can be screened in parallel (different proteins, mutants, etc.) with the T100 assuming a common buffer can be found
- GNE screening library tested for compound solubility >250 uM before inclusion in the library



Note, fragment screening by NMR also started at GNE.
Comparisons of the methods are good (99% agreement)

Best practices in data reduction

- Best practices in SPR data reduction to get the best quality data have been long established
- Each is critical to getting the most meaningful data
- Double referencing is the subtraction of buffer injections from the data to eliminate contributions from systematic injection artifacts (panel E→F)

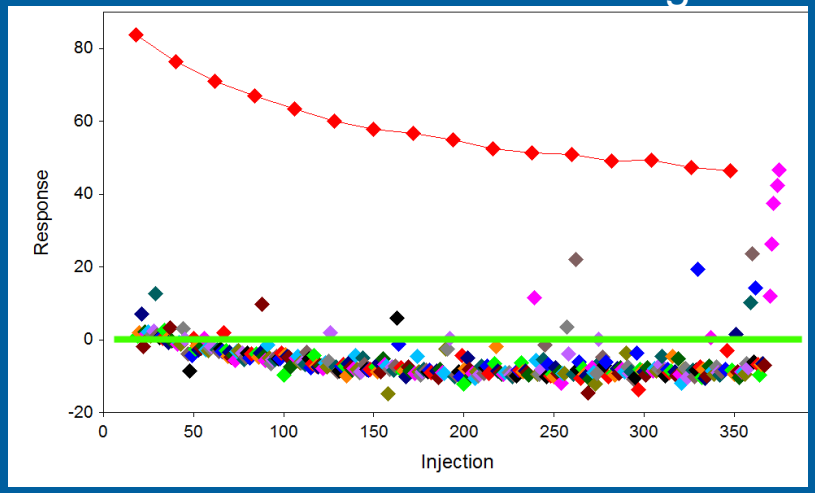


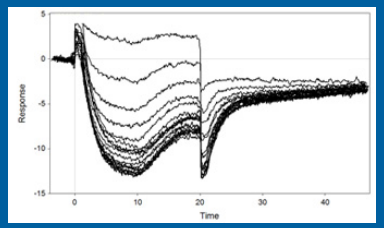
The requirement for double referencing fragment data

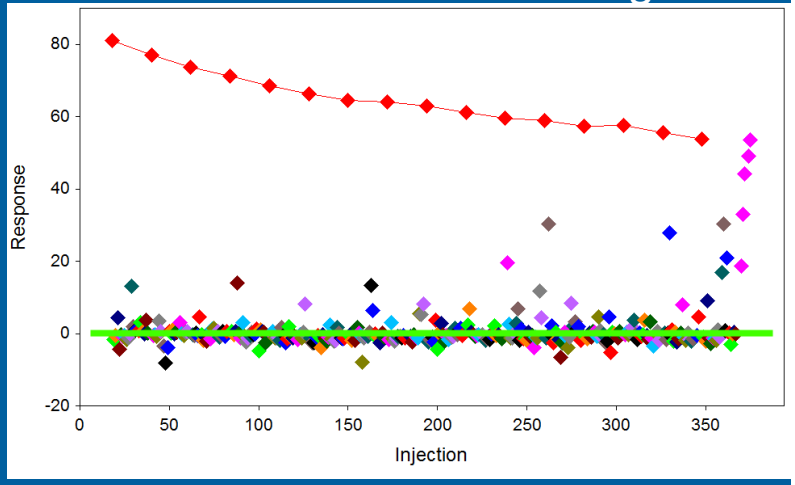
- Experimental runs are 18-22 hours long…systematic artifacts might drift
- Two different fragment plates from a screen, same target, instrument, and operator
- Nature and drift in systematic artifacts differs between the two days. Baseline standard deviation and skew statistics on the second plate are significantly improved after double referencing for both plates

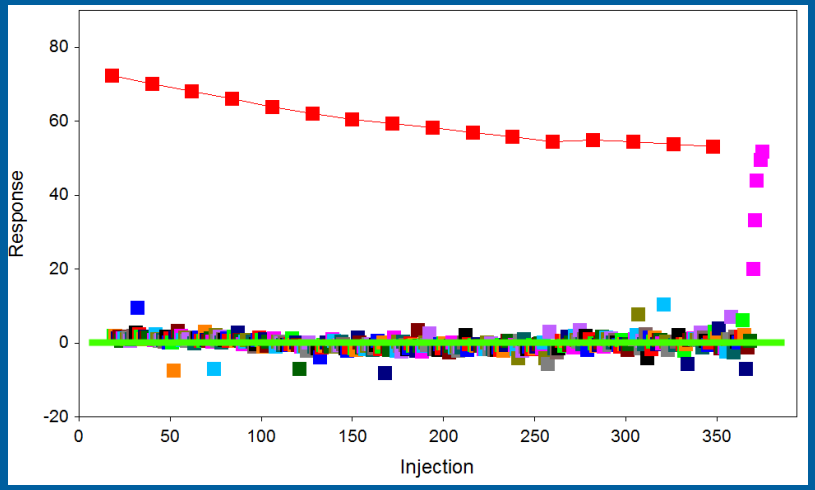
Before double referencing

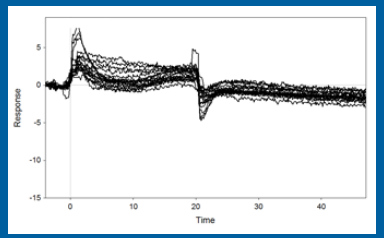
After double referencing



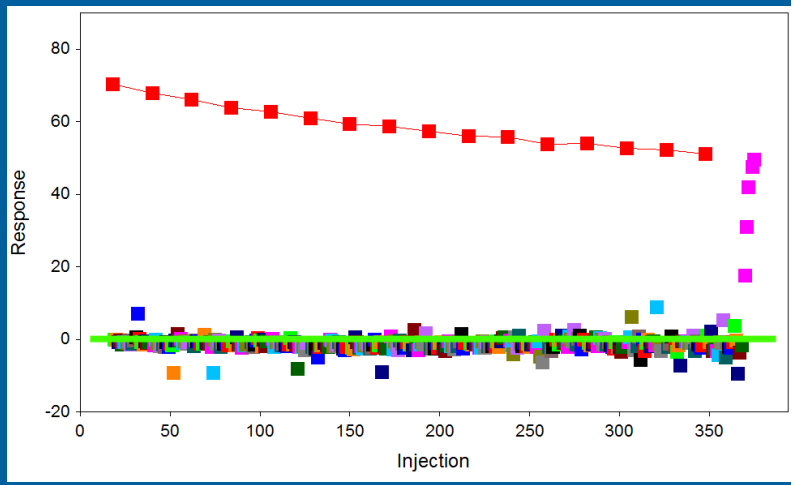








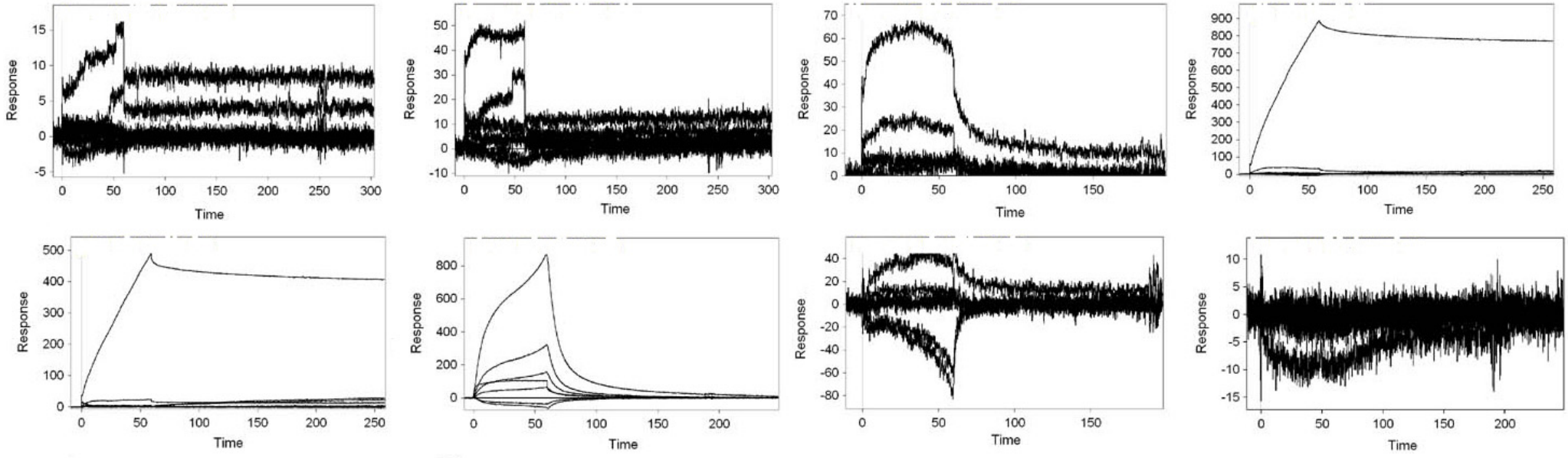
Blank injections represent <4% of the total screen



Practical considerations for promiscuous binders

- Promiscuous binders
  - Colliodal aggregates
  - Non-drug like and can be identified from sensorfram inspection
  - Promiscuous binding depends on compound, buffer, AND target, and thus cannot be cleared entirely from a library
- Pre-filtering a library to remove promiscuous binders requires pre-screening on every target and buffer, thus doubling screening time
- How significant a problem are promiscuous binders in fragment screening?

β-lactamase | HCVpol | HIV-RT | p38



JNK2 | IRAK | Neutravidin | Mock

Frequency of promiscuous binders across 13 fragment screens of the same library

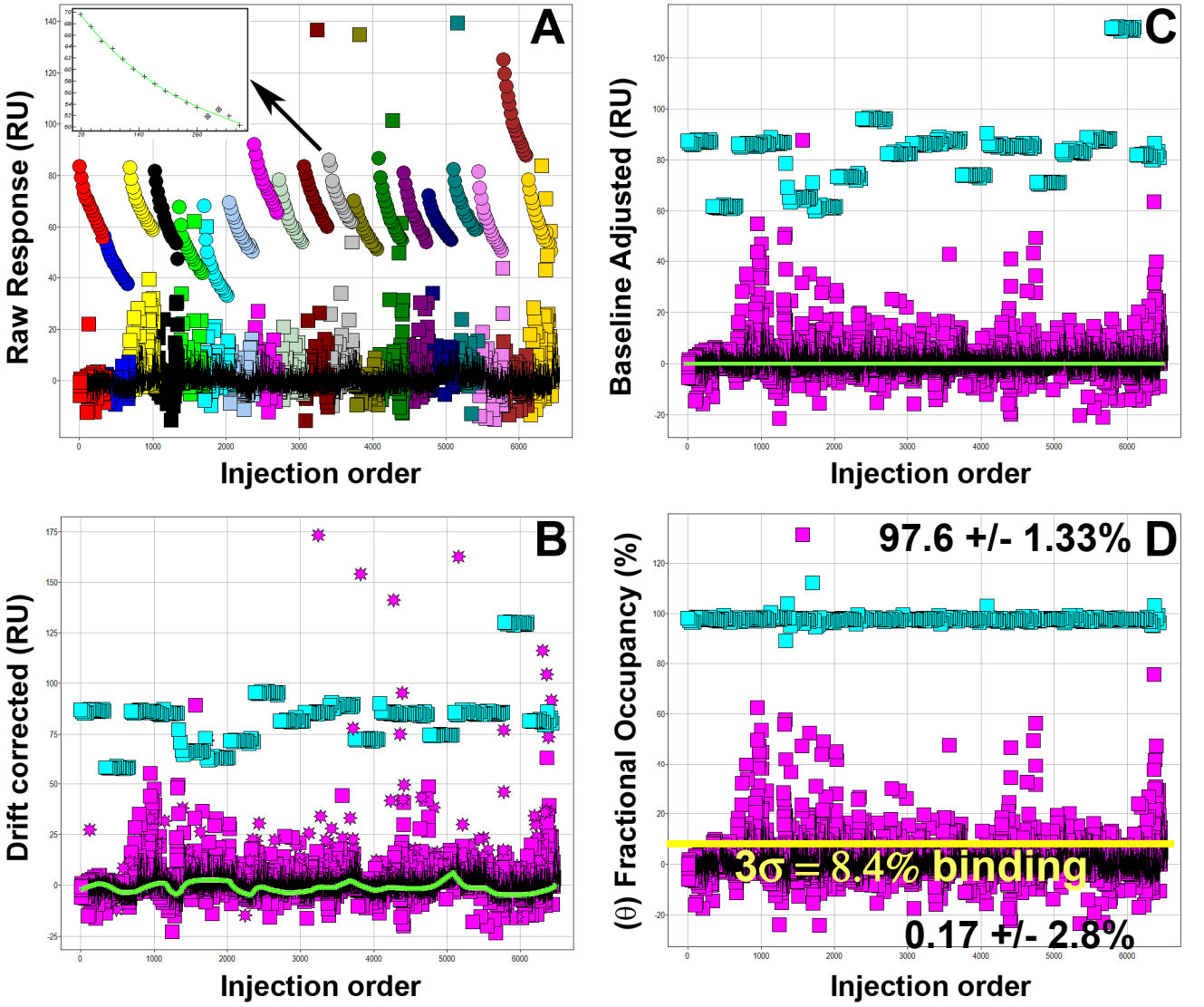
- Except for one screen carried out three pH units lower than the solubility testing assay used to filter the library, the number of promiscuous binders is always significantly lower than the number of true hits
- For many targets promiscuous binders represent ~3 or fewer compounds/plate
- Most fragments are NOT repeat offenders. Can't significantly clean the library by eliminating the repeat offenders (three strikes or more offenders are 1.5% of the library)
  - Exmples of compounds that behaved badly in one screen that are confirmed hits with crystal structures in other screens including one identified as badly behaved in multiple screens
- Visual QC of screening data is fast (< 5 min/plate or ~45 minutes of user time over a full screen), or inpsect only the statistical positives and eliminate compounds with poor sensorgrams

Screen ID	% Promiscuous Binders	% Hits
1	6.35	2.88
2	2.33	6.65
3	2.24	10.76
4	2.16	2.63
5	1.52	14.91
6	1.52	5.00
7	1.52	13.70
8	1.02	5.05
9	0.68	4.62
10	0.64	3.26
11	0.38	12.54
12	0.30	9.19
13	0.13	6.18

Frequency of compound identification as a promiscuous binder	Number of compounds
1	144
2	36
3	15
4	4
5	6
6	4
7	6
8	2
9	0
10 - 13	0

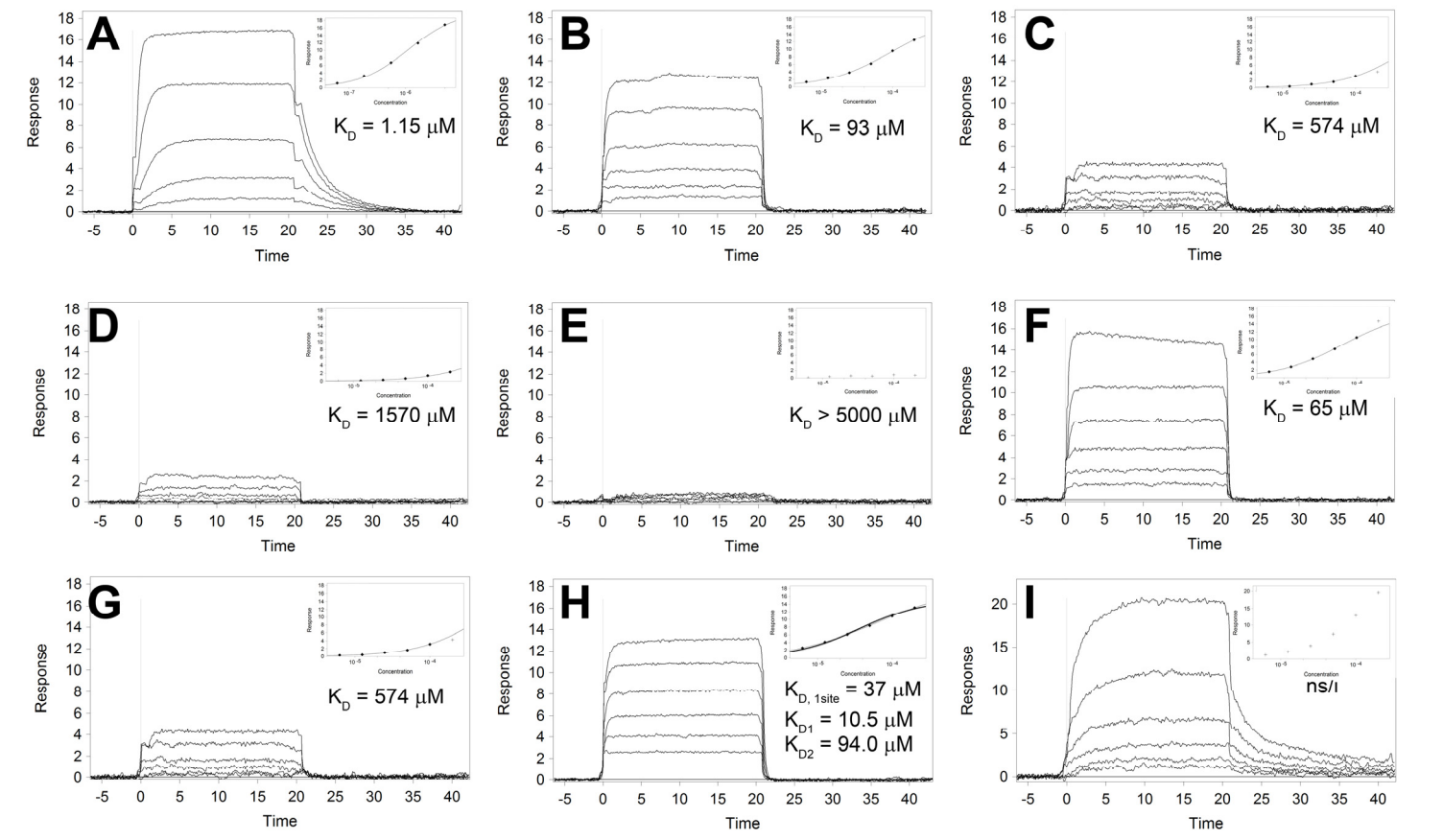
Scaling Fragment Data and Hit Selection

- The RU scale has many experimental dependencies separate from the binding fraction
- Raw data are corrected for drift in positive controls, centering the baseline at zero, and scaling based on the fraction of surface bound by the control replicates
- Result: Absolute scale allows compound comparison across targets with no experimental dependencies
- Hits can be selected more robustly and have selections based on the entire screen (no assumptions needed about instrument noise levels)
- Z' = 0.95 with 344 compounds (5.7% hti rate) > 3σ



Best Practices in Dose-Response Hit Confirmation

- Binding has 2 parameters, K_D and R_{max}. R_{max} is shared by all compounds in a single dose-response experiment and can be locked or globally fit by all the data $R = R_{max}*[cmpd]/([cmpd]+K_D)$
- Compounds with K_D < [top cmpd] can restrain their own R_{max}. Compounds weaker than the top tested concentration need to be globally fit
- As long as two concentrations are visibly above the noise and show a dose-response the K_D will be precise
- Visual sensorgram quality control is still required to weed out compounds with issues panel (I)

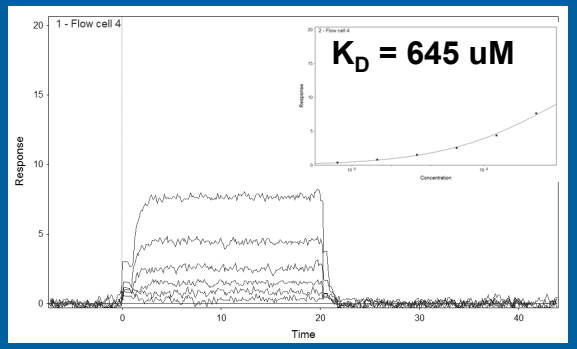


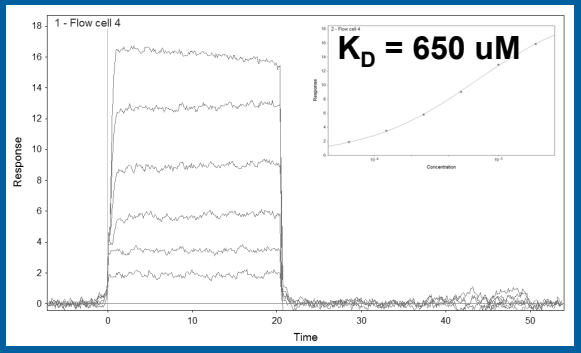
Locked R_{max} versus full dose response curves

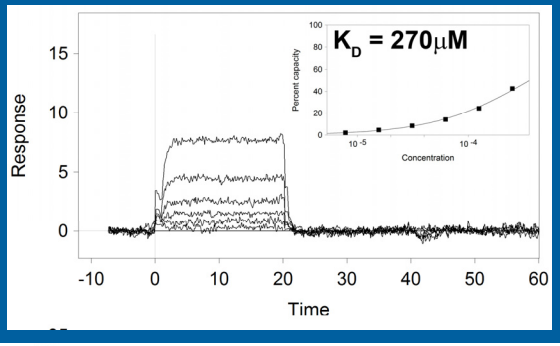
- Tested the results of the K_D extrapolation method using full dose-response curves
- 200 mM DMSO stock solutions made and dose-response tests performed with 250 uM and 2500 uM top concentrations
- Analysis is quite robust when compounds are soluble in the millimolar concentration range

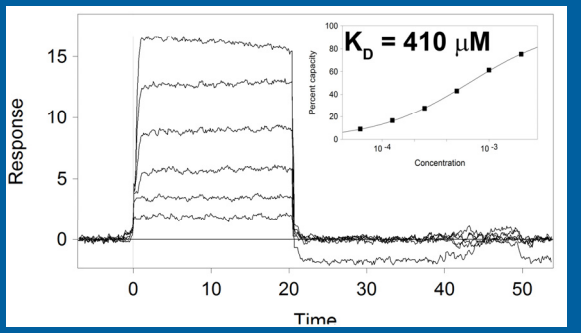
[top] = 250 uM

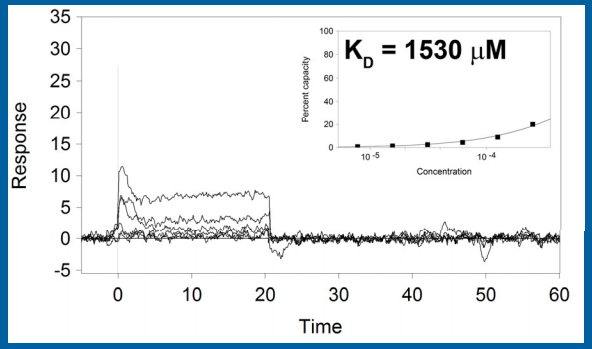
[top] = 2500 uM

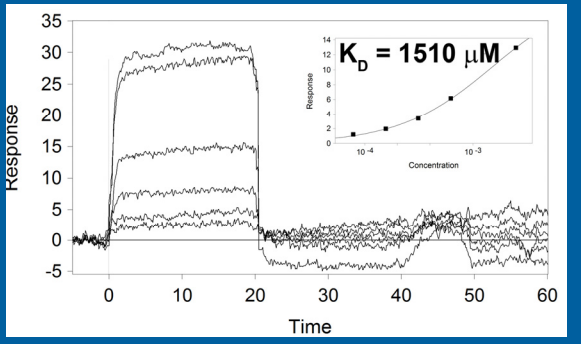












Why do compounds work in binding but fail in crystallization?

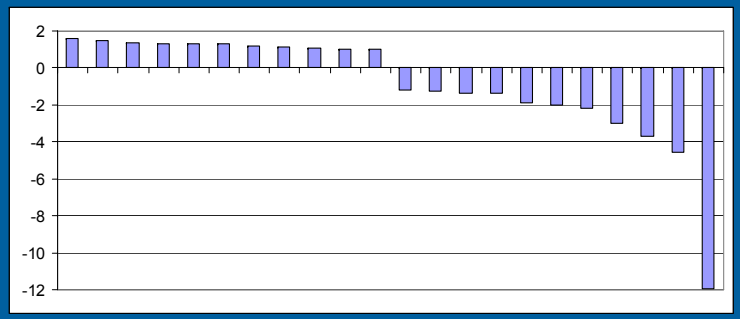
- Obtained TWO independent fresh powder weighouts of each compound
- Dissolved one at 10 mM and another at 100 mM in DMSO
- 250 uM data match historic results quite well
- When a K_D could be assigned they either matched well (~1-fold variation, N=17), or the K_D from the 100 mM stock was much weaker N=5.
- Precipitation in DMSO or buffer noted for a majority of failed crystallization compounds
- Poor DMSO and/or aqueous solubility can result in significant shifts in K_D due to wrongly assumed concentration. High concentration crystallographic stocks may be far from the desired concentration reducing actual soak concentrations considerably

Numbers are measured K_D in micromolar

10 mM DMSO 250 uM [top]	100 mM DMSO 2500 uM [top]	Precipitation in 100 mM DMSO Stock	Precipitation at 250 uM (aqueous)	Precipitation at 2500 uM (aqueous)
577	510			
530	406			
2120	1550			
1230	780			
1120	1110			
1640	2290			
3600	2480			
2670	2630			
2890	2190			
297	570			
780	1560			
2230	2060			
356	1070			x
558	2050			
940	1160			
1090	1510			x
87		x	x	x
2250	1750			
1240	2700			
201				x
1960		x		x
660	7900	x		
132				x
	10600			
2540	3100	x		
3900	3230			
200				x
78	354			
88			x	x
86				x

Co-crystal structure available

Failed co-crystallization



Fold difference

Practical affinity ranges for 20 SPR-based fragment screens on four target classes

- Hits identified using a primary screening concentration of 50 or 100 uM
- K_Ds determined using top concentration of 150 or 200 uM
- Allows for the rank ordering of compounds up to ~2 mM. Can go weaker using higher compound concentrations

Target Class	Most Potent K_D (uM)	Least Potent K_D (uM)
Cytokine	30	5000
Kinase	0.32	2800
Kinase	0.91	1090
Kinase	0.94	3520
Kinase	1.1	1180
Kinase	1.8	1700
Kinase	2.3	1990
Kinase	4.7	2600
Kinase	10.5	2010
Kinase	15.6	2900
Kinase	64	3400
Kinase	72	2400
Kinase	123	1230
Kinase	70	3900
Polymerase	65	4000
Protease	2.9	2300
Protease	44	2010
Protease	207	1900
Protease	210	3900
Protease	240	2900

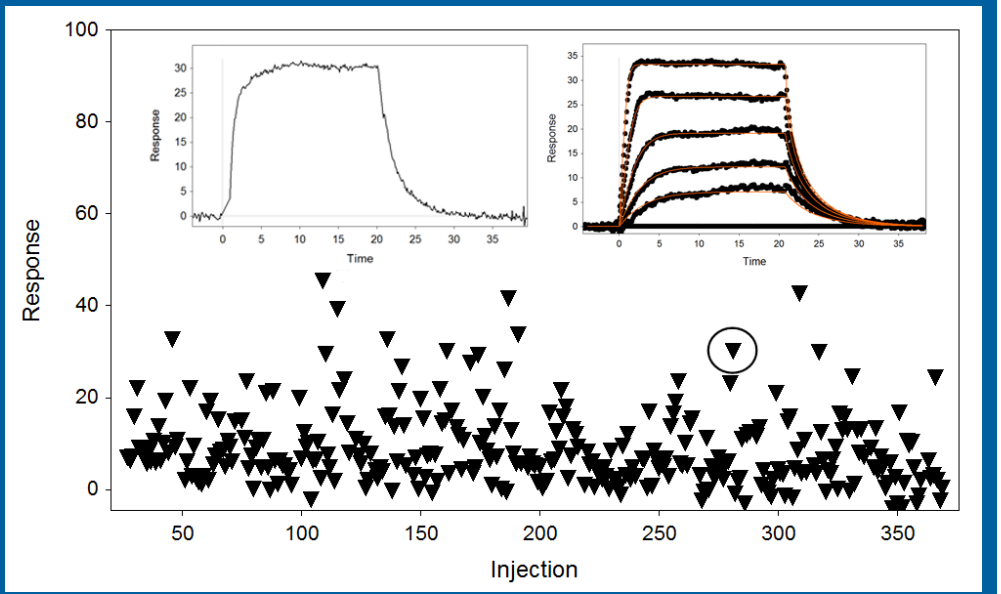
How small a fragment can you detect?

- There are a lot of theoretical calculations in the literature suggesting that compounds <150 Da cannot be detected by SPR to many proteins, especially heavier than ~50 KDa
- Calculations based on the RU (1 pg protein/mm^2) assuming compound refractive index increment is the same as protein
- Measured RII for compounds are often 20-200% higher than for proteins (why we use compound controls)
  - RII variation has minimal effect on K_D determination but significant ramifications for detectability
- Fragments binding to large proteins, or very small fragments binding to normal proteins has been detected
- Larger proteins may result in more false negatives for the smallest fragments, but SPR will still find hits

K_D= 2 uM

MW_{cmpd} = 296 Da

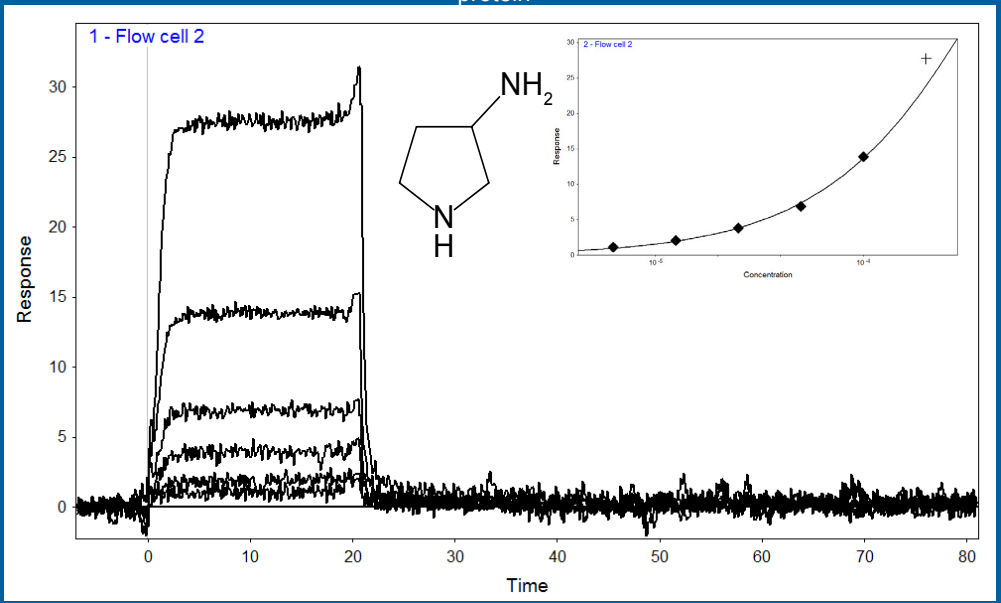
$MW_{protein}$ = 170 KDa



K_D= 600 uM

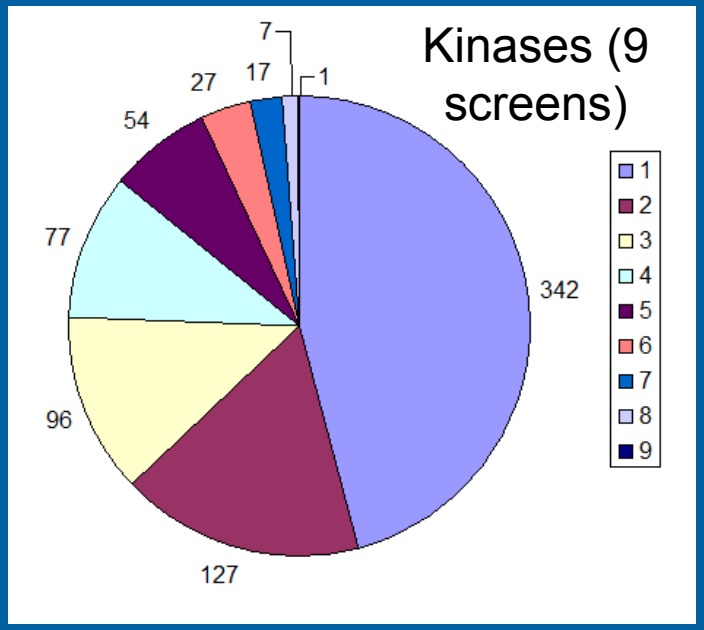
MW_{cmpd} = 86 Da

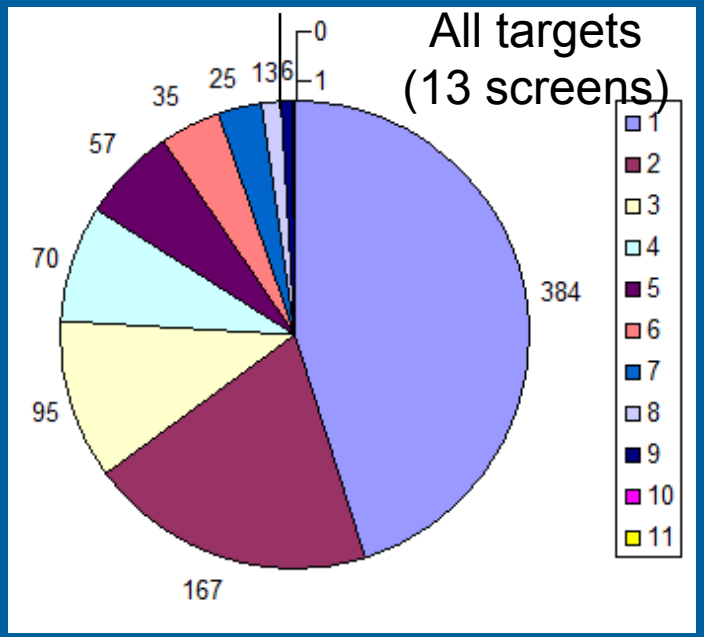
$MW_{protein}$ = 50 KDa



Number of fragments hitting a certain number of times in various screens of the same library

- ~45% of fragment hits from screens on 9 kinases show selectivity for just 1 kinase and and 63% of fragments hit only 1 or 2 kinases
- Selective fragments found for every kinase tested, though some have fewer compared to others
- Trend holds across when all targets included
- 13 screens on unique proteins yields 853 unique compounds as hits or 36% of the library
- Each screen will yield unique hits. What is unique about this 36% of the library?

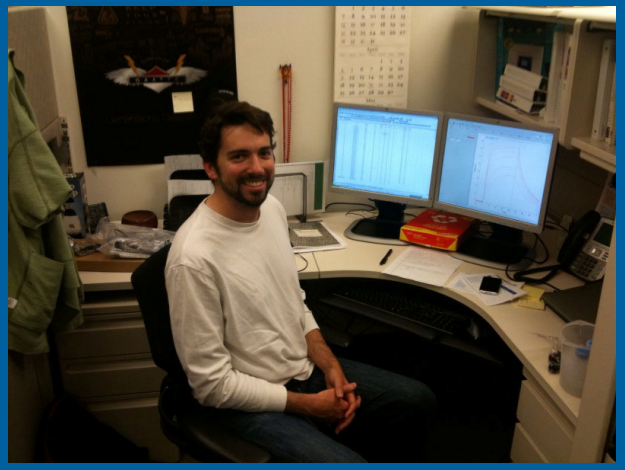




SPR Assays have excellent throughput

- Numbers of samples is not predictive of generating valuable data that drives decisions (but it helps!)
- 2009 (Fragment screening results only, not counting SAR work or assay development)
    - Single concentration screening interactions measured: 25,971
    - Dose-response interactions measured: 5,915
- 2010, Jan-September, Fragment screens and dose-response for follow-up and SAR work (not counting assay development time) on 9 targets
    - 29,522 fragment/target interactions measured in primary screening (last 3 months)
    - 8,349 dose-response curves
- Resources
    - 2 Biacore T100s, 1 Biacore 3000, 2 research associates, 1 scientist

Brandon Bravo



Keith Pitts

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  - Sarah Hymowitz
  - Micah Steffek
  - Lionel Rouge
- Protein Production
  - Jiansheng Wu
  - Hong Li
  - Maria Lorenzo
- Compound Management
  - Tim Dawes
  - Dan Hascall
  - Grady Howes
  - Steve Jones
  - Peter Thana
- Computational Chemistry
  - Jeff Blaney
  - Nick Skelton
  - Hans Purkey
  - Paul Gibbons
  - Alberto Gobbi
- Early Leads Chemistry/Chemistry
  - Steve Magnuson
  - Snahel Patel
  - Fred Cohen
  - Joachim Rudolph
- Roche
  - Ken Brameld
  - Harald Mauser
  - Eva-Maria Gutknecht
  - Achim Grenz
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  - **Rebecca Rich**
  - **Tom Morton (Scrubber2**

Backup slides

Table 4. K_D of fragment binding determined with the locked R_{max} method. The K_D in uM is listed followed in parentheses by the number of data points included in the fit. Data points were excluded from highest to lowest concentration. The "early" and "late" controls were collected as five-point dose response curves. Samples are sorted from lowest to highest potency and span the typical range for a fragment hit confirmation experiment. The greatest shift (1.8 fold) relative to $K_D(6)$ or failed fits are highlighted in bold.

Compound	$K_D(6)$	$K_D(5)$	$K_D(4)$	$K_D(3)$	$K_D(2)$	$K_D(1)$
Early control	1.15	1.15	1.15	1.15	1.15	1.152
Late control	1.18	1.18	1.18	1.18	1.18	1.84
fragment	82.30	82.30	79.30	75.90	74.80	74
fragment	105.30	99.30	95.00	90.10	88.30	88
fragment	108.30	105.50	104.80	99.20	91.90	90
fragment	138.40	142.10	143.30	144.00	159.00	161
fragment	144.00	152.60	146.40	142.10	148.00	138
fragment	177.30	177.30	176.60	172.00	171.00	174
fragment	186.60	200.80	192.00	184.00	**fail**	**fail**
fragment	206.80	205.90	202.00	197.00	187.00	174
fragment	217.00	216.60	212.00	202.00	198.00	202
fragment	258.00	258.00	255.00	257.00	244.00	260
fragment	280.00	280.00	261.00	234.00	226.00	230
fragment	290.00	275.00	275.00	280.00	334.00	360
fragment	323.00	326.00	311.00	291.00	**fail**	**fail**
fragment	367.00	365.00	373.00	360.00	349.00	320
fragment	373.00	320.00	323.00	322.00	370.00	480
fragment	464.00	464.00	456.00	454.00	**840.00**	**fail**
fragment	466.00	466.00	469.00	481.00	580.00	**fail**
fragment	527.00	510.00	507.00	520.00	**fail**	**fail**
fragment	534.00	534.00	542.00	500.00	570.00	470
fragment	595.00	545.00	537.00	530.00	**fail**	**fail**
fragment	1460.00	1380.00	1580.00	2000.00	**fail**	**fail**

Backup slides

Table 5. K_D reproducibility from independent experiments. 22 compounds were tested in duplicate on three forms of a kinase (full length, full length activated, and kinase domain only). Duplicates were run on different plates, different days, different machines, and by different users. Reported K_Ds were fit with the locked R_{max}method. K_Ds are reported in µM. Four compounds with more than a two-fold shift between replicates are highlighted in bold.

Fragment ID	K_D FL Inactive Replicate 1	K_D FL Active Replicate 1	K_D Kinase Domain Replicate 1	K_D FL Inactive Replicate 2	K_D FL Active Replicate 2	K_D Kinase Domain Replicate 2	FL Inactive Shift	FL Active Shift	Kinase Domain Shift
1	6.2	6.3	6.3	13	12.1	11.8	**2.10**	1.92	1.87
2	12.1	10.2	11.8	13	15	14	1.07	1.47	1.19
3	32	25	40	34	31	33	1.06	1.24	0.83
4	38	36	39	40	45	38	1.05	1.25	0.97
5	43	39	47	91	92	62	2.12	**2.36**	1.32
6	52	50	55	100	98	100	1.92	1.96	1.82
7	84	82	89	90	84	102	1.07	1.02	1.15
8	90	83	112	157	125	181	1.74	1.51	1.62
9	97	75	155	121	92	157	1.25	1.23	1.01
10	101	78	116	121	82	136	1.20	1.05	1.17
11	104	82	128	110	95	113	1.06	1.16	0.88
12	113	96	140	134	107	155	1.19	1.11	1.11
13	122	112	119	199	225	289	1.63	**2.01**	**2.43**
14	134	152	123	135	143	127	1.01	0.94	1.03
15	166	158	195	182	213	252	1.10	1.35	1.29
16	184	176	203	212	275	275	1.15	1.56	1.35
17	200	178	265	227	235	363	1.14	1.32	1.37
18	209	202	226	274	306	398	1.31	1.51	1.76
19	216	214	264	246	287	349	1.14	1.34	1.32
20	225	203	285	230	179	240	1.02	0.88	0.84
21	271	252	308	332	371	458	1.23	1.47	1.49
22	470	460	520	507	577	681	1.08	1.25	1.31