Supporting information for

KINATEST-ID™ : A PIPELINE TO DEVELOP PHOSPHORYLATION-DEPENDENT TERBIUM SENSITIZING KINASE ASSAYS

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Methods

Binding Affinity. Tb^{3+} binding to the phosphorylated and unphosphorylated forms of the various biosensors was measured using Tb^{3+} luminescence sensitized by the central tyrosine or phosphotyrosine residue of phosphorylated or unphosphorylated biosensors respectively. Tb^{3+} was added to 100 nM of the biosensors in either form at final concentrations ranging from 0 to 300 μM. All experiments were carried out in 10 mM HEPES and 100 mM NaCl (pH 7.5) at a volume of 100 μL. Luminescent emission spectra were collected between from 450 and 650 nm for 1 ms following a 50 μs dealy and 30 readings per data point after excitation of the samples at 266 nm (phosphorylated biosensors) or 275 nm (unphosphorylated biosensors). Background luminescence emission was subtratcted from the peptides in the absence of terbium. The area under each spectrum was integrated and used as the metric for quantification. The binding constant, K_d, was determined by fitting the data to equation [S1] using GraphPad Prism, where B_{max} is the maximum terbium luminescence based on the area under the emission spectra and X is the concentration of terbium.

$$Y = B_{max} * X / (K_d + X) \qquad [S1]$$

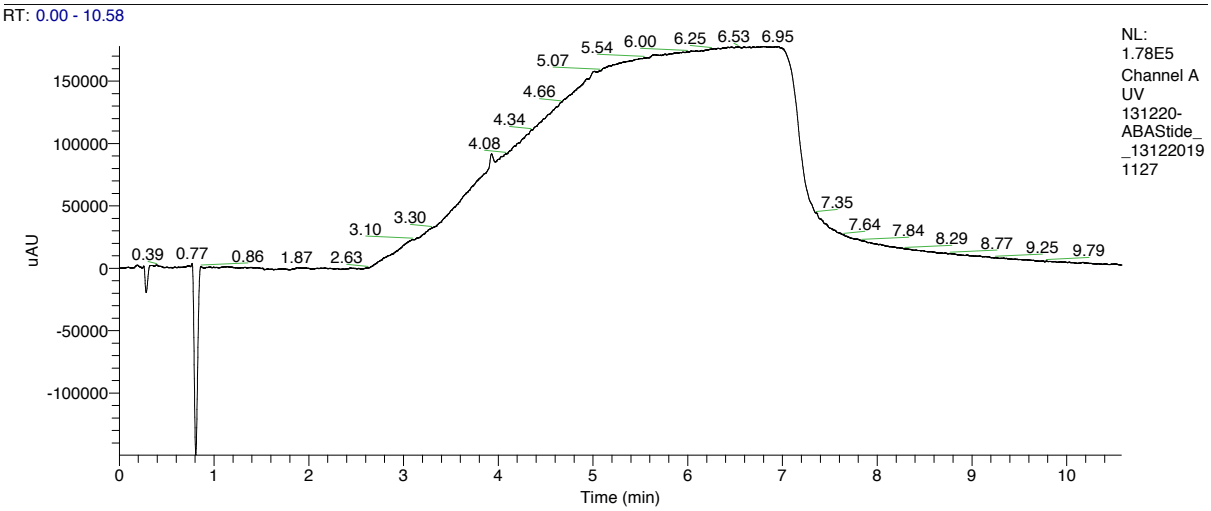
Luminescence Lifetime. Luminescence emission spectra were collected on a Biotek Synergy4 plate reader at 23°C in black 384-well plates (Greiner Fluortrac 200). Spectra were collected from 450-650 nm after excitation at 266 nm with a delay time starting at 100 μsec, increasing in steps of 100 μsec to 5000 μsec, and a collection time of 1 ms. Samples were prepared with 1 μM phosphorylated or unphosphorylated biosensor with 1 μM Tb^{3+} in 100 mM NaCl, 10 mM HEPES pH 7.5 in H_2O or D_2O in 100 μL total volume. The intensity of the emission peak at 549 nm was used as the metric for luminescence lifetime. Data were fitted to a single exponential decay curve using GraphPad Prism and lifetimes were determined. The luminescent decay rate of pKAStide:Tb^{3+} and KAStide:Tb^{3+} in H_2O and D_2O were determined and applied to the following equation [S2] to determine the hydration number (q):

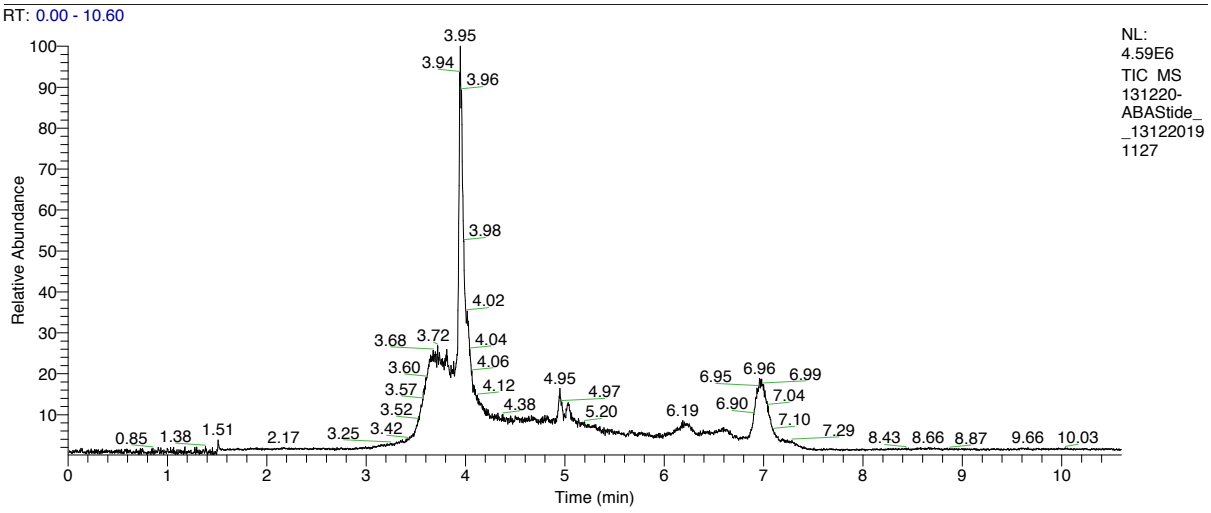
$$q = A'(k_{H2O} - k_{D2O} - 0.06) \qquad [S2]$$

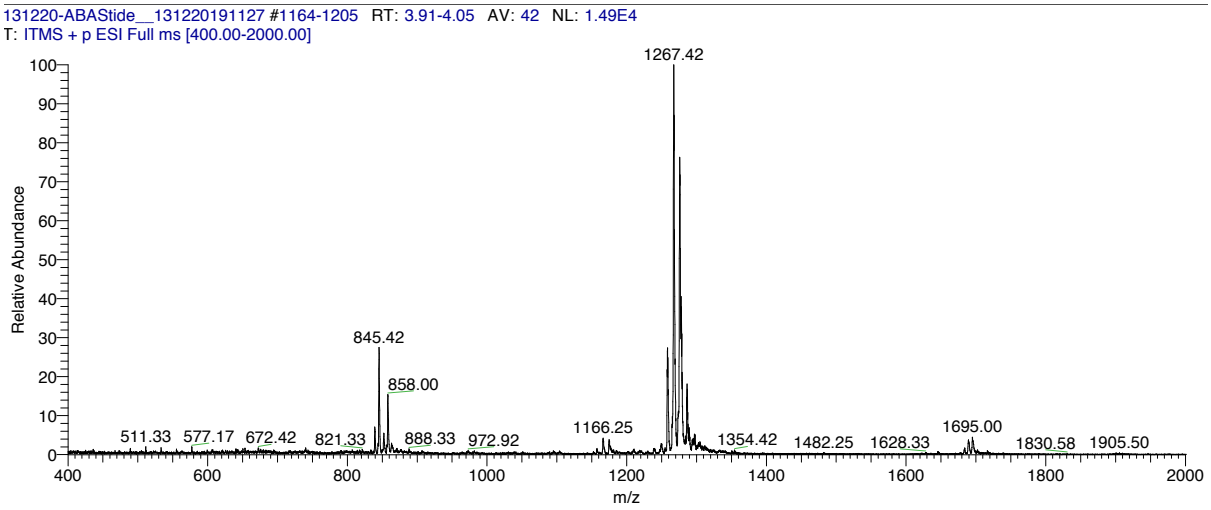
where k_{H2O} is the luminescence decay constant in H_2O and k_{D2O} is the luminescence decay constant in D_2O extrapolated from the plot. A' is the lanthanide specific constant, which is 5 for Tb^{3+}.

Peptides

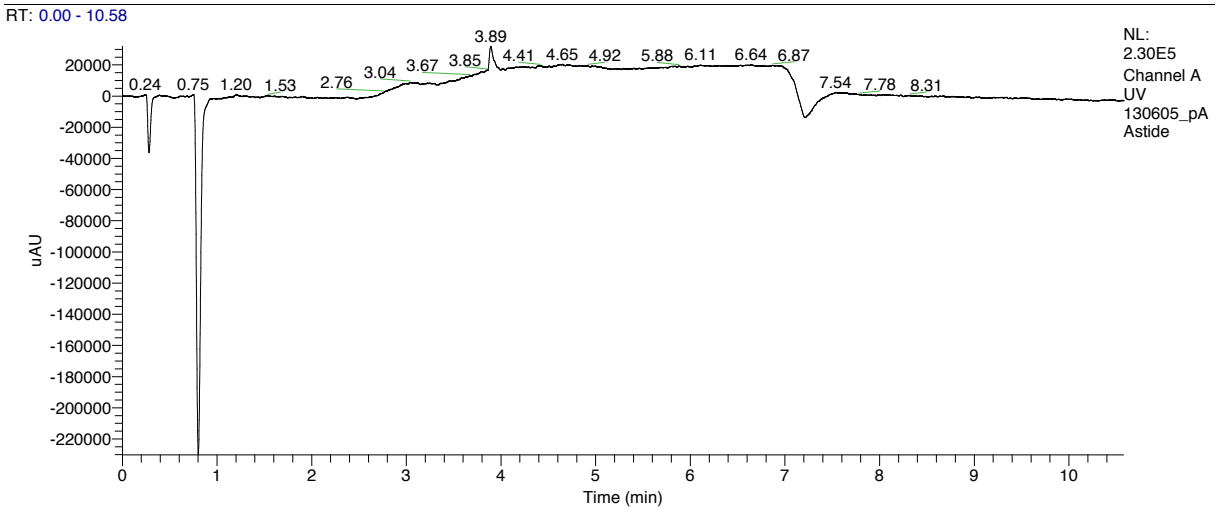
A. AbAStide







B. pAbAStide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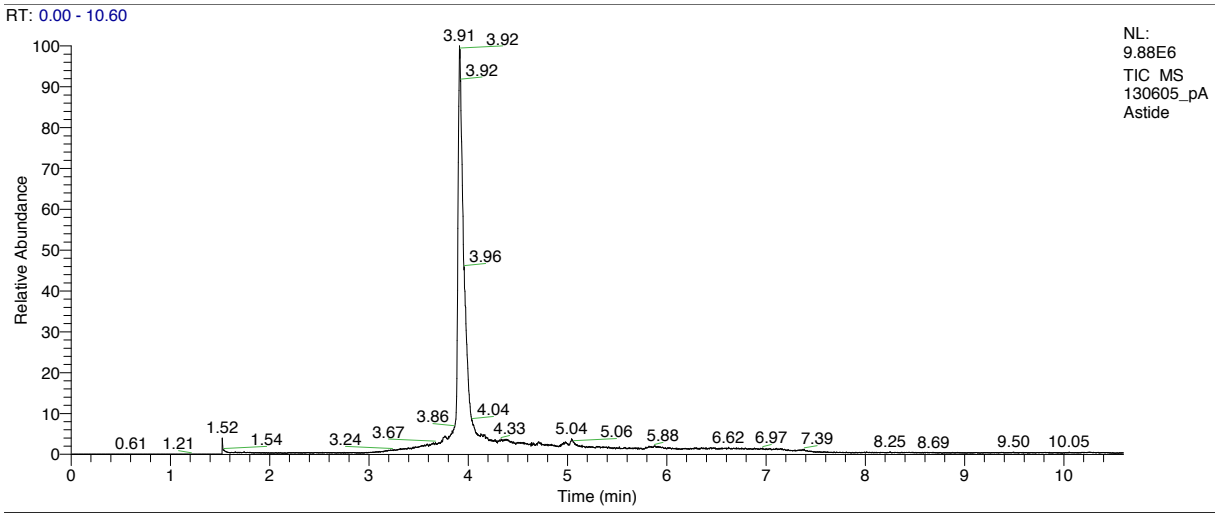
NL:
2.30E5
Channel A
UV
130605_pA
Astide
uAU
Time (min)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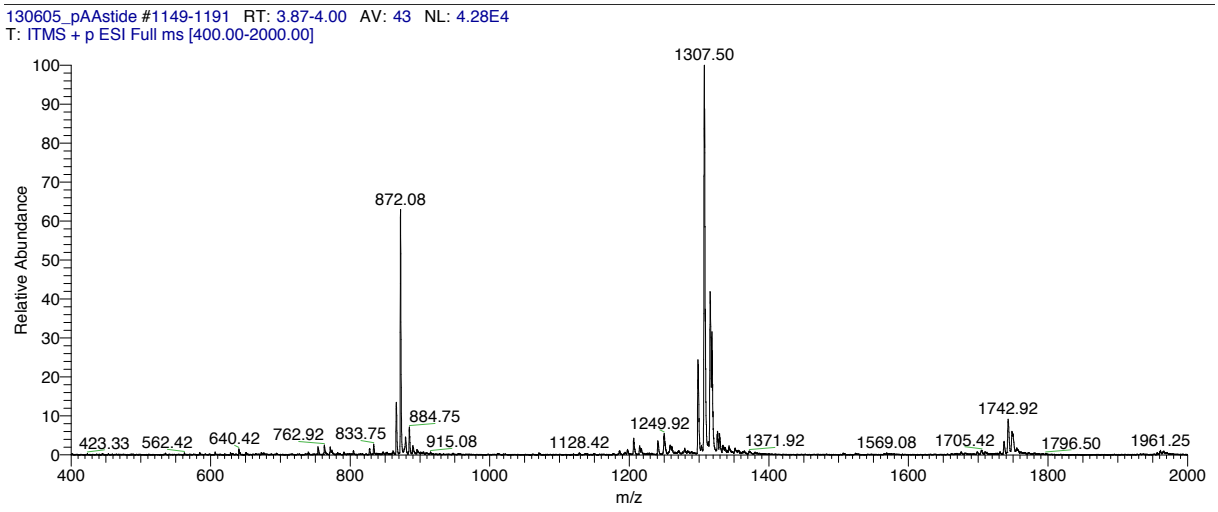
NL:
9.88E6
TIC MS
130605_pA
Astide
Relative Abundance
Time (min)


130605_pAAstide #1149-1191 RT: 3.87-4.00 AV: 43 NL: 4.28E4
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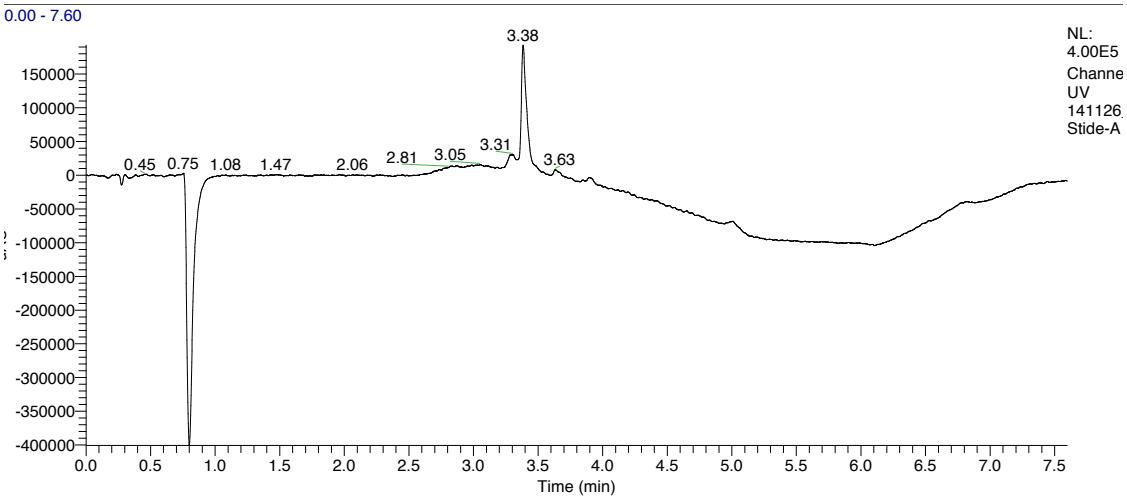
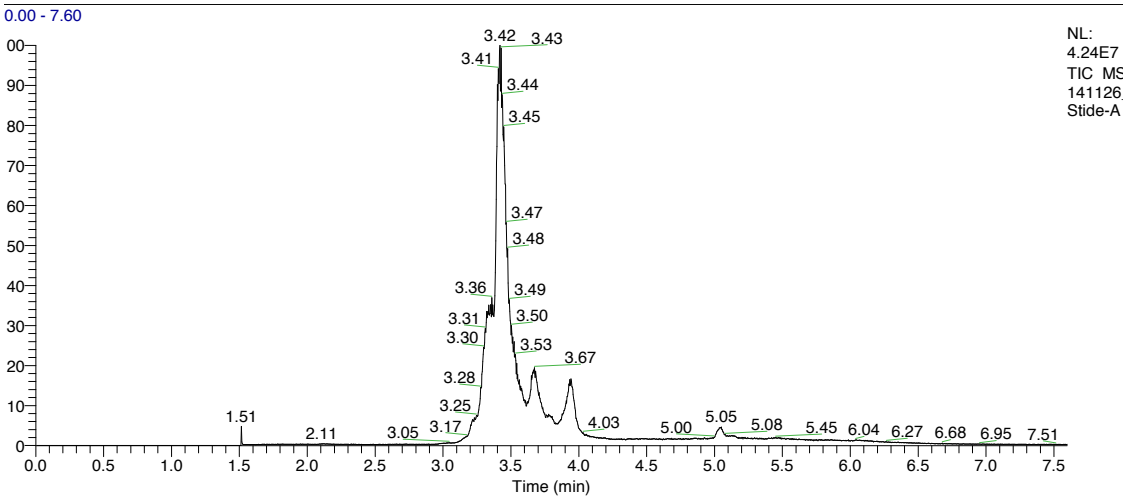
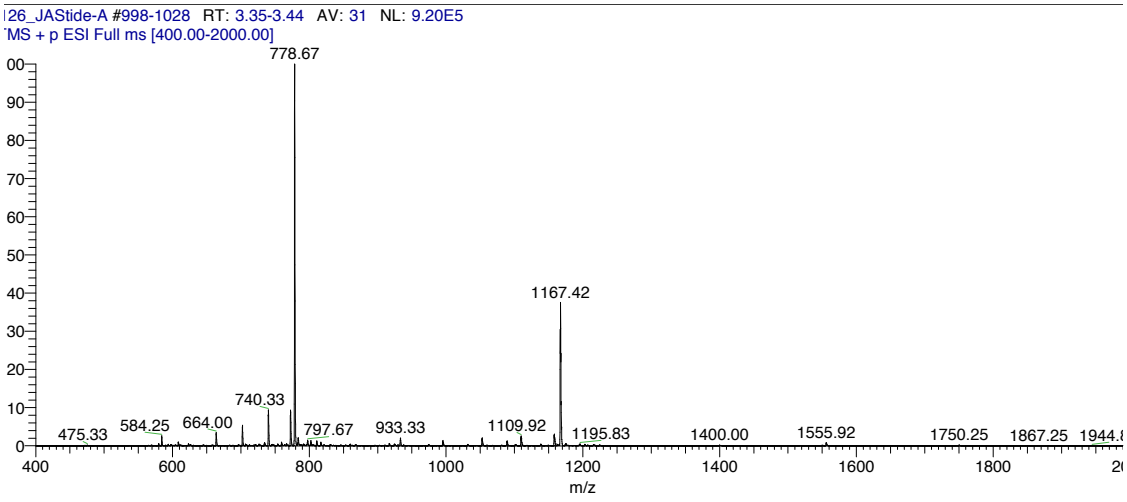
Relative Abundance
m/z

C. JAStide-A


0.00 - 7.60
NL:
4.00E5
Channe
UV
141126
Stide-A
3.38
0.45 0.75 1.08 1.47 2.06 2.81 3.05 3.31 3.63
Time (min)


0.00 - 7.60
NL:
4.24E7
TIC MS
141126
Stide-A
3.42 3.43 3.41 3.44 3.45 3.47 3.48 3.36 3.49 3.31 3.50 3.30 3.53 3.67 3.28 3.25 3.17 3.05 1.51 2.11 4.03 5.00 5.05 5.08 5.45 6.04 6.27 6.68 6.95 7.51
Time (min)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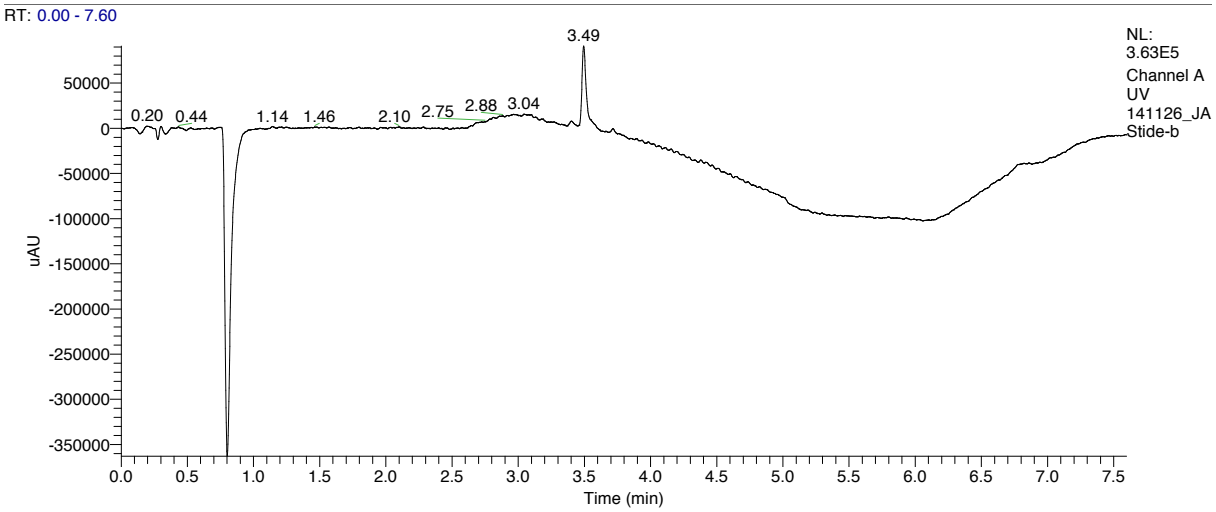
MS + p ESI Full ms [400.00-2000.00]
778.67
1167.42
740.33
475.33 584.25 664.00 797.67 933.33 1109.92 1195.83 1400.00 1555.92 1750.25 1867.25
m/z

D. JAStide-B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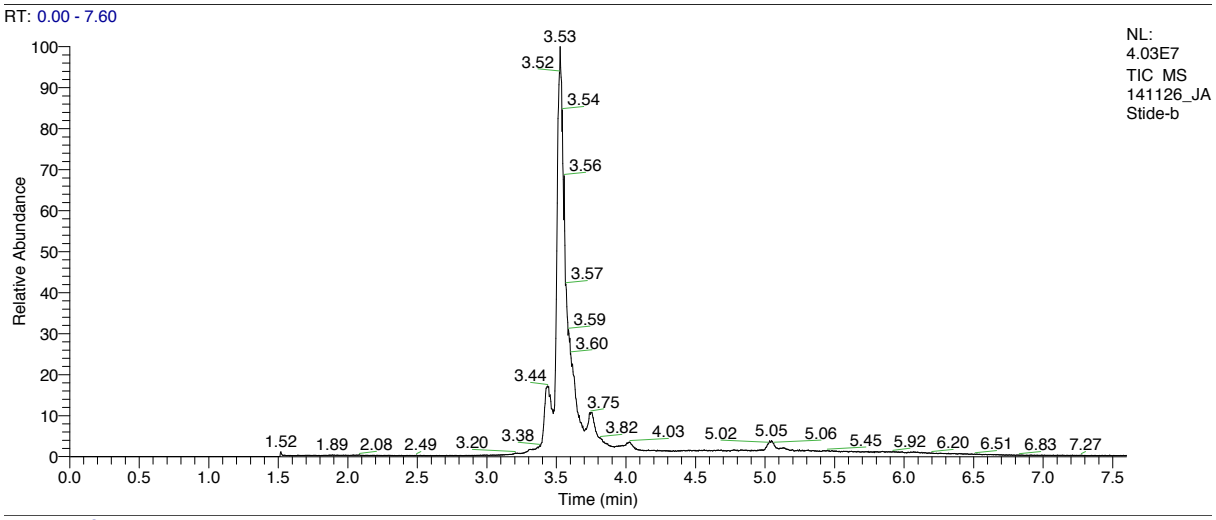
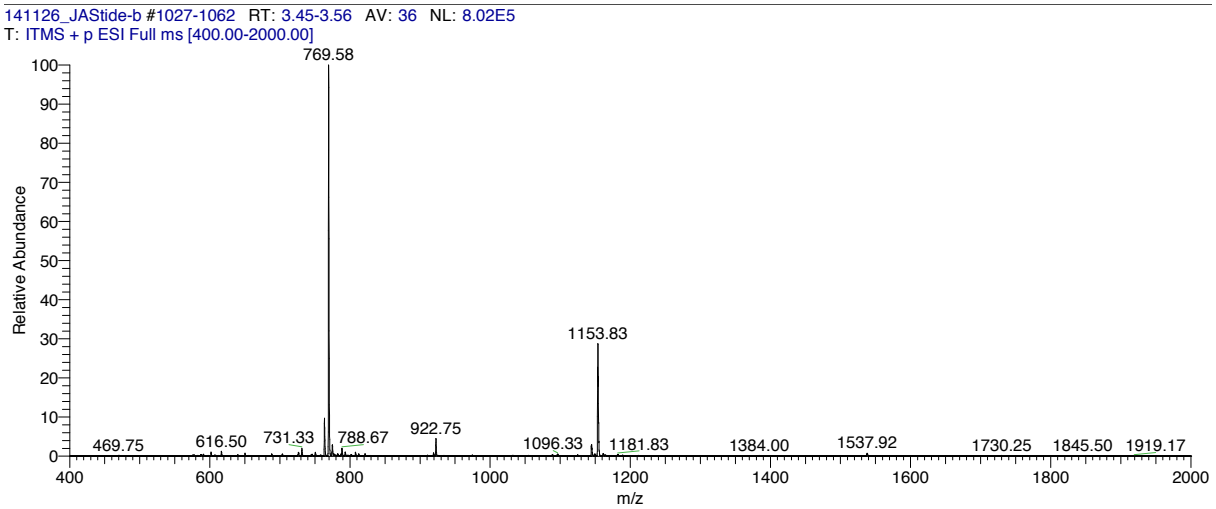
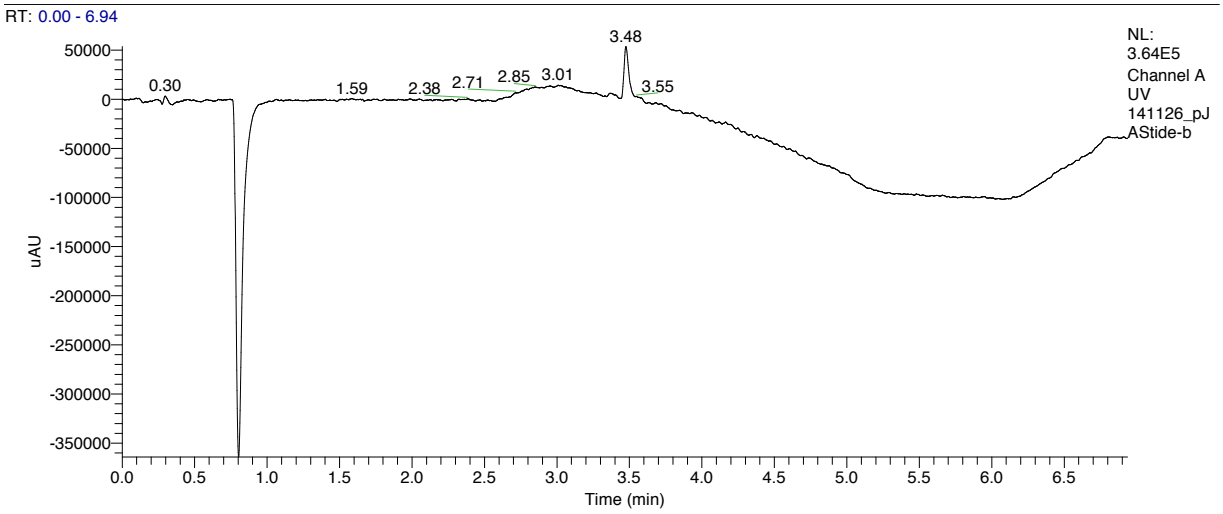
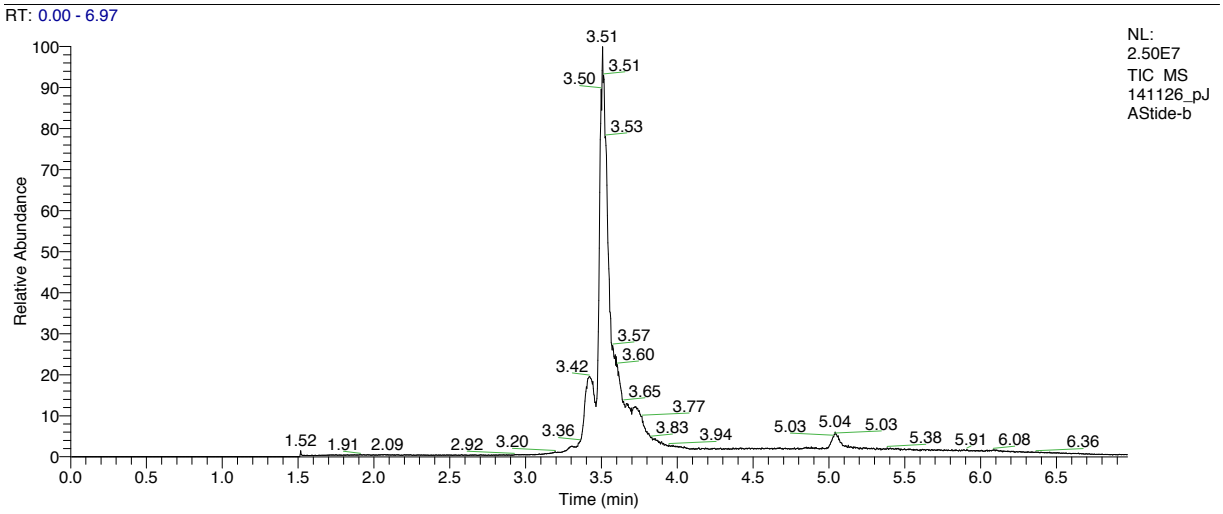
NL:
3.63E5
Channel A
UV
141126_JA
Stide-b
uAU
Time (min)


RT: 0.00 - 7.60
NL:
4.03E7
TIC MS
141126_JA
Stide-b
Relative Abundance
Time (min)


141126_JAStide-b #1027-1062 RT: 3.45-3.56 AV: 36 NL: 8.02E5
T: ITMS + p ESI Full ms [400.00-2000.00]
Relative Abundance
m/z

E. pJAStide-B


RT: 0.00 - 6.94
NL:
3.64E5
Channel A
UV
141126_pJ
AStide-b
uAU
Time (min)
0.30
1.59
2.38
2.71
2.85
3.01
3.48
3.55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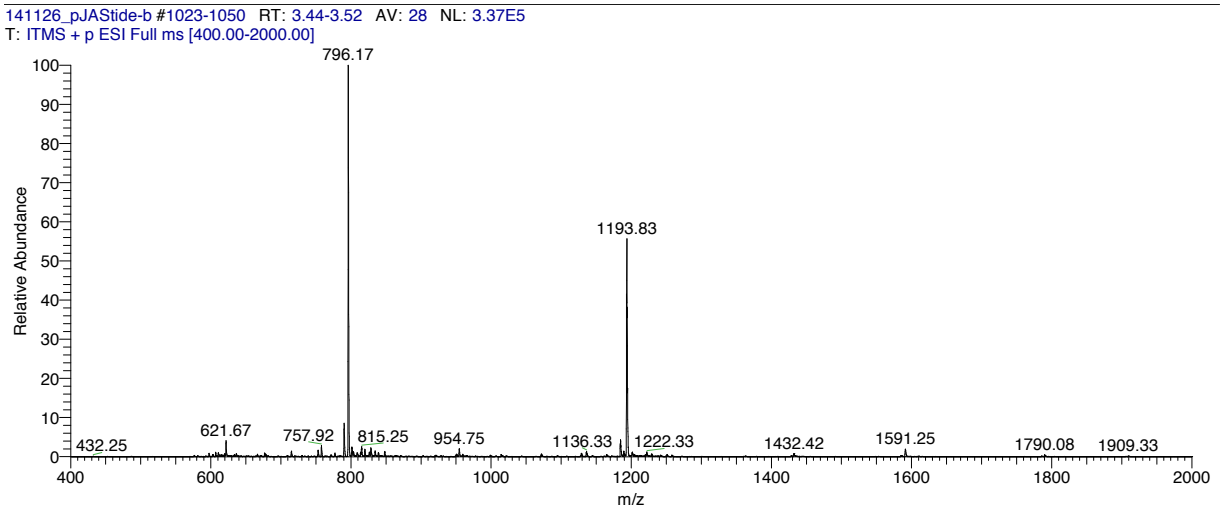
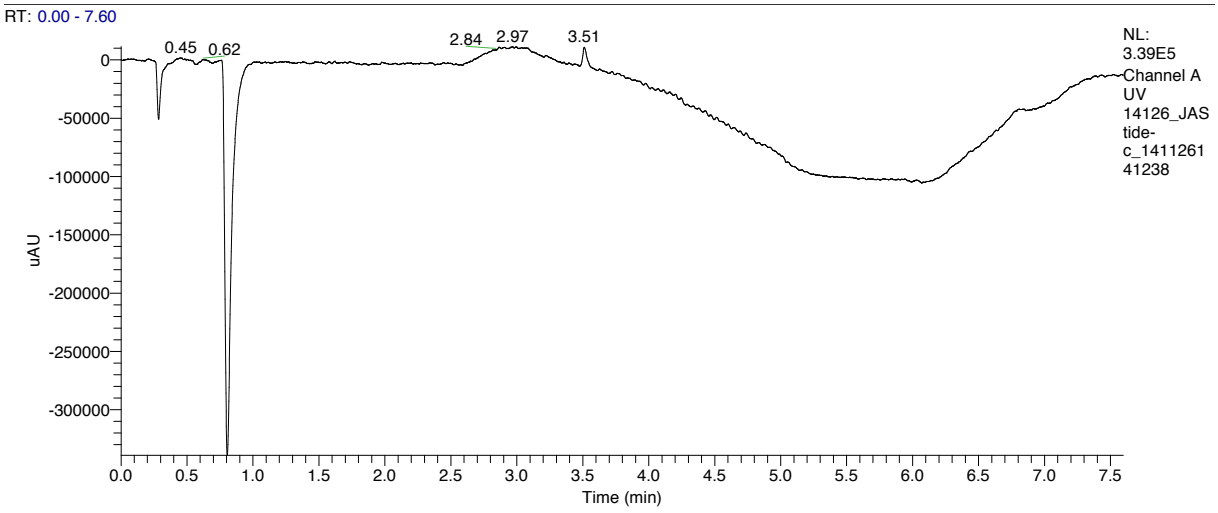
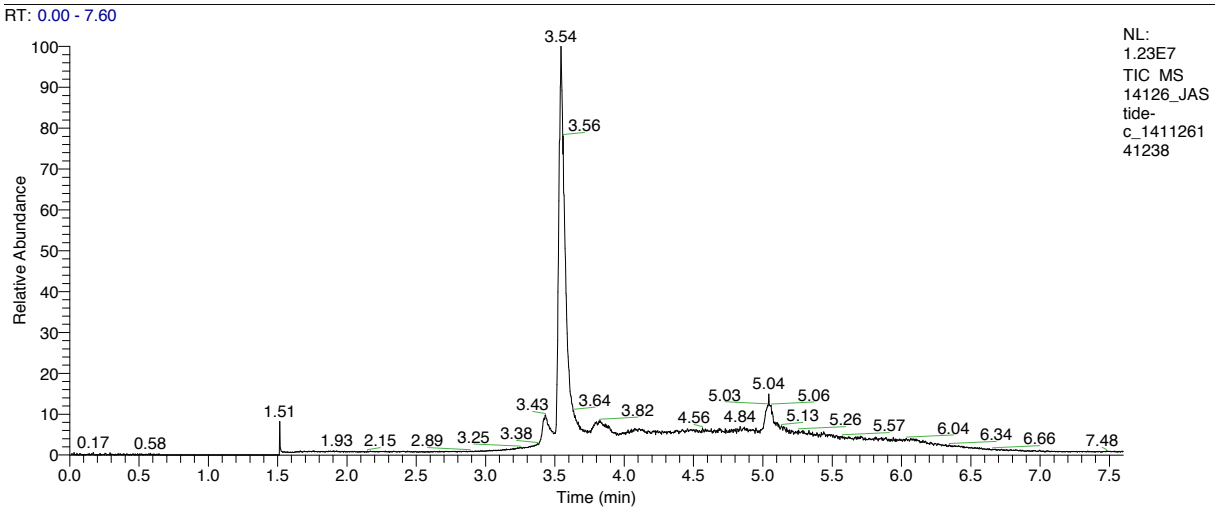
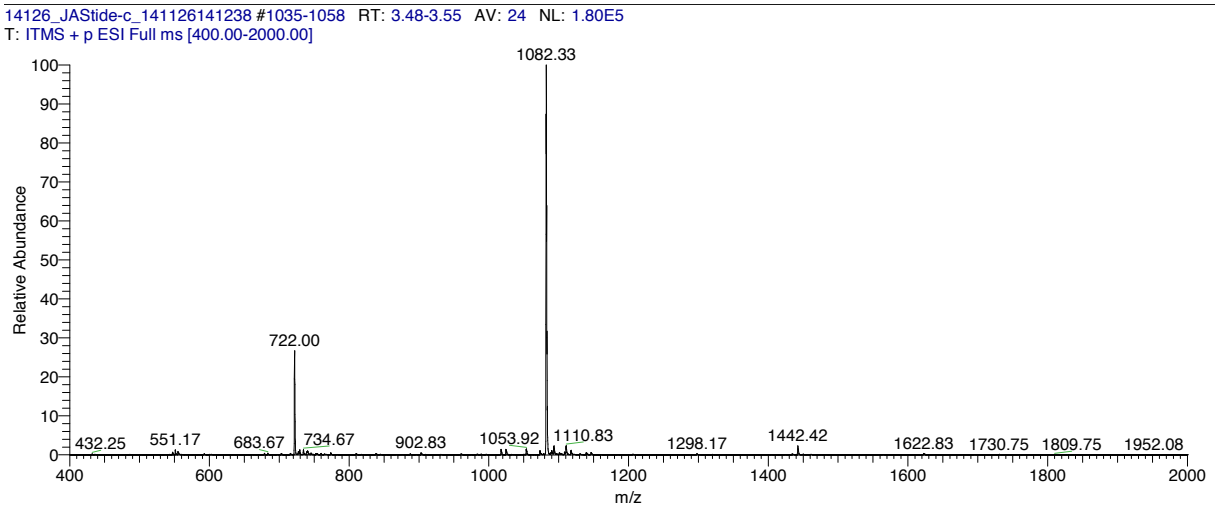
NL:
2.50E7
TIC MS
141126_pJ
AStide-b
Relative Abundance
Time (min)
3.51
3.50
3.53
3.42
3.57
3.60
3.65
3.77
3.83
3.94
3.36
3.20
2.92
2.09
1.91
1.52
5.03
5.04
5.03
5.38
5.91
6.08
6.36


141126_pJAStide-b #1023-1050 RT: 3.44-3.52 AV: 28 NL: 3.37E5
T: ITMS + p ESI Full ms [400.00-2000.00]
Relative Abundance
m/z
796.17
1193.83
432.25
621.67
757.92
815.25
954.75
1136.33
1222.33
1432.42
1591.25
1790.08
1909.33

F. JAStide-C


RT: 0.00 - 7.60
0.45 0.62
2.84 2.97
3.51
NL:
3.39E5
Channel A
UV
14126_JAS
tide-
c_1411261
41238
uAU
Time (min)


RT: 0.00 - 7.60
3.54
3.56
NL:
1.23E7
TIC MS
14126_JAS
tide-
c_1411261
41238
Relative Abundance
Time (min)


14126_JAStide-c_141126141238 #1035-1058 RT: 3.48-3.55 AV: 24 NL: 1.80E5
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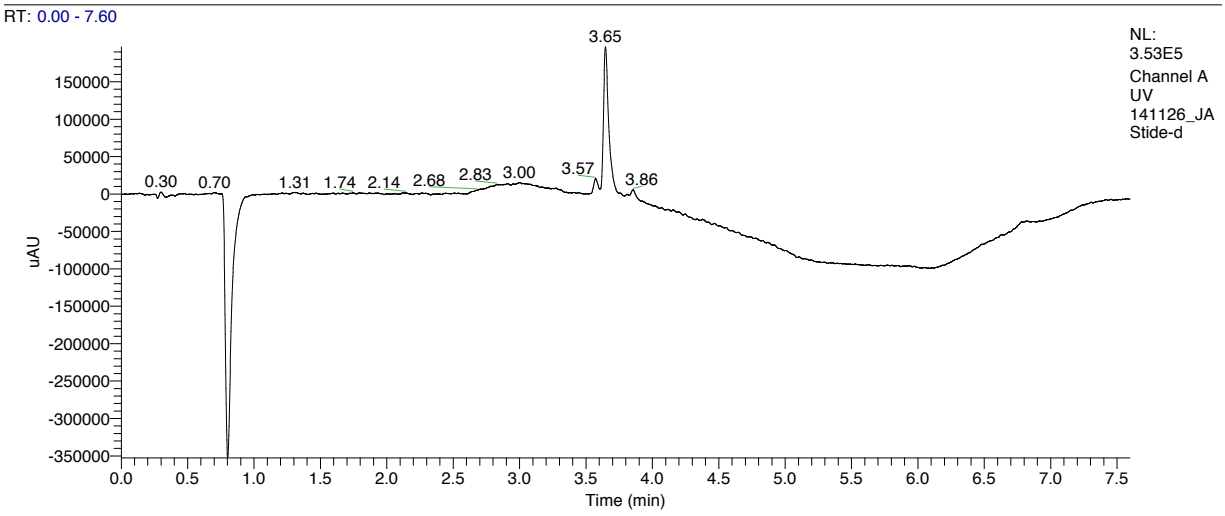
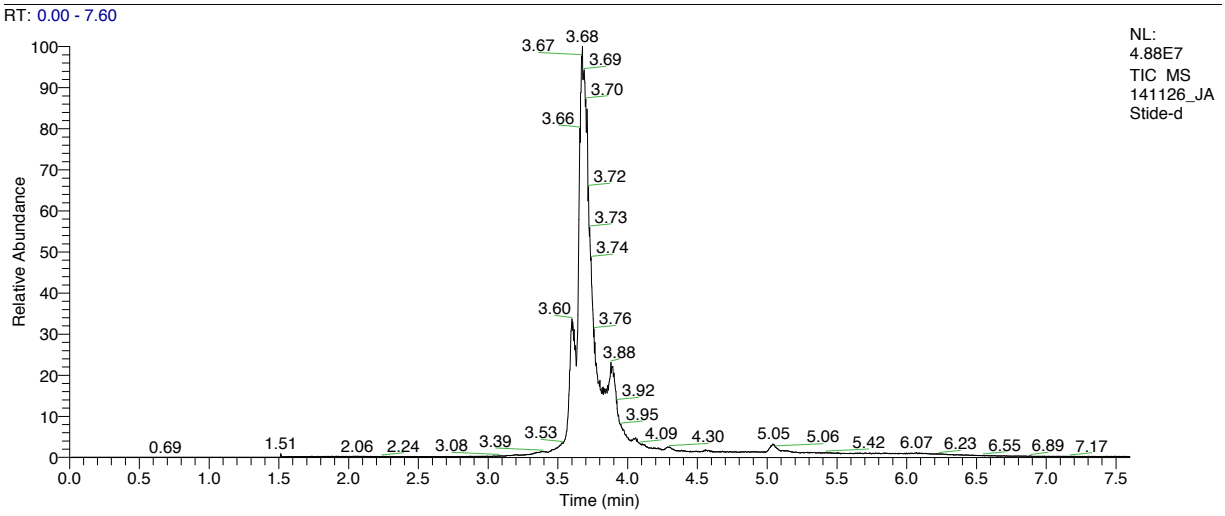
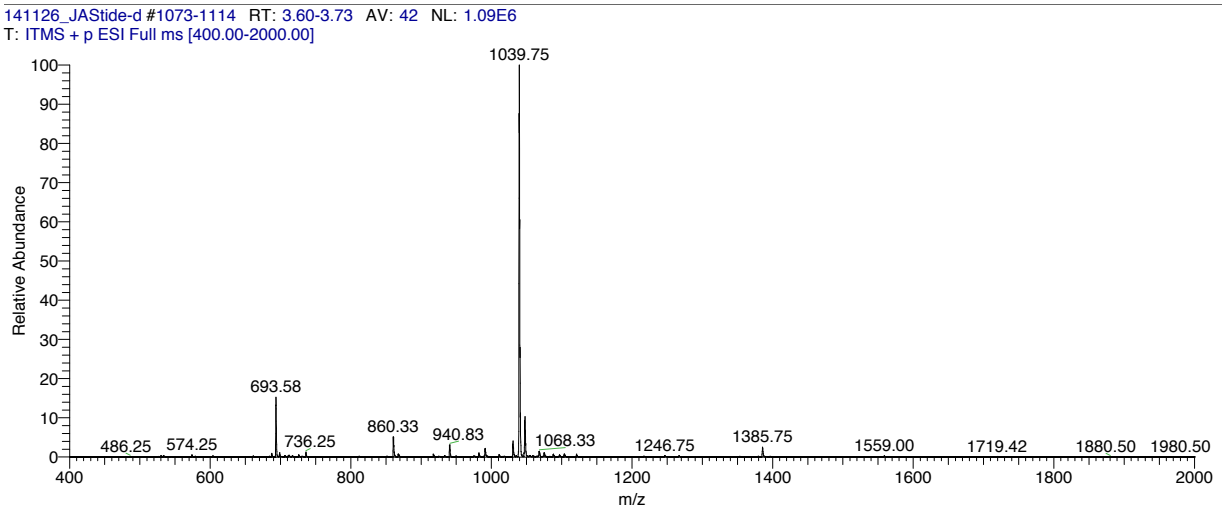
1082.33
722.00
Relative Abundance
m/z

G. JAStide-D


RT: 0.00 - 7.60
NL:
3.53E5
Channel A
UV
141126_JA
Stide-d
uAU
Time (min)


RT: 0.00 - 7.60
NL:
4.88E7
TIC MS
141126_JA
Stide-d
Relative Abundance
Time (min)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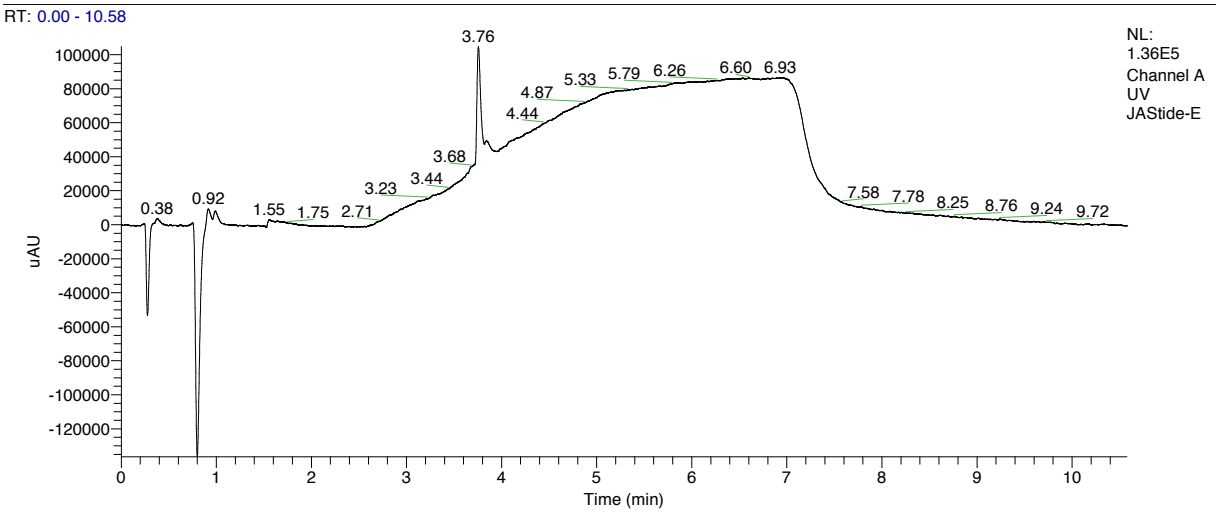
Relative Abundance
m/z

H. JAStide-E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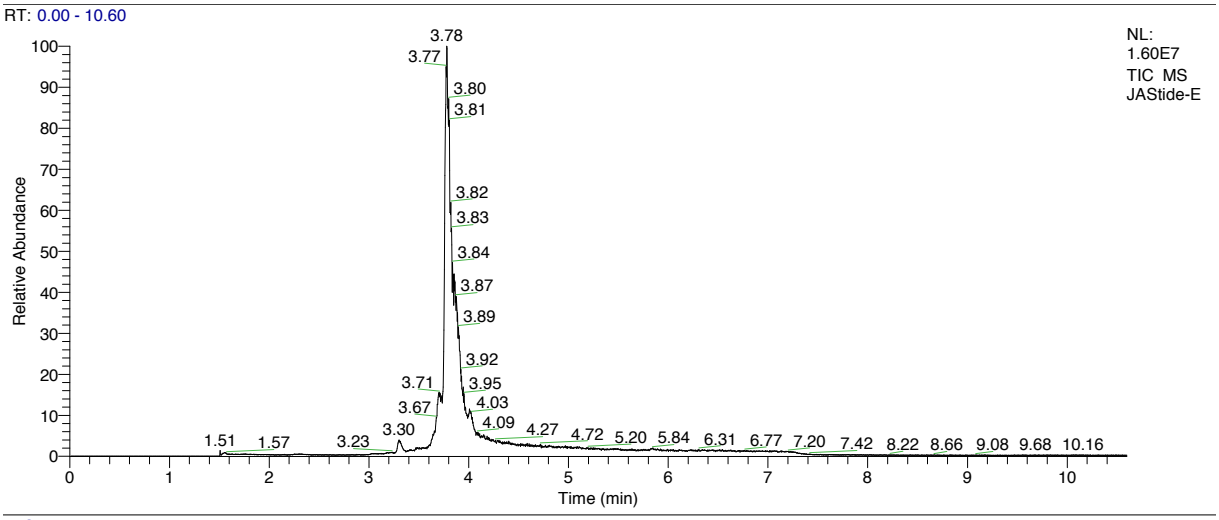
NL:
1.36E5
Channel A
UV
JAStide-E
uAU
Time (min)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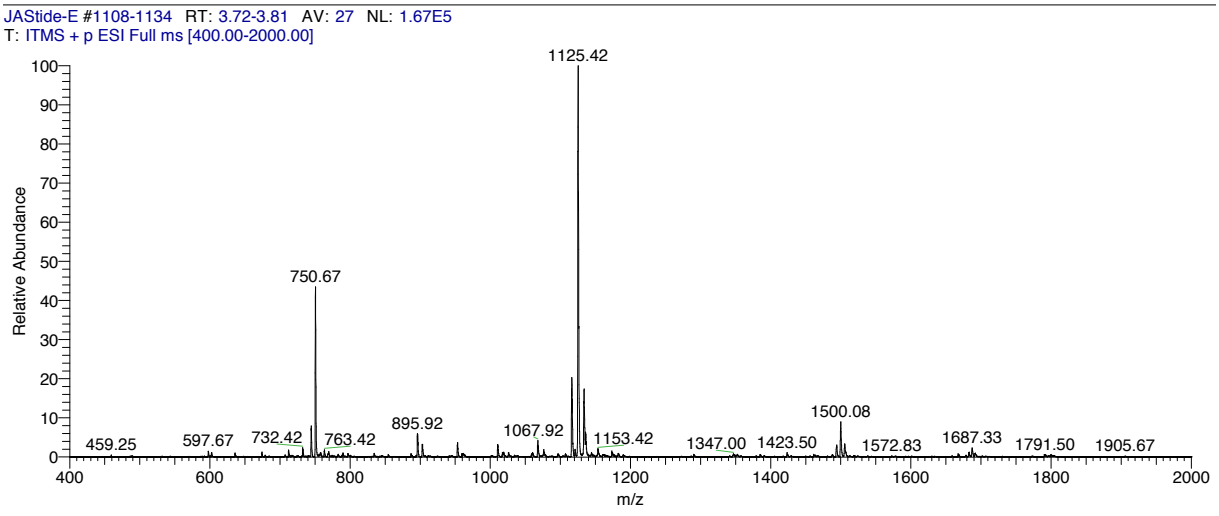
TIC MS
JAStide-E
Relative Abundance
Time (min)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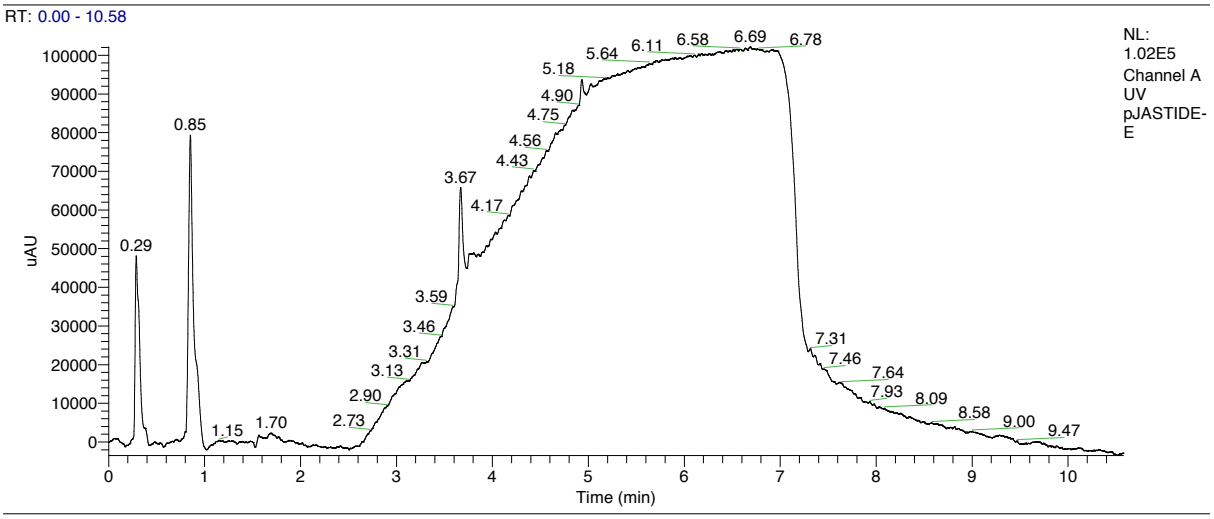
Relative Abundance
m/z

I. pJAStide-E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NL:
1.02E5
Channel A
UV
pJASTIDE-
E
uAU
Time (min)
0.29
0.85
1.15
1.70
2.73
2.90
3.13
3.31
3.46
3.59
3.67
4.17
4.43
4.56
4.75
4.90
5.18
5.64
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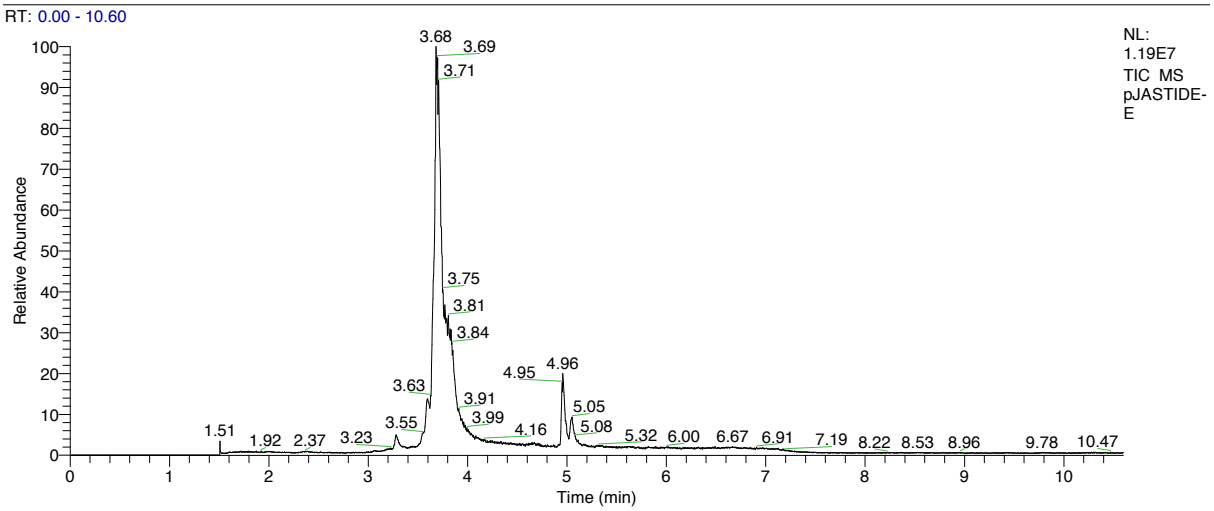
7.64
7.93
8.09
8.58
9.00
9.47


RT: 0.00 - 10.60
NL:
1.19E7
TIC MS
pJASTIDE-
E
Relative Abundance
Time (min)
1.51
1.92
2.37
3.23
3.55
3.63
3.68
3.69
3.71
3.75
3.81
3.84
3.91
3.99
4.16
4.95
4.96
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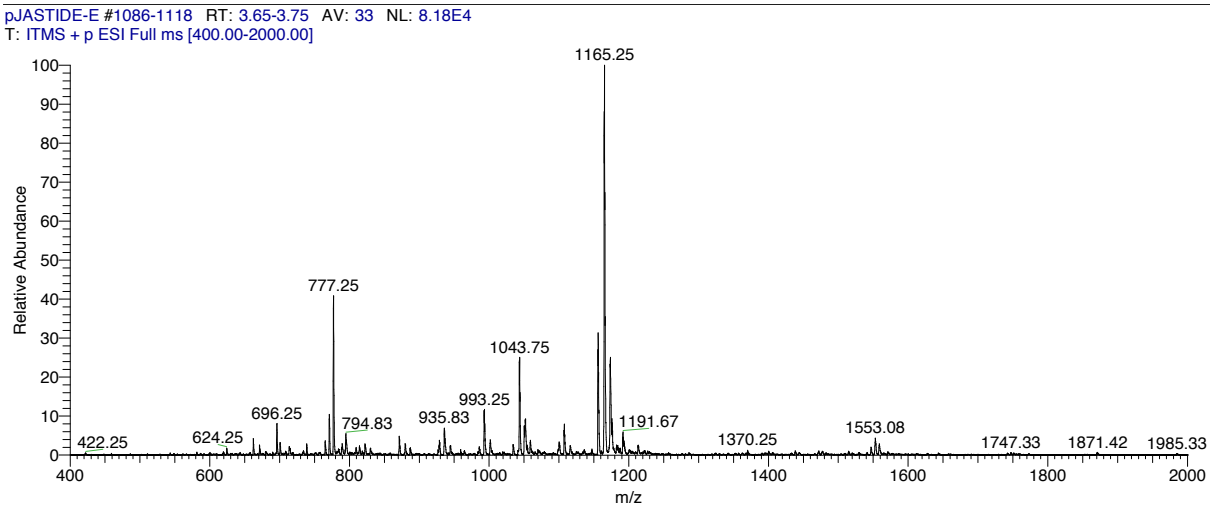
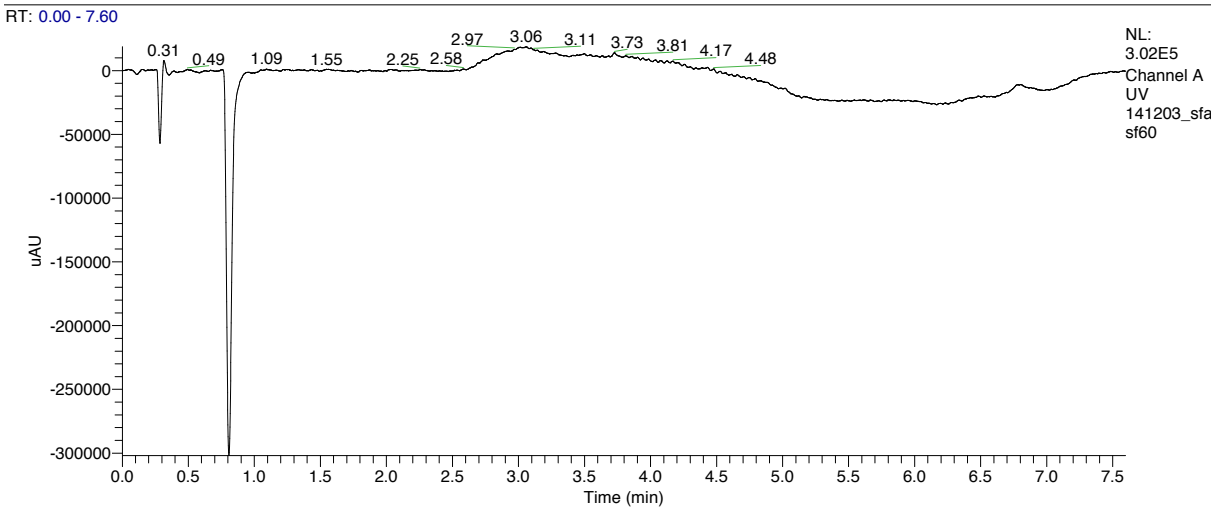
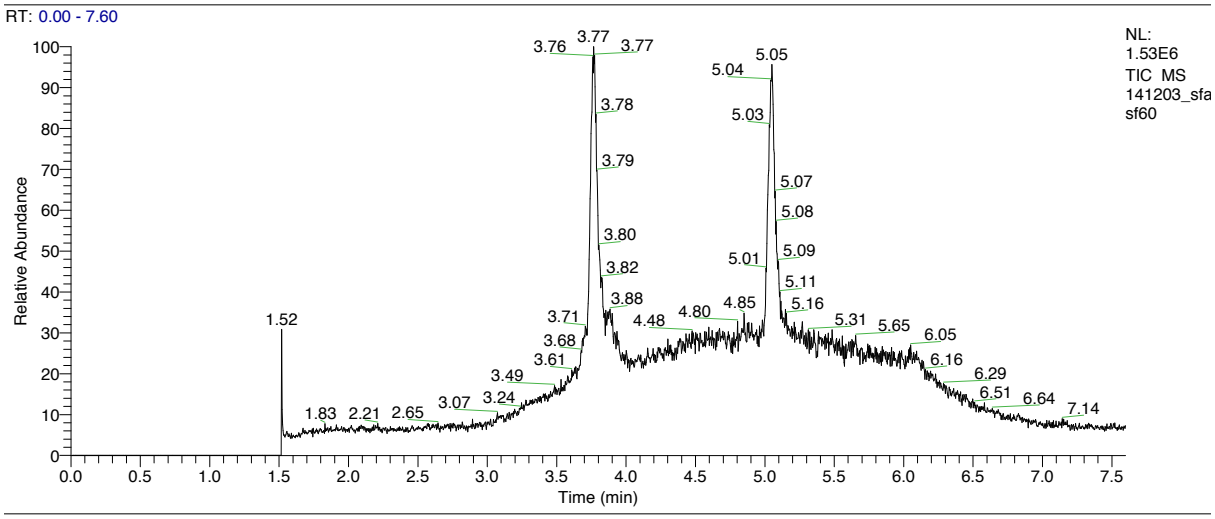
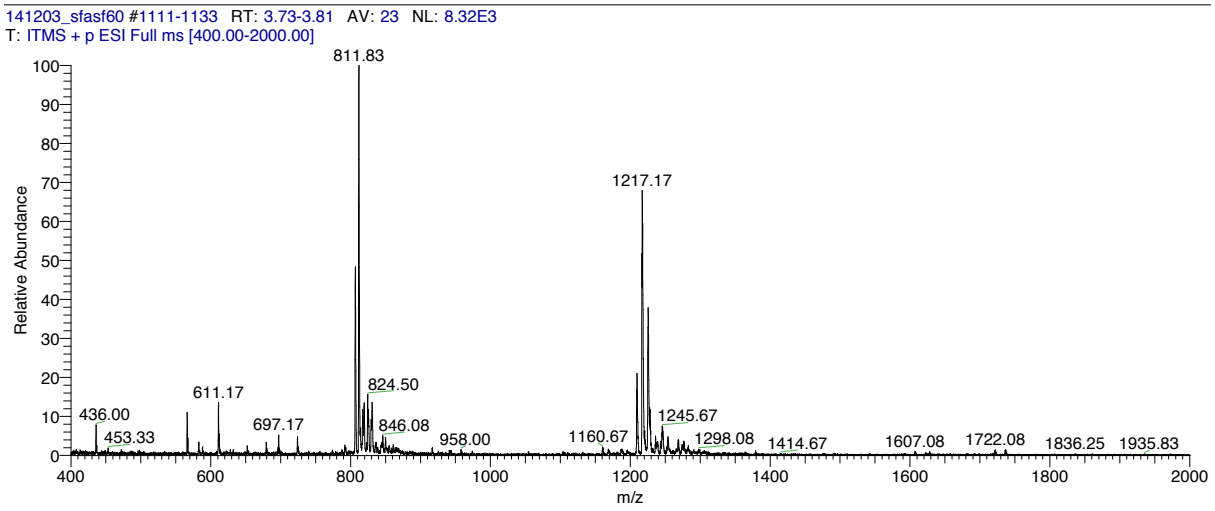
8.22
8.53
8.96
9.78
10.47


pJASTIDE-E #1086-1118 RT: 3.65-3.75 AV: 33 NL: 8.18E4
T: ITMS + p ESI Full ms [400.00-2000.00]
Relative Abundance
m/z
422.25
624.25
696.25
777.25
794.83
935.83
993.25
1043.75
1165.25
1191.67
1370.25
1553.08
1747.33
1871.42
1985.33

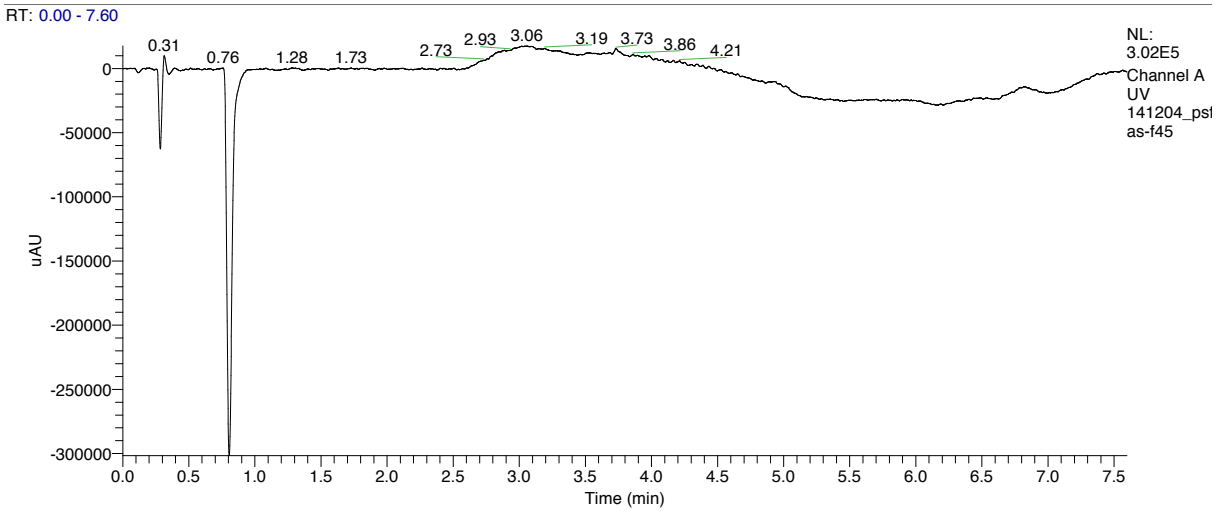
J. SFAStide-A







K. pSFAStide-A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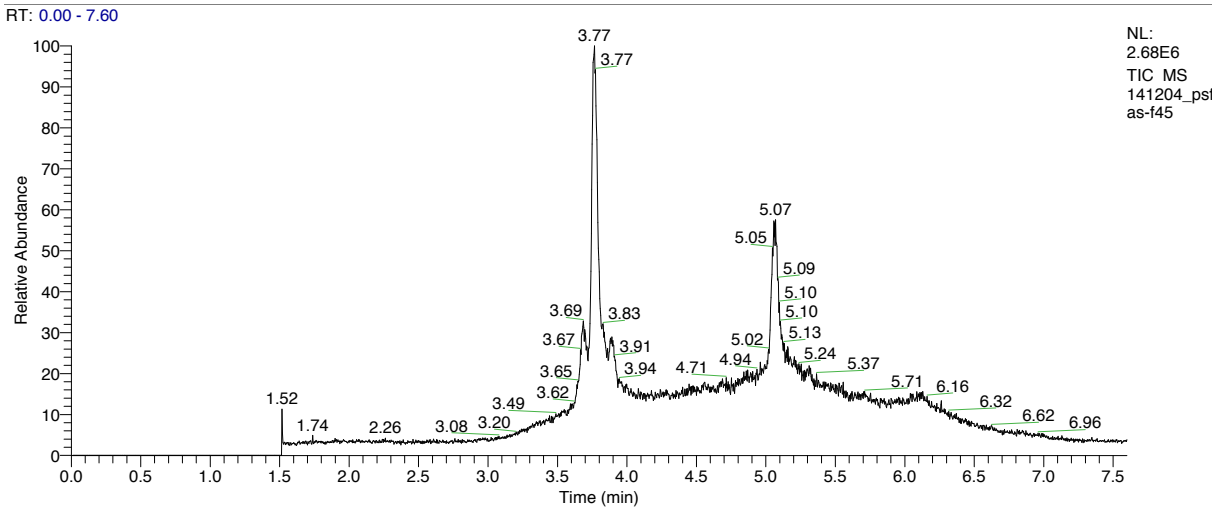
Channel A
UV
141204_psf
as-f45
uAU
Time (min)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NL:
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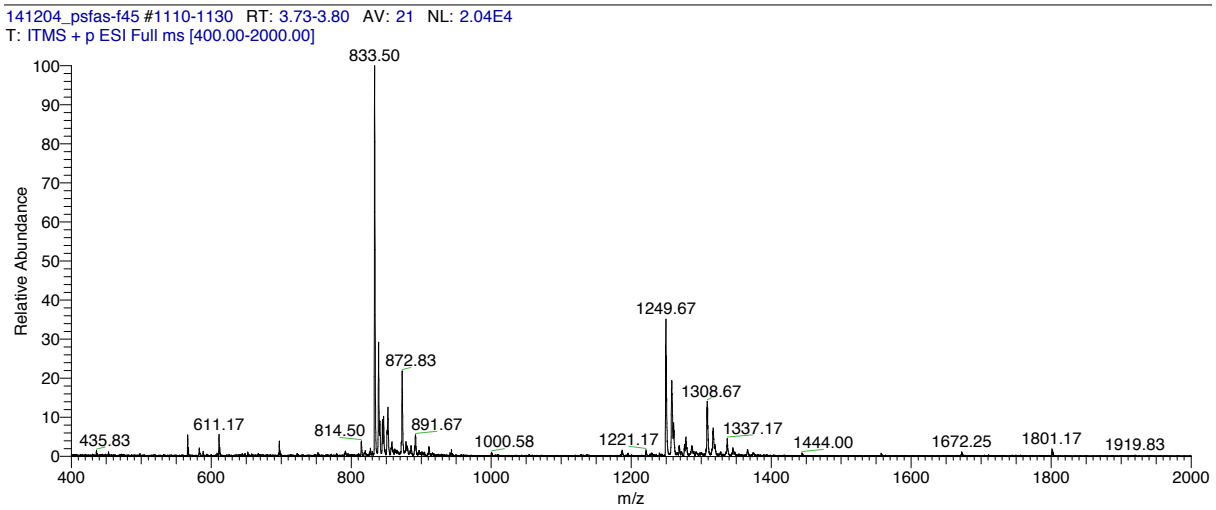
TIC MS
141204_psf
as-f45
Relative Abundance
Time (min)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T: ITMS + p ESI Full ms [400.00-2000.00]
Relative Abundance
m/z

L. SAStide

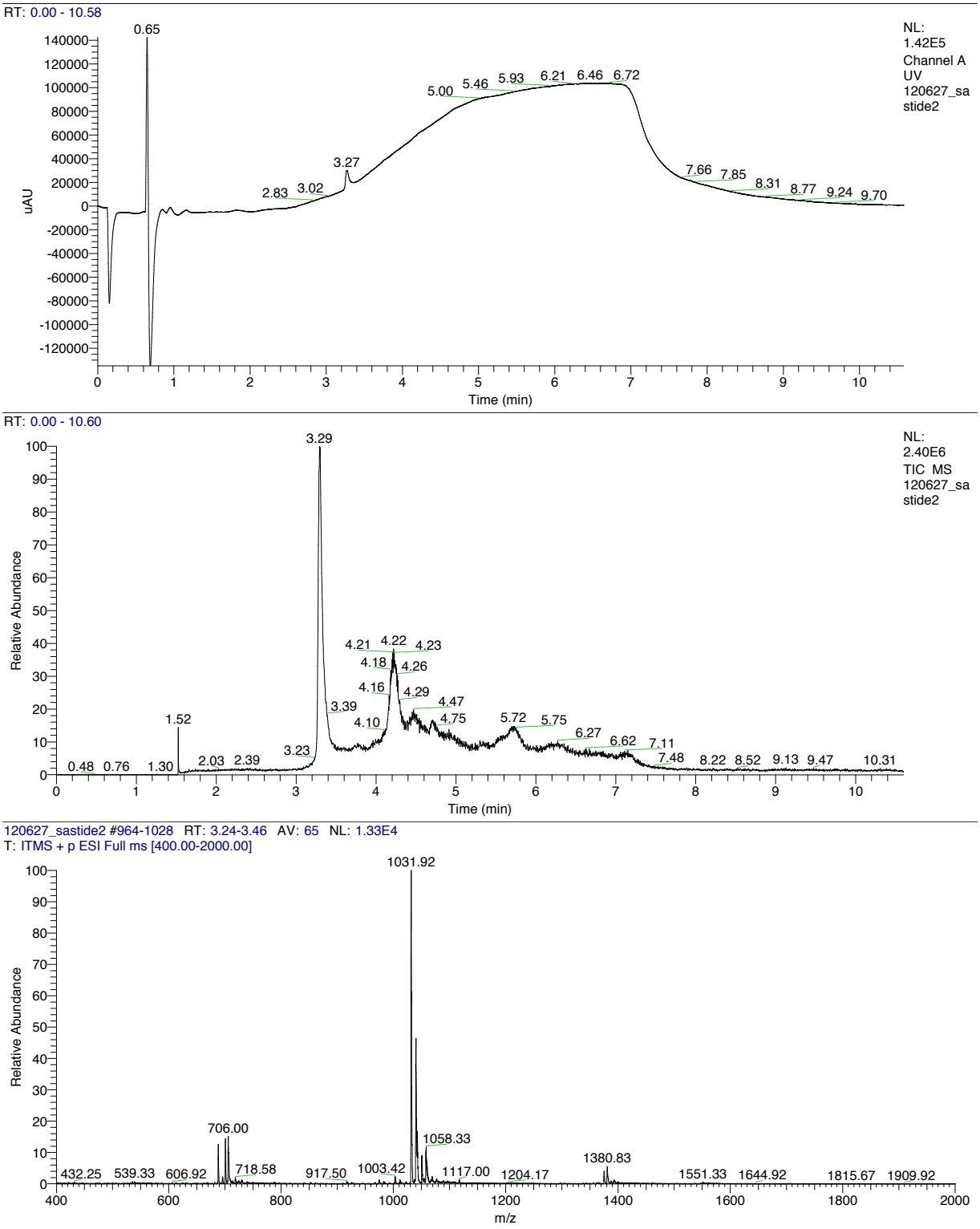

Table S1. Abl substrate motifs

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Abl	DPL	A	X	V	I/V/L/F	A	A	P/F	F	Songyang, Z. et al.

										Nature (1995), 373, 536-539
	Microarray	P	E/S	Q/N	I/L	A	F/S	P	R/W	Rychlewski, L. et al. J. Mol. Biol. (2004), 336, 307-311.
	NetPhorest	N/G/A/E/S	S/G/E/P/D/Q/A	P/G	V	A	Q/T/S	P/Y	V/S	Miller, M.L. et al. Sci Signal. (2008), 1, ra2
	One-bead-one-peptide		X	V	I	A	A	P	F	Wu, J.J. et al. Bioorg Med Chem Lett. (1998), 8, 2279-84.
			E	E	I	E	E	P	Y	
		E	D	A	I					
			E	X	I	X	X	P	X	
	KINATEST-ID	E/D/G	D/V/P/E/H	V/I/G/S	I/V/F/H	Q/A/D/I	T/P/Q/S	P/W/F/V/I	P/D/W	This work

Table S2. Btk substrate motifs

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Btk	M3	S/V/D/E/I	E/L/N/D	A/D/K/P	I/L/D/E	D/V/G/L	S/E/D/V	X	L/S/E/P	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	V/W/S/E	L/N/D/V	A/D	I/L	D/V	I/S/V/T/E	C/G/H/P	L/R/V/S	This work

Table S3. Csk substrate motifs

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Csk	DPL	X	E	P/V	I/V/L	M/I/F	F/I	F/I/L	F/I	Ruzzene, M. et al. Eur. J. Biochem. (1997), 246, 433-439
	M3	T/E/A/N/P	E/F	P/G/E/A/D	Q/I/V/D	Q/I/E/S	P/F/Q/V/E	G/Q/T/E/L	E/P/D/Y	Newman, R.H. et al. Mol. Syst. Biol. 9:655
	KINATEST-ID	T/D	E/Q	P/S/V/C	I/Q/V	Q/F/M/A	E/I/Q/S	F/P	F/K/N/T	This work

Table S4. Fes/Fer substrate motifs.

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Fes	DPL	E/A/D	E/A	E/A/G	I/E/V/D/L	E/D/A	E/D/I/V	I/V/E/M/L	E/I/V/D/G	Songyang, Z. et al. Nature (1995), 373, 536-539
	KINATEST-ID	N/M/D/T	D/E/Q	P/G	I/V/F/D	E/T/Q	D/N/T	V/I/T	E/G/R	This work

Table S5. Jak2 substrate motifs.

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Jak2	PAM Chip	E/D/A/L	D/N/G/V	A/D/P/H	E/D/T/H	L/I/Y/V/T	K/Y/I/R/Q	L/V/P/A	D/A/S/L	Sanz, A. et. al. PLoS One, (2011), 6,

										e18522.
	NetPhorest	T/A/L/S/I	S/D/Q/I	D/A/K/L	D/E/P	L/A/Y/V	K/V/T/P	V/L/T/E/P	S/K/V/D	Miller, M.L. et al. Sci Signal. (2008), 1, ra2
	M3	A	I/D	D/E	X	L/V/T	K/A/P/Q/V	V/P/L/M	D/L	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	K/D/R	D/I/N/M/P	D/M/P/K	D/E/R	L/I/V/F	I/K/Q/T/P/V	L/V/P/T	D/P/S/K	This work

Table S6. Src family kinase substrate motifs.

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Fyn	Phage Display			E	T/ϕ	G	X	I/V/L		Dente, L. et al. J. Mol. Biol. (1997) 269, 694-703
	M3	D/E	D/E	X	V/L/T	E/D	D/T	V/P/L	X	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	D/P/G/M/W/E	E/D/K/Q	N/D/G/S/E	I/V/L	G/D/E/S	T/S/D/M/I	F/I/V/L	X	This work
Lyn	DPL	D/E	E/D	E/D	I/V/L	E/G/D	E	L/IV	E	Ruzzene, M. et al. Eur. J. Biochem. (1997), 246, 433-439
	Phage Display		D/E	X	I/L	E/D	X	L	P	Schmitz, R. J. et. al. Mol.Biol. (1996), 260, 664-77.
	M3	E/D	E/K	D/N	D/T/L	E/L/D	D/N/T	V/P/M	D	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	D/E/S/K/G	E/D/K/V/I	D/N/G/T/E	I/V/L/T/F	E/G/D/A/V	F/S/T/E	L/I/V/P/W/F	P/W	This work
Hck	M3	E/T/D	G/P/K/S	L/S/A/D	X	D/Q/V/I	A/P/K	P/V/I/R	A/E/P/V	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	E/W/D/K	H/P/D/E	D/S/R/A	V/I/L	T/H/S/D/V	F/K/S/V/A	F/V/P/I/L	V/A/P/N	This work
Lck	DPL	X	E	X	I/V/L/F	G/A	V	L/V/F/I	F/L/V/I	Songyang, Z. et al. Nature (1995), 373, 536-

										539
	M3	E/D	D/E/L	D/S/E	X	D/E	X	L/P/V	X	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	V/D/Q/P	D/E/N/S	D/S/N/E/A	I/V/L	D/G/T/W/E	S/M/T/N	I/M/L/V	R/P/M/T	This work
Src	DPL	D	E/D	E/D/G	I/V/L	G/E/D	E	F/I/L/V	D/E	Songyang, Z. et al. Nature (1995), 373, 536-539
	One-bead-one-peptide			E/N/I/L	I/L/D/E	E/I/L	E/D			Kim, Y.G. et. al. Angew. Chem. Int. ed. Engl. (2007), 46, 5408-11.
	KINATEST-ID	E/D/N/P	E/D/S/N/A/Q	D/E/G/S	I/V/L/T/E/D	G/E/D	E/V/A/I	F/M/V/I	X	This work
Yes	M3	E/D/S/A	D/E/S	A/P/Q/T	X	E/A/G	A/T/P	P/V/A	A/E/P	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	D/E/T/A/S	E/D/S	D/E/G/H	I/V/L/T	E/S/T/G/D	V/A/T/S	V/L/P/F/I	G/N/W/H	This work

Table S7. Syk substrate motifs.

Kinase	Method	-4	-3	-2	-1	+1	+2	+3	+4	Ref
Syk	DPL	E/D	E/D	E	E/D/I	E/V/I	E/V/I	I/V/E	V/I/E	Ruzzene, M. et al. Eur. J. Biochem. (1997), 246, 433-439
	Positional Scanning	E/D	E/D	X	E/D	X	X			Kim, J.-Y. et al. Immuno. Lett. (2011), 135, 151-157
	NetPhorest	D/T/S/E	D/E/S/Q	D/E/S/N	D/E/P	E/V/D/L	D/E/T/N	V/P/L/A	D/N	Miller, M.L. et al. Sci Signal. (2008), 1, ra2
	Sequence Alignment	D	E	E	D	E	X	P	X	Geahlen, R.L. BBA (2009), 1793, 1115-27
	Phage Display	X	X	X	D	E	X	X	X	Schmitz, R. J. et. al. Mol.Biol. (1996), 260, 664-77.
	KALIP	L/E/D/P	E	N/E/L/D	D/E/A/L	E	A/D/E/S	V/I/L/S	G/K/I/L	Xue, L. et al. PNAS (2012), 109, 5615-20.
		D/E/A/S	D/E/A/P	E/D/G/A	D	E/V/D/L	E/V/D/L	D/K/L/P	V/K/P/T	
	M3	D/E/A/T	D/E/S/N	D/E/G/N	D/G/E	E/V/D	D/N/T	P/V/L/A	X	Newman, R.H. et al. Mol. Syst. Biol. (2013) 9:655
	KINATEST-ID	D	D/E	D/E	D	E	D/N	P/V	X	This work

Table S8. Site Selectivity Matrix (SSM).

Kinase	-4	-3	-2	-1	+1	+2	+3	+4
Abl	0.48	0.41	0.66	1.05	0.35	0.42	2.31	0.16
Arg	1.08	0.76	1.58	1.88	1.17	0.88	2.96	1.8
Btk	1.73	1	1.66	2.22	2.69	1.13	1.30	1.36
Csk	1.29	2.49	1.16	1.38	1.57	0.97	0.78	0.65
Fak	2.88	1.74	1.40	0.85	1.5	1.15	1.66	1.3
Fyn	0.51	0.57	0.40	0.68	0.99	1.25	0.93	0.67
Hck	1.36	1.02	0.76	1.24	1.13	0.87	1.41	1.38
Jak2	1.20	1.42	0.96	1.98	1.02	0.60	1.55	0.87
Lck	0.72	1.98	1.36	1.54	1.19	0.73	0.92	1.09
Lyn	1.26	1.44	0.80	0.52	1.28	1.25	1.61	0.82
Pyk2	1.36	1.55	1.24	2.13	1.92	1.64	3.13	1.56
Src	0.60	0.97	0.78	0.52	0.59	0.00	0.82	0.14
Syk	1.38	1.61	1.53	2.12	1.95	1.20	1.84	0.66
Yes	1.40	1.89	1.30	1.12	1.13	1.24	1.08	0.69
Zap70	1.45	1.71	1.46	2.22	2.20	1.60	3.06	1.77
Families								
Abl	0.43	0.3	0.64	0.88	0.15	0.42	2.38	0.19
Fes	2.02	2.00	1.37	2.00	1.90	2.00	2.07	1.62
Src	0.47	0.85	0.73	0.59	0.56	0.15	0.51	0.20

Table S9. Comparison of PSM performance.

Kinase	Proteomic sample size	PSPL sample size	MCC	Sensitivity	Specificity	Accuracy	Precision	EER	AROC	Threshold
Abl	73	20	0.509	0.4795	0.9774	94.40	0.6034	0.3637	0.9247	56
Arg	16	37	0.934	0.9375	0.9967	99.37	0.9375	0.0095	0.9988	82
Btk	21	34	0.668	0.7123	0.9835	97.23	0.6522	0.0567	0.9811	92
Csk	48	27	0.653	0.8542	0.9674	96.28	0.5256	0.0547	0.9785	23
Fyn	55	37	0.672	0.7818	0.8841	83.97	0.8431	0.0579	0.9518	14
Hck	31	18	0.579	0.9032	0.9243	92.22	0.4117	0.007	0.9522	14
Jak2	52	35	0.602	0.7308	0.9596	94.55	0.5428	0.1194	0.9582	45
Lck	38	24	0.602	0.7895	0.9630	95.55	0.4918	0.0663	0.9662	34
Lyn	48	47	0.53	0.4583	0.9856	95.44	0.6666	0.2532	0.9299	89
Pyk2	9	13	0.75	0.9200	0.9682	96.55	0.6389	0.0124	0.9862	30
Src	167	39	0.374	0.4970	0.9435	91.79	0.3487	0.7462	0.8255	20
Syk	66	13	0.67	0.6818	0.9848	96.83	0.6703	0.1995	0.9701	54
Yes	59	21	0.822	0.9661	0.875	90.9677	0.8260	-0.053	0.9601	11

“Proteomic sample size” was the number of curated endogenous substrates identified for the kinase, while the “PSPL sample size” is the number of amino acids that were identified as being significantly favorable at any position in the data from the Turk laboratory’s microarray experiments (Deng, Y.; Alicea-Velazquez, N. L.; Bannwarth, L.; Lehtonen, S. I.; Boggon, T. J.; Cheng, H. C.; Hytonen, V. P.; Turk, B. E. *J Proteome Res* 2014, *13*, 4339.)

“MCC” - Matthews correlation coefficient is a measure of the quality of the binary classification ranging from -1 to +1. A coefficient of +1 represents a perfect prediction, 0 is a random prediction and -1 is the classification is predicting the opposite of the observation in the data.

“Sensitivity” - the true positive rate.

“Specificity” – the true negative rate.

“Accuracy” - the proportion of true results in the classification.

“Precision” – the positive prediction value or the rate of true positives compared to all positives results in the classification.

“EER” – Equal Error Rate, which is the rate that the acceptance and rejection errors are equal. The lower the EER the more accurate the prediction.

“AROC” – Area under the Receiver-Operator Curve representing the Whitney-Mann coefficient.

“Threshold” – the value used to delineate the binary classification for each kinase prediction model.

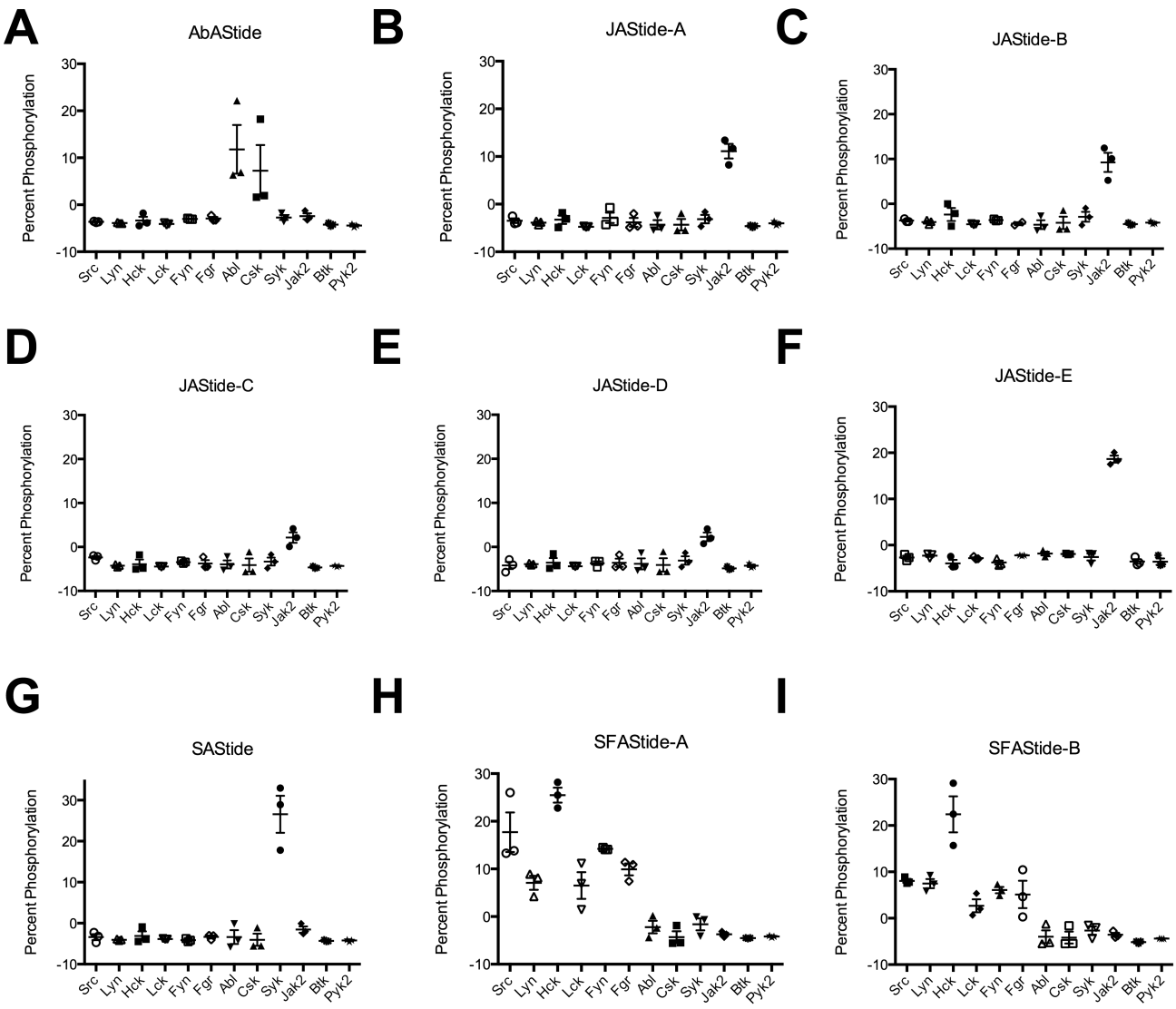

Figure S1. Percent phosphorylation of the various peptide biosensors by the collection of kinases used to generate the heat map in Figure 2B. A) AbAStide B) JAStide-A C) JAStide-B D) JAStide-C E) JAStide-D F) JAStide-E G) SAStide H) SFAStide-A I) SFAStide-B.

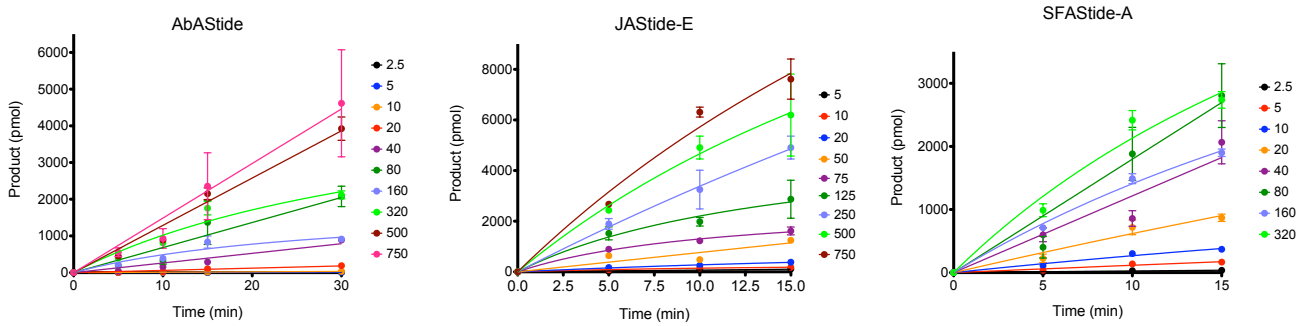

Figure S2. Kinetic rate plots of AbAStide with Abl, JAStide-E with Jak2, and SFAStide-A with Lyn.

Table S10. AbAStide initial rate equations from Abl kinase reaction progress curves. Equations represent the average of three replicates.

AbAStide		
Concentration (µM)	**Equation**	**R^2**
2.5	**y = 0.2621x + 0.3033**	**0.83**
5	**y = 0.3625x + 0.2056**	**0.91**
10	**y = 1.045x + 0.3476**	**0.68**
20	**y = 6.576x - 12.84**	**0.90**
40	**y = 30.78x - 93.78**	**0.78**
80	**y = 53.64x - 45.81**	**0.79**
160	**y = 73.78x - 116.8**	**0.82**
320	**y = 81.26x - 4.294**	**0.97**
500	**y = 88.55x + 1.871**	**0.97**
750	**y = 93.21x + 29.86**	**0.71**

Table S11. JAStide-E initial rate equations from Jak2 kinase reaction progress curves. Equations represent the average of three replicates.

JAStide-E		
Concentration (µM)	**Equation**	**R^2**
5	**y = 3.183x + 1.872**	**0.69**
10	**y = 16.33x + 6.170**	**0.85**
20	**y = 33.64x + 15.56**	**0.79**
50	**y = 127.5x + 27.34**	**0.94**
75	**y = 178.4x + 31.54**	**0.96**
125	**y = 304.0x + 90.35**	**0.90**
250	**y = 378.3x + 74.65**	**0.95**
500	**y = 486.0x + 38.00**	**0.99**
750	**y = 532.8x – 24.46**	**0.99**

Table S12. SFAStide-A initial rate equations from Lyn kinase reaction progress curves. Equations represent the average of three replicates.

SFAStide-A		
Concentration (μM)	**Equation**	**R^2**
2.5	y = 2.567x – 0.1921	0.70
5	y = 11.99x – 4.547	0.87
10	y = 25.99x + 0.5626	0.90
20	y = 62.01x -12.29	0.88
40	y = 129.1x – 87.81	0.82
80	y = 148.9x – 11.03	0.99
160	y = 188.2x – 179.5	0.75
320	y = 241.6x – 73.03	0.97

Table S13. Kinetic constants for kinase specific peptide biosensors

Sensor	K_m (μM)	V_{max} (pmol/min)	k_{cat} (min^{-1})	k_{cat}/K_m	R^2
AbAStide	99 ± 22	107 ± 7	1430 ± 96	14	0.92
JAStide-E	186 ± 27	672 ± 37	2690 ± 148	14	0.96
SFAStide-A	62 ± 10	270 ± 16	3600 ± 148	58	0.96

Table S14. Biosensor characterization of physical and photophysical properties

Sensor	Steady-State S:N	Time-Resolved S:N	K_d (μM)	τ^{-1}_{H2O} (ms)	τ^{-1}_{D2O} (ms)	q	Δq
SFAStide-A	2.1:1	10.6:1	1.4 ± 0.17	1.35	0.51	4	2
			9.8 ± 0.54	1.83	0.53	6	
SFAStide-B	2.1:1	6.2:1	8.2 ± 1.0	1.73	0.71	5	2
			54 ± 10	2.13	0.67	7	
JAStide-B	1.1:1	4.6:1	1.6 ± 0.21	1.26	0.81	2	2
			15 ± 0.46	1.63	0.71	4	
JAStide-E	1.9:1	6.8:1	7.9 ± 6.2	1.61	0.62	4	2
			67 ± 8.7	2.10	0.72	6	
AbAStide	1.3:1	7.3:1	12 ± 2.6	1.65	0.61	4	2
			26 ± 3.7	2.21	0.74	6	

S:N – signal to noise, K_d – dissociation constant, τ^{-1}_{H2O} – luminescence lifetime in the presence of H_2O, τ^{-1}_{D2O} – luminescence lifetime rate in the presence of D_2O, q – hydration number; the number of H_2O ligands in the inner coordination sphere at equilibrium, Δq – change in hydration number upon phosphorylation. The top row for each sensor represents the phosphorylated form while the bottom row represents the unphosphorylated form.

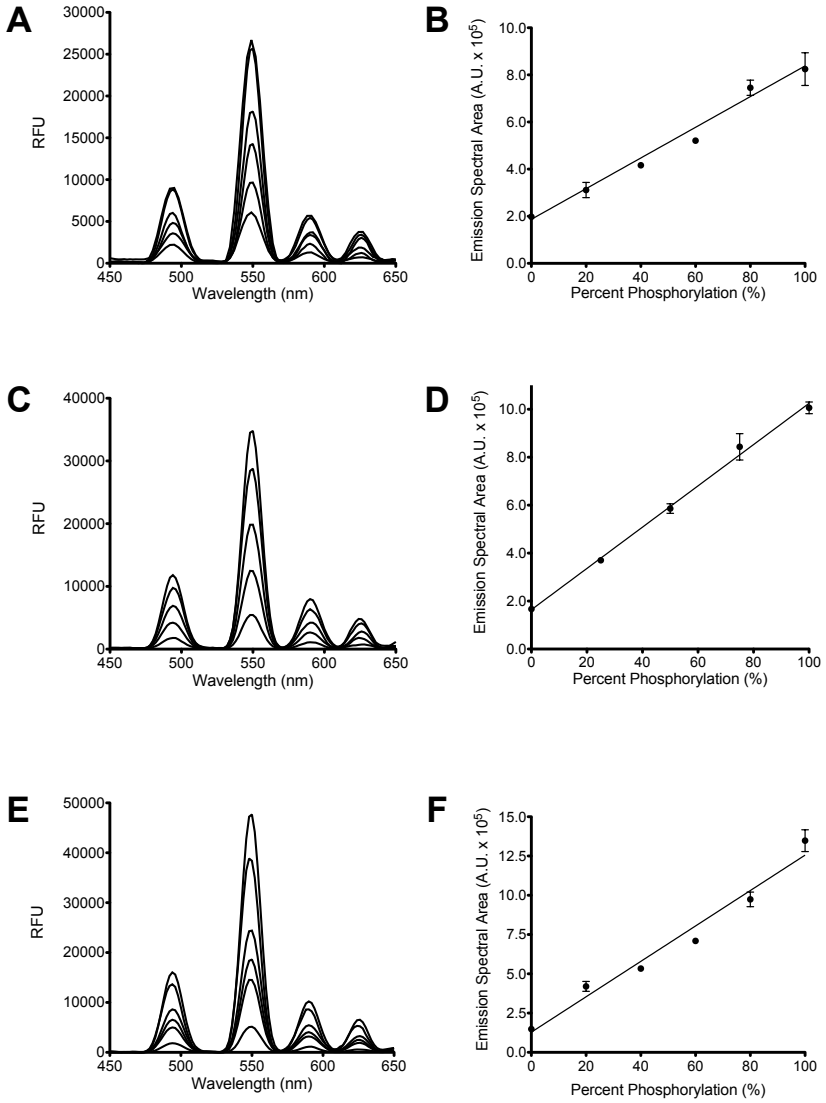

Figure S3. Calibration Curves. Synthetic phosphopeptide and unphosphorylated peptide were mixed in the proportions represented in the plots in (B-D); either 20%, 40%, 60%, 80% and 100% (A,B and E,F) or 25%, 50%, 75%, and 100% (C,D). (A) pAbAStide-Tb^{3+} luminescence emission spectra in the presence of quenched *in vitro* kinase buffer. (B) pAbAStide-Tb^{3+} luminescence emission spectral area calibration curve based on percent phosphorylation. (C) pJAStide-Tb^{3+} luminescence emission spectra in the presence of quenched in vitro kinase buffer. (D) pJAStide-E-Tb^{3+} luminescence emission spectral area calibration curve based on percent phosphorylation. (E) pSFAStide-A-Tb^{3+} luminescence emission spectral area calibration curve based on percent phosphorylation. (F) pSFAStide-A-Tb^{3+} luminescence emission spectral area calibration curve based on percent phosphorylation. Data indicate mean ±SEM for triplicate wells.

Table S15. Characterization of biosensor high-throughput screening parameters.

Sensor	Limit of Detection	Limit of Quantification	Z' factor	Signal Window	Signal:Noise	R^2
SFAStide-A	**7.9**	**21.8**	**0.79**	**13.7**	**9.1:1**	**0.95**
JAStide-E	**3.1**	**9.6**	**0.90**	**30.8**	**6.0:1**	**0.98**
AbAStide	**4.8**	**12.1**	**0.65**	**5.86**	**4.2:1**	**0.95**

Table S16. Characterization of high-throughput screening parameters in inhibitor assay. 500 nM of the inhibitor was used as the negative signal while all other concentrations were used as the positive signal with respect to the equations 2 and 3 in the methods section.

Inhibitor	Concentration (log nM)	Signal Window	Z' factor
Dasatinib	**-2**	**6.75**	**0.631**
	-1	**6.38**	**0.614**
	0	**2.82**	**0.387**
	1	**1.13**	**0.397**
	2	**-0.341**	**-0.284**
Imatinib	**-2**	**15.5**	**0.766**
	-1	**9.72**	**0.695**
	0	**5.25**	**0.838**
	1	**2.96**	**0.359**
	2	**-3.85**	**-1.97**
Ruxolitinib	**-2**	**5.20**	**0.701**
	-1	**4.78**	**0.647**
	0	**1.08**	**0.454**
	1	**-12.2**	**-0.518**
	2	**-21.05**	**-11.5**

Table S17. Percent inhibition of the top compounds in the high throughput screen and secondary screen.

Compound	HTS	Secondary Screen
SB-250715	76.87	47.34
GW829874X	79.09	58.12
GW442130X	100.8	64.28
GW809897X	89.47	66.16
GW406108X	90.96	67.67
GW305074X	77.69	67.83
GW589961A	77.82	67.91
GW280670X	78.28	70.68
GW768505A	79.57	72.89
GW827099X	86.35	76.62
GW513184X	84.55	80.32
GW806742X	76.74	80.63
GW643971X	78.76	80.81
GW612286X	80.39	84.23
GW711782X	86.67	87.32
GW410563A	78.84	89.68
GW693917A	83.62	97.54

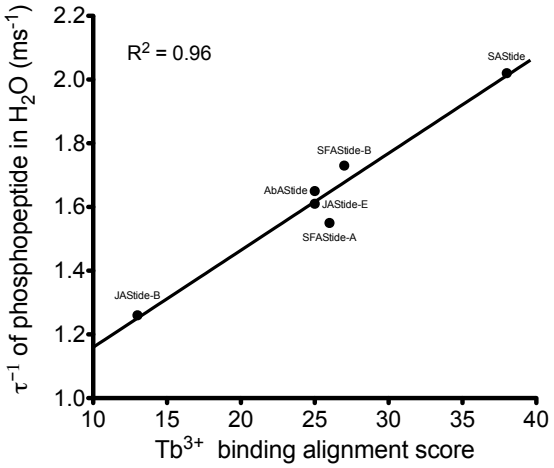

Figure S4. Correlation between sequence alignment score and phosphopeptide luminescent lifetime

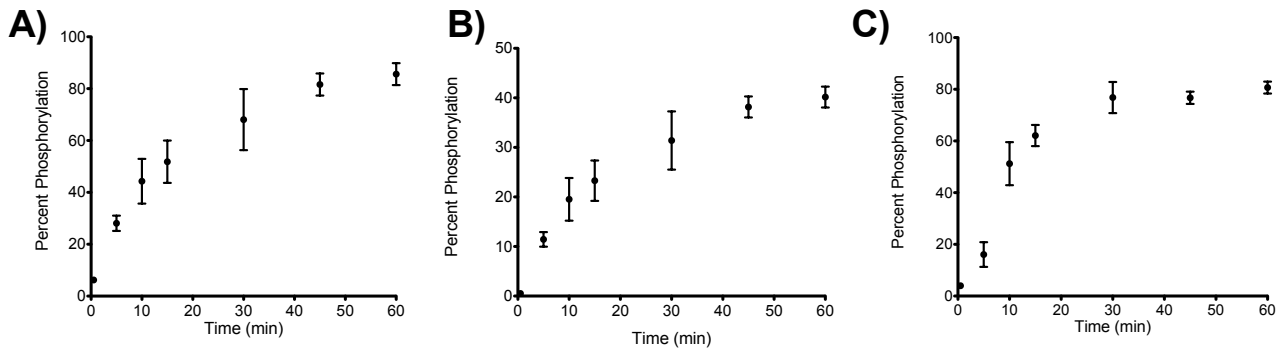

Figure S5. ELISA-based chemifluorescent validation of biosensor phosphorylation from *in vitro* assays for A) AbAStide B) JAStide-E and C) SFAStide-A.

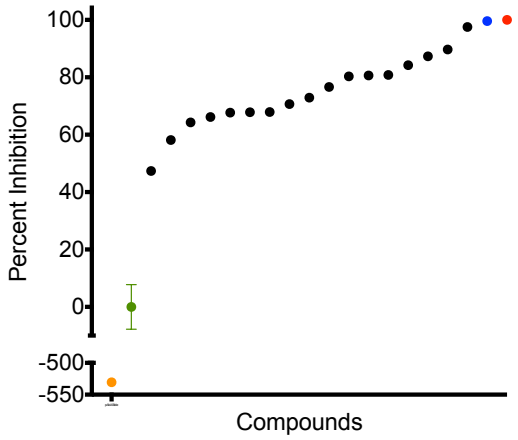

Figure S6. Secondary inhibitor screen validation of high-throughput screening hits using ELISA-based chemifluorescence detection. • DMSO, • pABAStide, • PKIS compounds, • AbAStide, and • imatinib.