Supplementary Figures

Supplementary Figures Legends

Supplementary Figure 1: Spectra of Detached, N- and O-linked Glycans from Serum and Recombinant Derived ITIH4

Supplementary Figure 2: Proteomic analyses and peptide identification of Serum and Recombinant ITIH4

Supplementary Figure 3: Extracted Ion Chromatogram (XIC) plots of ITIH4 glycopeptides treated with PNGaseF/$H_2{}^{18}O$ for site occupancy determination.

Supplementary Figure 4: Electron transfer dissociation (ETD) spectra of de-sialylated, mTRAQ-labeled glycopeptides IPKPEASFSPR+HexNAcHex and LAILPASAPPATSNPDPAVSR+HexNAc2Hex2.

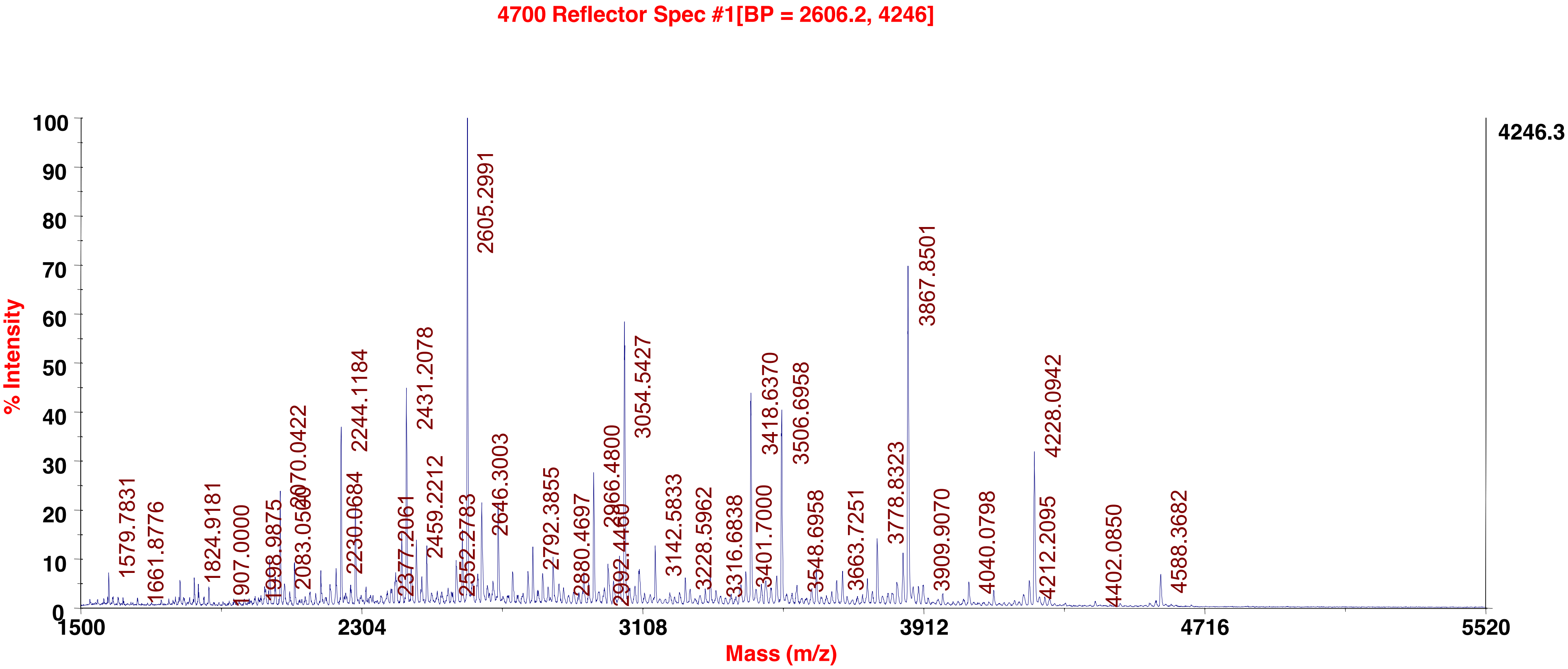
Supplementary Figure 5: Table of all observed recombinant and serum-derived ITIH4 glycopeptides from CID MS/MS spectra, including (a) no exoglycosidase treatment, (b) after treatment with 2/3,6,8 neuraminidase, or (c) after treatment with 2/3,6,8 neuraminidase and alpha 1-3,4 fucosidase.

Supplementary Figure 1

Supplementary Figure 1 A

MALDI-TOF (MS) Spectra of Permethylated N-Glycans

N-linked Glycans from Recombinant ITIH4

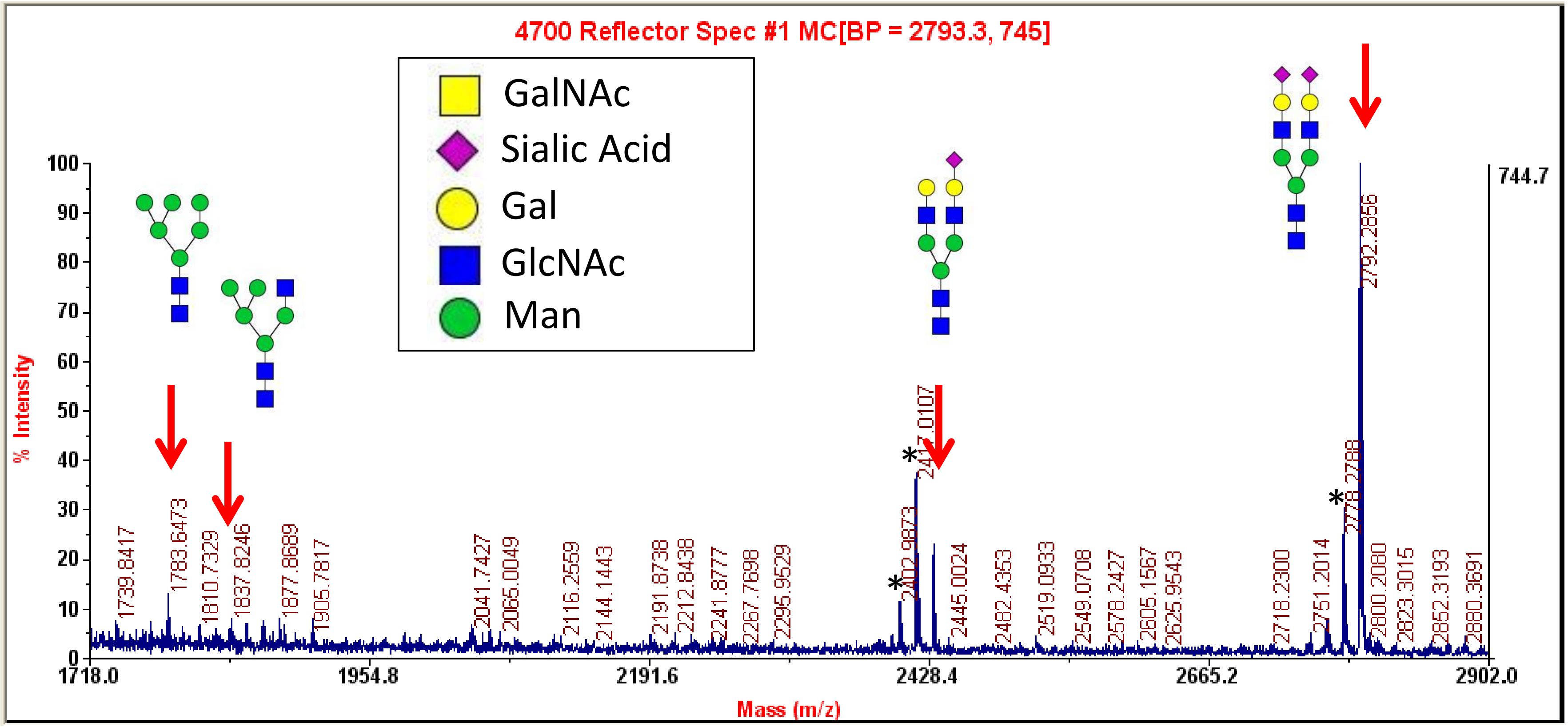


H:\GoldmanLab\KevinChandler\Data\KBC_274\KBC_274_280_MALDI_Glycans\KBC_280_07262013 D5_1_5500.T2D (HEK293 ITIH4 DETACHED, PERMETHYLATED N-GLYCANS)

Recombinant ITIH4 N-Glycan Relative Intensity

Peak (m/z)	Glycan	Relative Intensity (%)	Fuc (Y/N)	Fuc Intensity	NeuAc (Y/N)	NeuAc Intensity	Full NeuAc (Y/N)	Full NeuAc Intensity	2 Fuc (Y/N)	2 Fuc Intensity
1661.84	A2	0.1	0	0.0	0	0.0	0	0.0	0	0.0
2070.04	A2G2	1.3	0	0.0	0	0.0	0	0.0	0	0.0
2244.13	FA2G2	3.8	1	3.8	0	0.0	0	0.0	0	0.0
2285.15	FA3G1	1.4	1	1.4	0	0.0	0	0.0	0	0.0
2418.21	FA2F1G2	1.5	1	1.5	0	0.0	0	0.0	1	1.5
2431.21	A2G2S1	5.3	0	0.0	1	5.3	0	0.0	0	0.0
2489.25	FA3G2	1.4	1	1.4	0	0.0	0	0.0	0	0.0
2605.30	FA2G2S1	15.2	1	15.2	1	15.2	0	0.0	0	0.0
2646.33	FA3G1S1	2.5	1	2.5	1	2.5	0	0.0	0	0.0
2693.35	FA3G3	3.3	1	3.3	0	0.0	0	0.0	0	0.0
2792.38	A2G2S2	1.9	0	0.0	1	1.9	1	1.9	0	0.0
2850.43	FA3G2S1	1.4	1	1.4	1	1.4	0	0.0	0	0.0
2966.47	FA2G2S2	5.1	1	5.1	1	5.1	1	5.1	0	0.0
3054.53	FA3G3S1	10.4	1	10.4	1	10.4	0	0.0	0	0.0
3142.58	FA4G4	2.4	1	2.4	0	0.0	0	0.0	0	0.0
3228.61	FA3F1G3S1	1.2	1	1.2	1	1.2	0	0.0	1	1.2
3415.70	FA3G3S2	9.5	1	9.5	1	9.5	0	0.0	0	0.0
3503.75	FA4G4S1	7.4	1	7.4	1	7.4	0	0.0	0	0.0
3602.78	A3G3S3	2.0	0	0.0	1	2.0	1	2.0	0	0.0
3776.87	FA3G3S3	2.8	1	2.8	1	2.8	1	2.8	0	0.0
3864.93	FA4G4S2	13.6	1	13.6	1	13.6	0	0.0	0	0.0
4226.10	FA4G4S3	5.5	1	5.5	1	5.5	0	0.0	0	0.0
4587.27	FA4G4S4	0.8	1	0.8	1	0.8	1	0.8	0	0.0
		100.0		89.4		84.7		12.7		2.8

Detached Serum ITIH4 N-glycans

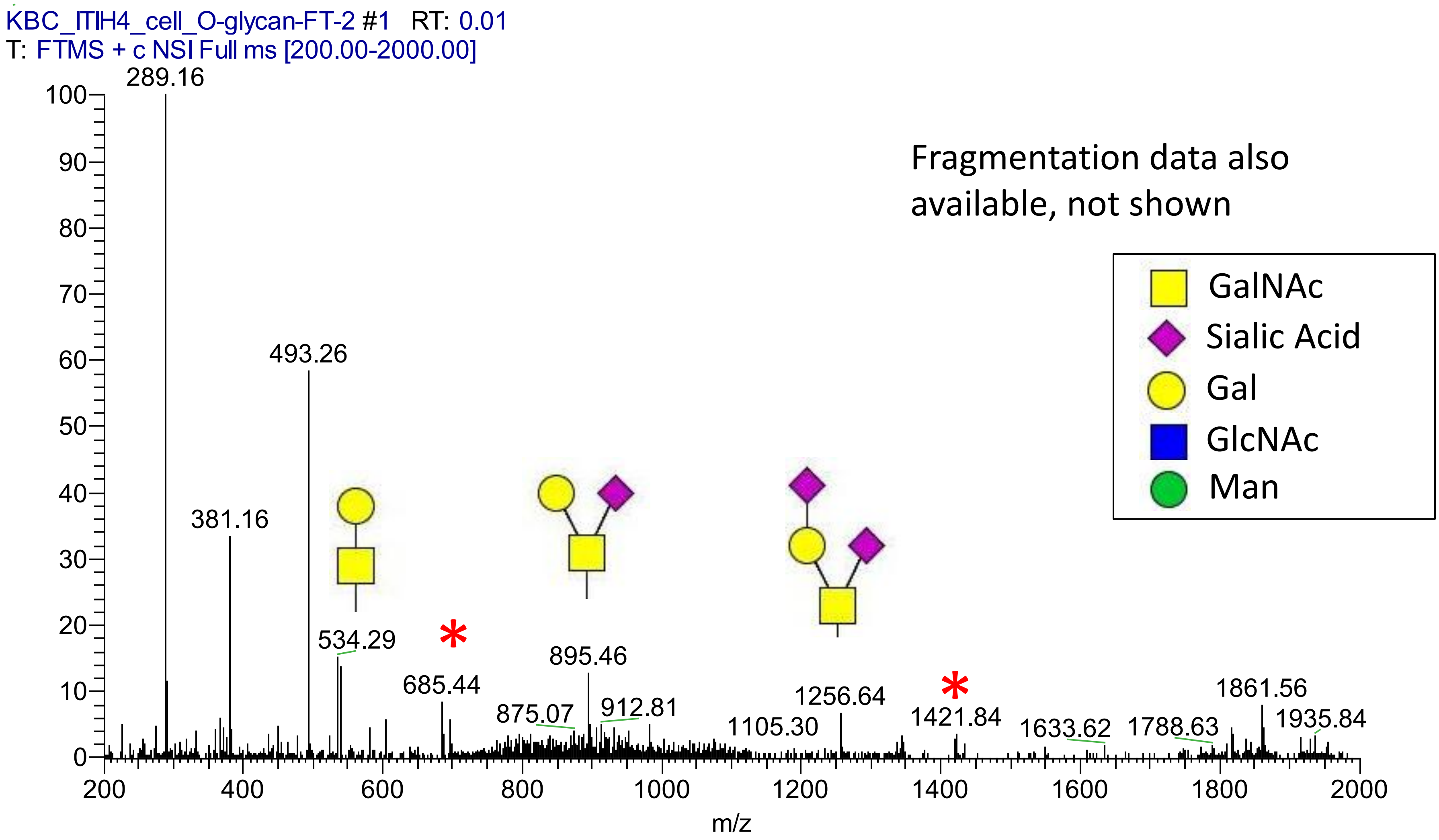


*incomplete permethylation

Supplementary Figure 1 B

Permethylated O-linked Glycans from Recombinant ITIH4

O-glycans - Full MS



Fragmentation data also available, not shown

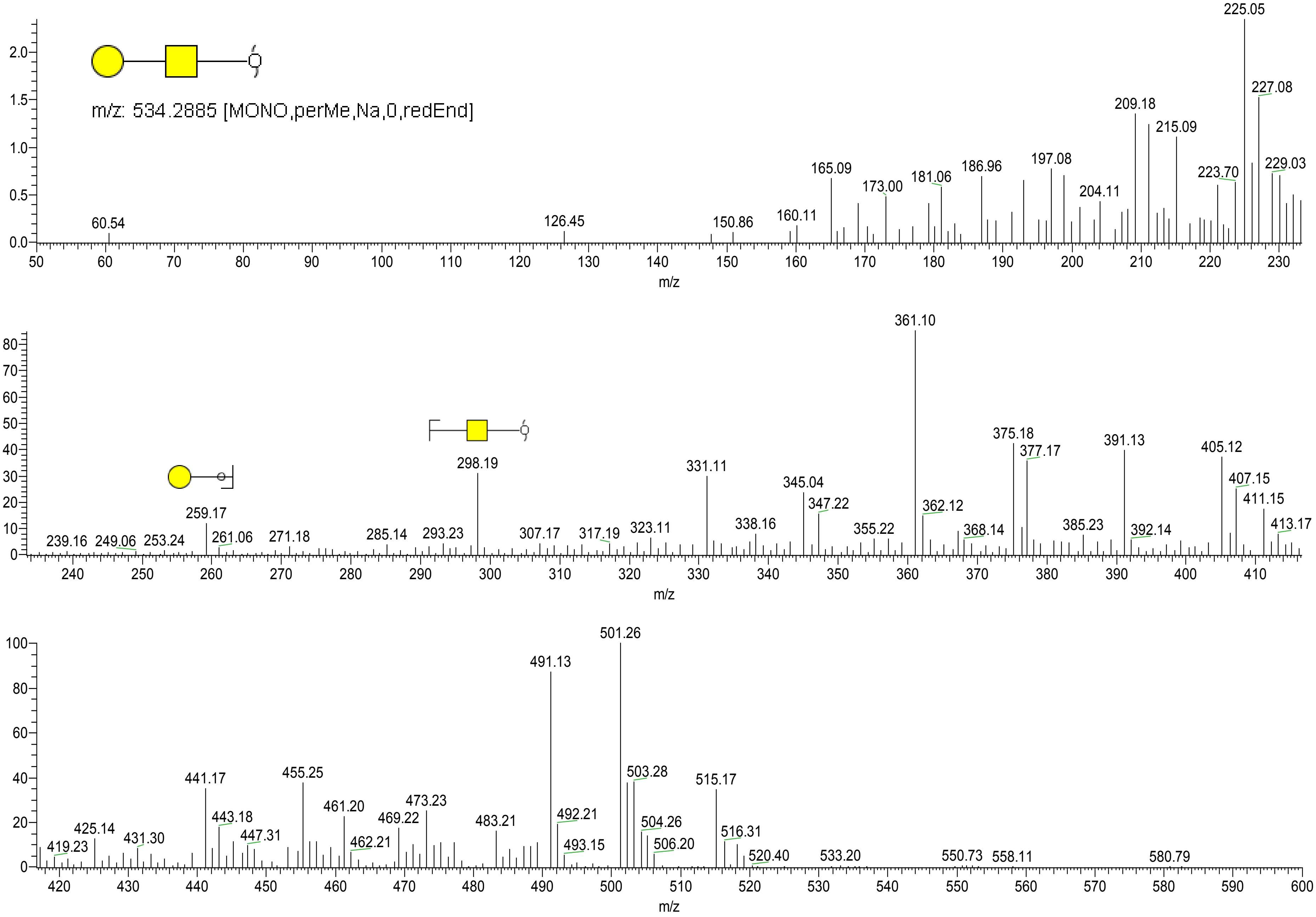
* non-glycan masses / contaminants

Supplementary Figure 1 C

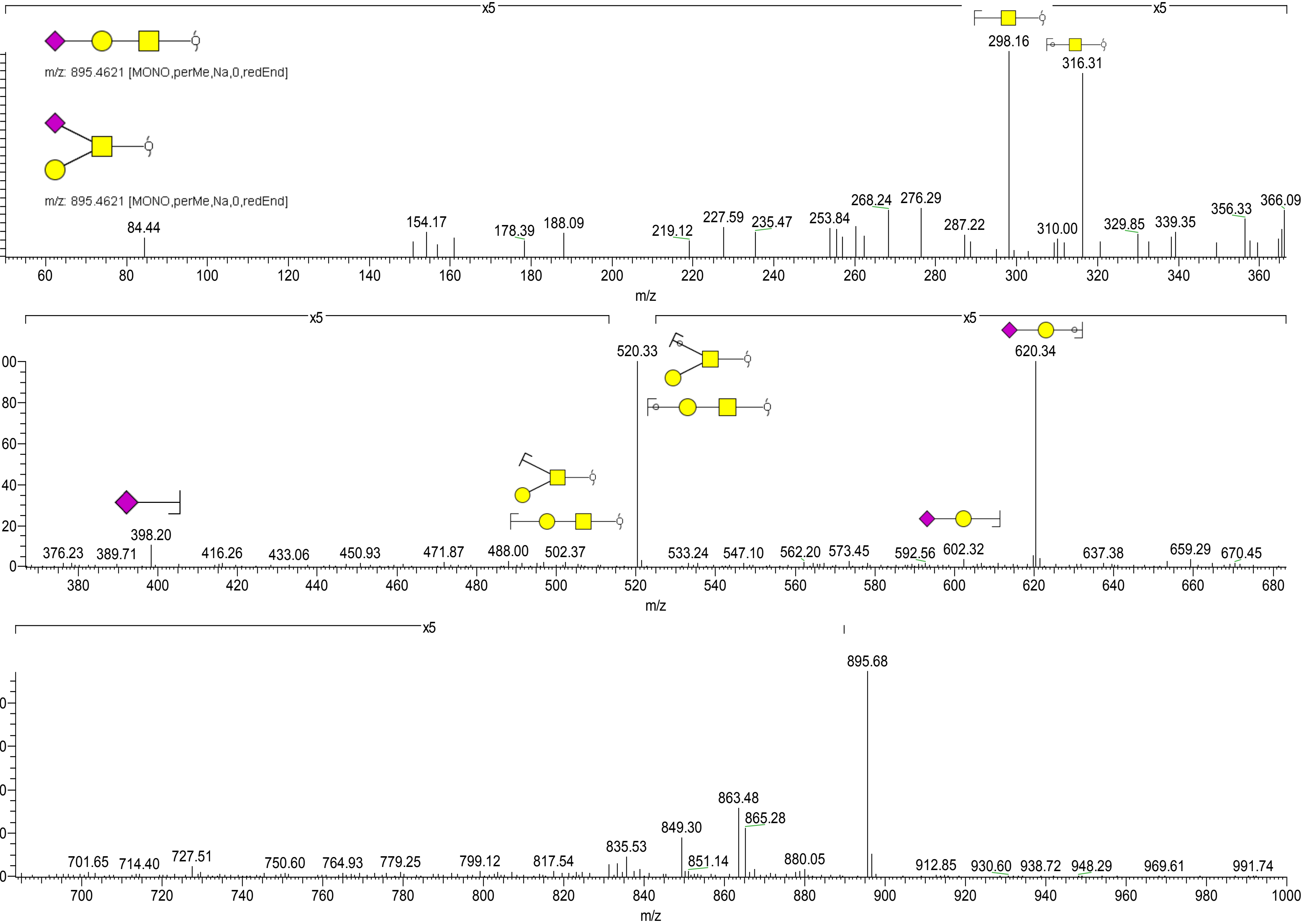
Permethylated O-linked Glycans from Serum ITIH4

Structure		Permethylated (Na)			
		z=1	z=2	z=3	z=4
(Hex)1(HexNAc)1-OMe	Major structures	534.2591	278.6391	193.4227	150.8145
(NeuAc)1(Hex)1(HexNAc)1-OMe		895.4722	459.2259	313.8139	241.1079
(NeuAc)2(Hex)1(HexNAc)1-OMe		1256.9248	639.8128	434.2051	331.4013
(NeuAc)2(Hex)2(HexNAc)2-OMe	Minor structures	1705.8619	864.4258	583.9471	443.7078
(NeuAc)2(Hex)3(HexNAc)3-OMe		2155.0880	1089.0389	733.6892	556.0143

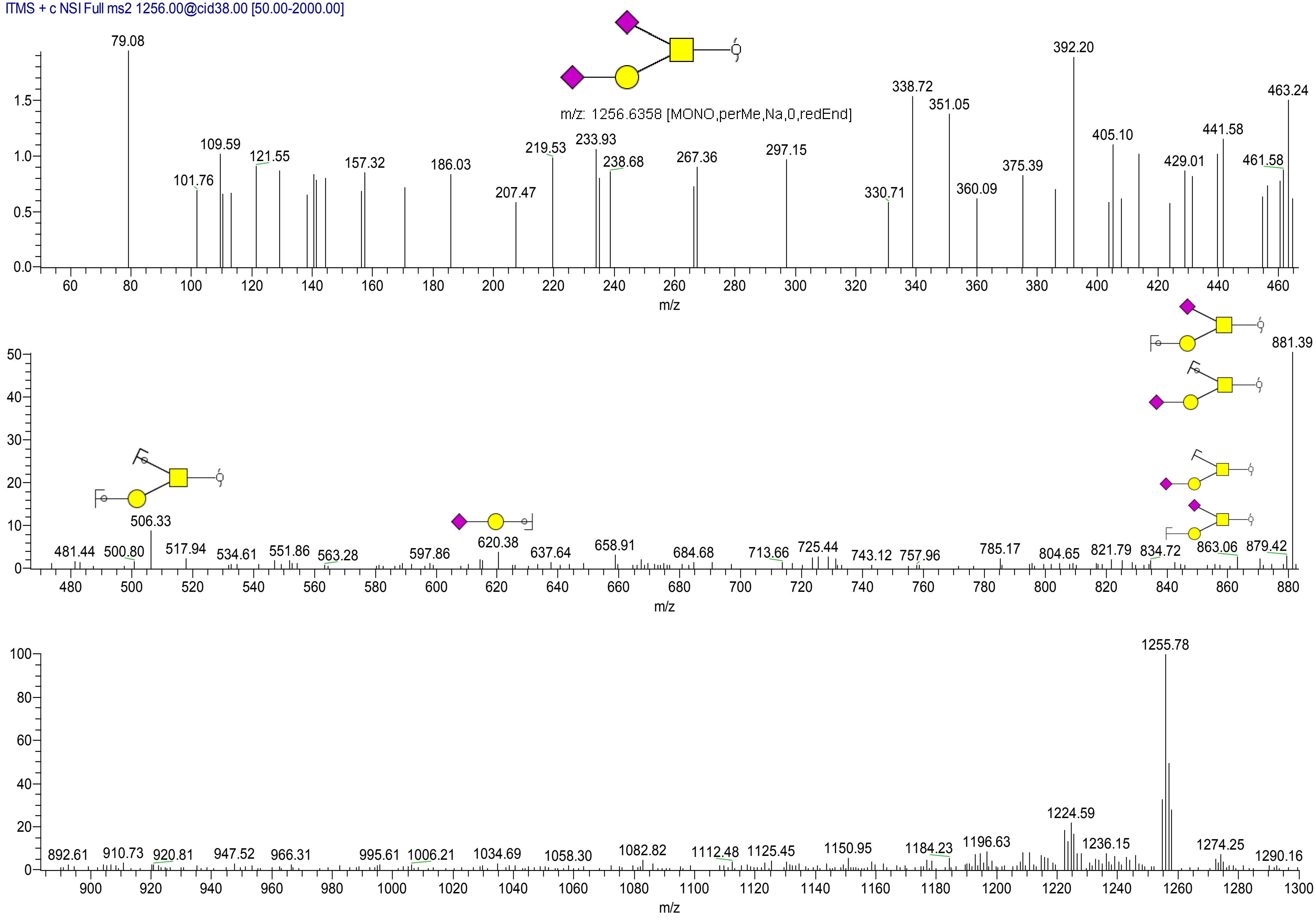
LZ-20130907-BE-Oglycan-Kevin-ITIH4dig-TIM_CID #68 RT: 1.79 AV: 1 NL: 3.26E3
T: ITMS + c NSI Full ms2 534.00@cid38.00 [50.00-2000.00]



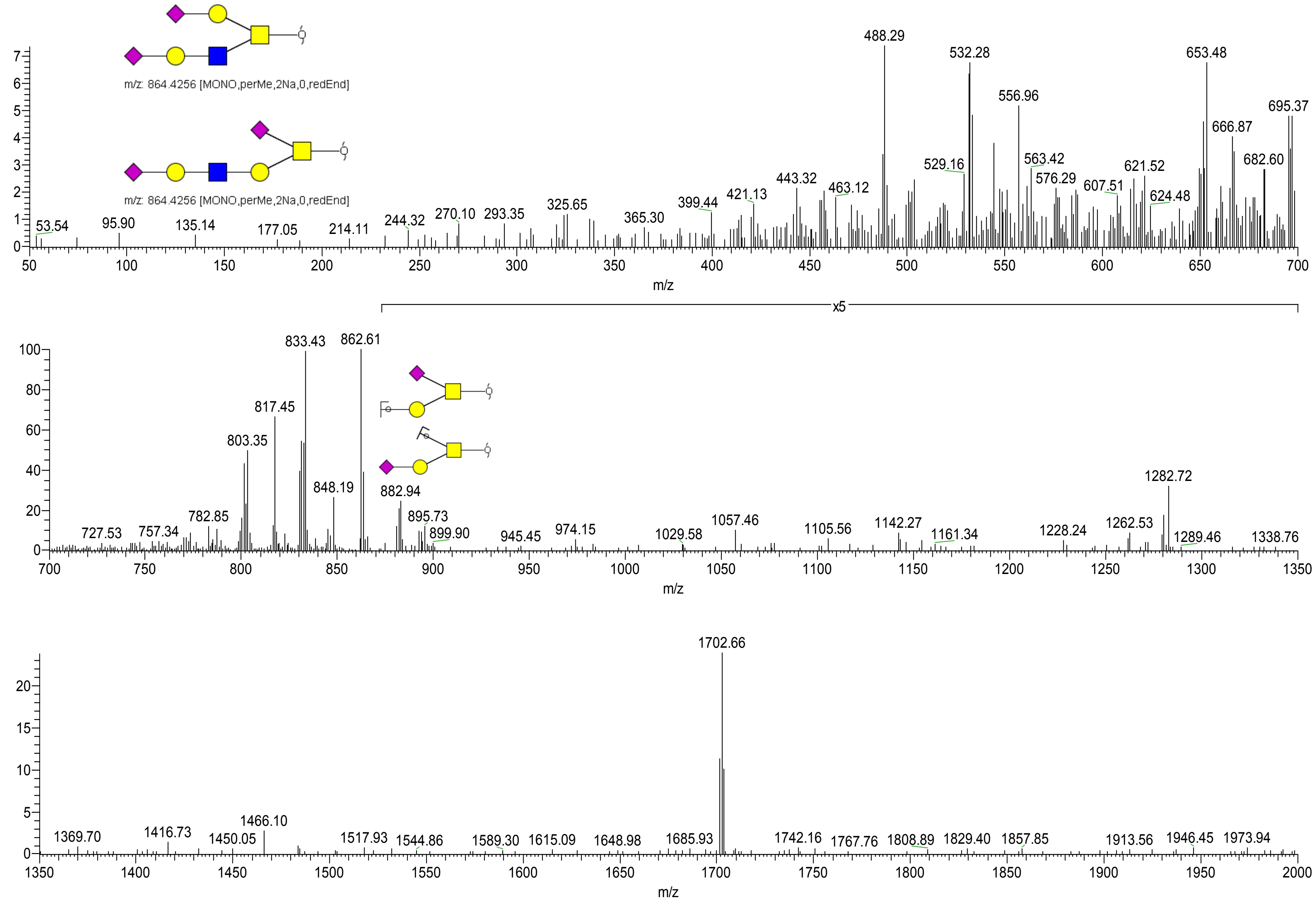
LZ-20130907-BE-Oglycan-Kevin-ITIH4dig-TIM_CID #249 RT: 7.39 AV: 1 NL: 2.95E3
T: ITMS + c NSI Full ms2 896.00@cid38.00 [50.00-2000.00]

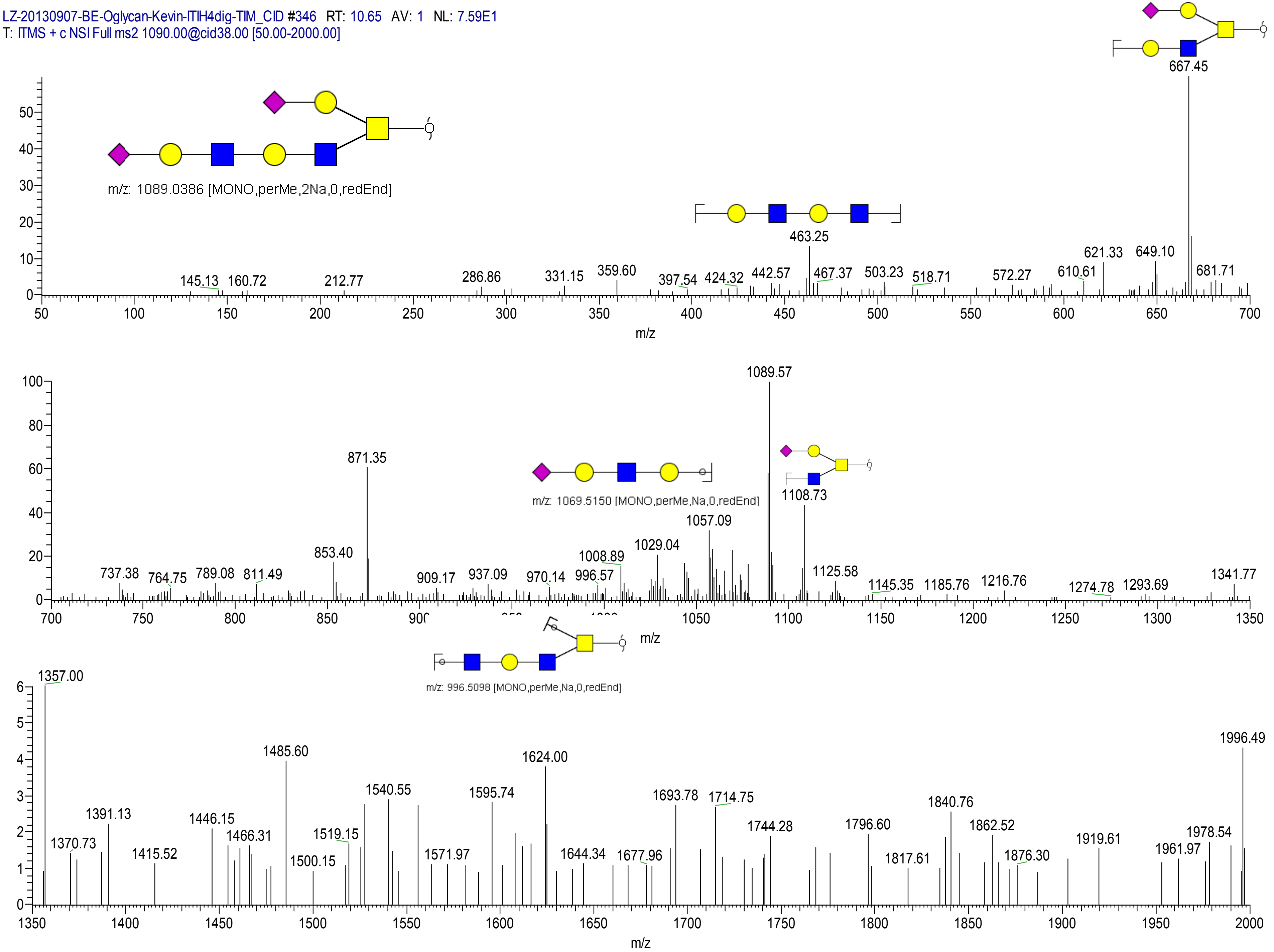


LZ-20130907-BE-Oglycan-Kevin-ITIH4dig-TIM_CID #429 RT: 13.48 AV: 1 NL: 1.24E2
T: ITMS + c NSI Full ms2 1256.00@cid38.00 [50.00-2000.00]



LZ-20130907-BE-Oglycan-Kevin-ITIH4dig-TIM_CID #233 RT: 6.87 AV: 1 NL: 4.45E2
T: ITMS + c NSI Full ms2 864.00@cid38.00 [50.00-2000.00]




LZ-20130907-BE-Oglycan-Kevin-ITIH4dig-TIM_CID #346 RT: 10.65 AV: 1 NL: 7.59E1
T: ITMS + c NSI Full ms2 1090.00@cid38.00 [50.00-2000.00]
m/z: 1089.0386 [MONO,perMe,2Na,0,redEnd]
667.45
463.25
145.13
160.72
212.77
286.86
331.15
359.60
397.54
424.32
442.57
467.37
503.23
518.71
572.27
610.61
621.33
649.10
681.71
m/z
1089.57
871.35
853.40
737.38
764.75
789.08
811.49
909.17
937.09
970.14
996.57
1008.89
1029.04
1057.09
1108.73
1125.58
1145.35
1185.76
1216.76
1274.78
1293.69
1341.77
m/z: 1069.5150 [MONO,perMe,Na,0,redEnd]
m/z
m/z: 996.5098 [MONO,perMe,Na,0,redEnd]
1357.00
1370.73
1391.13
1415.52
1446.15
1466.31
1485.60
1500.15
1519.15
1540.55
1571.97
1595.74
1624.00
1644.34
1677.96
1693.78
1714.75
1744.28
1796.60
1817.61
1840.76
1862.52
1876.30
1919.61
1961.97
1978.54
1996.49
m/z

Supplementary Figure 2

Figure 2A: Recombinant ITIH4 Protein Identification (Trypsin Digest, MS/MS)

N	%Cov(95)	Accession	Species	Peptides(95%)
1	52.9	sp|Q14624|ITIH4_HUMAN	HUMAN	166
2	22.51	sp|P08238|HS90B_HUMAN	HUMAN	19
3	29.64	sp|P08107|HSP71_HUMAN	HUMAN	21
4	33.07	sp|P63261|ACTG_HUMAN	HUMAN	14
4	29.33	sp|P60709|ACTB_HUMAN	HUMAN	13
5	12.12	sp|P13639|EF2_HUMAN	HUMAN	11
6	22.46	sp|P07195|LDHB_HUMAN	HUMAN	9
7	12.95	sp|P14625|ENPL_HUMAN	HUMAN	12
8	17.74	sp|P11021|GRP78_HUMAN	HUMAN	12
9	15.63	sp|P14618|KPYM_HUMAN	HUMAN	8
10	22.17	sp|Q71U36|TBA1A_HUMAN	HUMAN	9
10	22.17	sp|P68363|TBA1B_HUMAN	HUMAN	9
11	9.276	sp|O94985|CSTN1_HUMAN	HUMAN	8
12	13.33	sp|Q08380|LG3BP_HUMAN	HUMAN	9
13	4.885	sp|P01023|A2MG_HUMAN	HUMAN	9
14	15.21	sp|P06733|ENOA_HUMAN	HUMAN	6
15	2.589	sp|P49327|FAS_HUMAN	HUMAN	6
16	19.51	sp|P04075|ALDOA_HUMAN	HUMAN	6
17	13.29	sp|P07437|TBB5_HUMAN	HUMAN	6
18	4.972	sp|P14543|NID1_HUMAN	HUMAN	5
19	9.852	sp|P02768|ALBU_HUMAN	HUMAN	7
20	16.72	sp|P04406|G3P_HUMAN	HUMAN	4
21	10.71	sp|P16870|CBPE_HUMAN	HUMAN	5
22	17.49	sp|P07900|HS90A_HUMAN	HUMAN	14
23	17.18	sp|P11142|HSP7C_HUMAN	HUMAN	11
24	15.29	sp|P62258|1433E_HUMAN	HUMAN	4
25	24.6	sp|Q99880|H2B1L_HUMAN	HUMAN	4
25	24.6	sp|Q8N257|H2B3B_HUMAN	HUMAN	4
25	24.6	sp|Q16778|H2B2E_HUMAN	HUMAN	4
25	24.6	sp|P33778|H2B1B_HUMAN	HUMAN	4
25	24.6	sp|P23527|H2B1O_HUMAN	HUMAN	4
25	24.6	sp|P06899|H2B1J_HUMAN	HUMAN	4
25	24.6	sp|Q99879|H2B1M_HUMAN	HUMAN	4
25	24.6	sp|Q99877|H2B1N_HUMAN	HUMAN	4
25	24.6	sp|Q93079|H2B1H_HUMAN	HUMAN	4
25	24.6	sp|Q5QNW6|H2B2F_HUMAN	HUMAN	4
25	24.6	sp|P62807|H2B1C_HUMAN	HUMAN	4
25	24.6	sp|P58876|H2B1D_HUMAN	HUMAN	4
25	24.6	sp|P57053|H2BFS_HUMAN	HUMAN	4
25	24.6	sp|O60814|H2B1K_HUMAN	HUMAN	4
26	9.779	sp|Q8NHW5|RLA0L_HUMAN	HUMAN	3
26	9.779	sp|P05388|RLA0_HUMAN	HUMAN	3
27	9.354	sp|P10909|CLUS_HUMAN	HUMAN	3
28	21.36	sp|P62805|H4_HUMAN	HUMAN	3
29	4.027	sp|Q12906|ILF3_HUMAN	HUMAN	3

30	0.9423	sp|P13611|CSPG2_HUMAN	HUMAN	3
31	17.47	sp|P00338|LDHA_HUMAN	HUMAN	6
32	4.048	sp|P34932|HSP74_HUMAN	HUMAN	3
33	5.774	sp|P12277|KCRB_HUMAN	HUMAN	2
34	28.19	sp|P62158|CALM_HUMAN	HUMAN	3
35	2.93	sp|P22314|UBA1_HUMAN	HUMAN	3

Figure 2B: Serum ITIH4 Protein Identification (Trypsin Digest, MS/MS)

N	%Cov(95)	Accession	Species	Peptides(95%)
1	59.0	sp|Q14624|ITIH4_HUMAN	HUMAN	56
2	27.9	sp|P19823|ITIH2_HUMAN	HUMAN	20
3	12.6	sp|P01024|CO3_HUMAN	HUMAN	14
4	18.1	sp|P02768|ALBU_HUMAN	HUMAN	11
5	6.4	sp|P0C0L5|CO4B_HUMAN	HUMAN	7
5	6.4	sp|P0C0L4|CO4A_HUMAN	HUMAN	7
6	8.6	sp|P19827|ITIH1_HUMAN	HUMAN	6
7	7.1	sp|P04264|K2C1_HUMAN	HUMAN	4
8	20.6	sp|P01857|IGHG1_HUMAN	HUMAN	5
9	9.6	sp|P01876|IGHA1_HUMAN	HUMAN	3

Supplementary Figure 3

ITIH4 Occupancy Summary
Serum and Cell ITIH4

Part I:

N-glycosylation Site Occupancy in Serum-Derived ITIH4

- Serum-derived ITIH4 was treated with GluC and Trypsin, heated to denature proteases, then treated with PNGaseF in the presence of $H_2{}^{18}O$

- 0.1 ug of the digest was injected onto an Eksigent ChromXP C18-CL interfaced with a 5600 TripleTOF mass spectrometer (ABSCIEX)

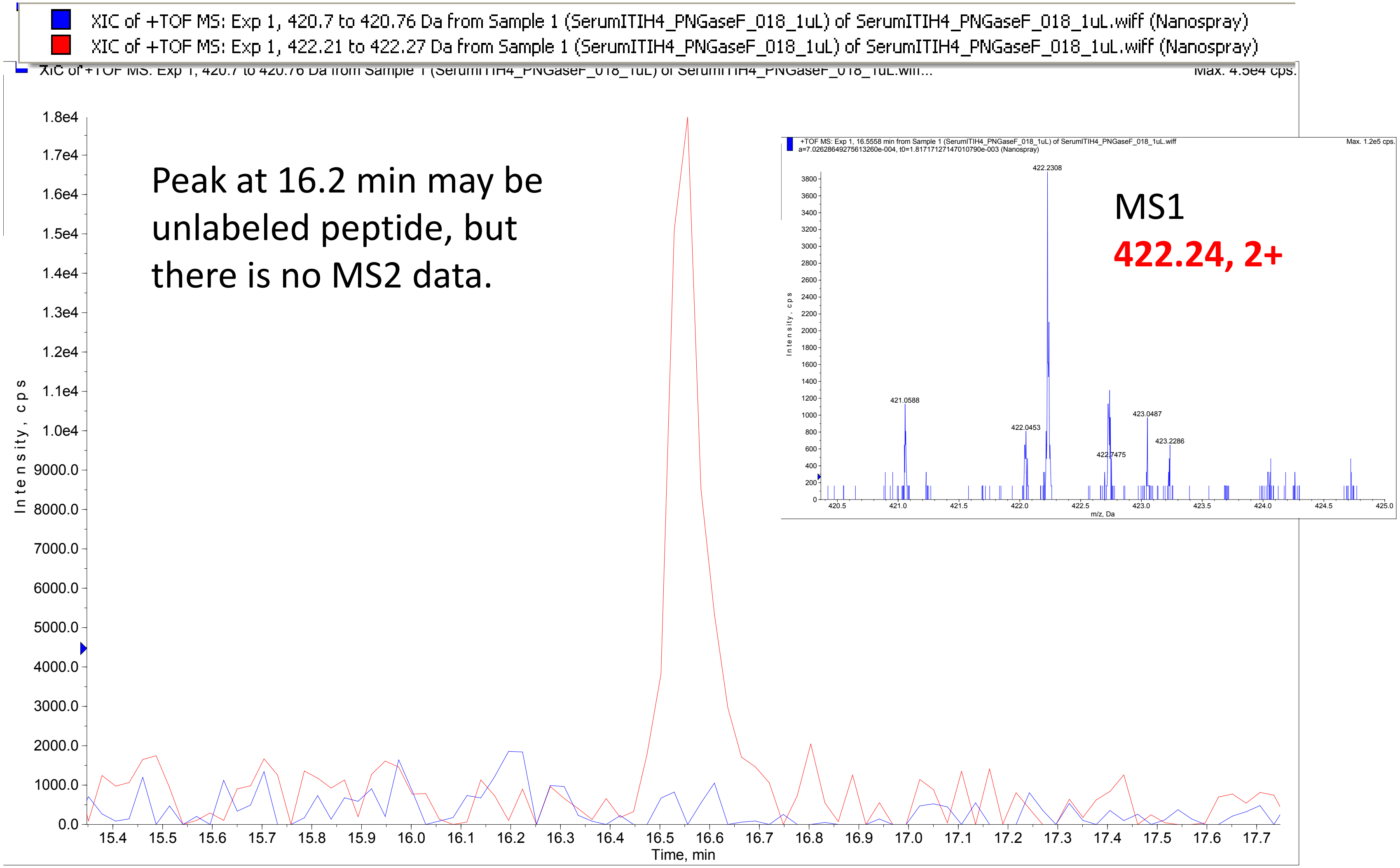
- Different chromatographic gradients were used (short gradient, 30 min; long gradient, 120 min)

Serum ITIH4 Occupancy Summary

Site	**Peptide(s)**	***m/z* (charge) Unlabeled**	***m/z* (charge) Labeled**	**Serum ITIH4**
N81	KAFITNF (T,G)	420.734 (2+)	422.240 (2+)	+
N207	STFMTNQLVDALTTWQNK (T,G)	700.014 (3+)	701.018 (3+)	>90%
N274	NVVFVIDK (T,G)	467.274 (2+)	468.780 (2+)	<1%
N517	LPTQNITFQTE (T,G)	646.330 (2+)	647.836 (2+)	>98%
N577	NQALNLSLAYSFVTPLTMVVTKPDDQE (T,G)	1027.853 (3+)	1028.857 (3+)	>95%

Serum ITIH4, N81

Site: N81 **(Peptide:** KAFITNF), Experiment: SerumITIH4_PNGaseF_O18_1uL.wiff
Unlabeled (**420.73, 2+**) not detected, labeled (**422.24, 2+**) detected
Retention Time: 16.5 min (short gradient, 30 min)



Peak at 16.2 min may be unlabeled peptide, but there is no MS2 data.

MS1
422.24, 2+

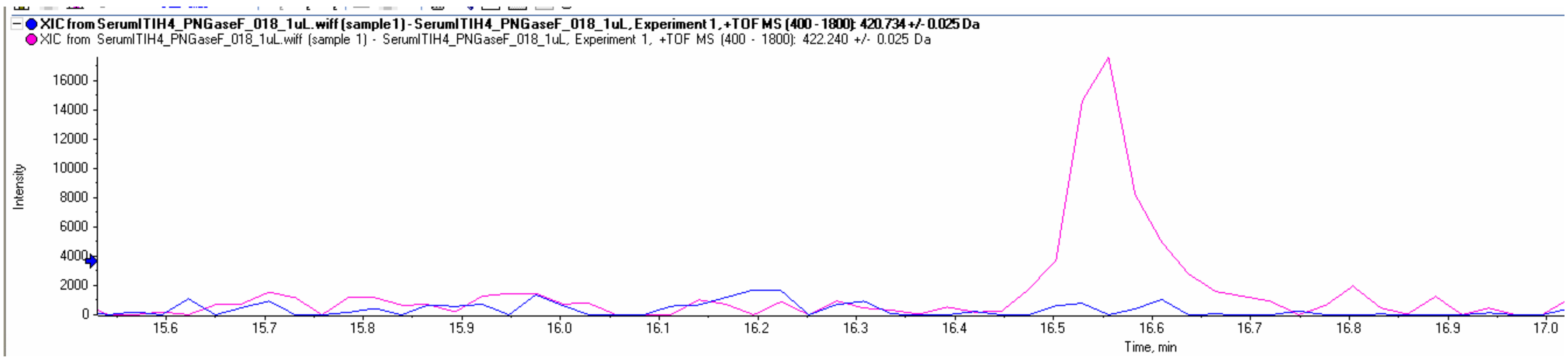
Note: In MS1, see 388 cps, or approx 7% of unlabeled peptide signal. Unlabeled ion may be at Retention Time 16.1957 min, *m/z* 420.73, 2+, but difficult to be confident because the isotope cluster is near the baseline

Serum ITIH4, N81

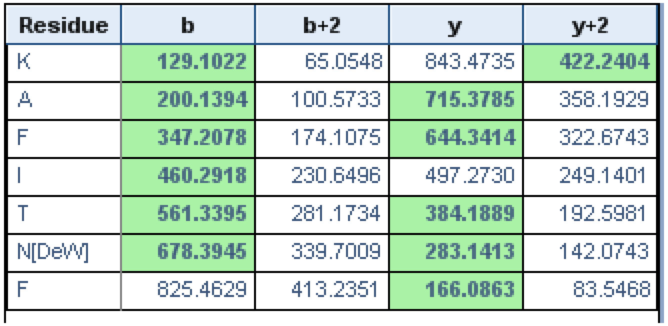
(N>D/O18)

KAFITNF.S

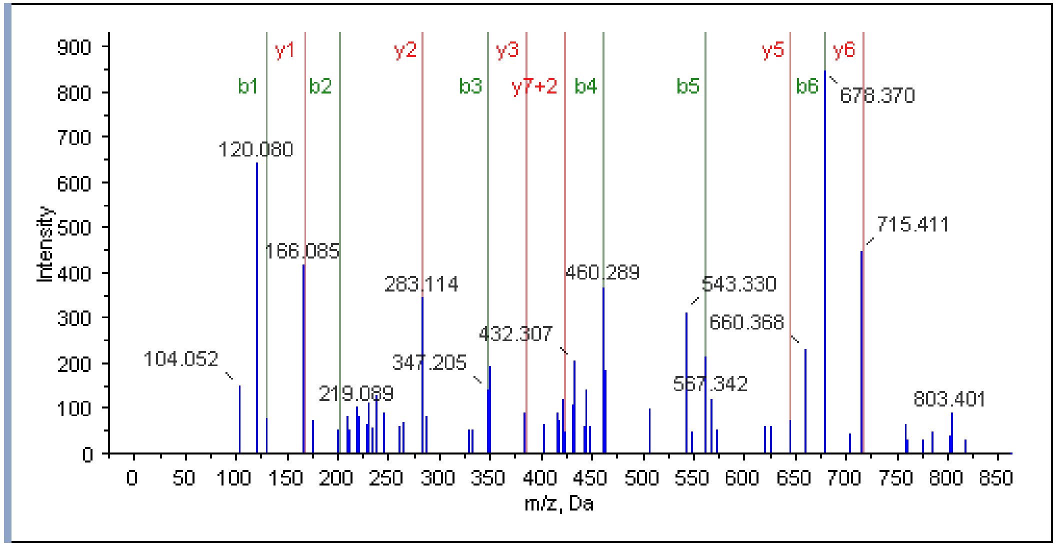
Labeled peptide observed, but low intensity. Unlabeled peptide (precursor and MS/MS) can not be found.



Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site



Residue	b	b+2	y	y+2
K	**129.1022**	65.0548	843.4735	**422.2404**
A	**200.1394**	100.5733	**715.3785**	358.1929
F	**347.2078**	174.1075	**644.3414**	322.6743
I	**460.2918**	230.6496	497.2730	249.1401
T	**561.3395**	281.1734	**384.1889**	192.5981
N[DeW]	**678.3945**	339.7009	**283.1413**	142.0743
F	825.4629	413.2351	**166.0863**	83.5468



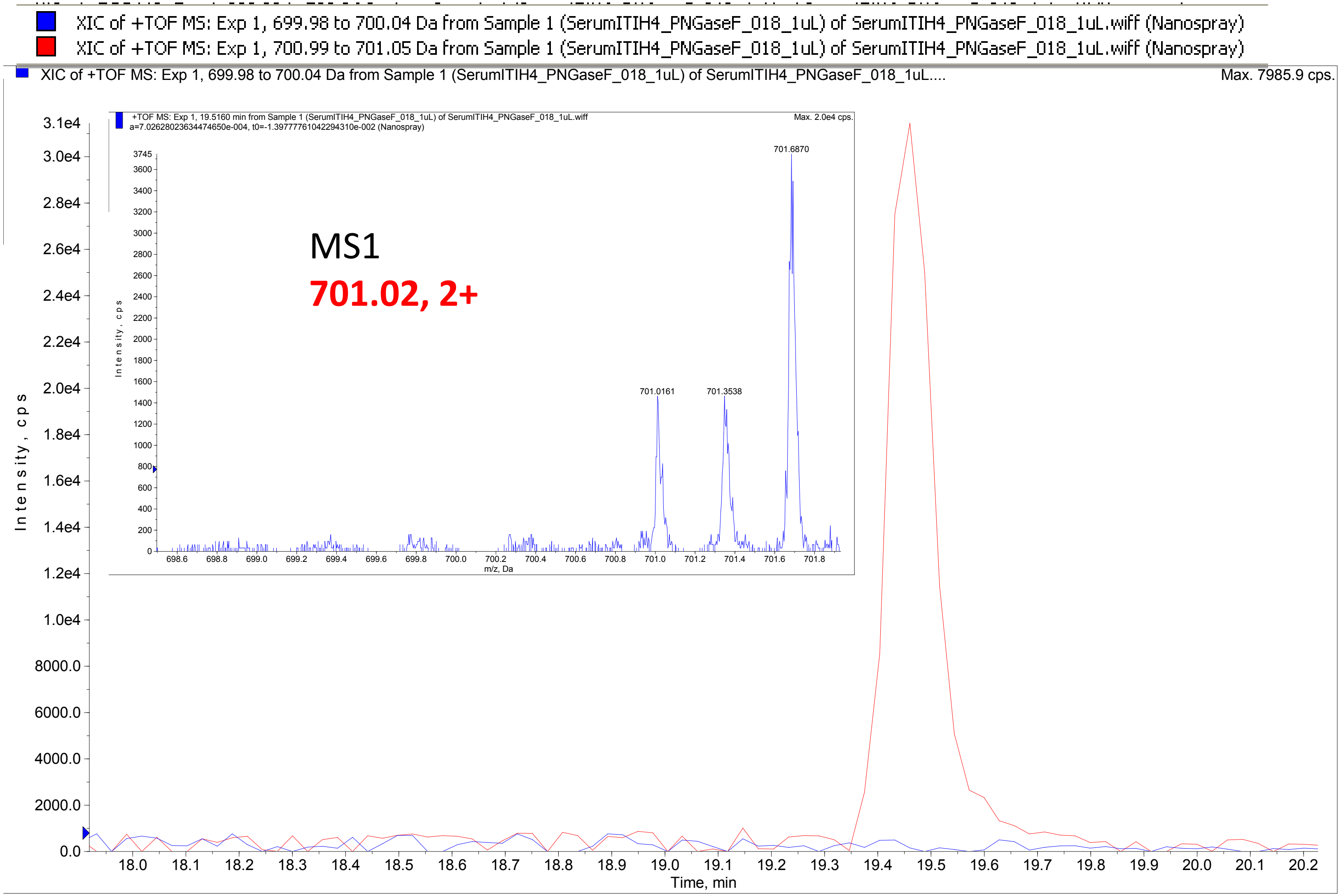
O18 labeled KAFITNFSMII also found, (NOT SHOWN), VERY low abundance

Serum ITIH4, N207

Site: N207 (Peptide: STFMTNQLVDALTTWQNK **)**   File: SerumITIH4_PNGaseF_O18_1uL.wiff

Unlabeled (**700.01, 3+**) not detected, labeled (**701.02, 3+**) detected

Retention Time: 19.5 min (short gradient, 30 min)

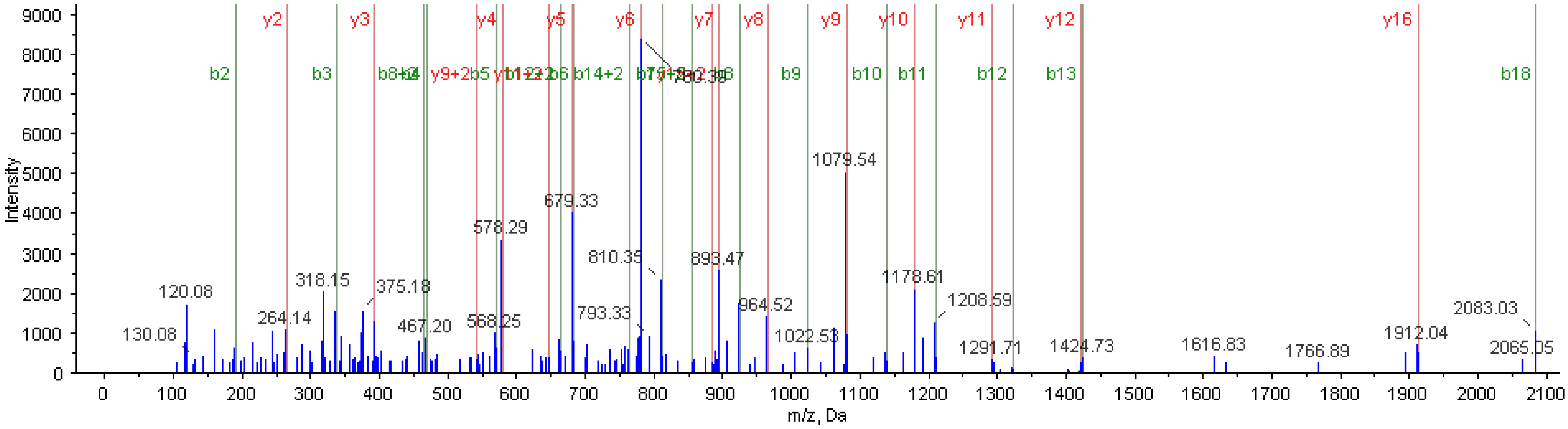


Note: In MS1, can't see unlabeled peptide. The labeled ion intensity max = 4000 cps at Retention Time 19.46 min. Can see an (unrelated) ion near, I=278 cps, good isotope cluster; if labeled peptide is present but lower than this ion, STFMTNQLVDALTTWQNK >90% occupancy

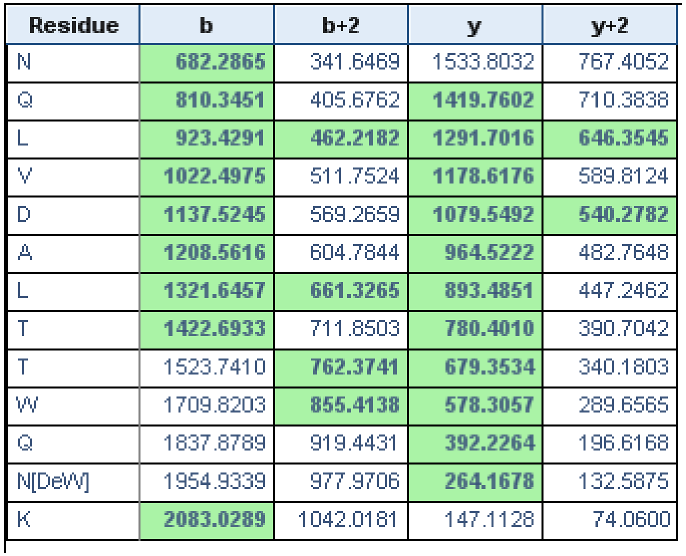
<u>Site: N207</u> (Peptide: STFMTNQLVDALTTWQNK) File: SerumITIH4_PNGaseF_O18_1uL.wiff

Labeled (**701.02, 3+**) detected

Retention Time: 19.5 min (short gradient, 30 min)

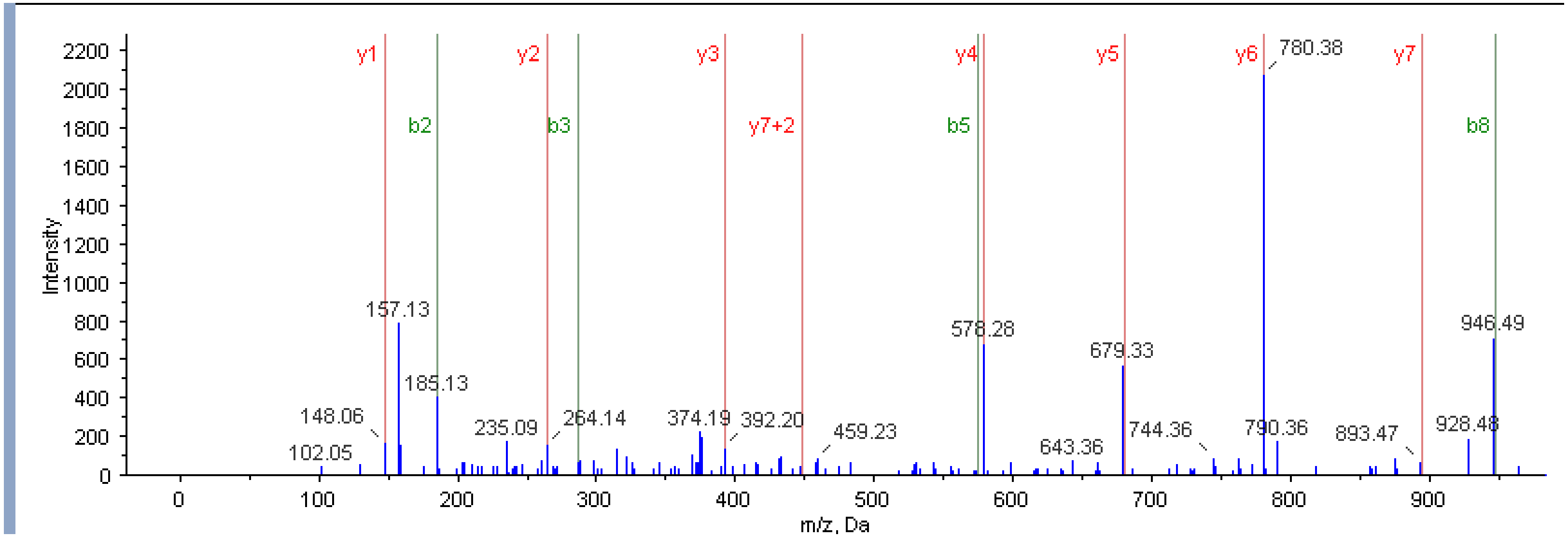


Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site

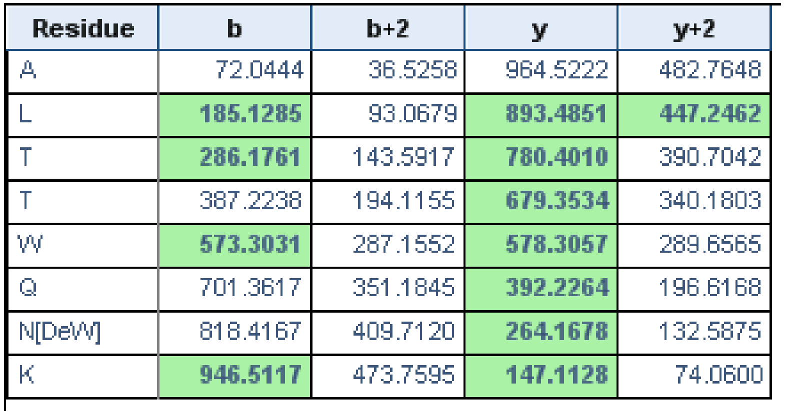


Residue	b	b+2	y	y+2
N	**682.2865**	341.6469	1533.8032	767.4052
Q	**810.3451**	405.6762	**1419.7602**	710.3838
L	**923.4291**	**462.2182**	**1291.7016**	**646.3545**
V	**1022.4975**	511.7524	**1178.6176**	589.8124
D	**1137.5245**	569.2659	**1079.5492**	**540.2782**
A	**1208.5616**	604.7844	**964.5222**	482.7648
L	**1321.6457**	**661.3265**	**893.4851**	447.2462
T	**1422.6933**	711.8503	**780.4010**	390.7042
T	1523.7410	**762.3741**	**679.3534**	340.1803
W	1709.8203	**855.4138**	**578.3057**	289.6565
Q	1837.8789	919.4431	**392.2264**	196.6168
N[DeW]	1954.9339	977.9706	**264.1678**	132.5875
K	**2083.0289**	1042.0181	147.1128	74.0600

Site: N207 (Peptide: ALTTWQNK **)** File: SerumITIH4_PNGaseF_O18_1uL.wiff



Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site



Residue	b	b+2	y	y+2
A	72.0444	36.5258	964.5222	482.7648
L	**185.1285**	93.0679	**893.4851**	**447.2462**
T	**286.1761**	143.5917	**780.4010**	390.7042
T	387.2238	194.1155	**679.3534**	340.1803
W	**573.3031**	287.1552	**578.3057**	289.6565
Q	701.3617	351.1845	**392.2264**	196.6168
N[DeW]	818.4167	409.7120	**264.1678**	132.5875
K	**946.5117**	473.7595	**147.1128**	74.0600

Serum ITIH4, N274

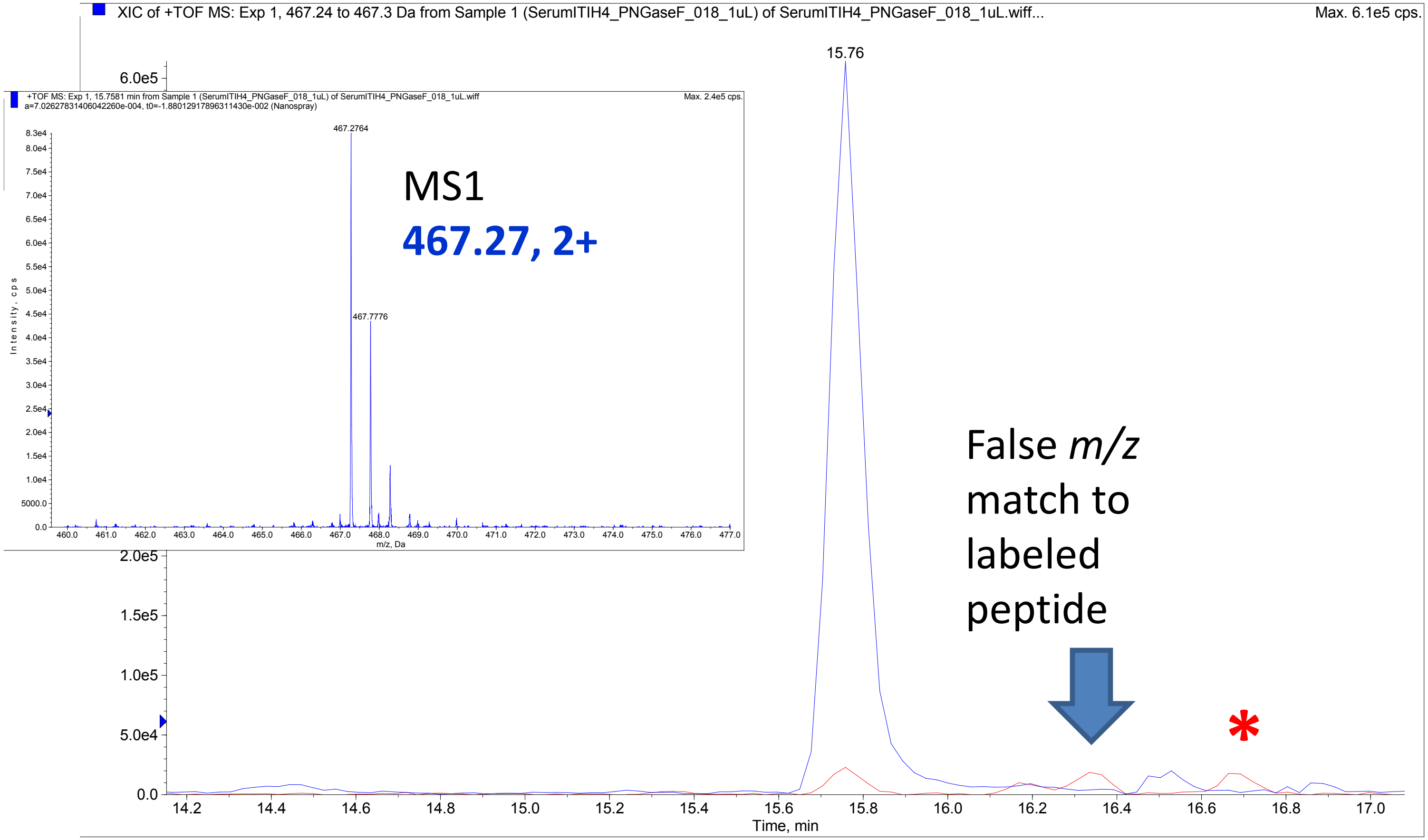
Site: N274 (Peptide: NVVFVIDK**)**   File: SerumITIH4_PNGaseF_O18_1uL.wiff

Unlabeled (**467.27, 2+**) detected, labeled (**468.78, 2+**) not detected

Retention Time: 15.8 min (short gradient, 30 min)

🟦 XIC of +TOF MS: Exp 1, 467.24 to 467.3 Da from Sample 1 (SerumITIH4_PNGaseF_018_1uL) of SerumITIH4_PNGaseF_018_1uL.wiff (Nanospray)

🟥 XIC of +TOF MS: Exp 1, 468.75 Da from Sample 1 (SerumITIH4_PNGaseF_018_1uL) of SerumITIH4_PNGaseF_018_1uL.wiff (Nanospray)



MS1

467.27, 2+

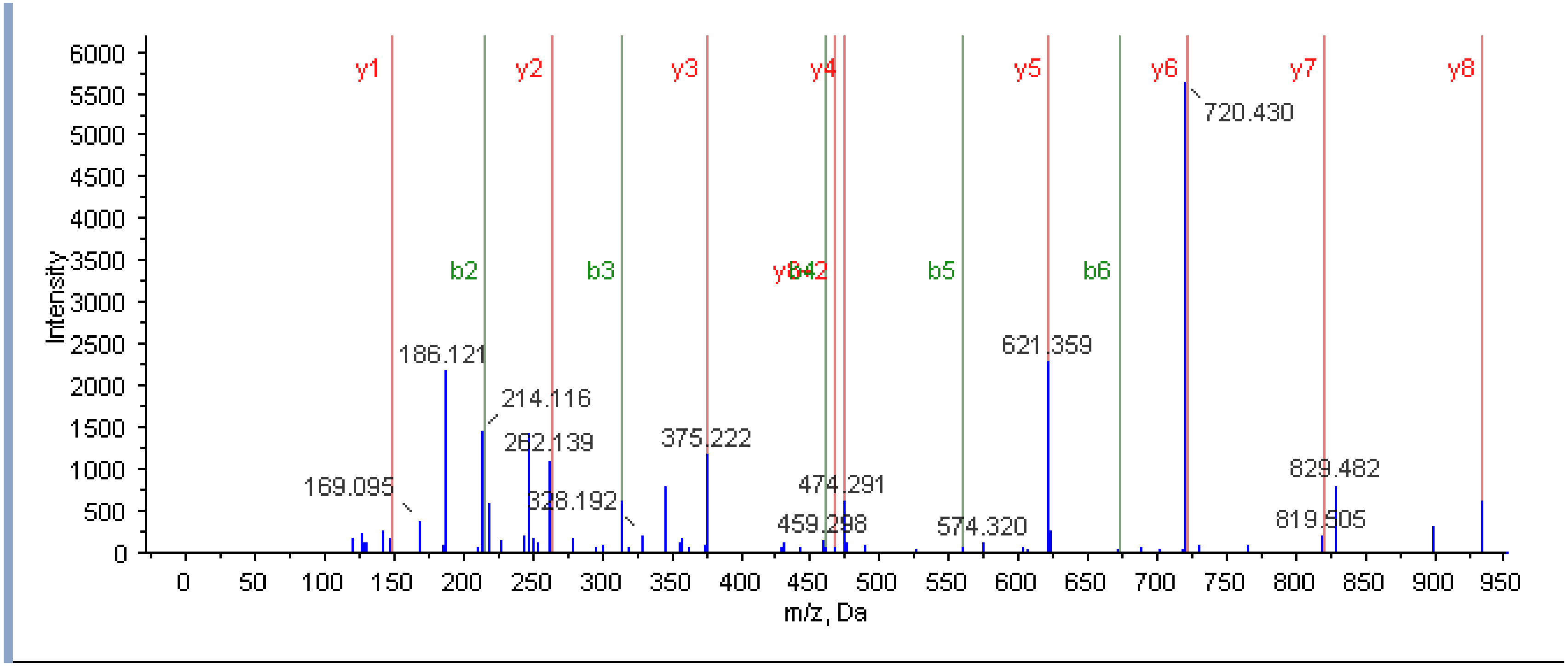
False *m/z* match to labeled peptide

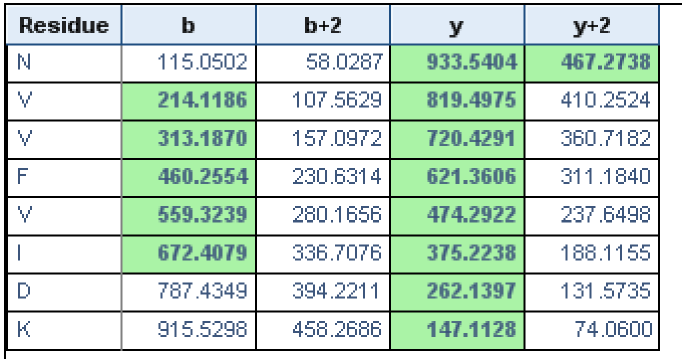
***Note:** In MS1, see 1000 cps, or approx 1% of labeled peptide signal, and 8.3e4 cps unlabeled peptide intensity. Therefore, NVVFVIDK ~1% occupancy. This is about 1 min difference in Retention Time, larger than expected for short gradient, so we indicate <1% occupancy.

N274: NVVFVIDK Occupancy (N>D/O18)

Unlabeled peptide:, 2+ (MW), 467.255 to 467.295

File: KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff



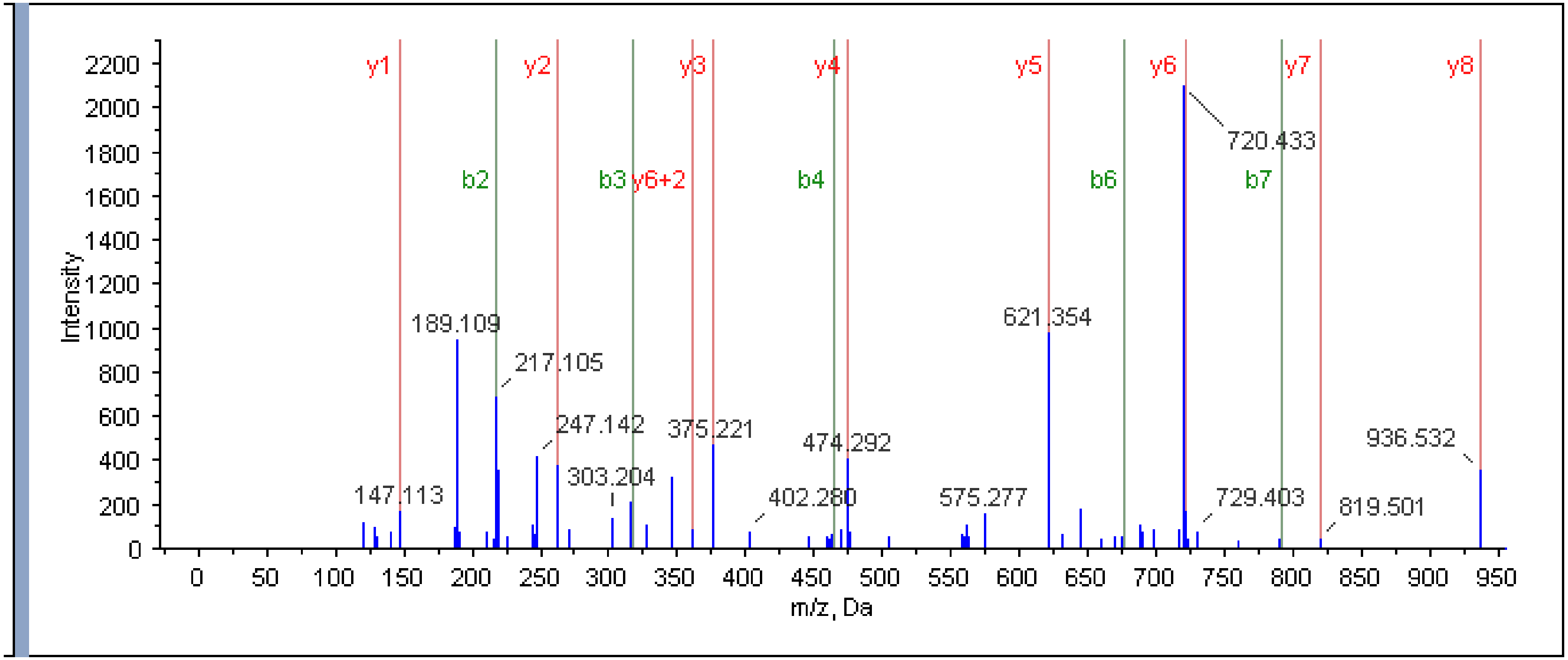


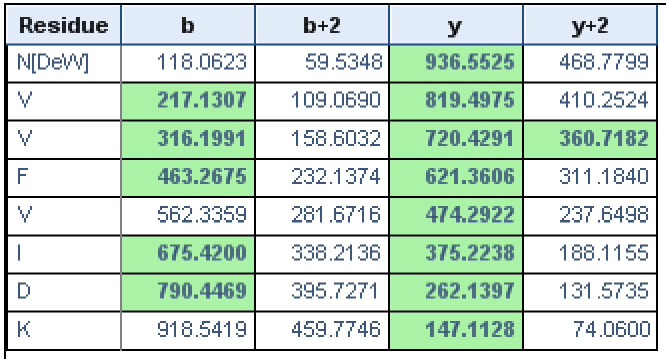
Residue	b	b+2	y	y+2
N	115.0502	58.0287	**933.5404**	**467.2738**
V	**214.1186**	107.5629	**819.4975**	410.2524
V	**313.1870**	157.0972	**720.4291**	360.7182
F	**460.2554**	230.6314	**621.3606**	311.1840
V	**559.3239**	280.1656	**474.2922**	237.6498
I	**672.4079**	336.7076	**375.2238**	188.1155
D	787.4349	394.2211	**262.1397**	131.5735
K	915.5298	458.2686	**147.1128**	74.0600

N274: NVVFVIDK Occupancy (N>D/O18)

Labeled peptide:, 2+ (MW), 468.740 to 468.780

File: KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff





Residue	b	b+2	y	y+2
N[DeW]	118.0623	59.5348	**936.5525**	468.7799
V	**217.1307**	109.0690	**819.4975**	410.2524
V	**316.1991**	158.6032	**720.4291**	**360.7182**
F	**463.2675**	232.1374	**621.3606**	311.1840
V	562.3359	281.6716	**474.2922**	237.6498
I	**675.4200**	338.2136	**375.2238**	188.1155
D	**790.4469**	395.7271	**262.1397**	131.5735
K	918.5419	459.7746	**147.1128**	74.0600

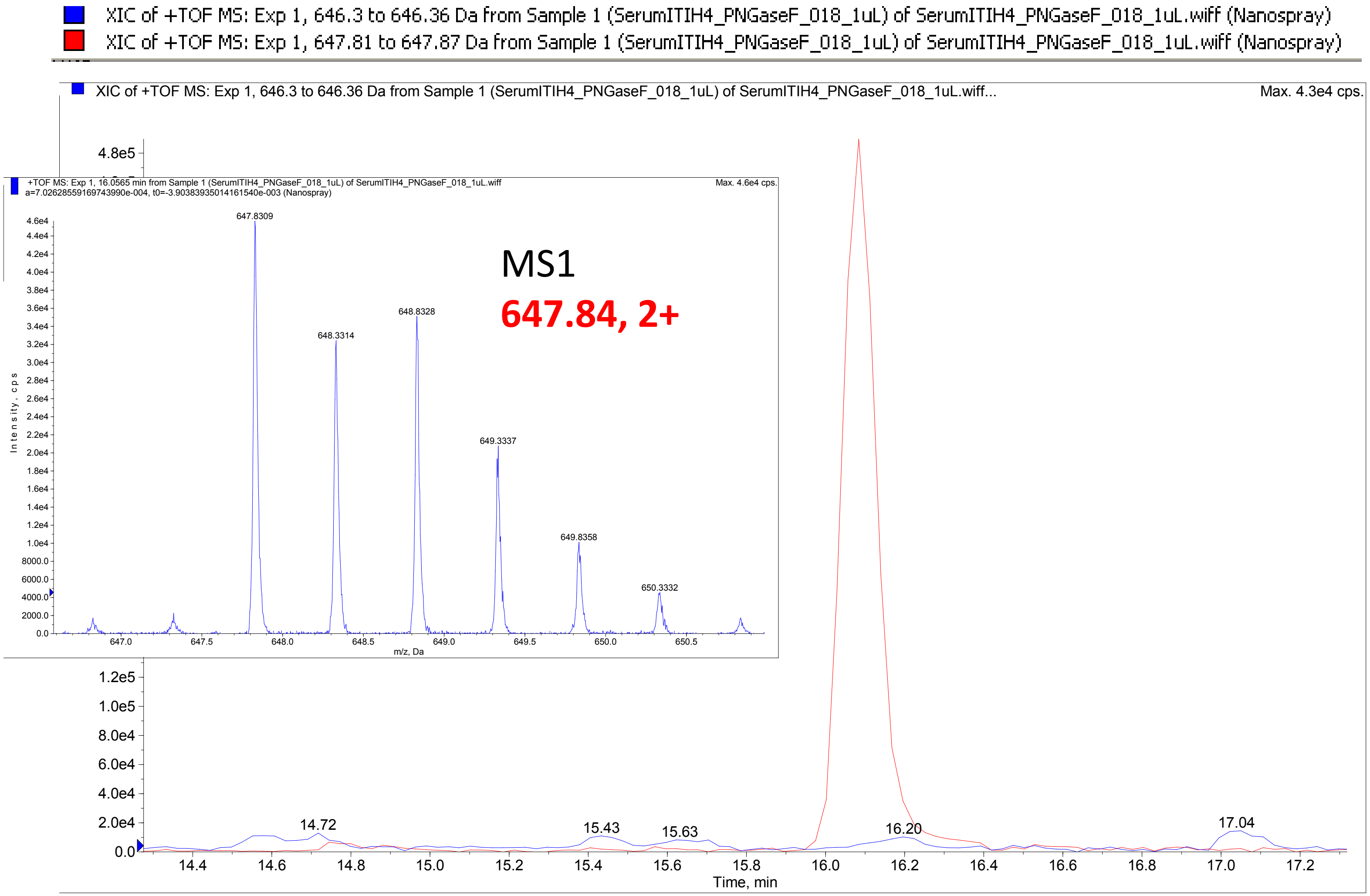
Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site

Serum ITIH4, N517

Site: N517 (Peptide: LPTQNITFQTE**)** File: SerumITIH4_PNGaseF_O18_1uL.wiff

LPTQNITFQTE – unlabeled (**646.33, 2+**) not detected, labeled (**647.84, 2+**) detected

Retention Time: 16.2 min (short gradient, 30 min)



MS1

647.84, 2+

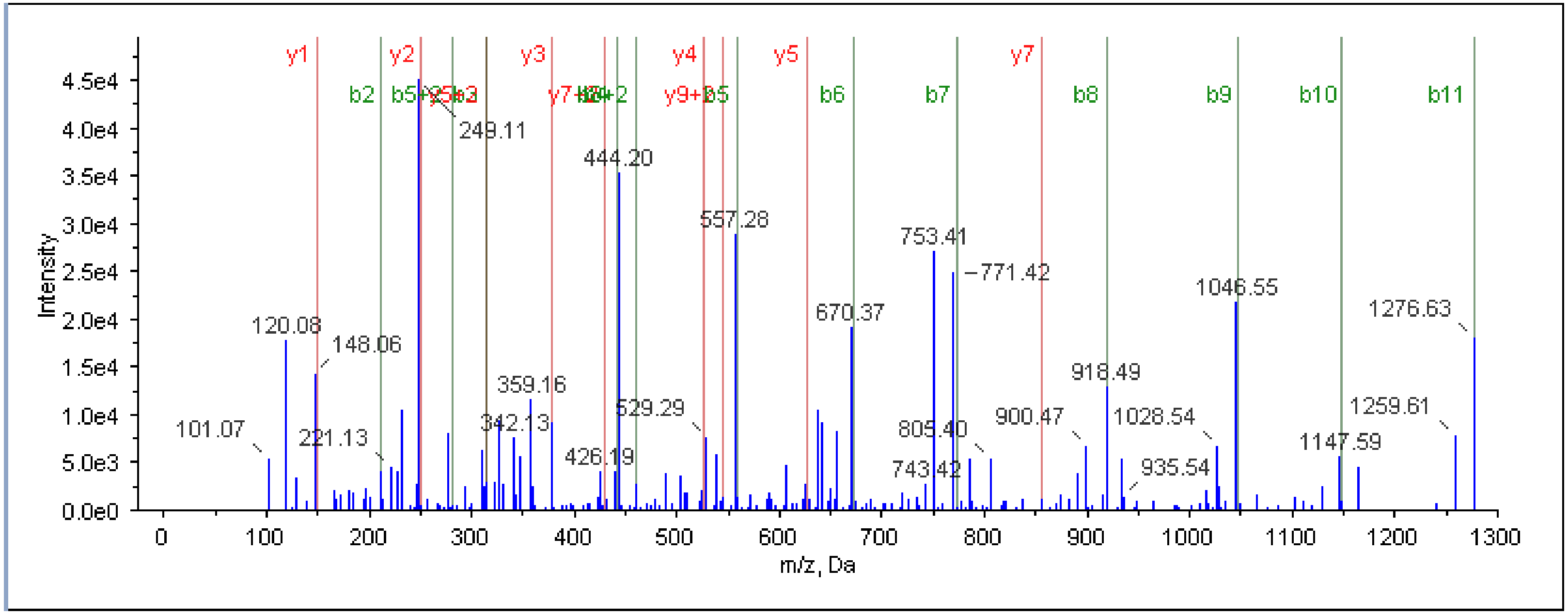
Note: In MS1, there may be unlabeled peptide present at Retention Time 15.68 min (close to baseline), *m/z* 646.33,2+,Intensity= 1000 cps. LABELED peptide 4.5e4 cps. Therefore, occupancy >98% (based on intensity).

Serum ITIH4, N517: LPTQNITFQTE Occupancy (N>D/O18)

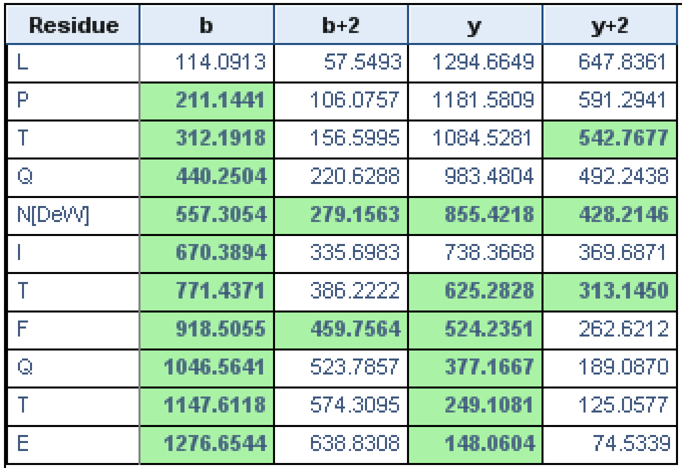
Unlabeled peptide: 646.3277, 2+ (MW 1290.6395), 646.2877 to 646.3577

Labeled peptide: 647.8249, 2+ (MW 1293.6340), 647.7949 to 647.8549

File: KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff



Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site



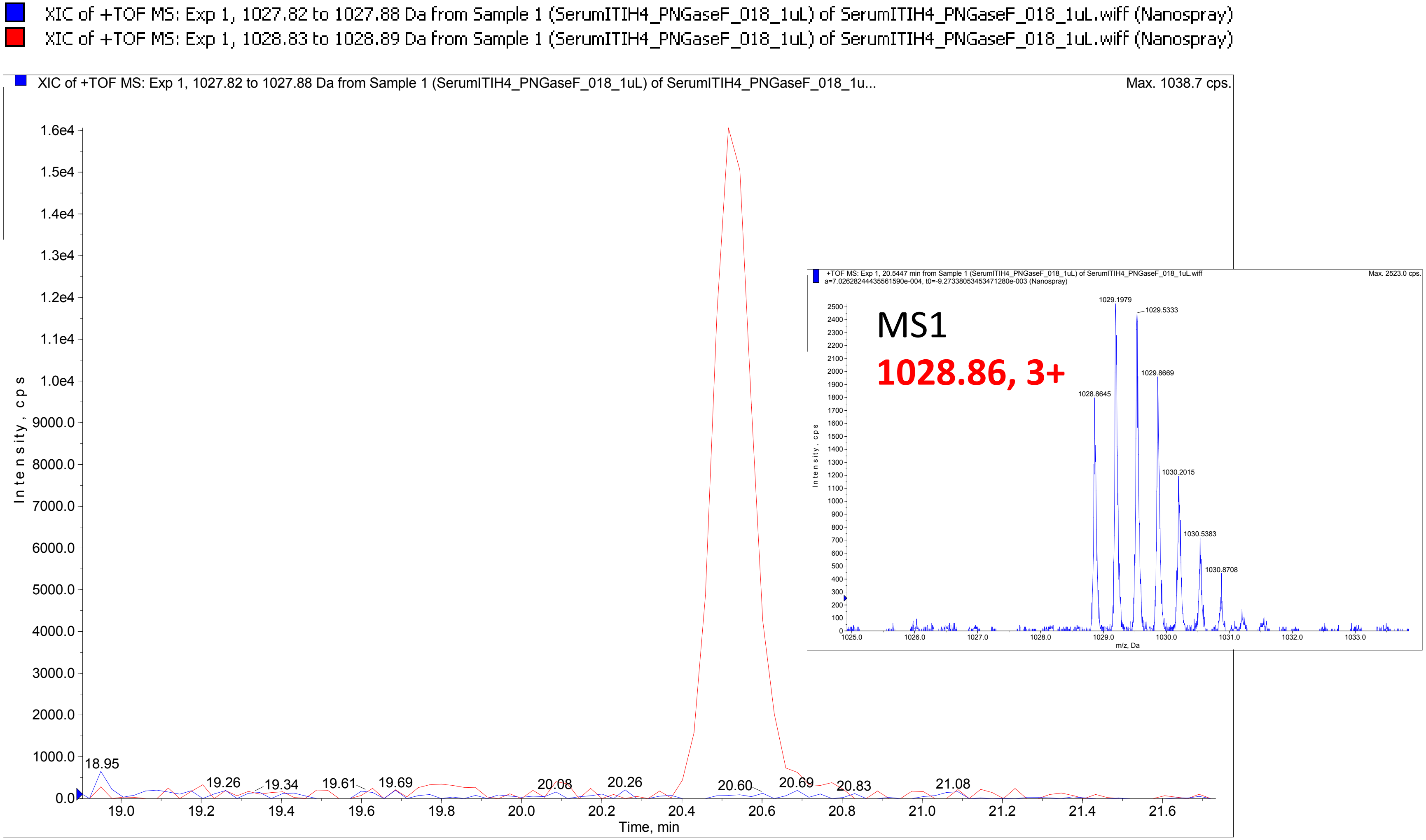
Residue	b	b+2	y	y+2
L	114.0913	57.5493	1294.6649	647.8361
P	**211.1441**	106.0757	1181.5809	591.2941
T	**312.1918**	156.5995	1084.5281	**542.7677**
Q	**440.2504**	220.6288	983.4804	492.2438
N[DeW]	**557.3054**	**279.1563**	**855.4218**	**428.2146**
I	**670.3894**	335.6983	738.3668	369.6871
T	**771.4371**	386.2222	**625.2828**	**313.1450**
F	**918.5055**	**459.7564**	**524.2351**	262.6212
Q	**1046.5641**	523.7857	**377.1667**	189.0870
T	**1147.6118**	574.3095	**249.1081**	125.0577
E	**1276.6544**	638.8308	**148.0604**	74.5339

Serum ITIH4, N577

Site: N577 (Peptide: NQALNLSLAYSFVTPLTMVVTKPDDQE**)** File: SerumITIH4_PNGaseF_O18_1uL.wiff

Unlabeled (**1027.85, 3+**) not detected, labeled (**1028.86, 3+**) detected

Retention Time: 20.45 min (short gradient, 30 min)

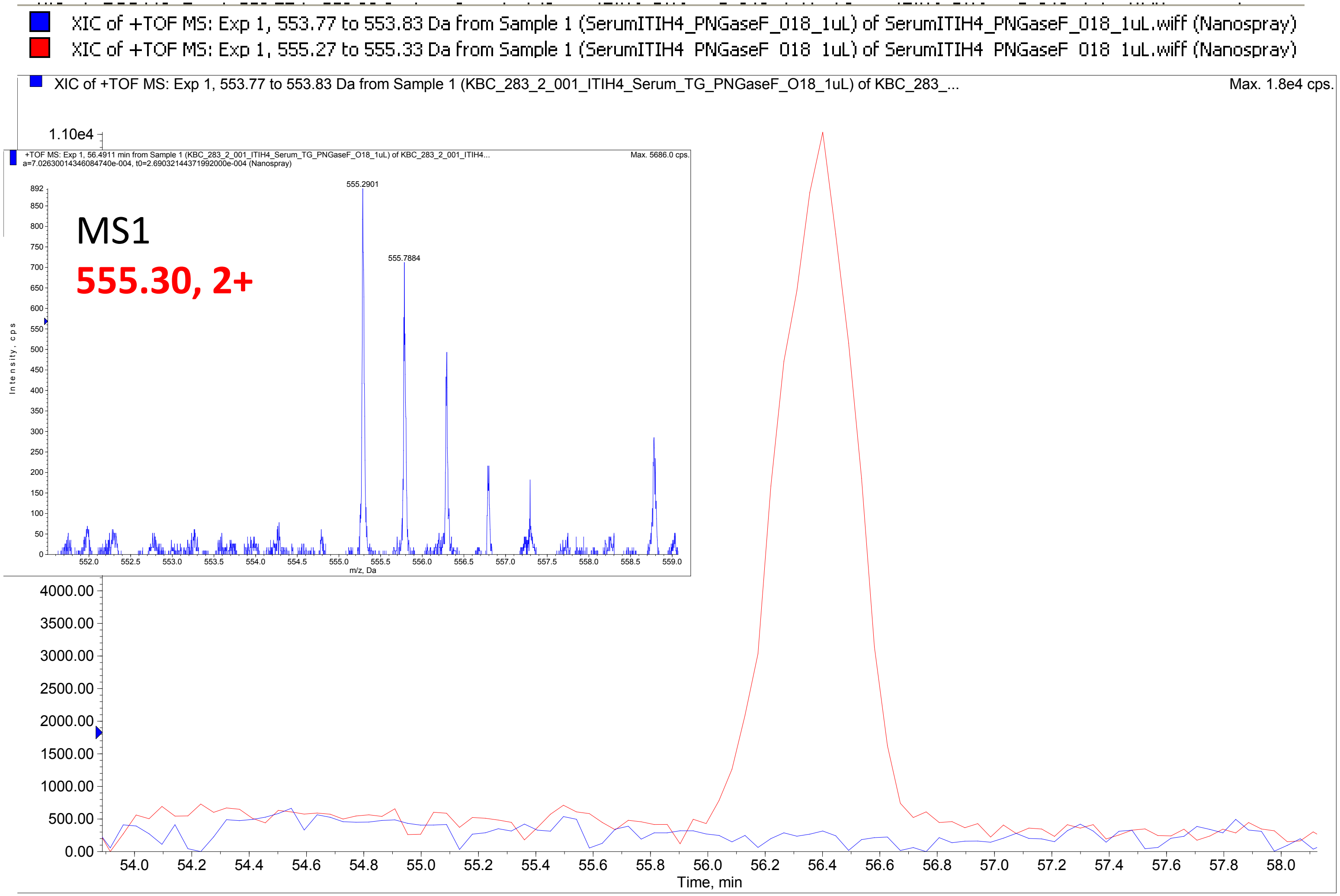


Note: In MS1, do not detect unlabeled peptide. Ions with a similar mass range at 150 cps can be observed. Therefore, if unlabeled peptide is below 150 cps, and LABELED peptide is at Intensity = 2734 cps, occupancy>95%.

Site: N577 (Peptide: NQALNLSLAY**)** File: KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff

Unlabeled (**553.80, 2+**) not detected, labeled (**555.30, 2+**) detected

Retention Time: 56.16-56.56 (long gradient, 120 min)



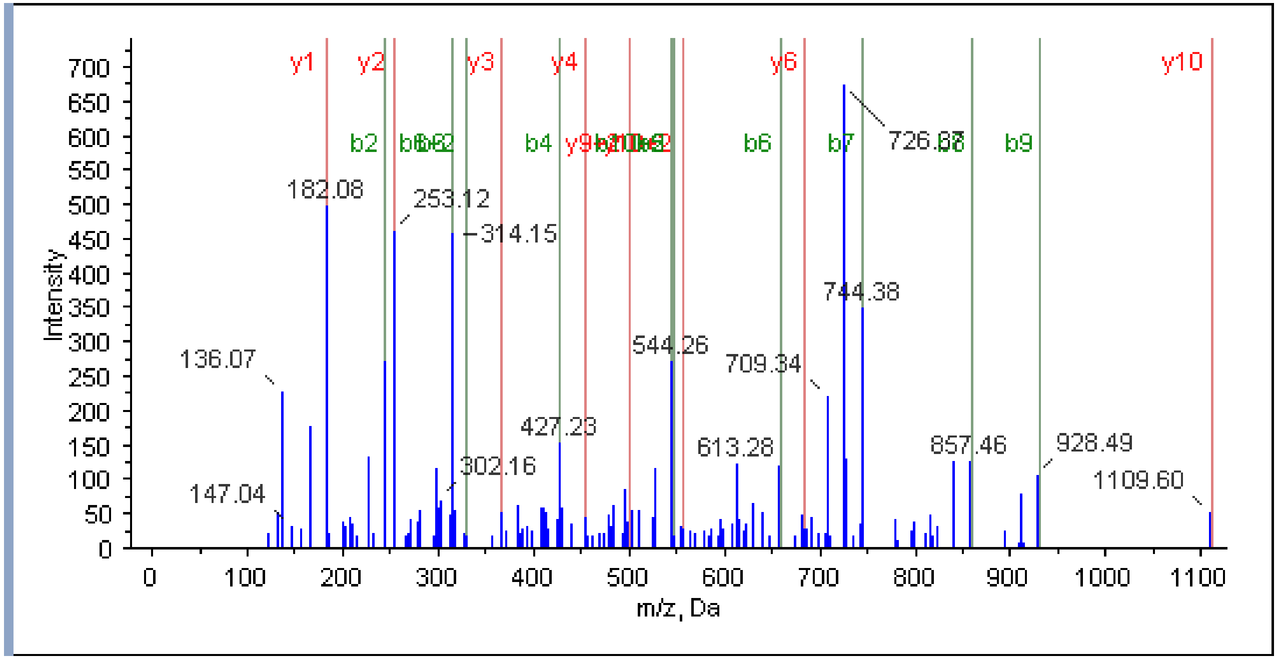
Note: Near the limit of detection. In MS1, the lowest ions could see the isotope cluster well were in Intensity = 150 cps range, or approx 10% of signal. So NQALNLSLAY >90% occupancy

Serum ITIH4, N577: NQALSNLSLAY Occupancy (N>D/O18)

Unlabeled peptide:, 2+ (MW), to

Labeled peptide:, 2+ (MW), to

File: KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff



Residue	b	b+2	y	y+2
N	115.0502	58.0287	1109.5961	555.3017
Q	243.1088	122.0580	995.5532	498.2802
A	314.1459	157.5766	867.4946	434.2509
L	427.2300	214.1186	796.4575	398.7324
N[DeW]	544.2850	272.6461	683.3734	342.1904
L	657.3690	329.1881	566.3184	283.6629
S	744.4010	372.7042	453.2344	227.1208
L	857.4851	429.2462	366.2023	183.6048
A	928.5222	464.7648	253.1183	127.0628
Y	1091.5856	546.2964	182.0812	91.5442

Fragmentation Evidence (peptide b- and y-ions) for N>D/O18 (3 Da shift) at glycosylation site

Only modified peptide can be detected, but at low intensity.

SerumITIH4_PNGaseF_O18_1uL.wiff (Trypsin + GluC, PNGaseF/18O)
Short gradient (30 min)
ProteinPilot results for peptides with N-glycosylation sites

Conf	Sequence	Modifications	Prec m/z	Theor m/z	Theor z	Time
0.1162	AFITNFSMIIDGMTYPGIIK	Delta:H(1)O(-1)18O(1)(N)@5; Oxidation(M)@8	751.0446	751.0553	3	20.6795
0.9892	KAFITNF	Delta:H(1)O(-1)18O(1)(N)@6	422.2301	422.2404	2	16.5347
1.245	KAFITNFSMIID	Delta:H(1)O(-1)18O(1)(N)@6	701.8627	701.8741	2	18.7392
61.41	LPTQNITFQTE	Delta:H(1)O(-1)18O(1)(N)@5	647.8237	647.8361	2	16.2111
23.91	NQALNLSLAY	Delta:H(1)O(-1)18O(1)(N)@5	555.2905	555.3017	2	17.7469
99	NQALNLSLAYSFVTPLTSMVVTKPDDQE	Delta:H(1)O(-1)18O(1)(N)@5	1028.849	1028.857	3	20.4536
99	NVVFVID		403.2258	403.2263	2	17.9113
99	NVVFVIDK		467.2743	467.2738	2	15.8195
99	STFMTNQLVDALTTWQNK	Delta:H(1)O(-1)18O(1)(N)@17	701.0089	701.018	3	19.4746

KBC_283_2_001_ITIH4_Serum_TG_PNGaseF_O18_1uL.wiff
Long gradient (120 min)
ProteinPilot results for peptides with N-glycosylation sites

Conf	Sequence	Modifications	dMass	Prec m/z	Theor m/z	Theor z	Time
0.5414	ALTTWQNK	Delta:H(1)O(-1)18O(1)(N)@7	-0.02938	482.7501	482.7647	2	24.147
1.579	KAFITNF	Delta:H(1)O(-1)18O(1)(N)@6	-0.02425	422.2282	422.2404	2	47.1421
92.19	LPTQNITFQTE	Delta:H(1)O(-1)18O(1)(N)@5	-0.02375	647.8242	647.8361	2	45.648
0.005132	LPTQNITFQTE		-0.00621	646.327	646.3301	2	34.8314
42.69	NQALNLSLAY	Delta:H(1)O(-1)18O(1)(N)@5	-0.02232	555.2905	555.3017	2	56.2904
97.26	NVVFVID		-0.00266	403.225	403.2263	2	55.0064
2.741	NVVFVIDK	Delta:H(1)O(-1)18O(1)(N)@1	-0.02401	468.7679	468.7799	2	47.8217
99	NVVFVIDK		0.000511	467.2741	467.2738	2	43.4274
0.5379	TQNITFQTE	Delta:H(1)O(-1)18O(1)(N)@3	-0.02376	542.7558	542.7677	2	33.0738

Part II:

N-Glycosylation Site Occupancy In Recombinant ITIH4

- Recombinant ITIH4 was treated with GluC and Trypsin, (or Trypsin and Chymotrypsin) heated to denature proteases, then treated with PNGaseF in the presence of $H_2{}^{18}O$

- 0.1 ug of the digest was injected onto an Eksigent ChromXP C18-CL interfaced with a 5600 TripleTOF mass spectrometer (ABSCIEX)

- Different chromatographic gradients were used (short gradient, 30 min; long gradient, 120 min)

Recombinant ITIH4 Occupancy Summary

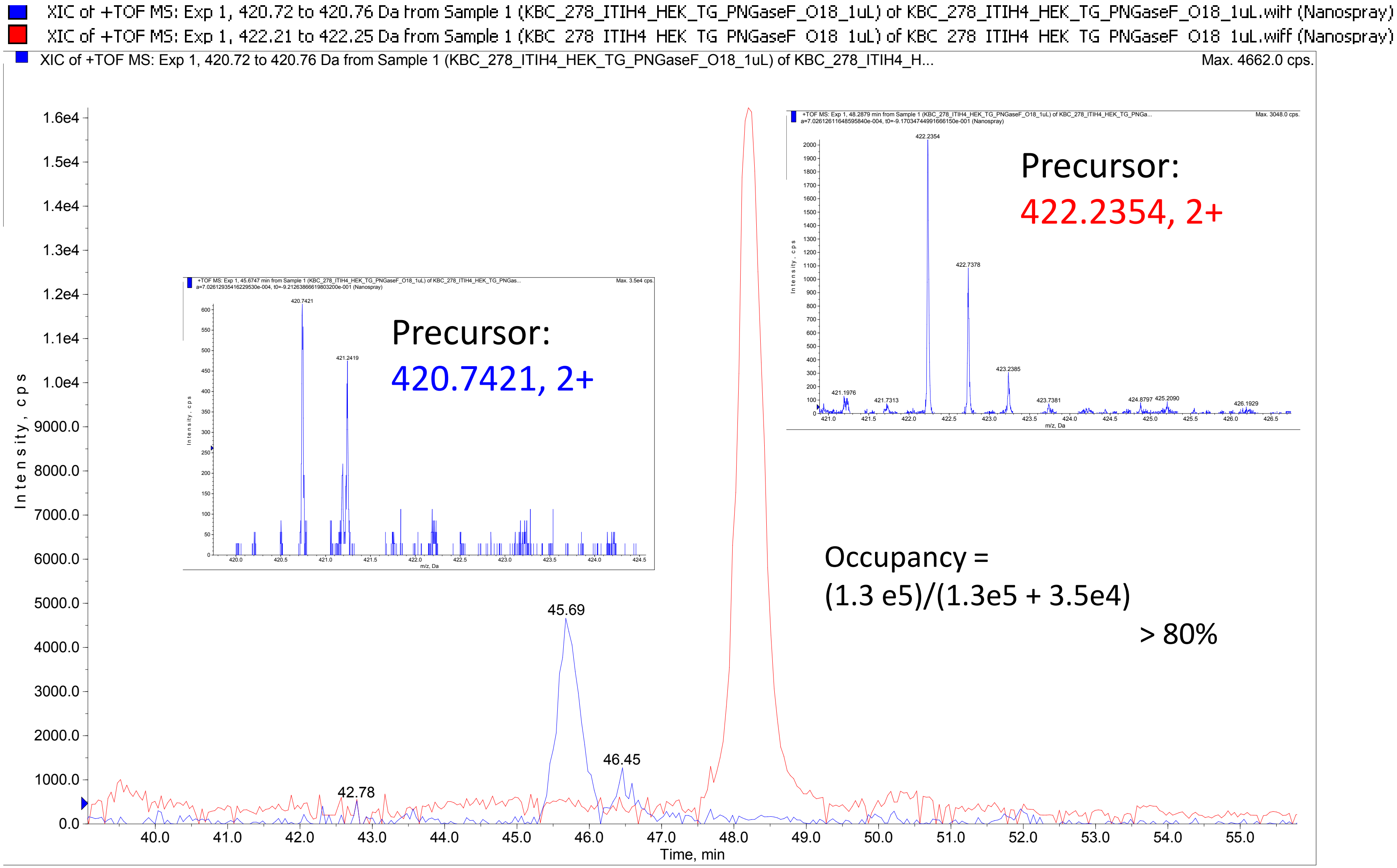
Site	**Peptide(s)**	***m/z* (charge) Unlabeled**	***m/z* (charge) Labeled**	**Cell ITIH4**
N81	KAFITNF (T,G)	420.734 (2+)	422.240 (2+)	>80%
N207	MTNQLVDALTTWQNK (T,C)	881.943 (3+)	883.449 (3+)	>97%
N274	NVVFVIDK (T,G)	467.274 (2+)	468.780 (2+)	<1%
N517	LPTQNITFQTE (T,G)	646.330 (2+)	647.836 (2+)	>99%
N577	NQALNLSLAY (T,C)	553.796 (2+)	555.302 (3+)	>90%

Recombinant ITIH4, N81

Site: N81 (Peptide: KAFITNF) File: KBC_278_ITIH4_HEK_TG_PNGaseF_O18_1uL.wiff

Unlabeled peptide: 420.7360, 2+; Labeled peptide: 422.2291, 2+

Retention Time: 48 min (long gradient)



Precursor:
420.7421, 2+

Precursor:
422.2354, 2+

Occupancy =
(1.3 e5)/(1.3e5 + 3.5e4)
> 80%

Note: The unlabeled and labeled peptides differ in retention time by a larger-than-expected time. There is MS/MS evidence of the labeled peptide, therefore, we conclude that the Occupancy > 80%

Recombinant ITIH4, N207

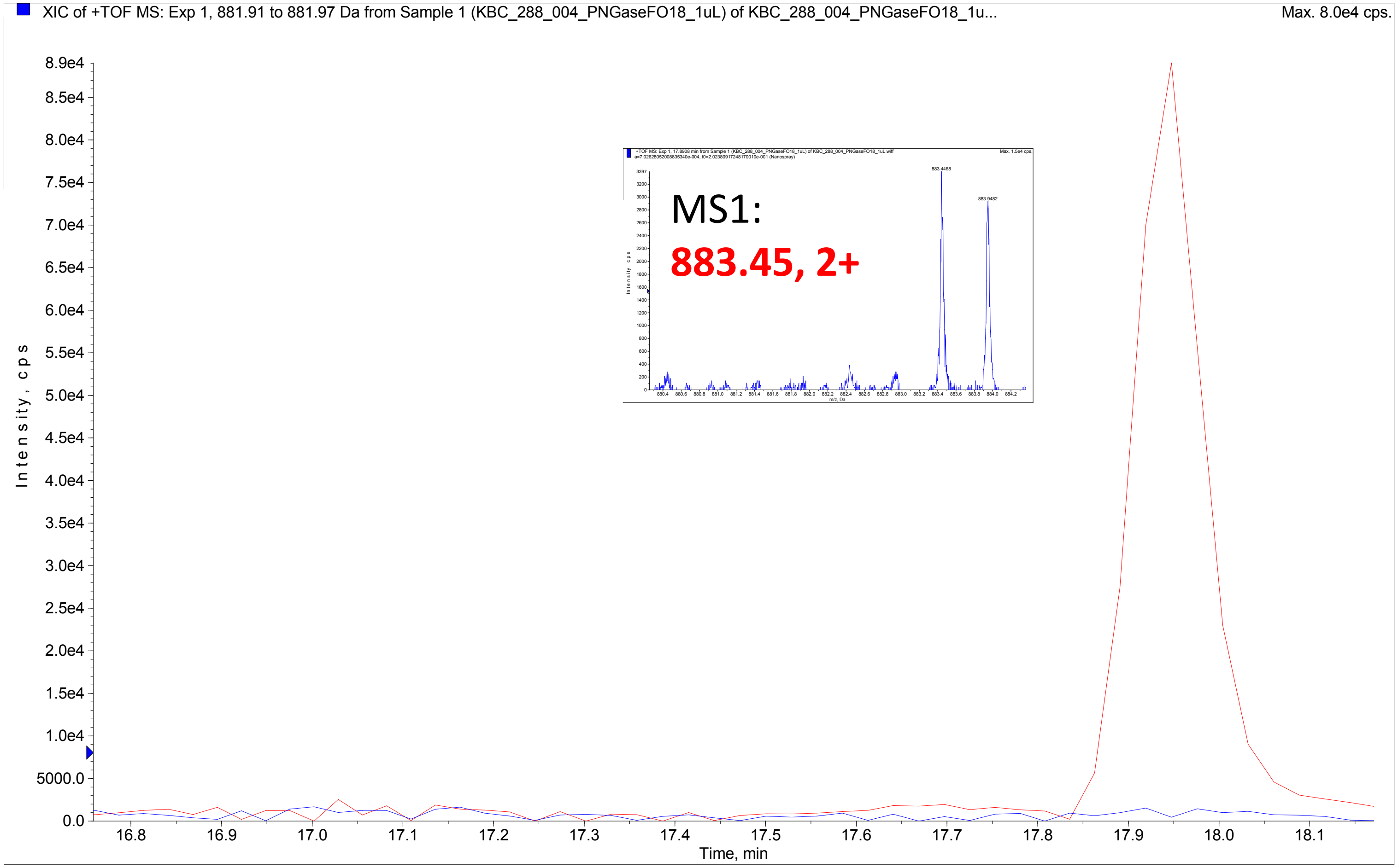
Site N207: MTNQLVDALTTWQNK

KBC_288_004: HEK ITIH4 + Trypsin + Chymotrypsin + PNGaseF / O18

Unlabeled: 881.9432, 2+ not detected; Labeled: 883.4493, 2+

🟦 XIC of +TOF MS: Exp 1, 881.91 to 881.97 Da from Sample 1 (KBC_288_004_PNGaseFO18_1uL) of KBC_288_004_PNGaseFO18_1uL.wiff (Nanospray)

🟥 XIC of +TOF MS: Exp 1, 883.42 to 883.48 Da from Sample 1 (KBC_288_004_PNGaseFO18_1uL) of KBC_288_004_PNGaseFO18_1uL.wiff (Nanospray)



NOTE: Labeled peptide detected at retention time 17.95 min, 9498 cps. Unlabeled peptide is not detected. In the MS spectrum near the expected retention time and m/z, I can see ions as low as about 284 cps. Therefore, estimate that non-labeled peptide is at most 3%, so occupancy is > 97%.

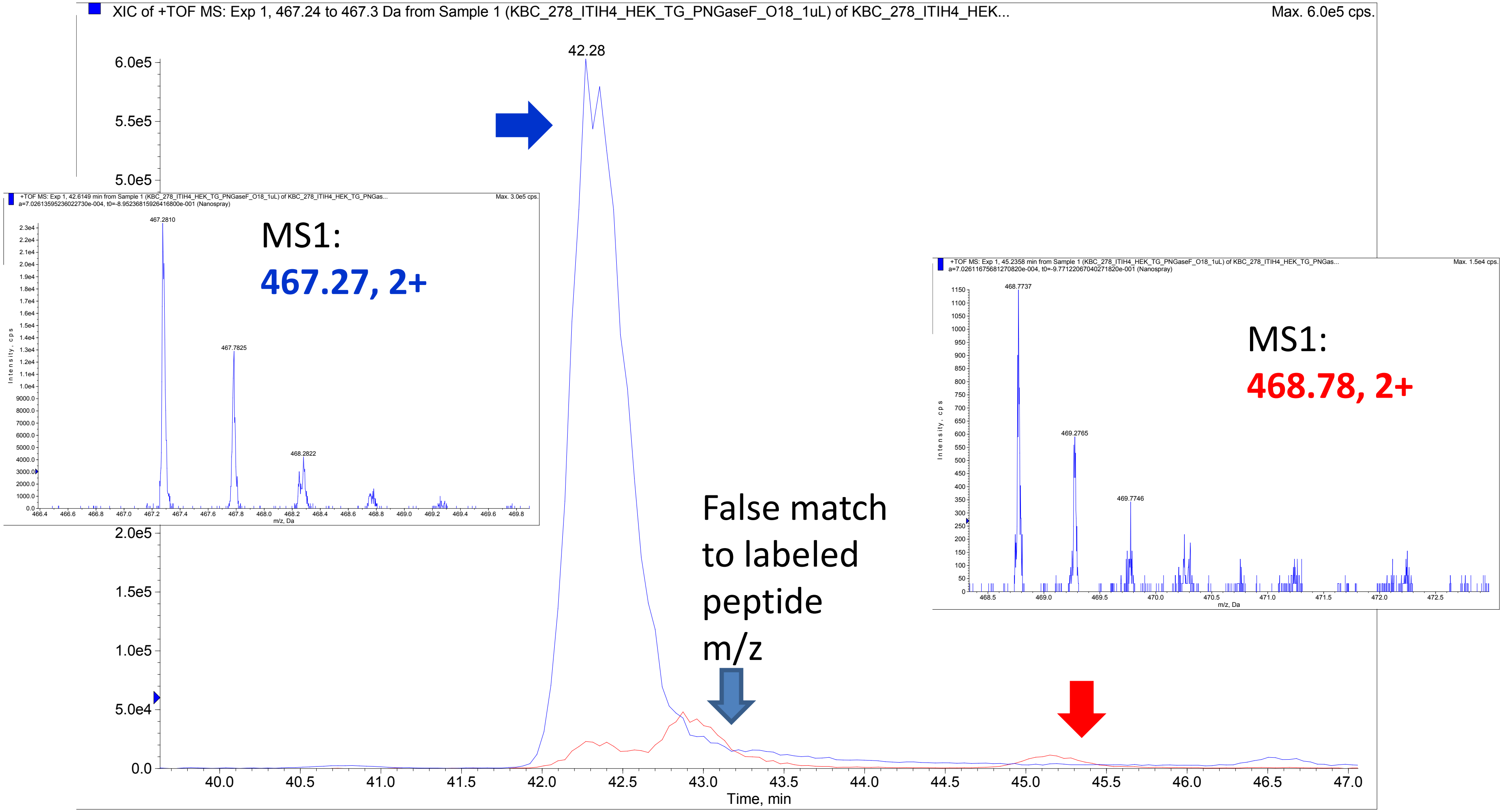
Recombinant ITIH4, N274

Site: N274 (Peptide: NVVFVIDK**)** File: KBC_278_ITIH4_HEK_TG_PNGaseF_O18_1uL.wiff

Unlabeled (**467.27, 2+**) detected, labeled (**468.78, 2+**)

Retention Time: 42.5 min (long gradient, 120 min)

🟦 XIC of +TOF MS: Exp 1, 467.24 to 467.3 Da from Sample 1 (HEKITIH4_PNGaseF_O18_1uL) of HEKITIH4_PNGaseF_O18_1uL.wiff (Nanospray)
🟥 XIC of +TOF MS: Exp 1, 468.75 to 468.81 Da from Sample 1 (HEKITIH4_PNGaseF_O18_1uL) of HEKITIH4_PNGaseF_O18_1uL.wiff (Nanospray)



MS1:
467.27, 2+

False match to labeled peptide m/z

MS1:
468.78, 2+

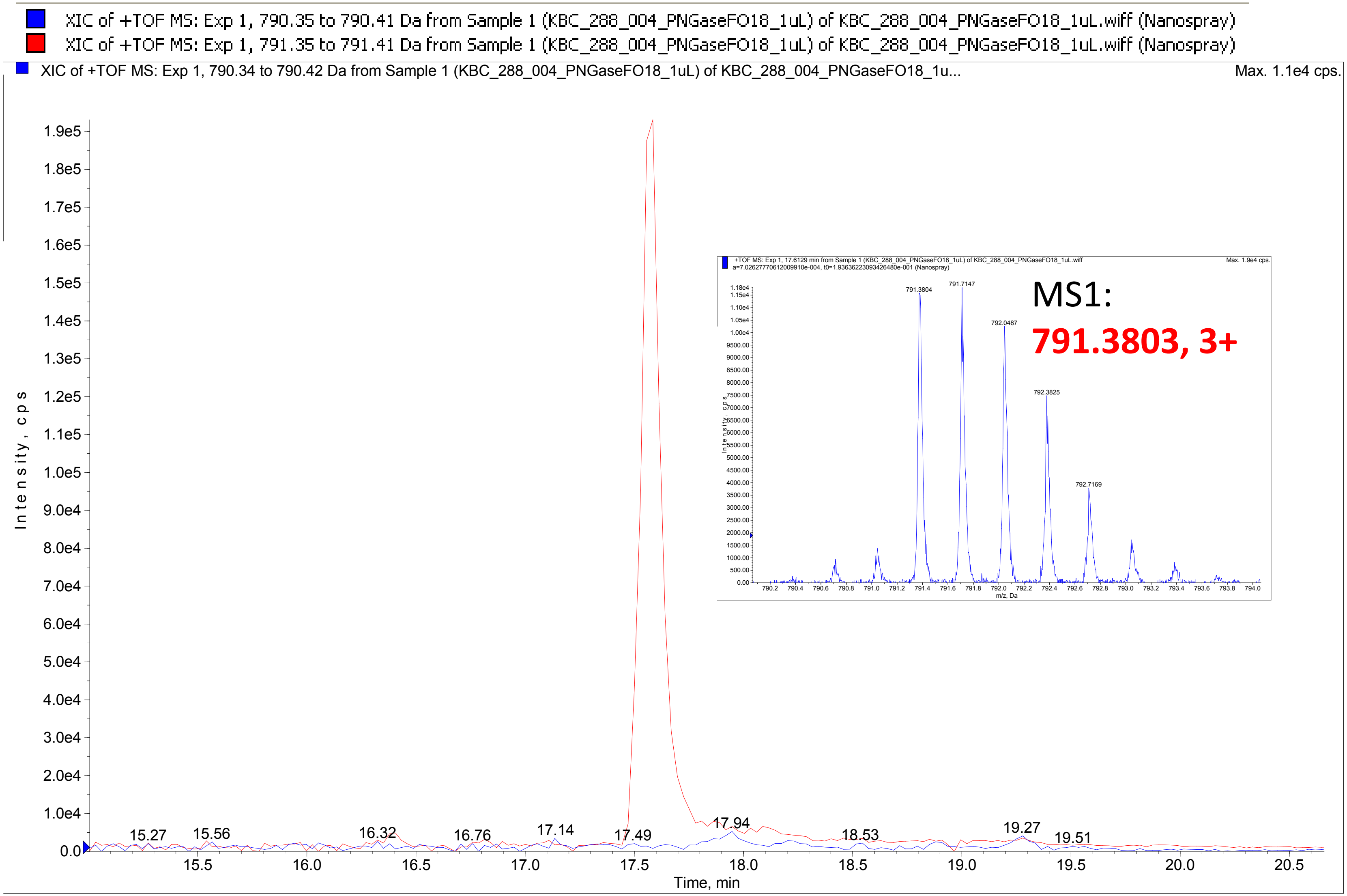
NOTE: Intensity of UNlabeled peptide is 7.7e4 (RT 42.3 min), and intensity of labeled peptide is 1e3 (RT 45.1 min). Therefore, occupancy = 2%. Different in retention time between unlabeled and labeled peptide is greater than expected. At N274, occupancy <1%

Recombinant ITIH4, N517

Site N517: LPTQNITFQTESSVAEQEAEF

KBC_288_004: HEK ITIH4 + Trypsin + Chymotrypsin + PNGaseF / O18

Unlabeled: 790.3763, 3+ not detected; **Labeled: 791.3803, 3+**



MS1:
791.3803, 3+

NOTE: Intensity of labeled peptide is 1.9e5, cannot detect unlabeled peptide. Therefore, occupancy is >99%.

Recombinant ITIH4, N577

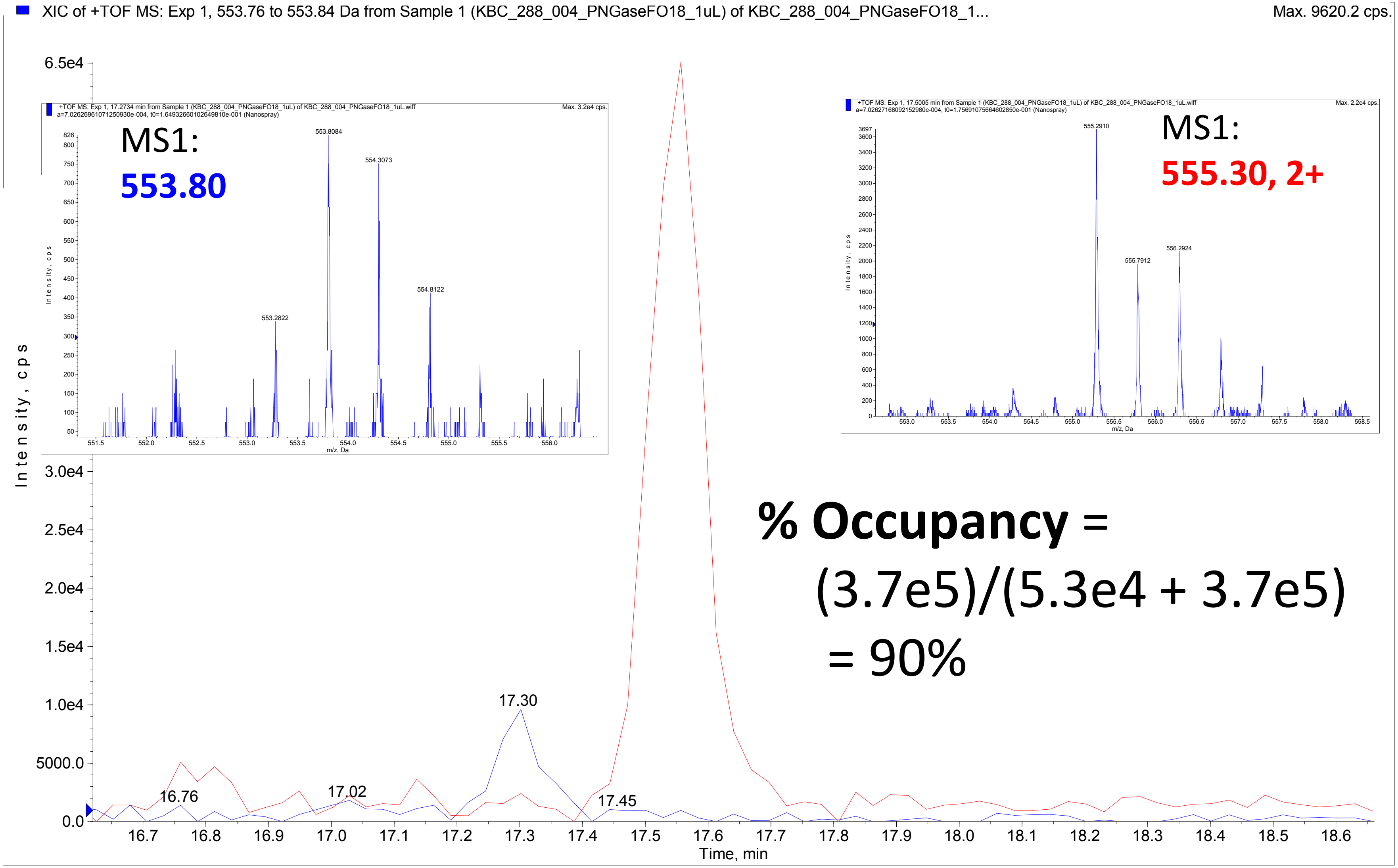
Site N577: NQALNLSLAY

KBC_288_004: HEK ITIH4 + Trypsin + Chymotrypsin + PNGaseF / O18

Unlabeled: 553.796. 2+: Labeled: 555.302. 2+

🟦 XIC of +TOF MS: Exp 1, 553.76 to 553.84 Da from Sample 1 (KBC_288_004_PNGaseFO18_1uL) of KBC_288_004_PNGaseFO18_1uL.wiff (Nanospray)

🟥 XIC of +TOF MS: Exp 1, 555.26 to 555.34 Da from Sample 1 (KBC_288_004_PNGaseFO18_1uL) of KBC_288_004_PNGaseFO18_1uL.wiff (Nanospray)



% Occupancy =

(3.7e5)/(5.3e4 + 3.7e5)

= 90%

HEK ITIH4 + Trypsin + GluC

Conf	Sequence	Modifications	Prec MW	Prec m/z	Theor MW	Theor m/z	Theor z	Time
99.0	LPTQNITFQTE	Delta:H(1)O(-1)18O(1)(N)@5	1293.63	647.82	1293.66	647.84	2	15.93
99.0	STFMTNQLVDALTTWQNK	Delta:H(1)O(-1)18O(1)(N)@17	2100.01	701.01	2100.03	701.02	3	19.32
45.6	NQALNLSLAY	Delta:H(1)O(-1)18O(1)(N)@5	1108.57	555.29	1108.59	555.30	2	17.60
0.5	KAFITNF	Delta:H(1)O(-1)18O(1)(N)@6	842.44	422.23	842.47	422.24	2	16.30

HEK ITIH4 + Trypsin + ChymoT

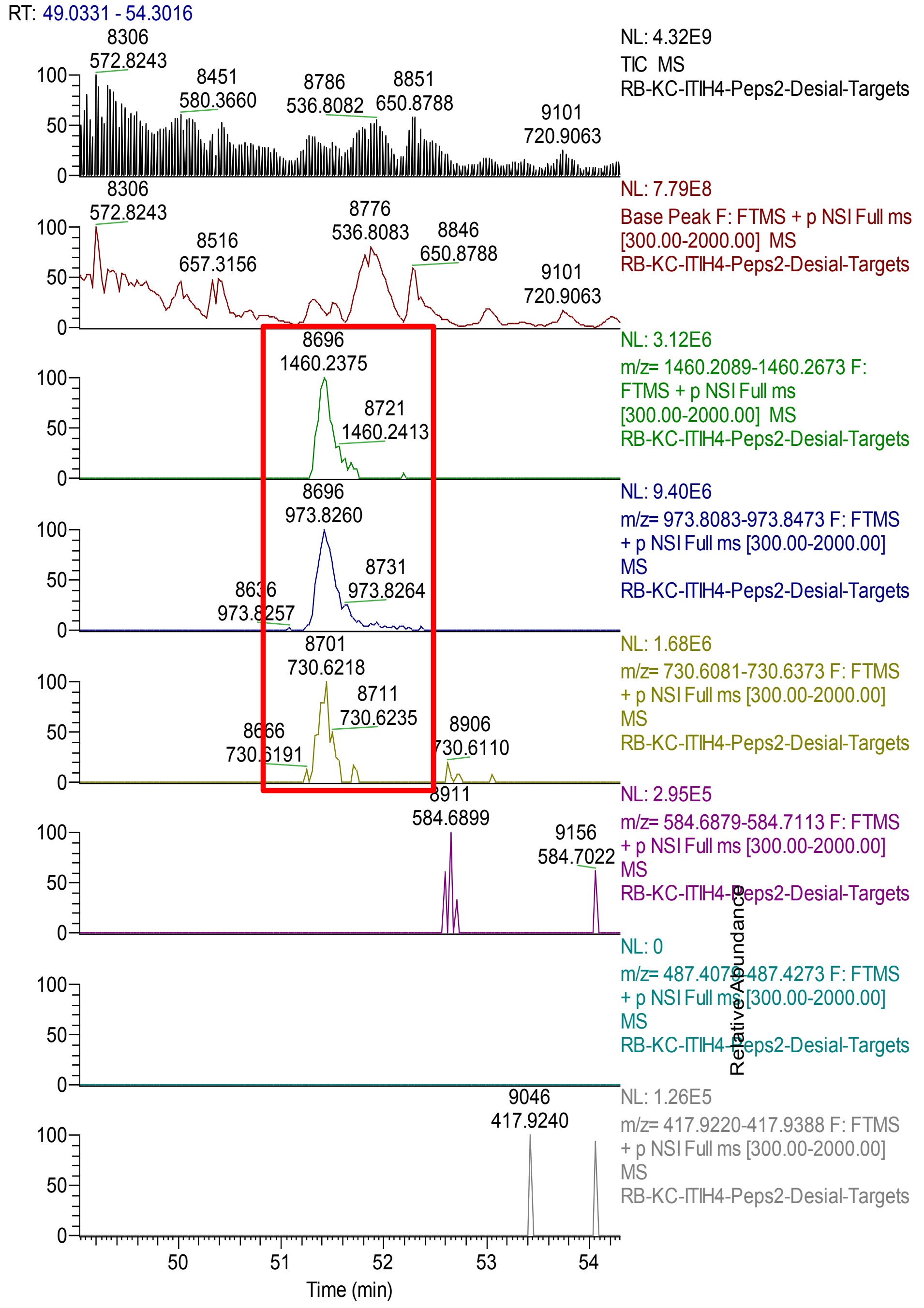
Conf	**Sequence**	**Modifications**	**Prec MW**	**Prec m/z**	**Theor MW**	**Theor m/z**	**Theor z**	**Time**
99.0	LPTQNITFQTESSVAEQEAEF	Delta:H(1)O(-1)18O(1)(N)@5	2371.10	791.37	2371.12	791.38	3	17.52
99.0	MTNQLVDALTTWQNK	Delta:H(1)O(-1)18O(1)(N)@14	1764.86	883.44	1764.88	883.45	2	17.97
99.0	NQALNLSLAY	Delta:H(1)O(-1)18O(1)(N)@5	1108.57	555.29	1108.59	555.30	2	17.54
98.9	RNQALNLSLAY	Delta:H(1)O(-1)18O(1)(N)@6	1264.67	633.34	1264.69	633.35	2	16.36

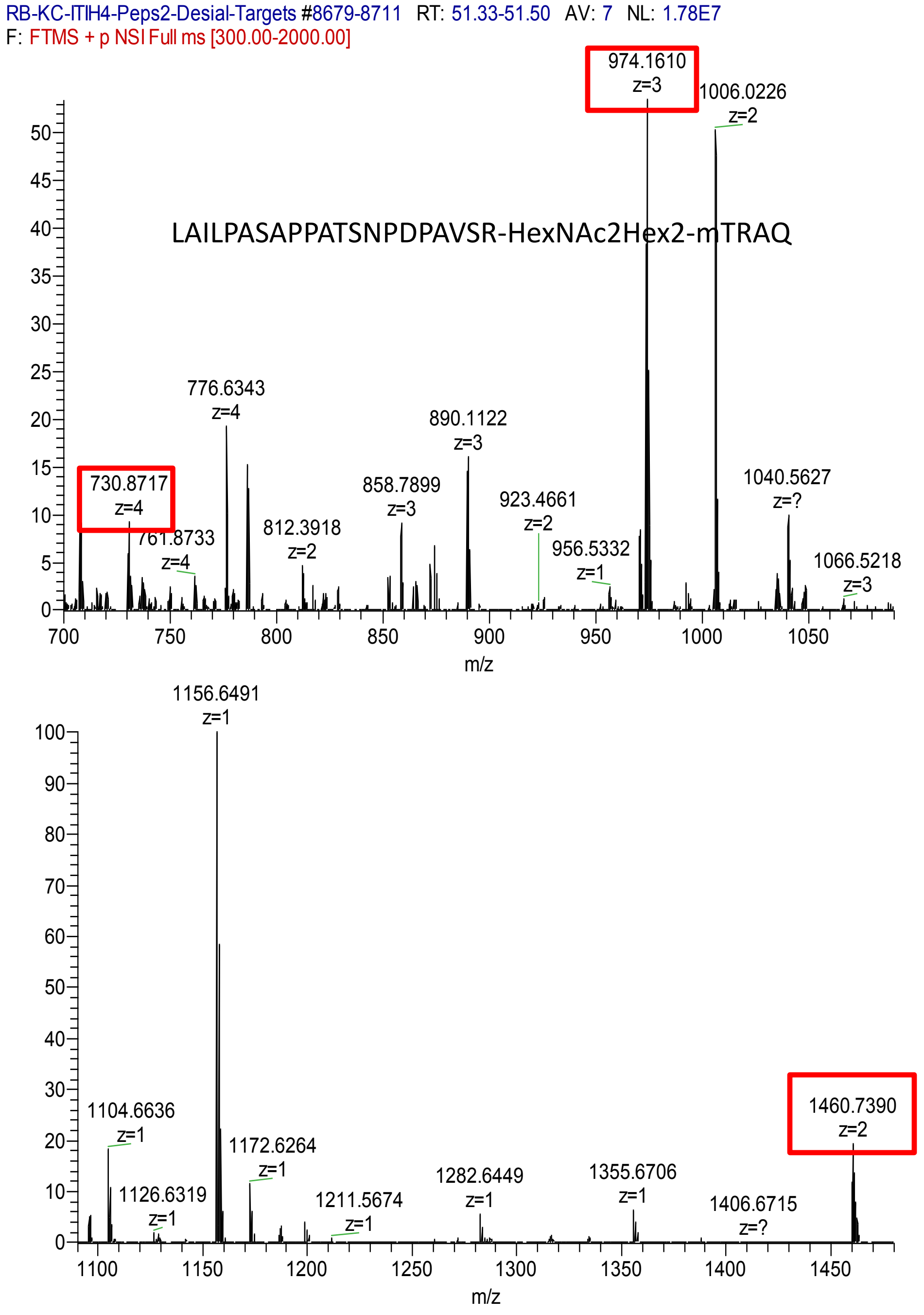
Supplementary Figure 4

Serum-Derived ITIH4 Glycopeptides (Desialylated) with ETD Fragmentation

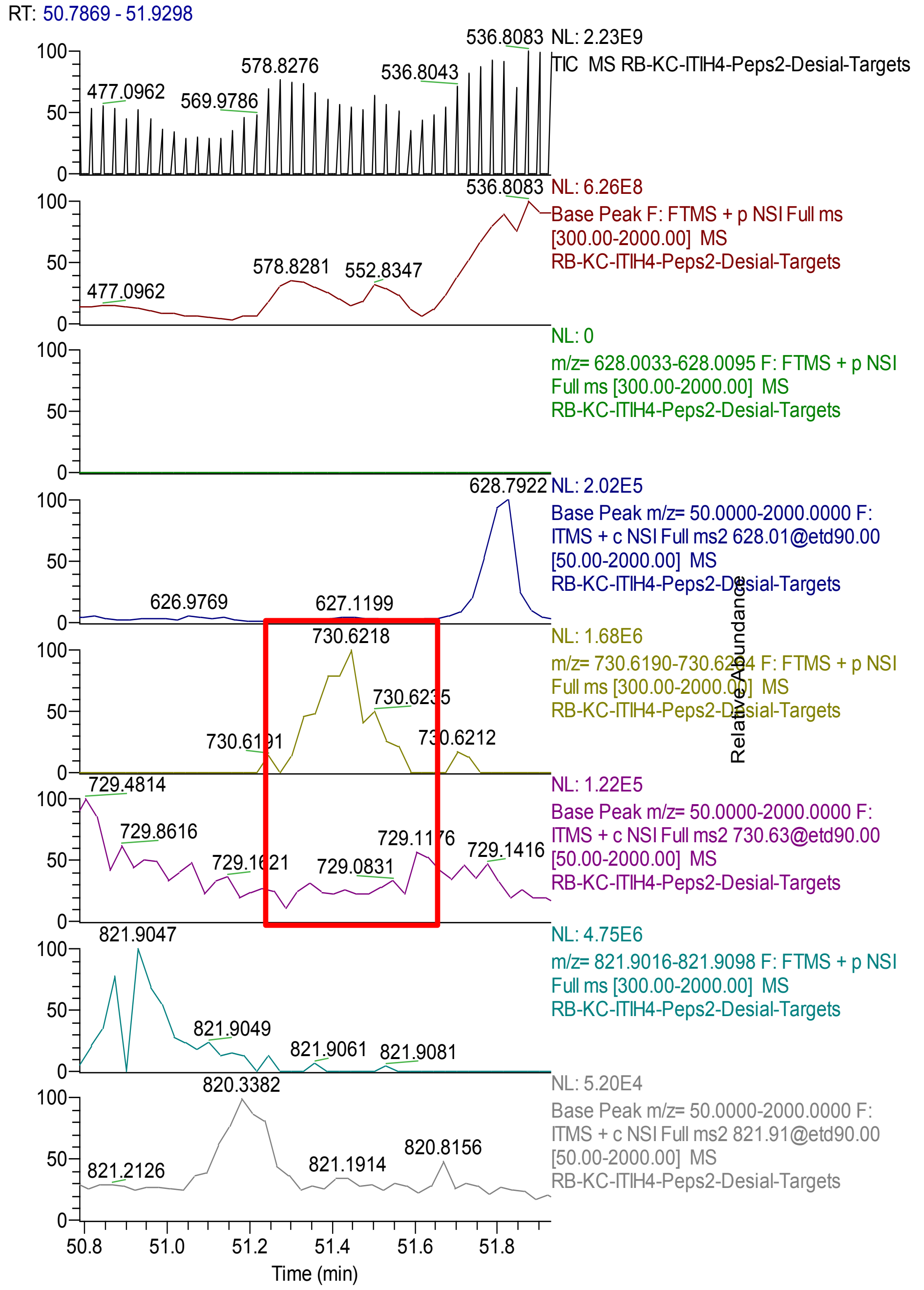
IPKPEASFSPR + 2mTRAQ + Hex-HexNAc
L AILPASAPPATSNPDPAVSR + mTRAQ + Hex2HexNAc2

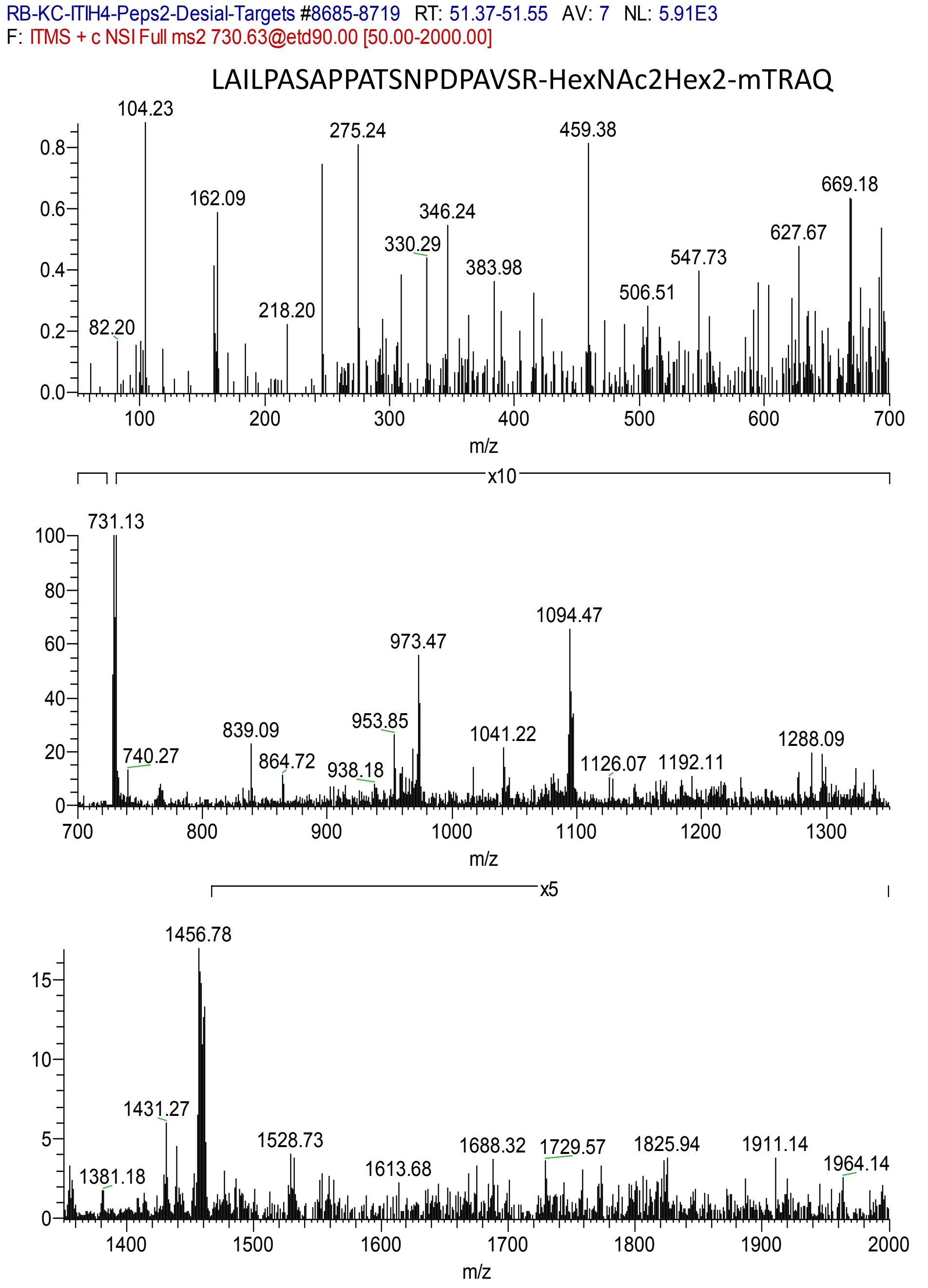
RB-KC-ITIH4-Peps2-Desial-Targets 10/1/2013 10:07:39 AM

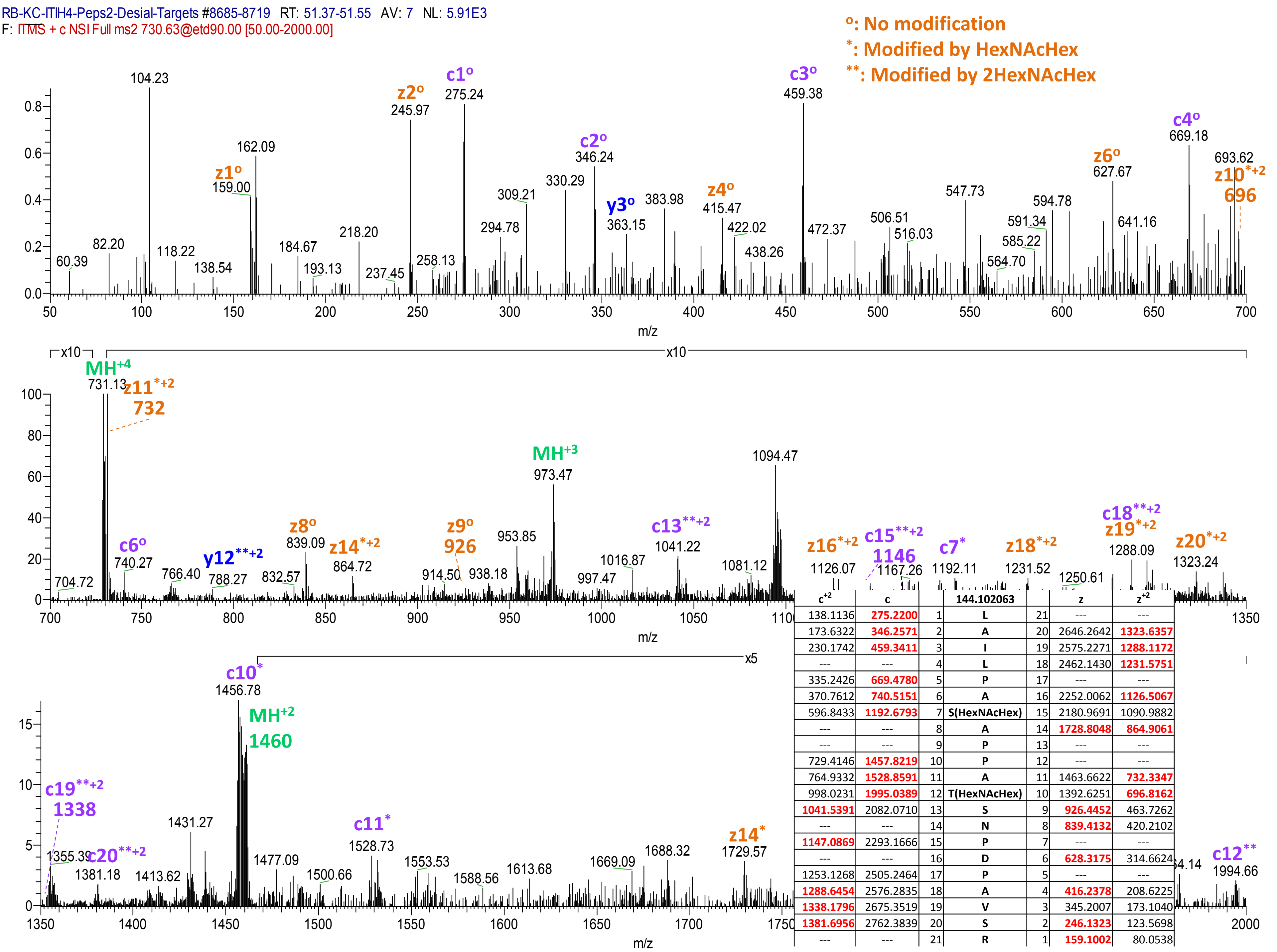




RB-KC-ITIH4-Peps2-Desial-Targets 10/1/2013 10:07:39 AM

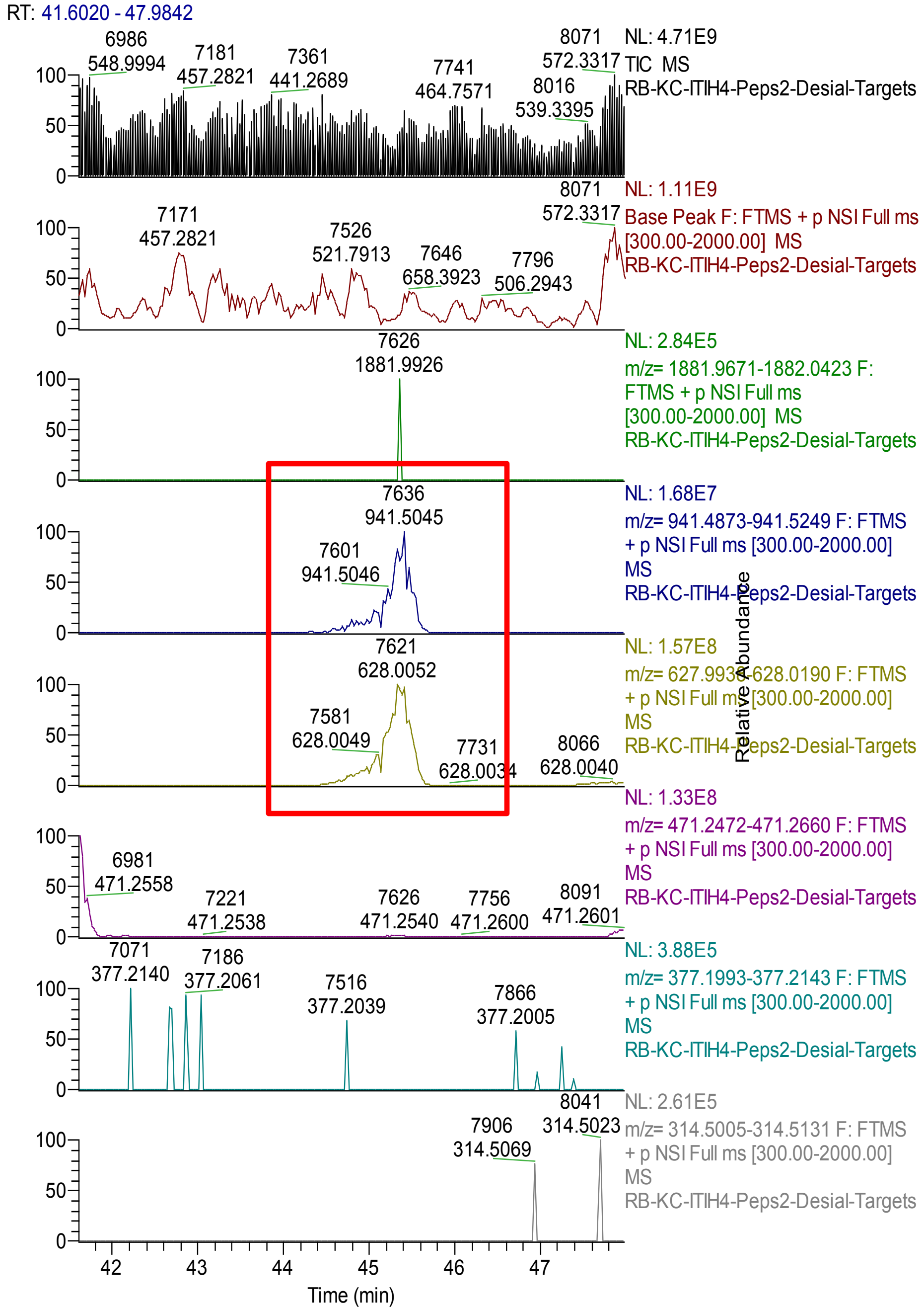


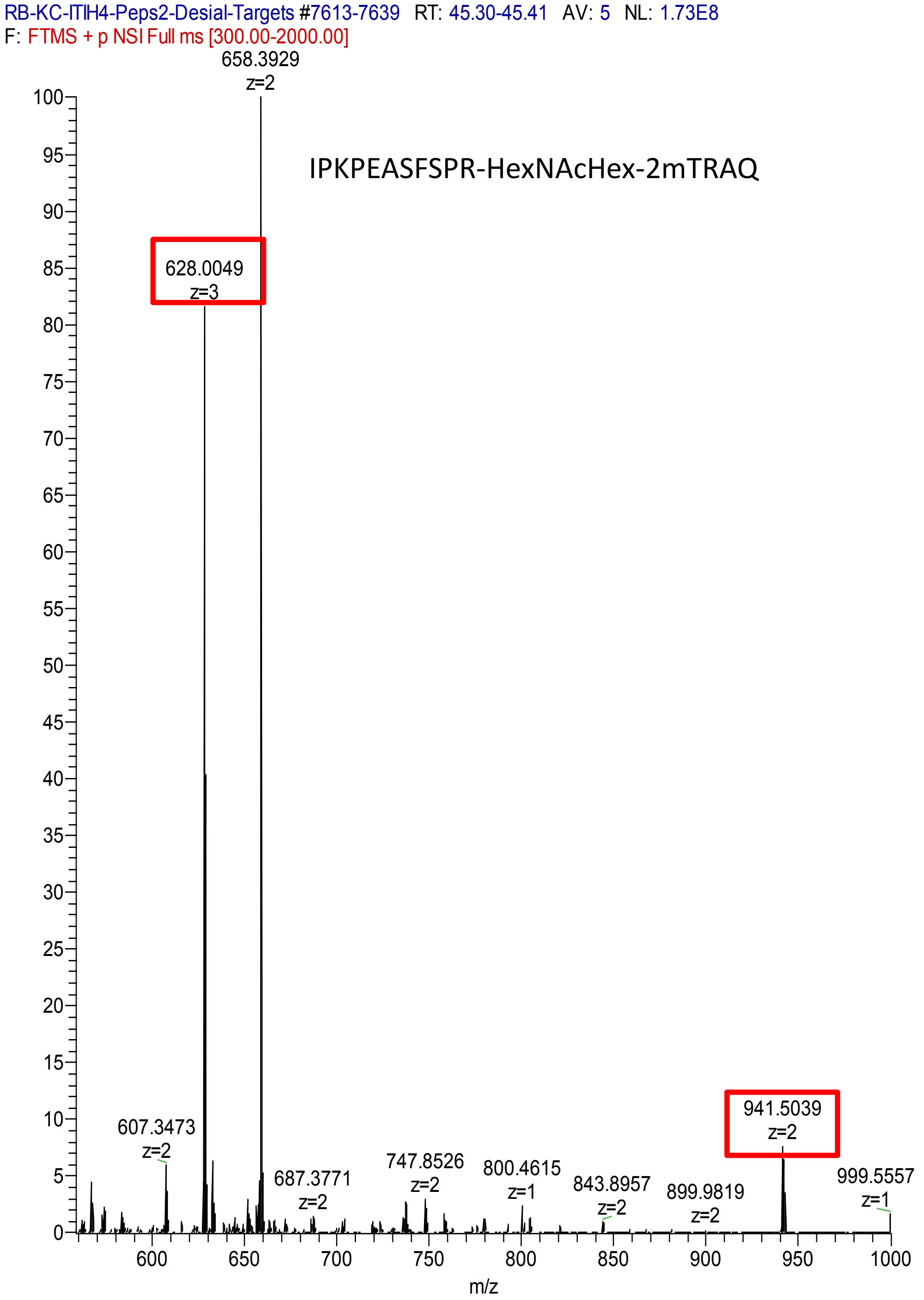




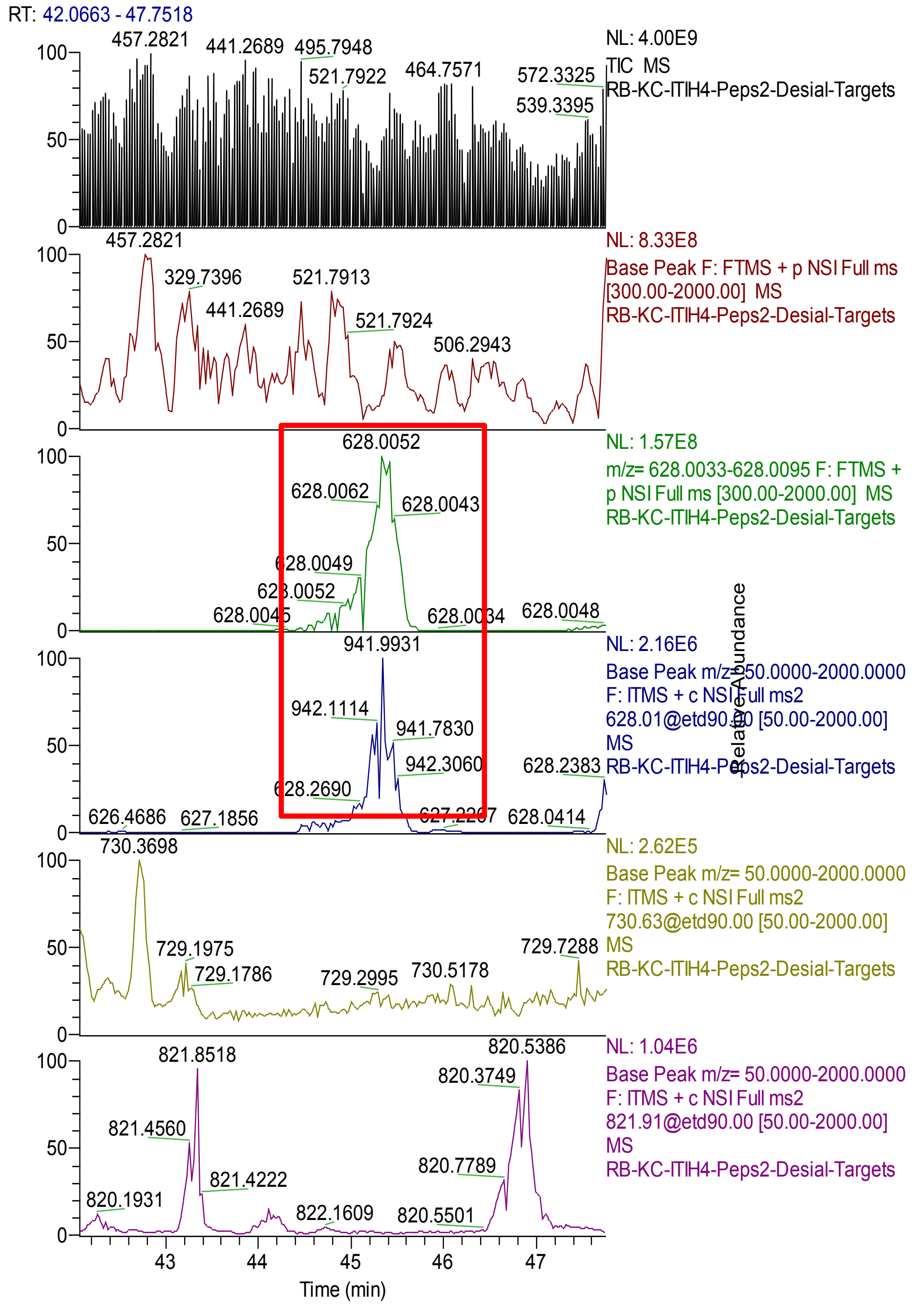
c+2	c		144.102063		z	z+2
138.1136	**275.2200**	1	L	21	---	---
173.6322	**346.2571**	2	A	20	2646.2642	**1323.6357**
230.1742	**459.3411**	3	I	19	2575.2271	**1288.1172**
---	---	4	L	18	2462.1430	**1231.5751**
335.2426	**669.4780**	5	P	17	---	---
370.7612	**740.5151**	6	A	16	2252.0062	**1126.5067**
596.8433	**1192.6793**	7	S(HexNAcHex)	15	2180.9691	1090.9882
---	---	8	A	14	**1728.8048**	**864.9061**
---	---	9	P	13	---	---
729.4146	**1457.8219**	10	P	12	---	---
764.9332	**1528.8591**	11	A	11	1463.6622	**732.3347**
998.0231	**1995.0389**	12	T(HexNAcHex)	10	1392.6251	**696.8162**
1041.5391	2082.0710	13	S	9	**926.4452**	463.7262
---	---	14	N	8	**839.4132**	420.2102
1147.0869	2293.1666	15	P	7	---	---
---	---	16	D	6	**628.3175**	314.6624
1253.1268	2505.2464	17	P	5	---	---
1288.6454	2576.2835	18	A	4	**416.2378**	208.6225
1338.1796	2675.3519	19	V	3	345.2007	173.1040
1381.6956	2762.3839	20	S	2	**246.1323**	123.5698
---	---	21	R	1	**159.1002**	80.0538

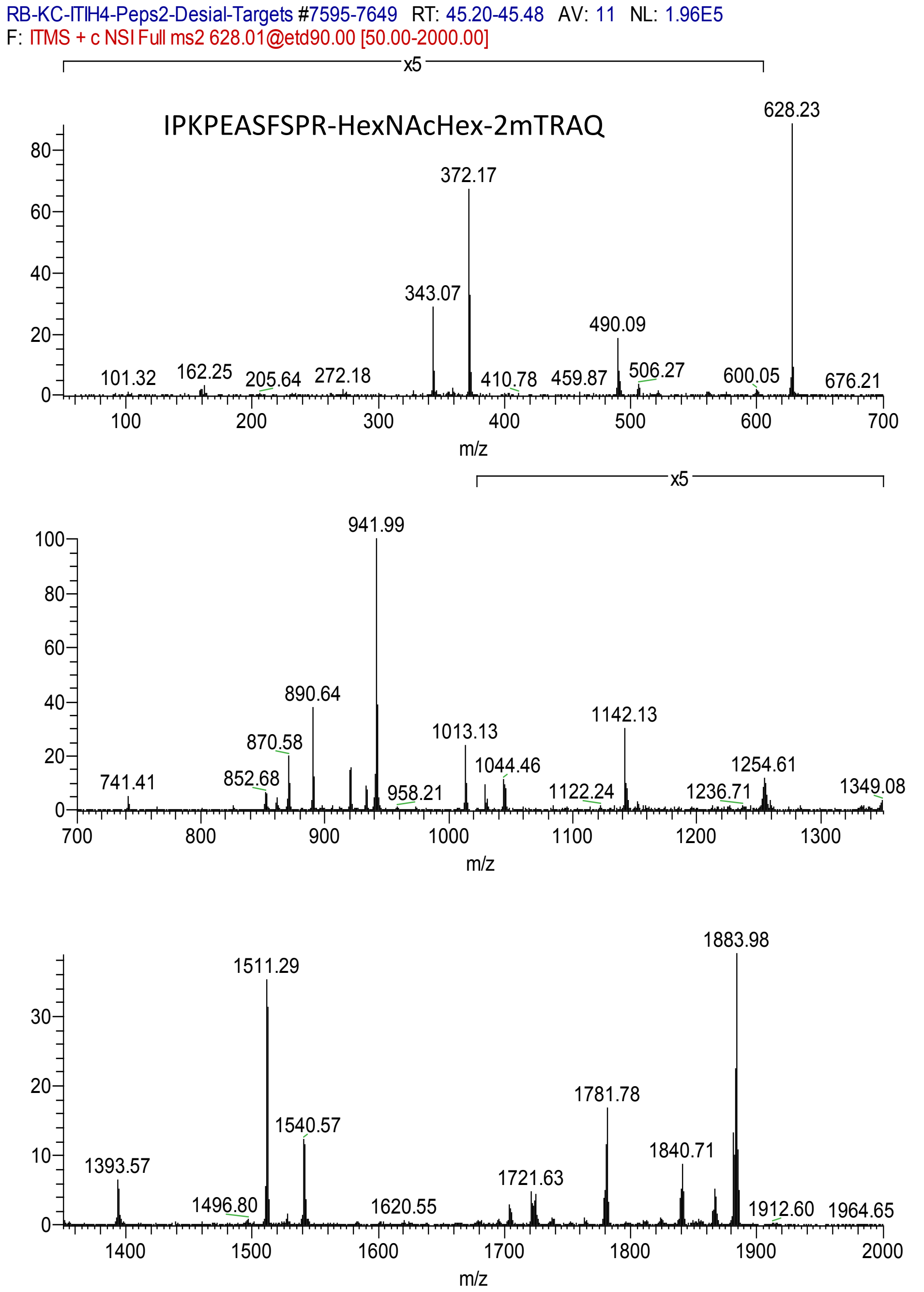
RB-KC-ITIH4-Peps2-Desial-Targets 10/1/2013 10:07:39 AM

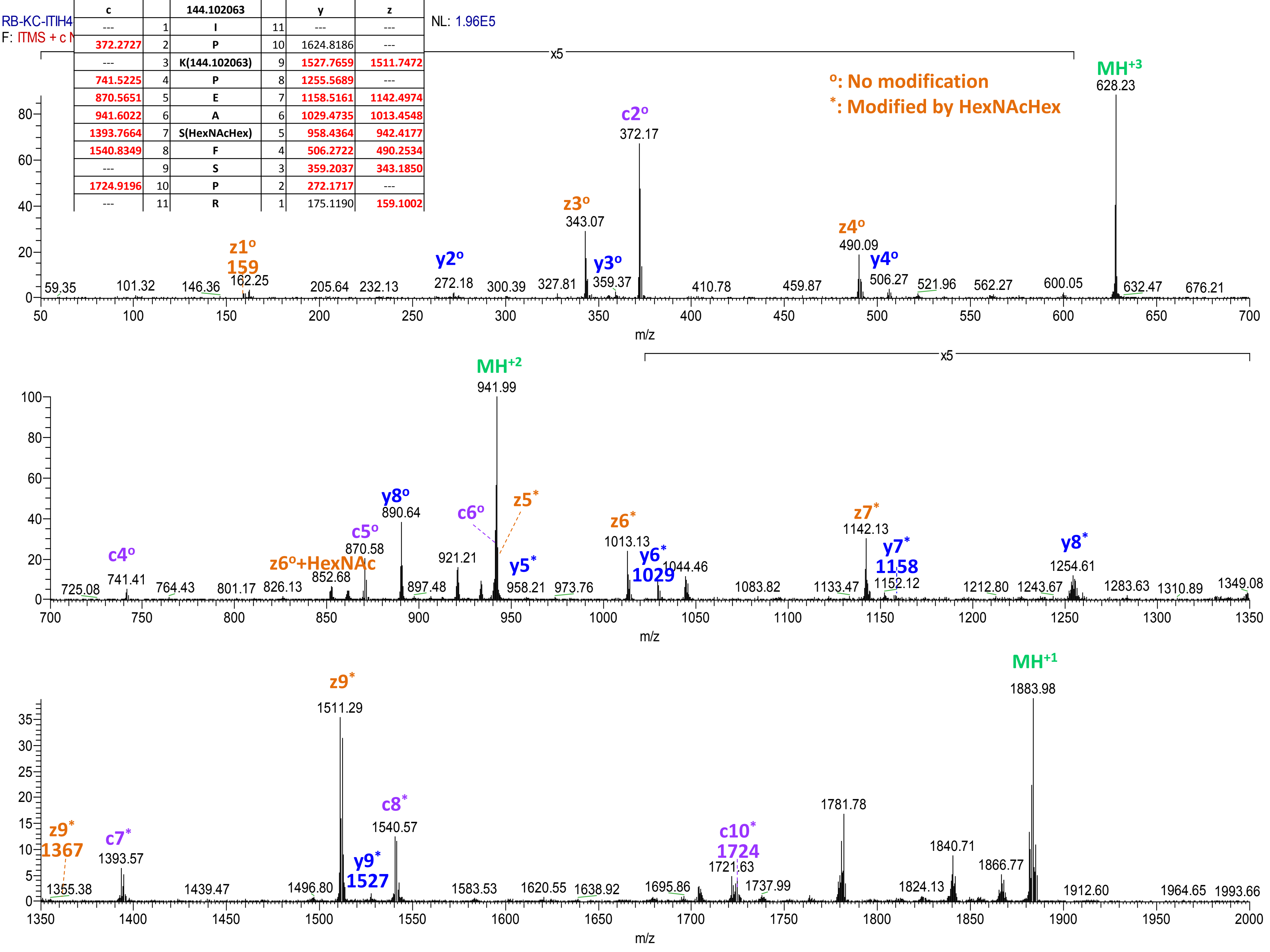




RB-KC-ITIH4-Peps2-Desial-Targets 10/1/2013 10:07:39 AM







c		144.102063		y	z
---	1	**I**	11	---	---
372.2727	2	**P**	10	1624.8186	---
---	3	**K(144.102063)**	9	**1527.7659**	**1511.7472**
741.5225	4	**P**	8	**1255.5689**	---
870.5651	5	**E**	7	**1158.5161**	**1142.4974**
941.6022	6	**A**	6	**1029.4735**	**1013.4548**
1393.7664	7	**S(HexNAcHex)**	5	**958.4364**	**942.4177**
1540.8349	8	**F**	4	**506.2722**	**490.2534**
---	9	**S**	3	**359.2037**	**343.1850**
1724.9196	10	**P**	2	**272.1717**	---
---	11	**R**	1	175.1190	**159.1002**

Supplementary Figure 5

Table of all observed recombinant and serum-derived ITIH4 glycopeptides from CID MS/MS spectra, including (a) no exoglycosidase treatment, (b) after treatment with 2/3,6,8 neuraminidase, or (c) after treatment with 2/3,6,8 neuraminidase and alpha 1-3,4 fucosidase.

Cell ITIH4 + Neuraminidase

AFIT N FS (N81)

Oxford	***m/z* (charge)**	**%**
M5	964.90 (2+)	3
M6	1045.93 (2+)	2
A2	670.96 (3+)	1
	711.29 (3+)	5
A2G1	724.97 (3+)	2
	765.31 (3+)	4
A2G2	778.99 (3+)	64
A3G1	792.68 (3+)	3
FA2G2	827.67 (3+)	5
FA3G1	841.35 (3+)	4
A3G2	846.69 (3+)	3
A3G3	900.70 (3+)	4

ALTTWQ N K (N207)

Oxford	***m/z* (charge)**	**%**
FA2	802.69 (3+)	2
FA2G1	856.70 (3+)	3
FA2G2	910.72 (3+)	40
FA3G1	924.4 (3+)	23
	951.03 (3+)	8
FA2F1G2	959.41 (3+)	3
FA3F1G1	973.08 (3+)	16
FA3G2	978.42 (3+)	4
	986.76 (3+)	1

N VVFVIDK (N274)

Oxford	***m/z* (charge)**	**%**
M5	717.33 (3+)	15
M6	771.34 (3+)	62
M7	825.36 (3+)	14
M8	879.38 (3+)	9

LPTQ N ITFQTE (N517)

Oxford	***m/z* (charge)**	**%**
FA2	912.73 (3+)	3
FA2G1	966.75 (3+)	3
FA3	980.43 (3+)	<1
FA2G2	1020.77 (3+)	40
FA2F1G2	1069.46 (3+)	2
FA3G1	776.09 (4+)	3
FA3G1	1034.43 (3+)	9
FA3F1G1	1083.13 (3+)	4
FA3G2	1088.46 (3+)	4
FA3F1G2	1137.15 (3+)	<1
FA3G3	857.11 (4+)	17
FA3F1G3	1191.17 (3+)	<1
FA4G2	867.37 (4+)	3
FA4G4	948.40 (4+)	12

NQAL N LSLAY (N577)

Oxford	***m/z* (charge)**	**%**
FA2	851.04 (3+)	3
FA2G1	905.06 (3+)	3
FA2G2	959.07 (3+)	44
FA3G1	972.73 (3+)	15
FA3G3	810.85 (4+)	15
FA4G2	821.10 (4+)	5
FA4G4	902.13 (4+)	5
FA4F1G4	938.63 (4+)	7

HexNAc3Hex7Fuc1	999.41 (3+)	3
IPKPEASFSPR (S640, S642)		
Oxford	***m/z* (charge)**	**%**
HexNAc1Hex1	531.95 (3+)	93
HexNAc1Hex4	694.04 (3+)	7
GPDVLTATVSGK (T506, T508, S510)		
Oxford	***m/z* (charge)**	**%**
HexNAc1Hex1	755.37 (2+)	6
HexNAc2Hex2	625.63 (3+)	67
HexNAc1Hex4	665.96 (3+)	4
HexNAc1Hex1NeuAc1	600.96 (3+)	23
Cell ITIH4 + Neuraminidase + a1-3,4 Fucosidase		
Oxford	***m/z* (charge)**	**%**
M5	964.90 (2+)	3
M6	1045.93 (2+)	2
A2	670.96 (3+)	1
	711.29 (3+)	5
A2G1	724.97 (3+)	3
	765.31 (3+)	3
A2G2	778.99 (3+)	66
A3G1	792.68 (3+)	5
FA2G2	827.67 (3+)	1
FA3G1	841.35 (3+)	3
A3G2	846.69 (3+)	4
A3G3	900.70 (3+)	4
ALTTWQ N K (N207)		
Oxford	***m/z* (charge)**	**%**
A2	754.00 (3+)	<1
FA2	802.69 (3+)	2
A2G1	808.01 (3+)	<1
FA2G1	856.70 (3+)	2
A2G2	862.03 (3+)	1
FA2G2	910.72 (3+)	42
A3G1	875.71 (3+)	<1
FA3G1	924.4 (3+)	39
	951.03 (3+)	5
FA2F1G2	959.41 (3+)	<1
A3G2	929.73 (3+)	<1
FA3G2	978.42 (3+)	3
HexNAc6Hex3Fuc1	938.07 (3+)	5
N VVFVIDK (N274)		
Oxford	***m/z* (charge)**	**%**
M5	717.33 (3+)	15
M6	771.34 (3+)	66
M7	825.36 (3+)	11
M8	879.38 (3+)	9
LPTQ N ITFQTE (N517)		
Oxford	***m/z* (charge)**	**%**
A2	864.05 (3+)	<1
FA2	912.73 (3+)	3
A2G1	918.07 (3+)	<1
FA2G1	966.75 (3+)	3
A2G2	972.08 (3+)	<1
FA2G2	1020.77 (3+)	41
FA2F1G2	1069.46 (3+)	<1
FA3	980.43 (3+)	1
FA3G1	776.09 (4+)	13

FA3F1G1	1083.13 (3+)	<1
FA3G2	1088.46 (3+)	4
FA3F1G2	1137.15 (3+)	<1
A3G3	1093.79 (3+)	<1
FA3G3	857.11 (4+)	15
FA3F1G3	1191.17 (3+)	<1
A4G2	867.37 (4+)	3
A4G4	1215.52 (3+)	<1
FA4G4	948.40 (4+)	14
NQAL N LSLAY (N577)		
Oxford	***m/z* (charge)**	**%**
FA2	851.04 (3+)	3
FA2G1	905.06 (3+)	3
FA2G2	959.07 (3+)	46
FA3G1	972.73 (3+)	10
A3G3	774.34 (4+)	2
FA3G3	810.85 (4+)	15
A4G2	784.59 (4+)	1
FA4G2	821.10 (4+)	3
A4G4	853.47 (4+)	2
FA4G4	902.13 (4+)	6
FA4F1G4	938.63 (4+)	6
HexNAc3Hex7Fuc1	999.41 (3+)	3
IPKPEASFSPR (S640, S642)		
Oxford	***m/z* (charge)**	**%**
HexNAc1Hex1	531.95 (3+)	95
HexNAc1Hex4	694.04 (3+)	5
GPDVLTATVSGK (T506, T508, S510)		
Oxford	***m/z* (charge)**	**%**
HexNAc1Hex1	755.37 (2+)	4
HexNAc2Hex2	625.63 (3+)	66
HexNAc1Hex4	665.96 (3+)	4
HexNAc1Hex1NeuAc1	600.96 (3+)	26

Serum ITIH4 + Neuraminidase

KAFITNF (N81)

Oxford	***m/z* (charge)**	**%**
A2G2	821.70 (3+)	82
A2F1G2	870.37 (3+)	7
A3G3	943.39 (3+)	11

ALTTWQNK (N207)

Oxford	***m/z* (charge)**	**%**
A2G2	862.04 (3+)	100

NVVFVIDK (N274)

Oxford	***m/z* (charge)**	**%**
M5	717.32 (3+)	25
M6	771.34 (3+)	75

LPTQNITFQTE (N517)

Oxford	***m/z* (charge)**	**%**
A2G2	729.31 (4+) 972.08 (3+)	64
FA2G2	765.82 (4+) 1020.77 (3+)	3
A3G3	820.59 (4+) 1093.78 (3+)	23
FA3G3	857.11 (4+) 1142.48 (3+)	9
FA3F1G3	893.63 (4+) 1191.17 (3+)	<1
A4G4	911.89 (4+) 1215.52 (3+)	<1

NQALNLSLAY (N577)

Oxford	***m/z* (charge)**	**%**
A2G2	683.04 (4+) 910.38 (3+)	84
A3G3	774.34 (4+) 1032.34 (3+)	16
A3F1G3	1080.80 (3+)	<1

Serum ITIH4 + Neuraminidase
+ a1-3,4 Fucosidase

KAFITNF (N81)

Oxford	***m/z* (charge)**	**%**
A2G2	821.70 (3+)	87
FA2G2	870.37 (3+)	3
A3G3	943.39 (3+)	10

ALTTWQNK (N207)

Oxford	***m/z* (charge)**	**%**
A2G2	862.04 (3+)	100

NVVFVIDK (N274)

Oxford	***m/z* (charge)**	**%**
M5	717.32 (3+)	30
M6	771.34 (3+)	70

LPTQNITFQTE (N517)

Oxford	***m/z* (charge)**	**%**
A2G2	729.31 (4+) 972.08 (3+)	65
FA2G2	765.82 (4+) 1020.77 (3+)	3

A3G3	820.59 (4+)	29
FA3G3	857.11 (4+) 1142.48 (3+)	2
FA3F1G3	893.63 (4+) 1191.17 (3+)	<1
A4G4	911.89 (4+) 1215.52 (3+)	1
NQALNLSLAY (N577)		
Oxford	***m/z* (charge)**	**%**
A2G2	683.04 (4+) 910.39 (3+)	84
A3G3	774.34 (4+) 1032.34 (3+)	16