Characterization of Protein Serotonylation via Bioorthogonal Labeling and Enrichment

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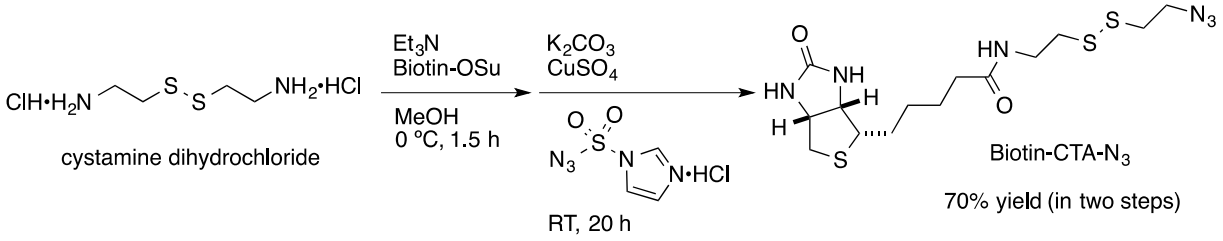
KEYWORDS: Click chemistry, serotonylation, protein modifications, proteomics

SUPPORTING EXPERIMENTAL SECTION

General Materials. SW480 colorectal carcinoma cell line was obtained from ATCC. The standard peptide Hras 57-68 was synthesized by solid phase synthesis by the Peptide Synthesis Lab at the Institute of Biological Chemistry, Academia Sinica. Culture media were sourced from Cellgro and fetal bovine sera were purchased from Biological Industries, Inc. Other chemicals were purchased from Sigma-Aldrich and solvents were acquired from Merck Chemicals, unless otherwise specified.



Scheme S1. Synthesis of Biotin-CTA-N_3.

Synthesis of Biotin-CTA-N_3. All reactions were performed under a nitrogen atmosphere in oven-dried glassware unless otherwise indicated. Reaction products were purified by column chromatography on silica gel. Organic solutions were concentrated under reduced pressure in a water bath (<40 ˚C). Analytical TLC was performed on pre-coated Merck Kieselgel 60 F254 glass plates and was detected by UV visualization (254 nm) and/or by charring with stains that contained cerium molybdate (for general use) or ninhydrin (for amino-group-containing samples). Column chromatography was performed on silica gel (Merck, 230–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-400 (400 MHz) by using DMSO-d_6 (δ_H=2.5 ppm, central line of a septet) as internal standards. ^{13}C NMR spectra were recorded on Bruker AV-400 (100 MHz) by using DMSO-d_6 (δ_C=39.5 ppm, central line of a septet). 2D NMR spectra (including 1H-1H

COSY, 1H-13C HMQC, and HMBC) were acquired on Bruker AV-400 spectrometers. High-resolution mass spectroscopy (HRMS) was performed on Bruker Bio-TOF III (ESI-TOF) spectrometers. Optical rotations were measured at the sodium D-line (589 nm) at 25 ˚C on a PerkinElmer Model 341 Polarimeter. Specific rotations were reported as $[\alpha]_D^{25}$ after dividing the observed values by concentration (*C*, in g mL^{-1}) and path length (*l*, in dm).

To a solution of cystamine dihydrochloride (132 mg, 0.59 mmol) and triethylamine (61 μL, 0.44 mmol) in anhydrous methanol (6 mL) at 0 ˚C under nitrogen atmosphere was added biotin-OSu (100 mg, 0.29 mmol)[1] portionwise over 10 minutes. The mixture was stirred at 0 ˚C for 1.5 h. At this moment, K_2CO_3 (324 mg, 2.34 mmol), anhydrous $CuSO_4$ (1.90 mg, 11.7 μmol) and imidazole-1-sulfonyl azide hydrochloride (246 mg, 1.17 mmol) [2]were consecutively added to the same reaction vessel, and the resulting mixture was allowed to stir at room temperature for another 20 h. The reaction mixture was filtered through a short pad of Celite 545 and the filtrate was concentrated *in vacuo* to afford a yellow crude. The crude compound was purified by column chromatography on silica gel (EtOAc/MeOH/H_2O, 7:2:1) to afford desired product (82 mg, 70% yield in two steps) as a white solid. R_f= 0.68 (EtOAc/MeOH/H_2O, 7:2:1, v/v/v); $[\alpha]_D^{25}$ = +52.9 (*c* = 1.0, MeOH); ^{1}H NMR (400 mHz, DMSO-d_6): δ = 7.98 (t, *J* = 5.34 Hz, 1H, amide NH), 6.42 (s, 1H, urea NH), 6.36 (s, 1H, urea NH), 4.32-4.29 (m, 1H, bridged H), 4.14-4.11 (m, 1H, bridged H), 3.60 (t, *J* = 6.41 Hz, 2H, -SSCH$_2$C<u>H$_2$</u>N$_3$), 3.34-3.30 (m, 2H, overlapped with HOD, -NHC<u>H$_2$</u>CH$_2$SS-), 3.12-3.07 (m, 1H, -SCH-), 2.93 (t, *J* = 6.41 Hz, 2H, -SSC<u>H$_2$</u>CH$_2$N$_3$), 2.82 (dd, *J* = 12.4, 5.05 Hz, 1H, -SCH$_2$-), 2.79 (t, *J* = 6.78 Hz, 2H, -NHCH$_2$C<u>H$_2$</u>SS-), 2.57 (d, *J* = 12.4 Hz, 1H, -SCH$_2$-), 2.06 (t, *J* = 7.36 Hz, 2H, -C<u>H$_2$</u>CONH-), 1.64-1.22 ppm (m, 6H, alkyl chain H); ^{13}C NMR (100 mHz, DMSO-d_6): δ = 172.2 (C=O), 162.7 (C=O), 61.0 (bridged CH), 59.2 (bridged

CH), 55.4 (-SCH-), 49.2 (-SSCH$_2$<u>C</u>H$_2$N$_3$), 39.8 (-SCH$_2$-), 37.7 (-NH<u>C</u>H$_2$CH$_2$SS-), 37.4 (-NHCH$_2$<u>C</u>H$_2$SS-), 36.9 (-SS<u>C</u>H$_2$CH$_2$N$_3$), 35.1 (-NHCOCH$_2$-), 28.2 (CH$_2$), 28.0 (CH$_2$), 25.2 ppm (CH$_2$) ; HRMS (ESI-TOF): *m/z* calcd for $C_{14}H_{25}N_6O_2S_3$: 405.1196 $[M+H]^+$; found: 405.1195 and $C_{14}H_{24}N_6O_2S_3Na$: 427.1015 $[M+Na]^+$; found: 427.1013.

Cell cultures. SW480 cells were ordinarily cultured under 5% CO_2 at 37 ℃ and passaged in DMEM supplemented with fetal bovine serum (10%), 1% penicillin-streptomycin (1%) and sodium pyruvate (1%). Since typical DMEM contains $CaCl_2$ (~2 mM), the growth medium was replaced by nutrient-supplemented calcium-free DMEM (Gibco) 24 h before further experimentation.

Lysis and immunoblotting. SW480 cells were detached by trypsin-EDTA (0.25%) and collected by centrifugation, washed several times and reconstituted in calcium-free Dulbecco's PBS (DPBS) on ice. Cells were lysed by rapid freeze/thaw cycles followed by passage through 23-gauge syringe needles on ice; cell debris was pelleted by centrifugation at 4 ℃. For immunoblotting, samples were desalted using Zeba spin columns (Thermo) and subject to SDS-PAGE on 4-20% gradient gel (NuSep) followed by a transfer onto PVDF membrane (GE Healthcare). Blocking was achieved with bovine serum albumin (30 mg mL^{-1}) solubilized in Tris-buffered saline solution with Tween 20 (TBS-T, 0.1%) for 3 h, followed by incubations with alkaline phosphatase-conjugated streptavidin (1:10,000). Membranes are washed with TBS-T for 1 h (20 min, 3 times). Blots are imaged by chemilluminescence with CDP-Star (Perkin Elmer) for alkaline phosphatase-conjugated streptavidin and antibodies or luminol (Millipore) for horseradish peroxidase-

conjugated antibodies. Visualization is performed on a Fujifilm LAS-4000 image acquisition system.

Metabolic labeling of Hsp40. SW480 cells were treated with 2 mM 5-HT or 2 mM 5-PT with or without 5 mM CaCl2 for 3 h before collection and lysis with RIPA buffer. Samples were quantified and 5% of the lysates were retained as inputs. Samples were then diluted with 1 mL DPBS to reduce the concentration of surfactants. Lysates were pre-cleared in the presence of protein-G magnetic beads (GE Healthcare) for 1 h at 4 ℃, then decanted and incubated overnight with 12 μL anti-Hsp40 antibody (Cell Signaling) for ligand formation at 4 ℃. Samples were then co-incubated with protein-G Sepharose beads (GE Healthcare) overnight at 4 ℃ for precipitation. After DPBS washes, beads were resuspended in DPBS and clicked with 0.2 mM Biotin-CTA-N_3 in the presence of 1 mM TBTA, 1 mM sodium ascorbate and 0.3 mM $CuSO_4$ for 2 h. After washing with Dulbecco's PBS (DPBS, Cellgro) for 3 times, samples are subject to SDS-PAGE and immunoblotting with alkaline phosphatase-conjugated streptavidin (Invitrogen), anti-Hsp40 or anti-GAPDH antibody (Genetex).

Protein association analysis. Protein hits from Mascot database search results were entered into STRING (ver 9.1) by accession numbers to generate a protein association network model. The resultant confidence network with the following parameters: required confidence = high (0.700), no more than 5 interactors and no additional nodes. The computed results were then imported into into Cytoscape (ver 3.1.0) to generate a hierarchical network based on interacting pairs and the numerical combined association scores. Protein clustering and functions were assigned based on gene ontology classifications listed on UniprotKB and reclassified by their general categories.

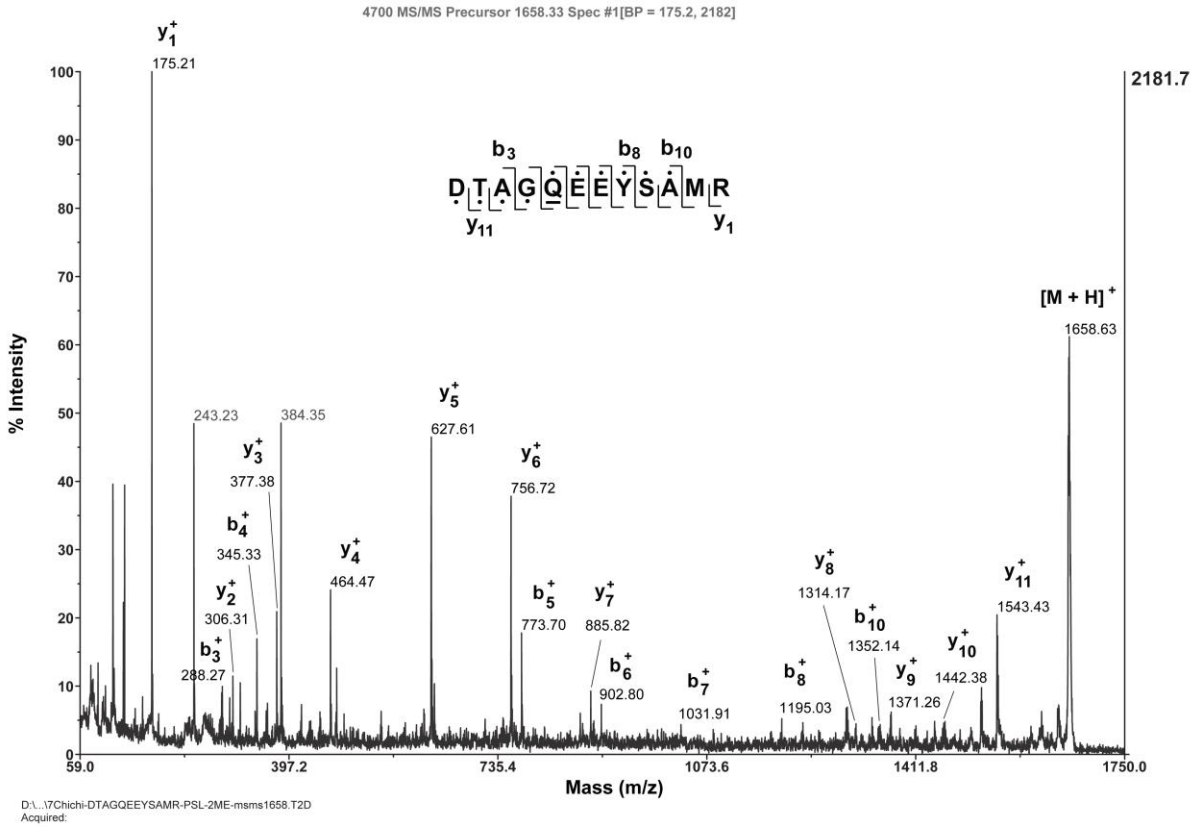
Serotonylation motif analysis. Protein hits from Mascot database search results were entered into Motif-X (ver 1.2)[3] by amino acid sequences with serotonylated sites labeled as "Q*" to generate 13-bit alignment information with the following parameters: occurrences = 20, significance = 0.000001, foreground = MS/MS sequences extended from the IPI Human Proteome. The background database is also set to the IPI Human Proteome. Upon acquiring alignment information, sequence logos are generated using WebLogo (ver. 2.8.2)[4] as a frequency plot.

Analysis of 5-PT standard peptide serotonylation by MALDI MS and MS/MS. For MALDI-TOF MS profiling, peptides solubilized in 50% acetonitrile, 0.1% TFA were mixed 1:4 v:v with the matrix solution (5 mg/ml α-cyano-4-hydrocinnamic acid in 75 % acetonitrile and 0.1 % formic acid) prior to spotting onto the 100-well format sample plate. Data acquisition was performed manually on a MALDI-TOF/TOF 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA), equipped with an Nd:YAG laser (355-nm wavelength, 500-ps pulse, and 200-Hz repetition rate) with 5000 shots accumulated in reflectron positive ion mode. MS survey data were acquired with automated target plate movement and automatically processed by the Applied Biosystems 4700 Explorer software. A typical data acquisition comprises a total of 20 sub-spectra of 125 laser shots each with laser energy normally set at 3200. In MS/MS operation, 1-keV collisions with argon were used to generate the high-energy CID spectra using a source voltage of 8 kV, a collision cell voltage of 7 kV, and a second accelerating voltage of 15 kV. MS/MS spectral assignments for de novo sequencing and identification of site-specific glutamine modification were performed manually.

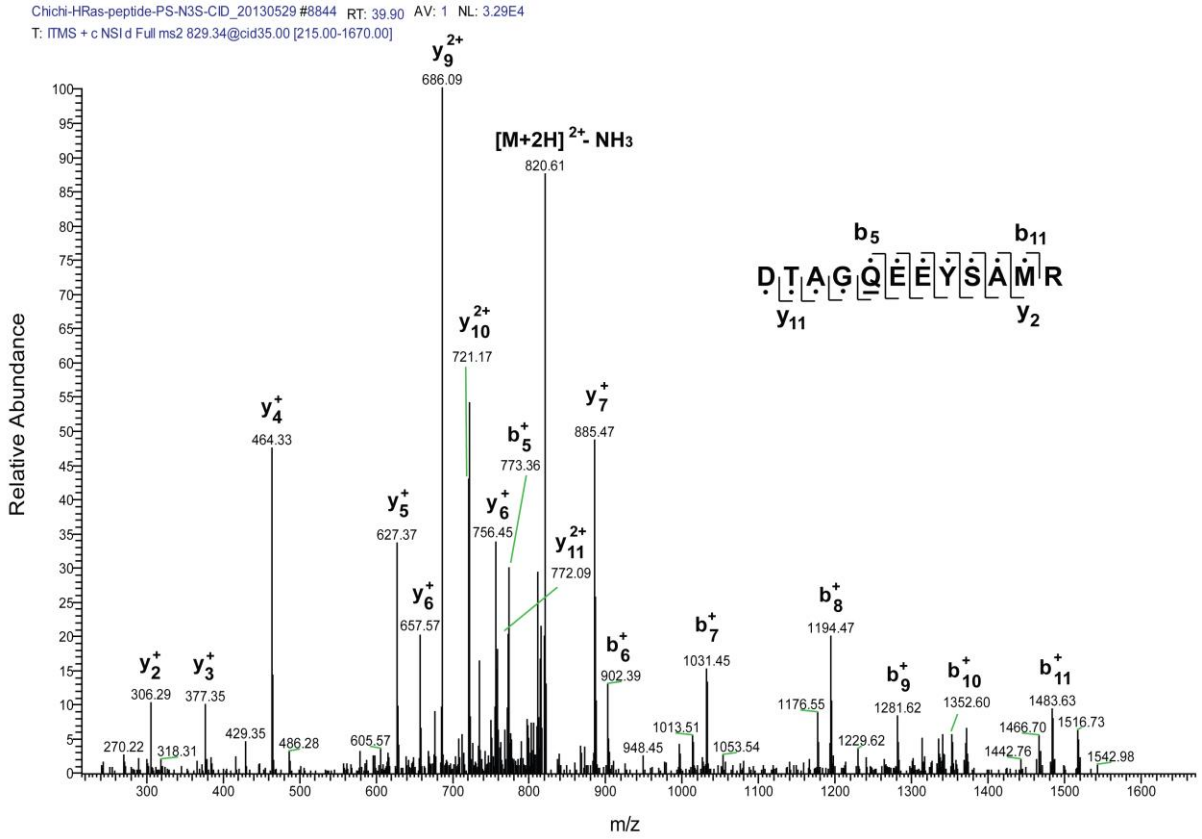
SUPPORTING FIGURES

Figure S1. MALDI and ESI-MS/MS spectra of 5-PT-serotonylated standard peptide Hras 57-68. Precursor ions for each spectrum is described separately. Serotonylated residue is underlined. Filled circles above the labeled ions indicate serotonylated product ions with a mass increment of *m/z* 376.1 in the presence of 2-ME or *m/z* 300.1 without 2-ME.

S1a. MALDI-MS/MS for thiolated precursor ion with $[M + H]^+$ at *m/z* 1658.63



S1b. ESI-MS/MS for thiolated precursor ion with $[M + 2H]^{2+}$ at *m/z* 829.34



S1c. ESI-MS/MS for 2-ME-linked precursor ion with $[M + 2H]^{2+}$ at *m/z* 867.34

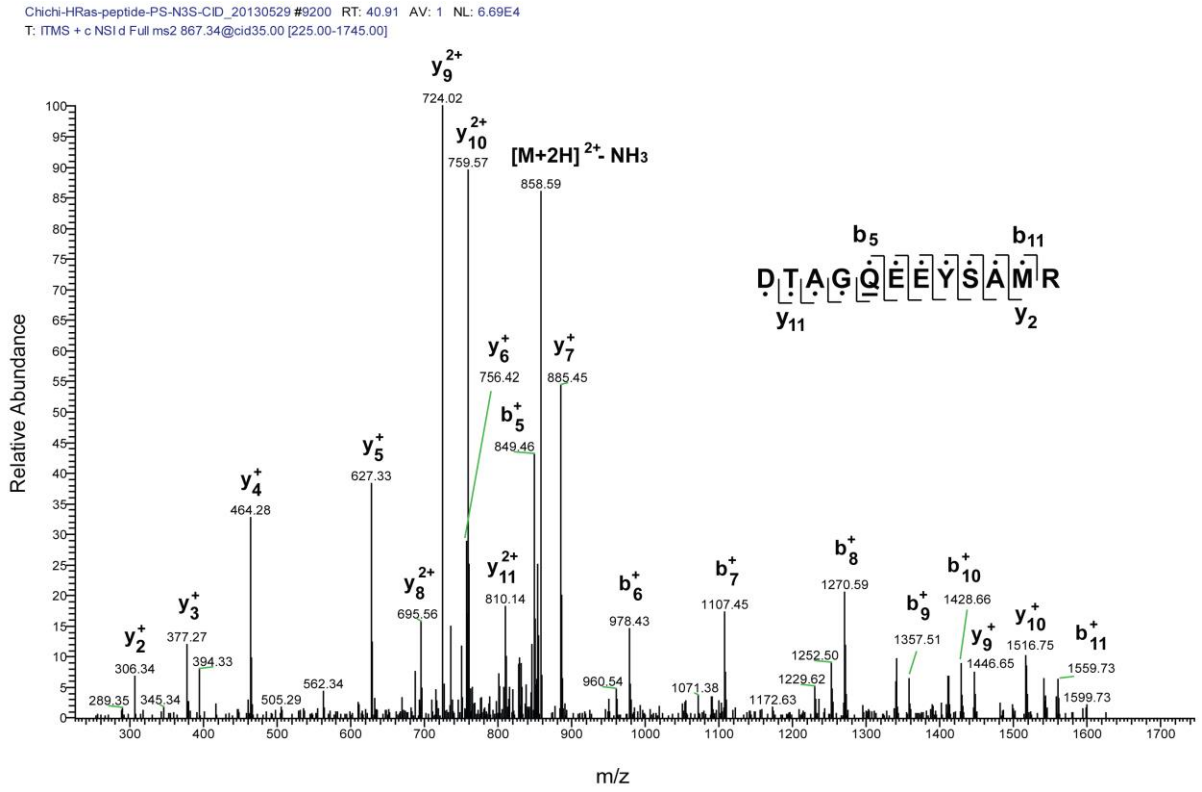
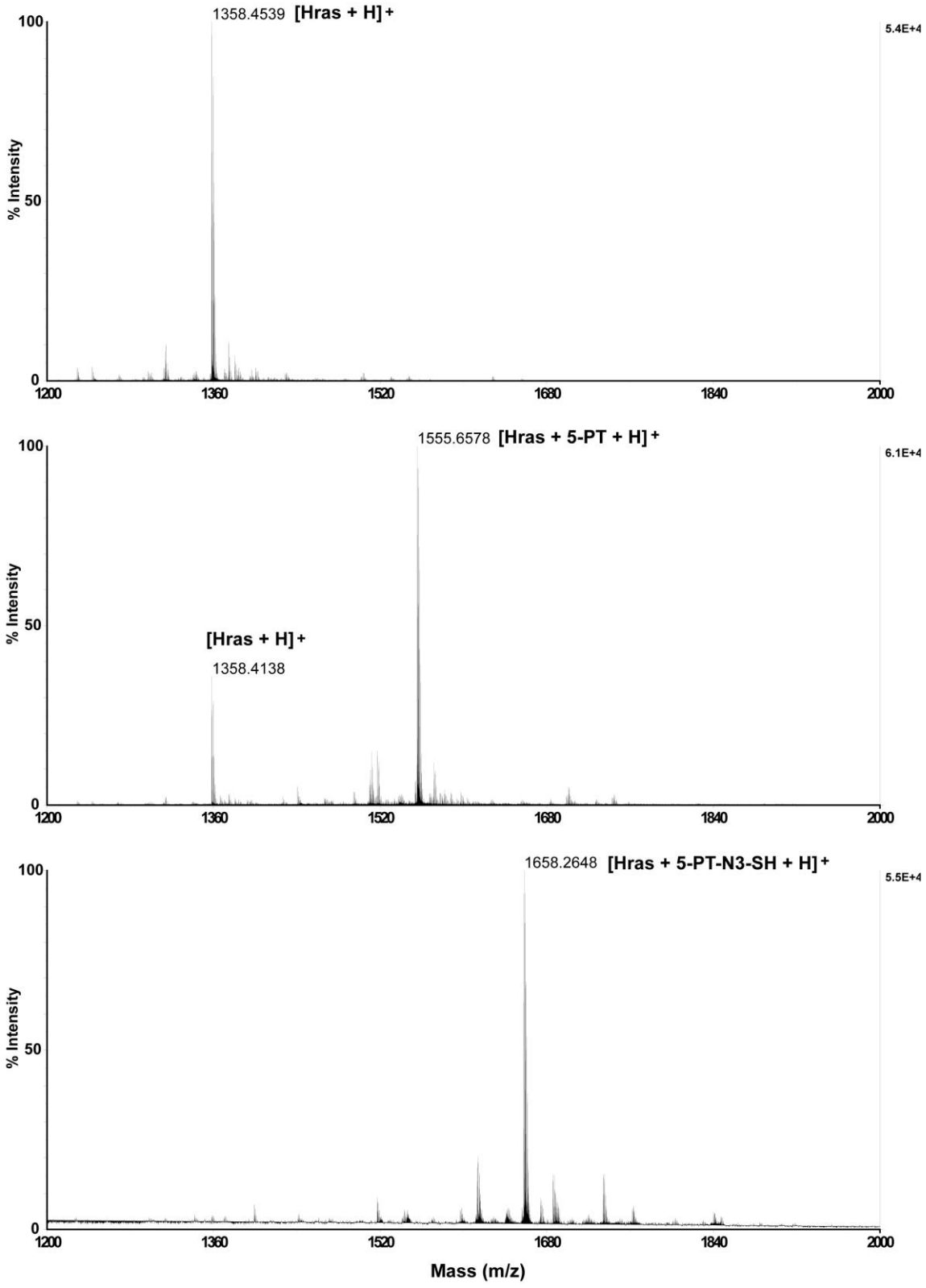


Figure S2. Modification Yields and Recovery of Serotonylated Peptide. (a) Evaluation of relative incorporation of efficiency of 5-PT onto peptide Hras 57-68 (*Hras*) before and after modification (top and middle spectra, respectively) and conjugation of Biotin-CTA-N_3 onto modified *Hras* (bottom spectrum) by MALDI-MS. Comparison suggests an approximate relative efficiency of 70% for 5-PT serotonylation, while the conjugation of Biotin-CTA-N_3, post-elution by 2-ME, was essentially near completion. (b) Evaluation of enrichment yields of *Hras* at various stages of modification and enrichment process, including serotonylation (top spectrum) as well as before (middle spectrum) and after streptavidin enrichment (bottom spectrum) as observed by MALDI-MS. A comparison of ion intensity suggests an approximate 52% recovery by enrichment. *Hras + 5-PT* denotes 5-PT modified standard peptide; *Hras + 5-PT-N3-SH* indicates thiolated Biotin-CTA-N_3-conjugated adduct post elution by 2-ME, and *Hras + 5-PT-2ME* indicates 2-ME-thiolated Biotin-CTA-N_3-conjugated adduct post elution.

S2a. MALDI-MS spectra for the evaluation of 5-PT incorporation efficiency



S2b. MALDI-MS spectra for the evaluation of recovery efficiency

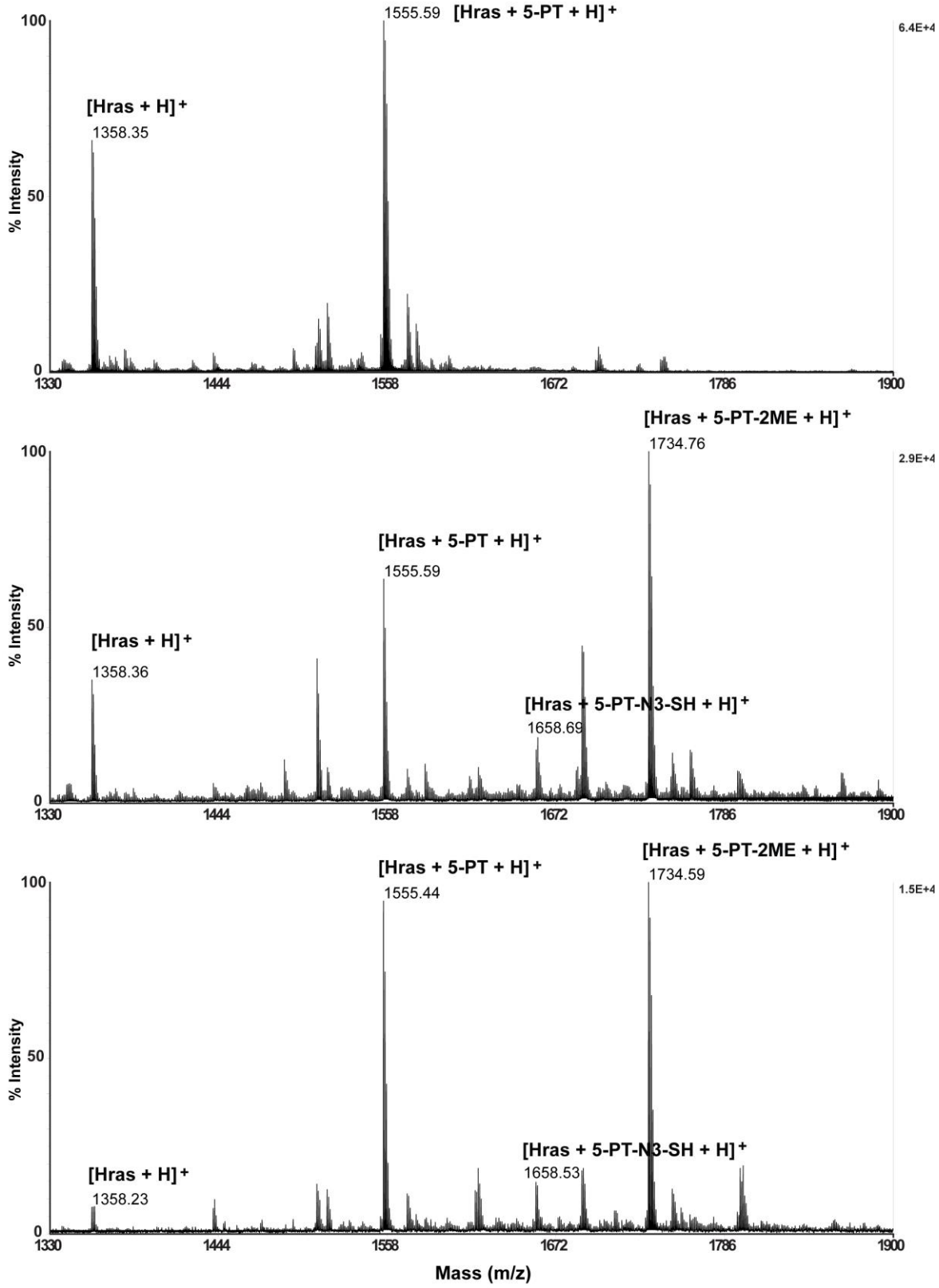
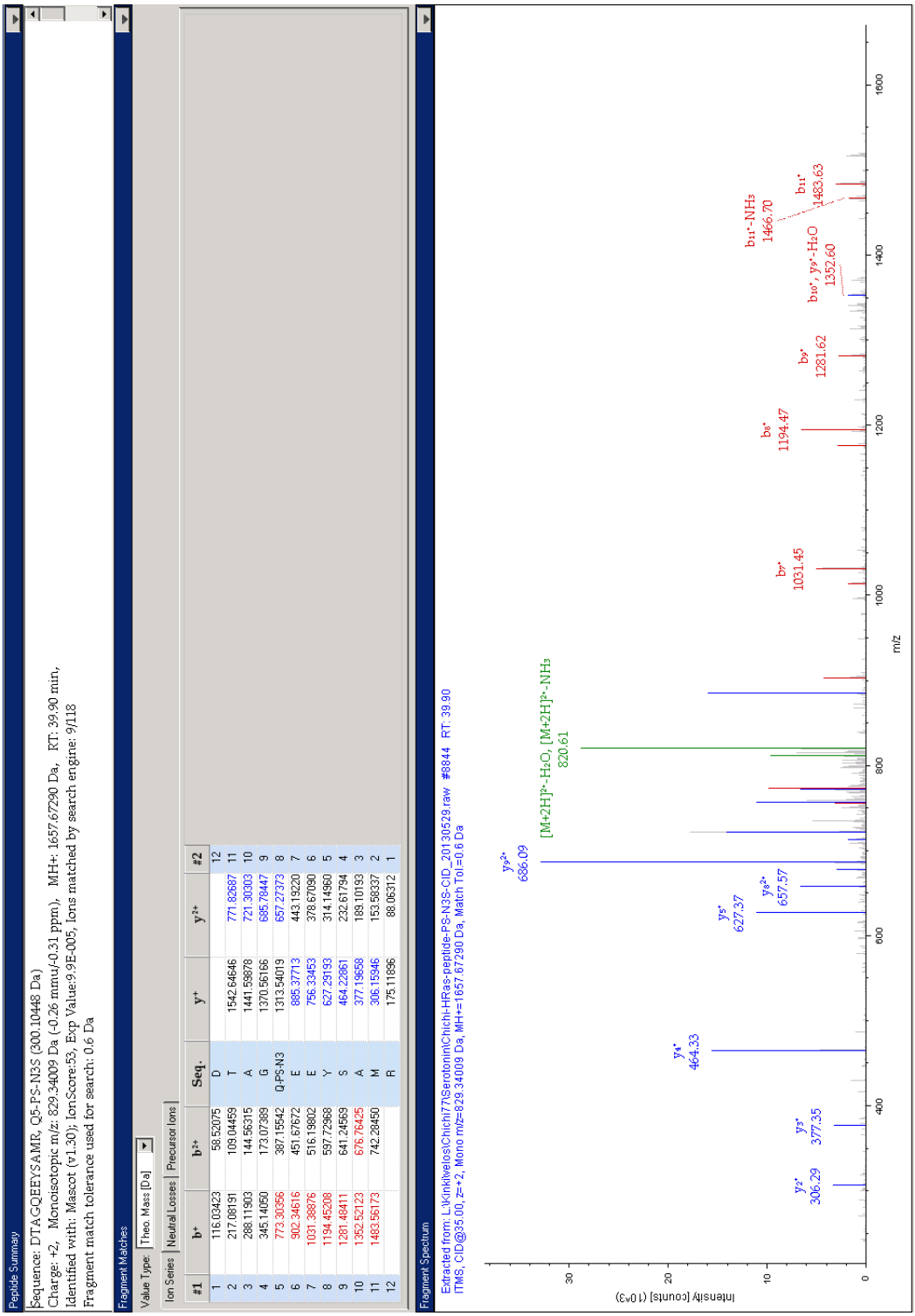
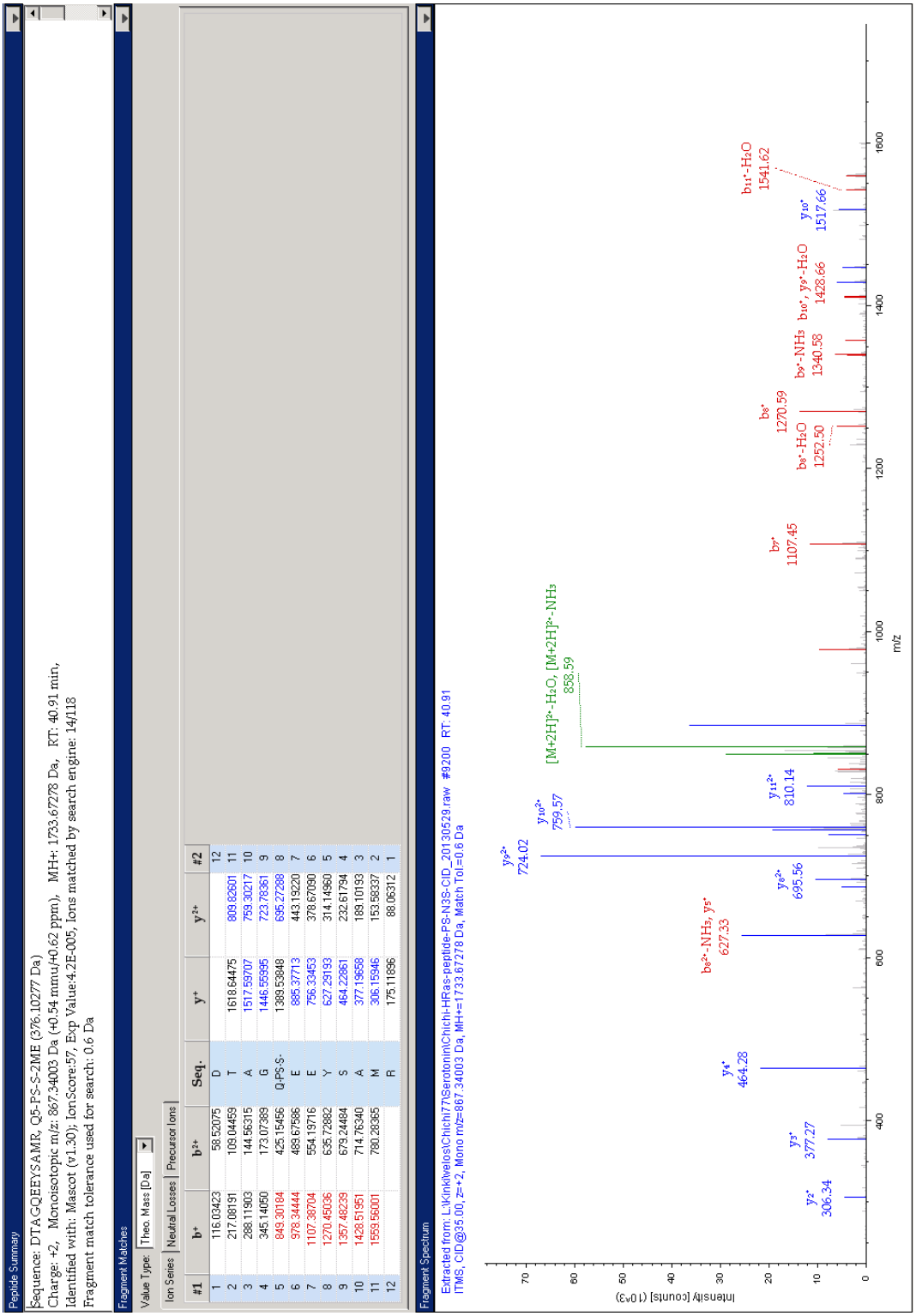


Figure S3. A comparison of spectra of 5-PT-serotonylated *Hras* peptide obtained by CID vs. HCD dissociation.

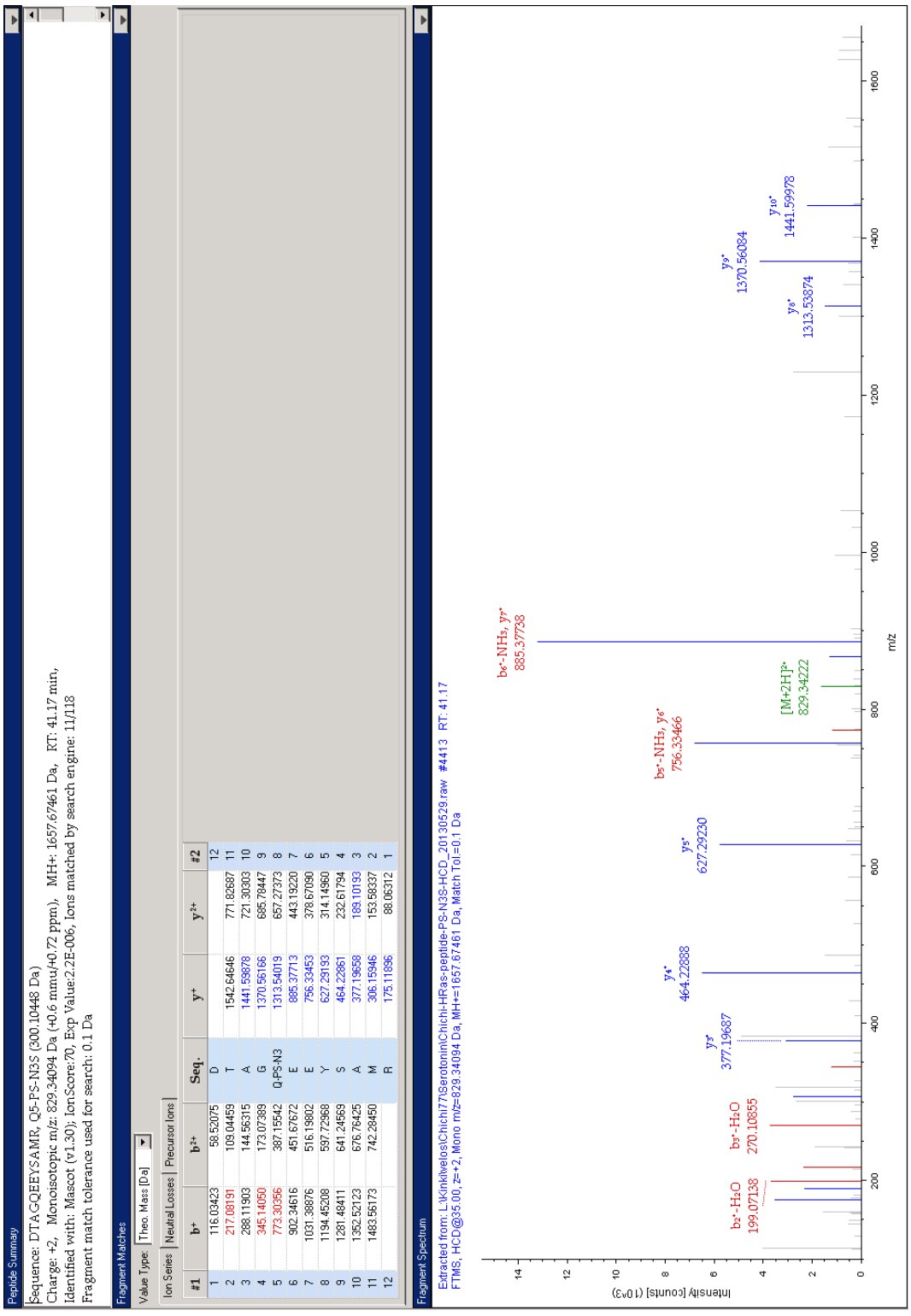
S3a. Q5-PS-N3SH by CID



S3b. Q5-PS-S-2ME by CID



S3c. Q5-PS-N3SH by HCD



S3d. Q5-PS-S-2ME by HCD

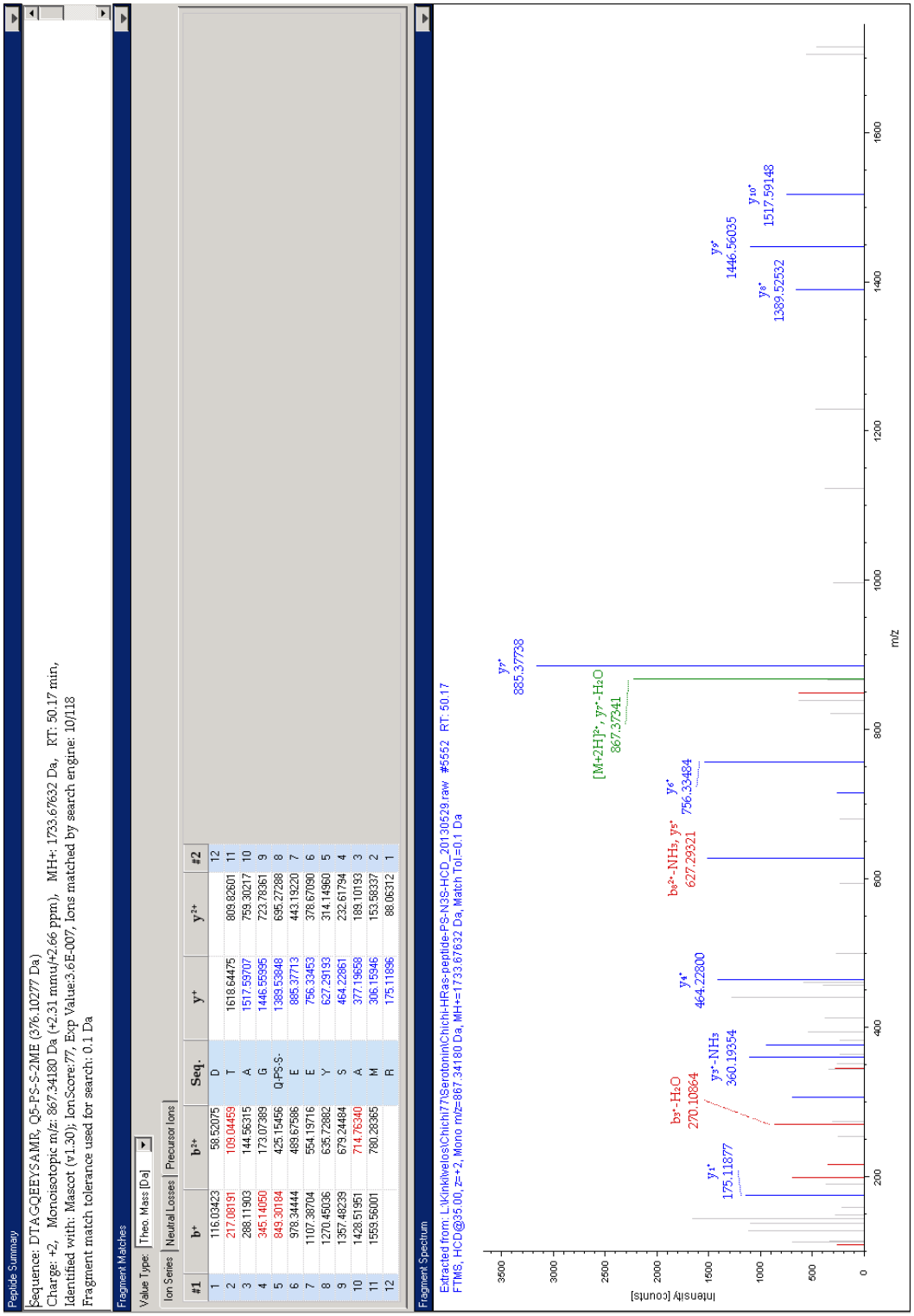
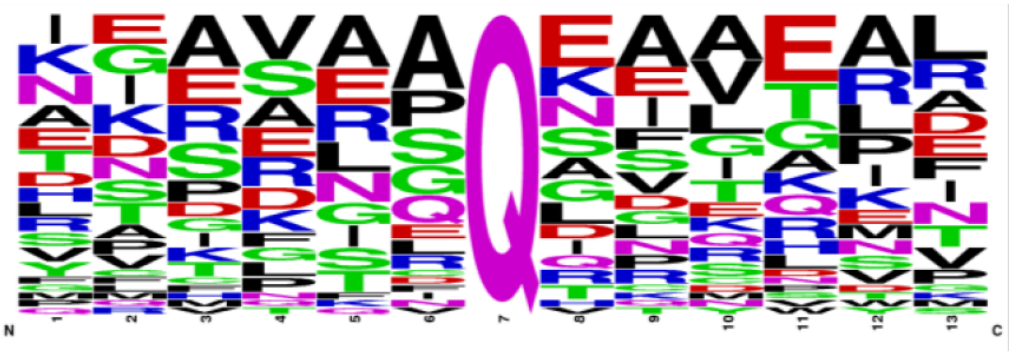
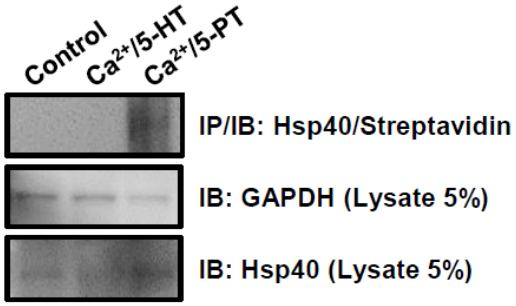


Figure S4. Additional implications of protein Serotonylation. (a) alignment of potential linear motifs for protein serotonylation, as generated by Motif-X and WebLogos. Sequence suggests a likelihood of preferential serotonylation sites among stretches of non-polar and aliphatic residues such as valine, alanine and proline. (b) metabolic labeling of Hsp40 serotonylated by 5-PT in vivo in SW480 cultures. Hsp40 is immunoprecipitated after treatment with calcium chloride (5 mM) and either 5-HT or 5-PT (2 mM), labeling with Biotin-CTA-N_3 on-beads and blotted against alkaline phosphatase-conjugated streptavidin. 5% of lysates were retained as input controls and blotted against GAPDH and Hsp40.

a)



b)



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4. Crooks, G. E.; Hon, G.; Chandonia, J. M.; Brenner, S. E., WebLogo: a sequence logo generator. *Genome Res* **2004,** 14, (6), 1188-90.

Table S1. Summary of Identified Proteins and Sites of Serotonylation. Hits were identified by accession numbers through searches against the UniProtKB databank. Modification glutamine residues are marked in red; scores marked (a) denote those carrying a mass increment of 376.1 Da indicative of a 2-ME-thiolated product ion, and (b) denote those carrying a mass increment of 300.1 Da indicative of a thiol product ion.

Protein name	Accession number	Mass (kDa)	Mascot peptide score	Peptide sequence[b]	Expect value	Mass deviation (ppm)	$[M+H]^+$ (Da)	Charge
Stathmin/Phosphoprotein p19	P16949	17.3	53	ASGQ^{18}AFELILSPR	1.6E-04	1.83	1764.8593 [a]	2
			64		1.3E-05	1.04	1688.8695 [b]	2
Protein canopy homolog 2	Q9Y2B0	20.6	53	INPDGSQ^{64}SVVEVPYAR	1.8E-04	0.32	2030.9760 [b]	2
Tubulin-folding cofactor B	Q99426	27.3	41	AQ^{140}QEAEAAQR	1.0E-03	1.9	1477.6340 [a]	2
			47		2.8E-04	0.61	1401.6337 [b]	2
26S proteasome non-ATPase regulatory subunit 9	O00233	24.7	27	ANYDVLESQ^{47}K	2.0E-02	3.38	1542.6768 [a]	2
			47		3.9E-04	0.63	1466.6742 [b]	2
Myosin light chain 3	P60660	16.9	44	ALGQ^{41}NPTNAEVLK	1.2E-03	0.73	1730.8366 [a]	2
			46		5.5E-04	2.01	1654.8404 [b]	2

Protein G2	P48634	228.7	45	ESAEQSSGPGPSLRPQ[202]NSTTWR	1.4E-03	1.49	2672.2311 [b]	3
Oxidative stress-associated Src activator	Q9NZB2	121.8	45	SQ[934]GGVQPIPSQGGK	8.7E-04	1.68	1639.8038 [b]	2
Splicing factor 3B subunit 4	Q15427	44.4	43	NQ[11]DATVYVGGLDEK	1.6E-03	1.17	1808.8294 [b]	2
14-3-3 protein zeta/delta	P63104	27.7	30	LAEQ[15]AER	1.4E-02	2.28	1192.5265 [a]	2
			42		7.7E-04	1.34	1116.5270 [b]	2
			29	SVTEQ[32]GAELSNEER	3.0E-02	2.11	1848.8221 [b]	2
Far upstream element (FUSE)-binding protein 2	Q92945	73.1	33	DAFADAVQ[79]R	5.9E-03	1.28	1368.5842 [a]	2
			41		2.2E-03	1.72	1292.5864 [b]	2
			36	KDAFADAVQ[79]R	6.9E-03	1.57	1420.6813 [b]	2
			29	GSPQ[482]QIDHAK	2.2E-02	1.84	1380.6503 [b]	2
40S ribosomal protein S19	P39019	16.1	26 [a]	DVNQQ[12]EFVR	4.4E-02 [a]	1.15 [a]	1510.6584 [a]	2 [a]
			41 [b]		1.8E-03 [b]	1.72 [b]	1434.6583 [b]	2 [b]

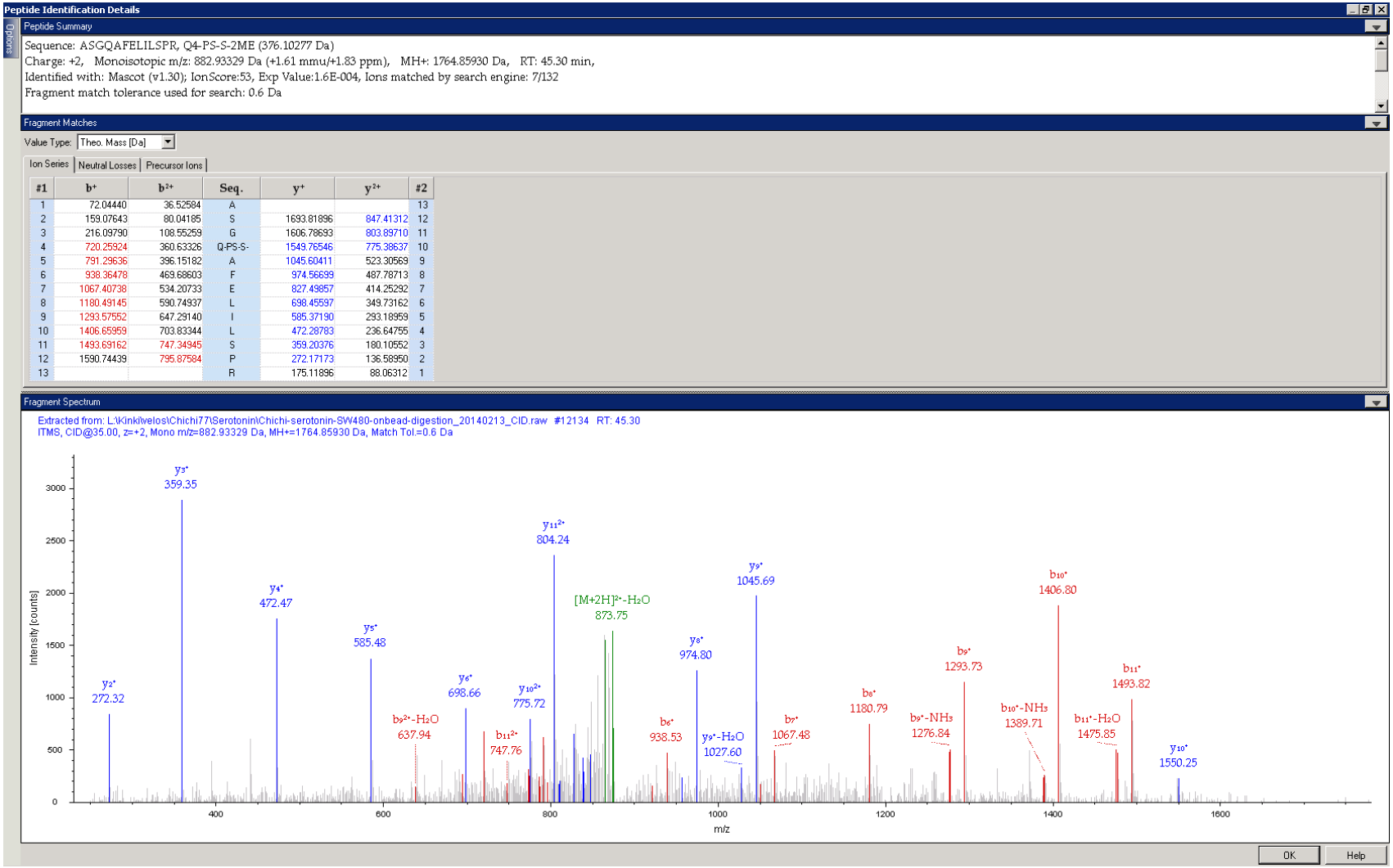
Ezrin	P15311	69.4	41	SQEQ[416]LAAELAEYTAK	2.1E-03	1.96	2027.9242 [a]	3
Vimentin	P08670	53.6	41	VELQ[108]ELNDR	1.3E-03	2.22	1415.6768 [b]	2
Actin	P60709	41.7	39	DSYVGDEAQ[59]SK	1.1E-03	2.28	1498.6304 [b]	2
Heat shock protein A5	P11021	72.3	30	ITITNDQ[530]NR	2.1E-02	0.2	1450.6569 [a]	2
			38		3.3E-03	0.47	1374.6590 [b]	2
Mitochondrial 39S ribosomal protein L12	P52815	21.3	38	LVESLPQ[170]EIK	2.5E-03	1.44	1455.7680 [b]	2
Zinc finger protein 9	P62633	19.4	38	GFQ[44]FVSSSLPDICYR	3.9E-03	1.55	2151.9483 [a]	2
Prelamin-A/C	P02545	74.1	37	SGAQ[15]ASSTPLSPTR	5.4E-03	1.13	1735.7911 [a]	2
PDZ and LIM domain protein 2	Q96JY6	37.4	37	LLQ[246]EALEAEER	4.8E-03	-1.54	1600.7764 [b]	2
Bcl-2-binding protein Bis	O95817	61.6	37	SQ[172]SPAASDCSSSSSSASLPSSGR	3.6E-03	1.17	2500.0494 [b]	3
Thymosin beta-4	P62328	5.0	36	KTETQ[24]EK	7.0E-03	1.39	1163.5530 [b]	2

Triosephosphate isomerase	P60174	30.8	36	IAVAAQ[102]NCYK	5.5E-03	0.21	1437.6769 [b]	2
Desmoyokin	Q09666	628.7	36	SEDGVEGDLGETQ[147]SR	3.3E-03	-0.17	1878.7920 [b]	2
Y-box transcription factor	P67809	35.9	36	NEGSESAPEGQ[181]AQQR	3.7E-03	1.34	1887.8064 [b]	2
Radixin	P35241	68.5	35	AFAAQ[453]EDLEK	5.6E-03	2.63	1497.6541 [a]	2
Ran-specific GTPase-activating protein (Ran-binding protein 1)	P43487	23.3	35	FLNAENAQ[149]K	5.2E-03	1.02	1410.6308 [a]	2
Valyl-tRNA synthetase	P26640	140.4	34	LQQ[1245]TEAELR	9.4E-03	-0.08	1387.6786 [b]	2
Filamin-C	Q14315	290.8	34	TSQ[1956]LNVGTSTDVSLK	1.2E-02	2.9	1925.9152 [a]	2
Chromatin-modifying protein 4b	Q9H444	24.9	30	GGPTPQ[23]EAIQR	1.6E-02	2.04	1529.7020 [a]	2
			32		1.2E-02	0.63	1453.7015 [b]	2
SH3 domain-binding protein 1	Q9H299	10.4	32	SQQ[21]SEVTR	9.7E-03	1.51	1234.5652 [b]	2

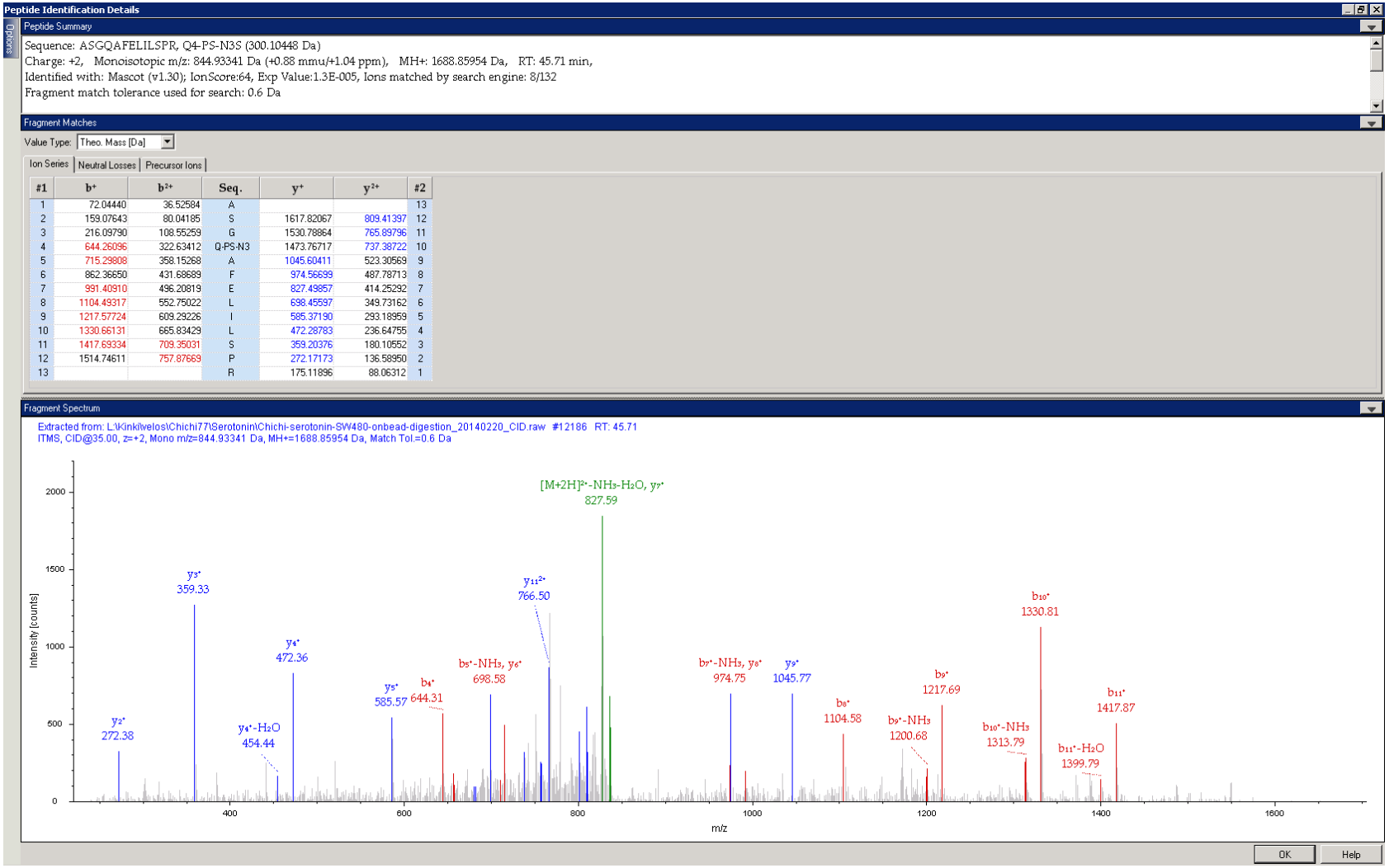
Eukaryotic translation initiation factor 4H	Q15056	27.4	32	EEVVQ[244]KEQE	9.0E-03	0.83	1417.6429 [b]	2
Heat shock protein 40	P25685	38.0	32	NPFDTFFGQ[118]R	5.3E-03	0.88	1604.6760 [a]	2
Plectin	Q15149	531.5	31	LSVAAQ[2425]EAAR	2.1E-02	3.09	1391.6602 [a]	2
Mitochondrial ATP synthase subunit delta	P30049	17.5	31	AEIQ[154]IR	1.3E-02	1.37	1029.5313 [b]	2
DNA helicase V	Q96AE4	67.5	31	IAQ[324]ITGPPDR	1.6E-02	1.49	1367.6910 [b]	2
Alpha-2-macroglobulin	P01023	163.2	31	Q[854]TVSWAVTPK	1.6E-02	0.84	1416.7105 [b]	2
Na(+)/K(+) ATPase alpha(III) subunit	P13637	111.7	30	VDNSSLTGESEPQ[215]TR	2.6E-02	0.63	1919.8565 [b]	2
Calvasculin	P26447	11.7	24	TDEAAFQ[56]K	2.5E-02	2.42	1285.5372 [a]	2
			29		1.8E-02	1.46	1209.5375 [b]	2
dUTP pyrophosphatase	P33316	26.5	29	IAQ[219]LICER	2.5E-02	1.76	1302.6469 [b]	2

Caldesmon	Q05682	93.2	29	EAEGAPQ[529]VEAGK	2.1E-02	2.27	1485.6825 [b]	2
Zinc finger protein 162	Q15637	68.3	29	AVEQ[221]LR	2.0E-02	1.9	1015.5162 [b]	2
Mitochondrial import inner membrane translocase subunit Tim10	P62072	10.3	27	VQQ[85]SSGPA	1.9E-02	1.82	1073.4853 [b]	2
Stress-induced-phosphoprotein 1	P31948	62.6	26	NPVIAQ[529]K	2.6E-02	1.05	1069.5623 [b]	2
CD34	P28906	40.7	26	Q[377]HVVADTEL	3.4E-02	1.0	1311.6164 [b]	2
DNA polymerase delta catalytic subunit	P28340	123.6	24	CQ[1062]GSLHEDVICTSR	3.4E-02	2.02	1980.8144 [a]	2
JKT41-binding protein	O14979	46.4	19	NQQ[145]DDGK	4.0E-02	2.37	1104.4554 [b]	2

Stathmin/Phosphoprotein p19_ ASGQ[18]AFELILSPR_2-ME-thiolated product ion

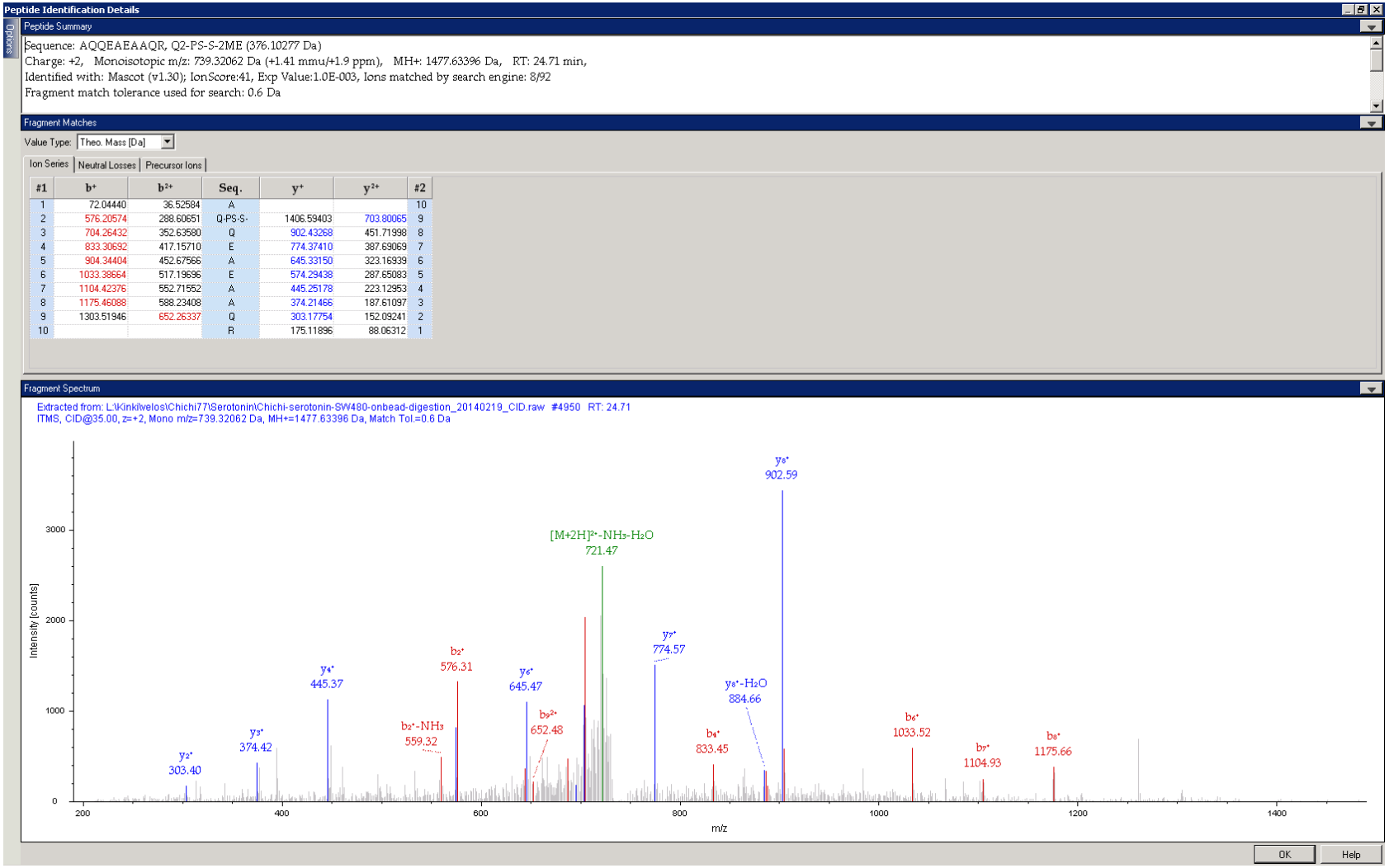


Stathmin/Phosphoprotein p19_ ASGQ[18]AFELILSPR_thiol product ion

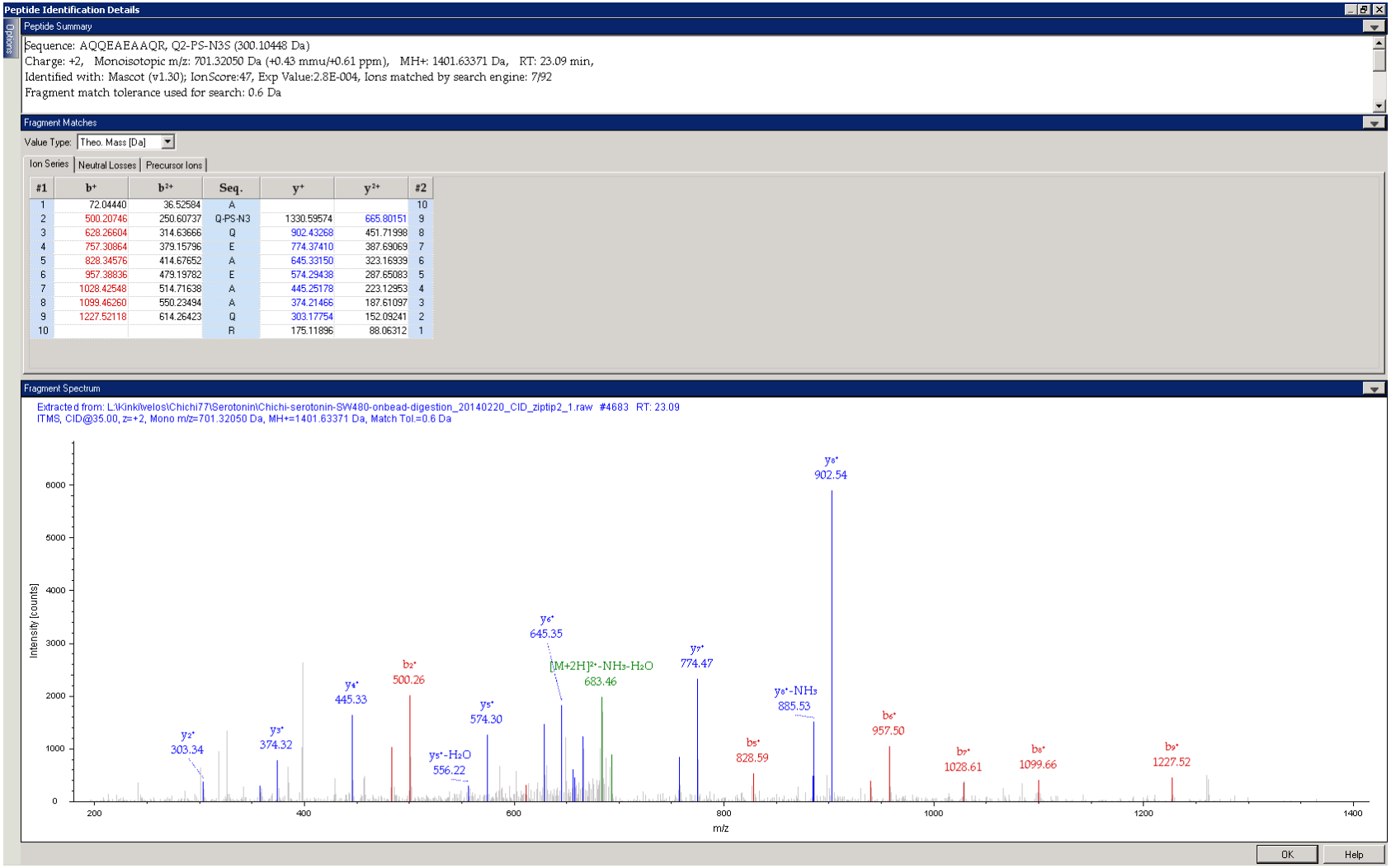


Protein canopy homolog 2_ INPDGSQ[64]SVVEVPYAR_thiol product ion

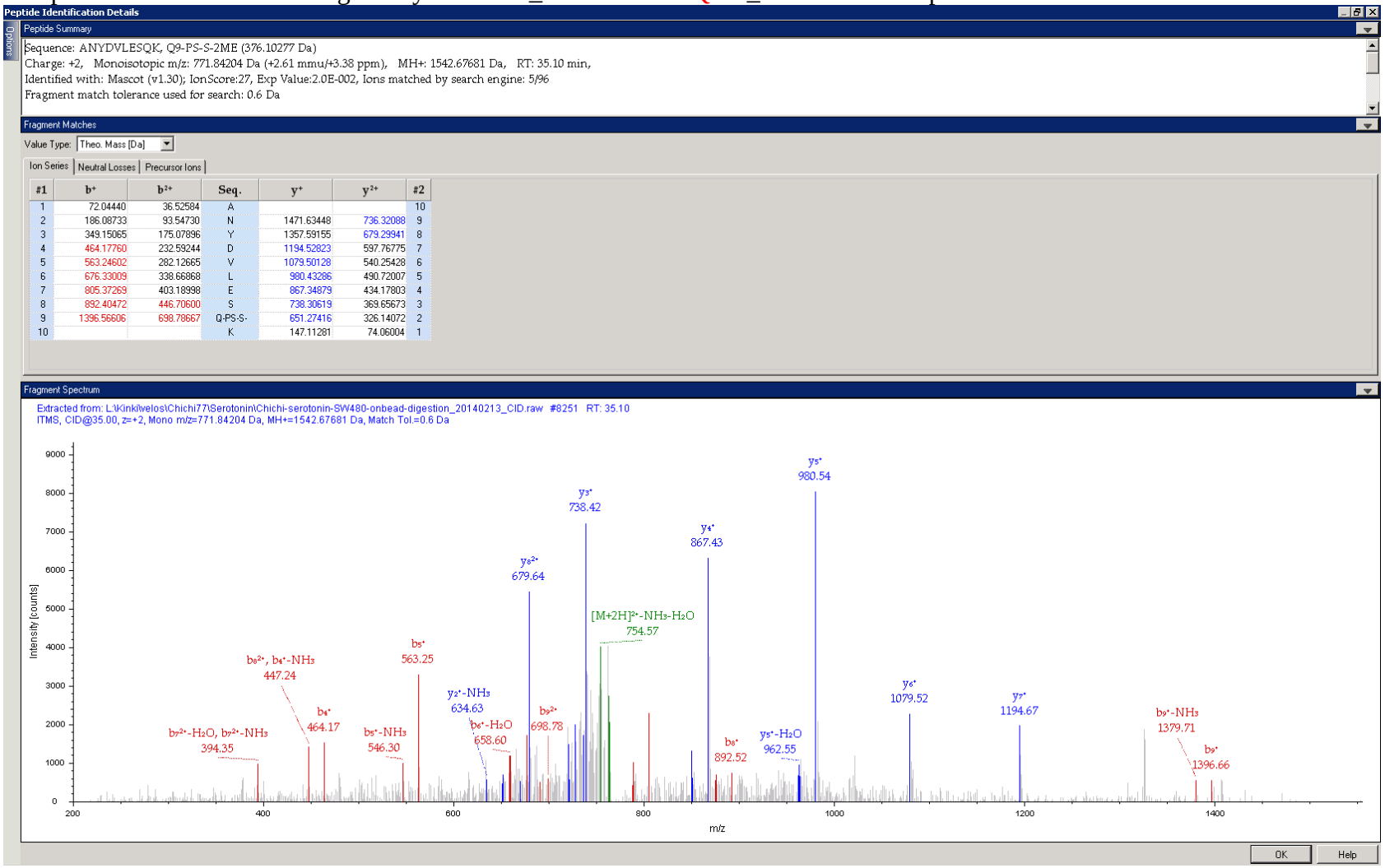
Tubulin-folding cofactor B_ AQ^{140}QEAEAAQR_2-ME-thiolated product ion



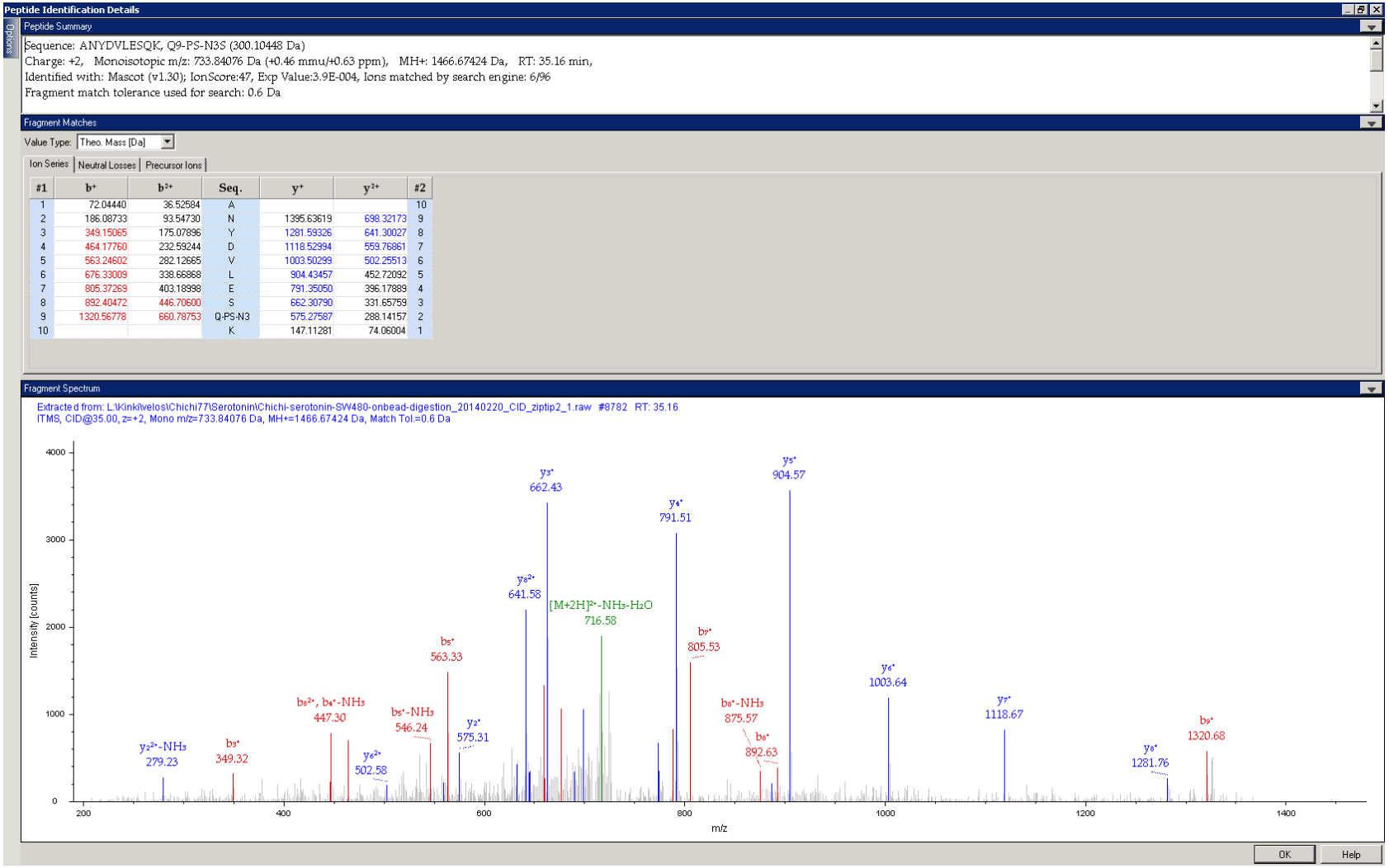
Tubulin-folding cofactor B_ AQ[140]QEAEAAQR_thiol product ion



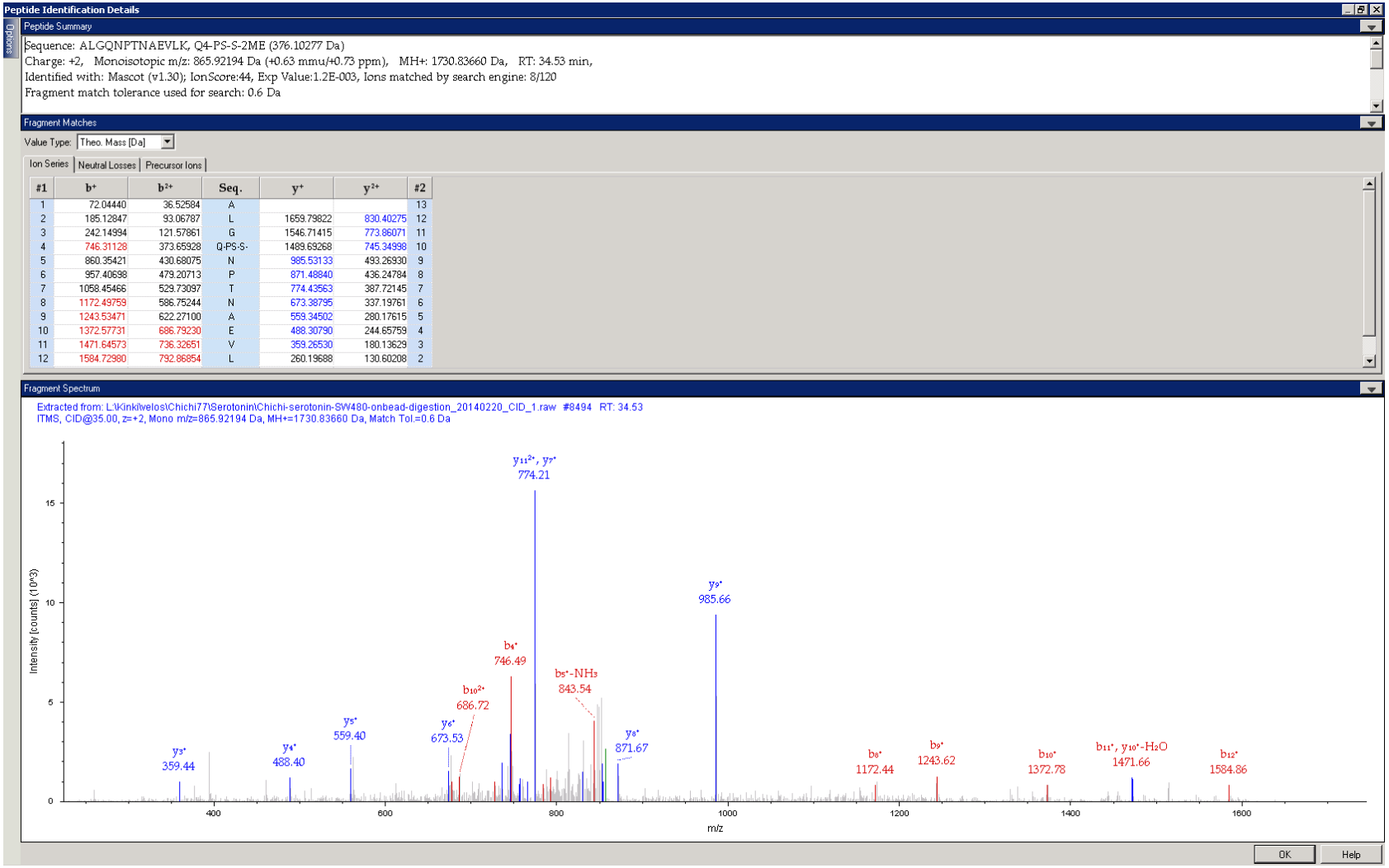
26S proteasome non-ATPase regulatory subunit 9_ ANYDVLESQ[47]K_2-ME-thiolated product ion



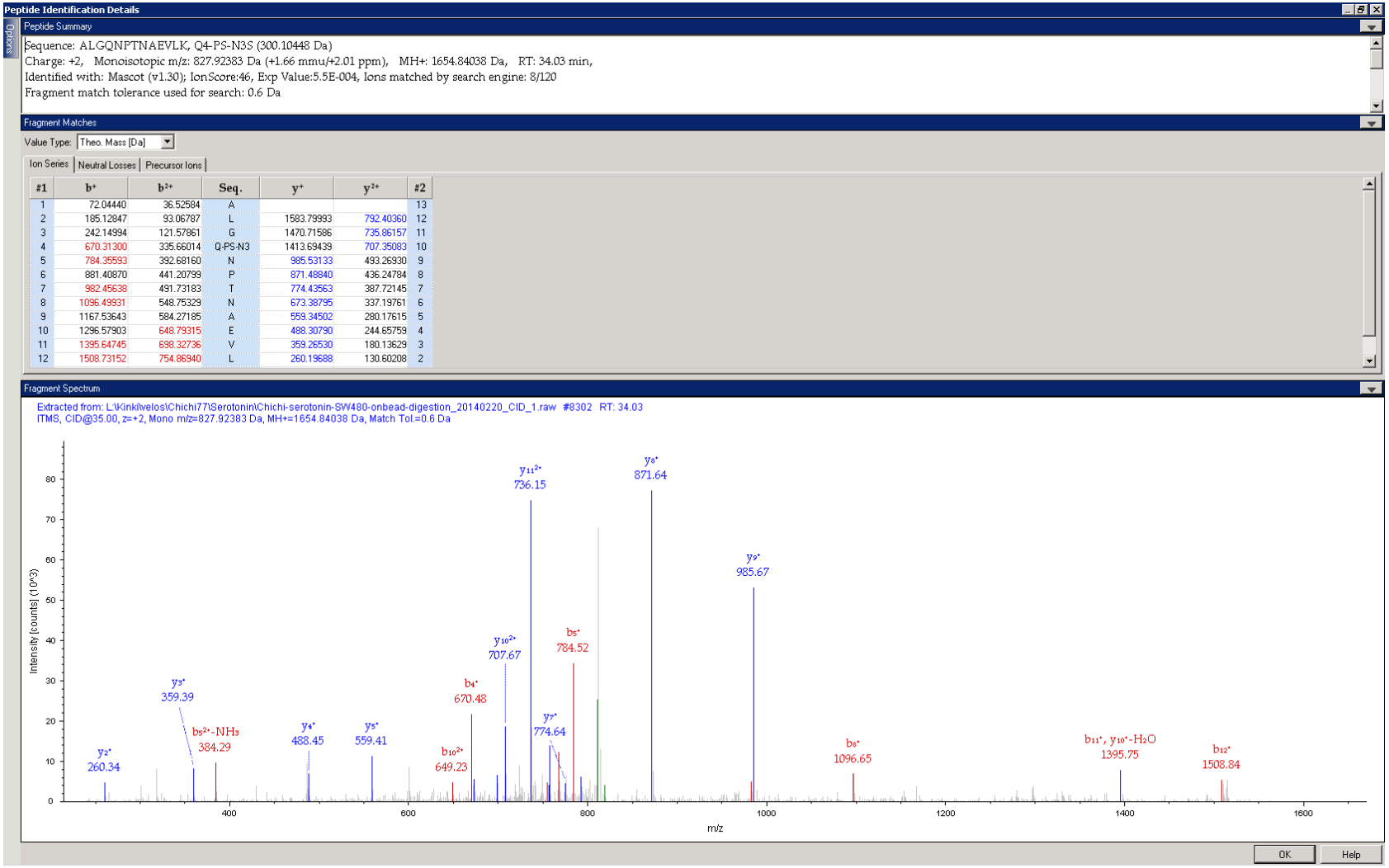
26S proteasome non-ATPase regulatory subunit 9_ ANYDVLESQ[47]K_thiol product ion



Myosin light chain 3_ ALGQ[41]NPTNAEVLK_2-ME-thiolated product ion

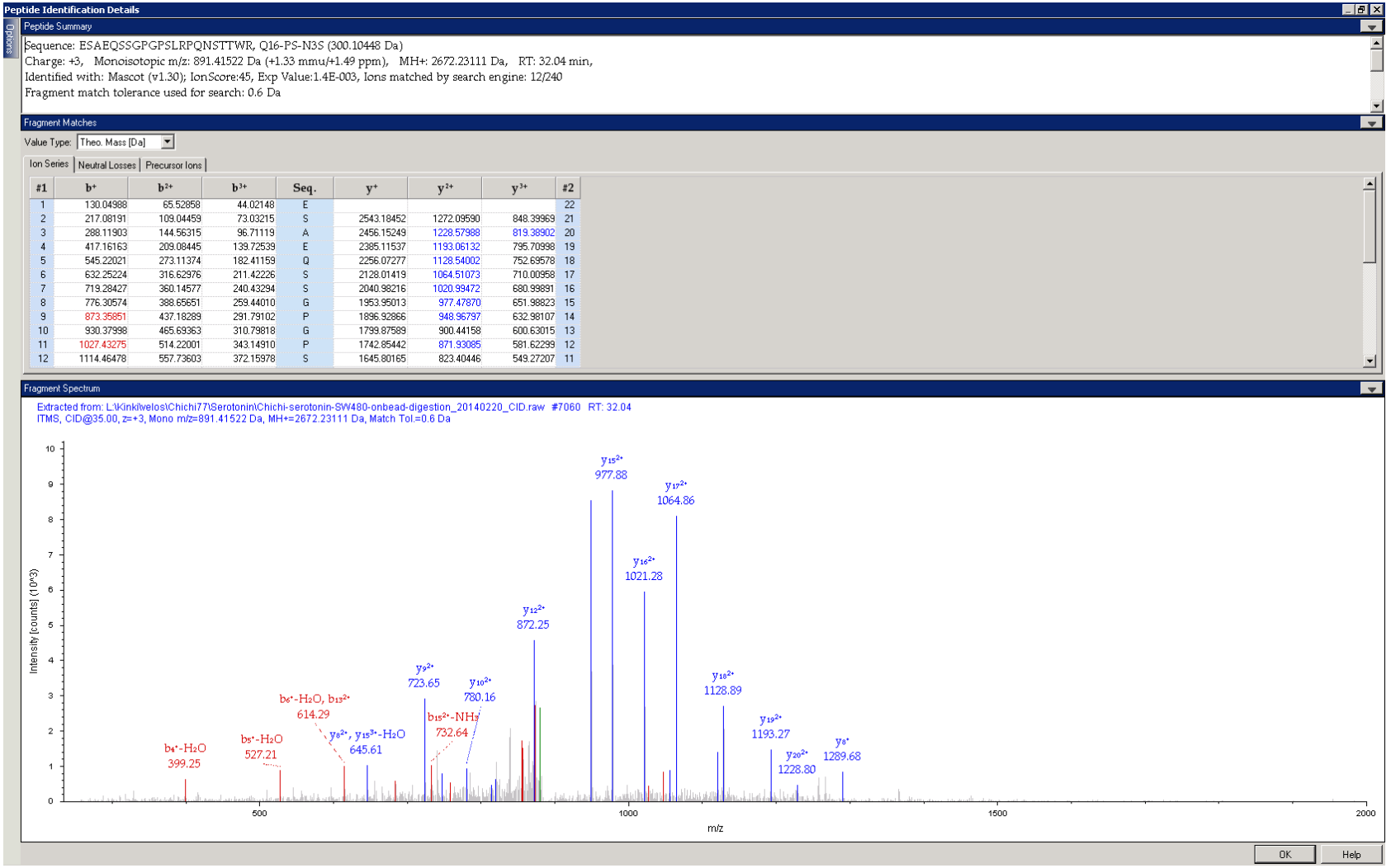


Myosin light chain 3_ ALGQ[41]NPTNAEVLK_thiol product ion

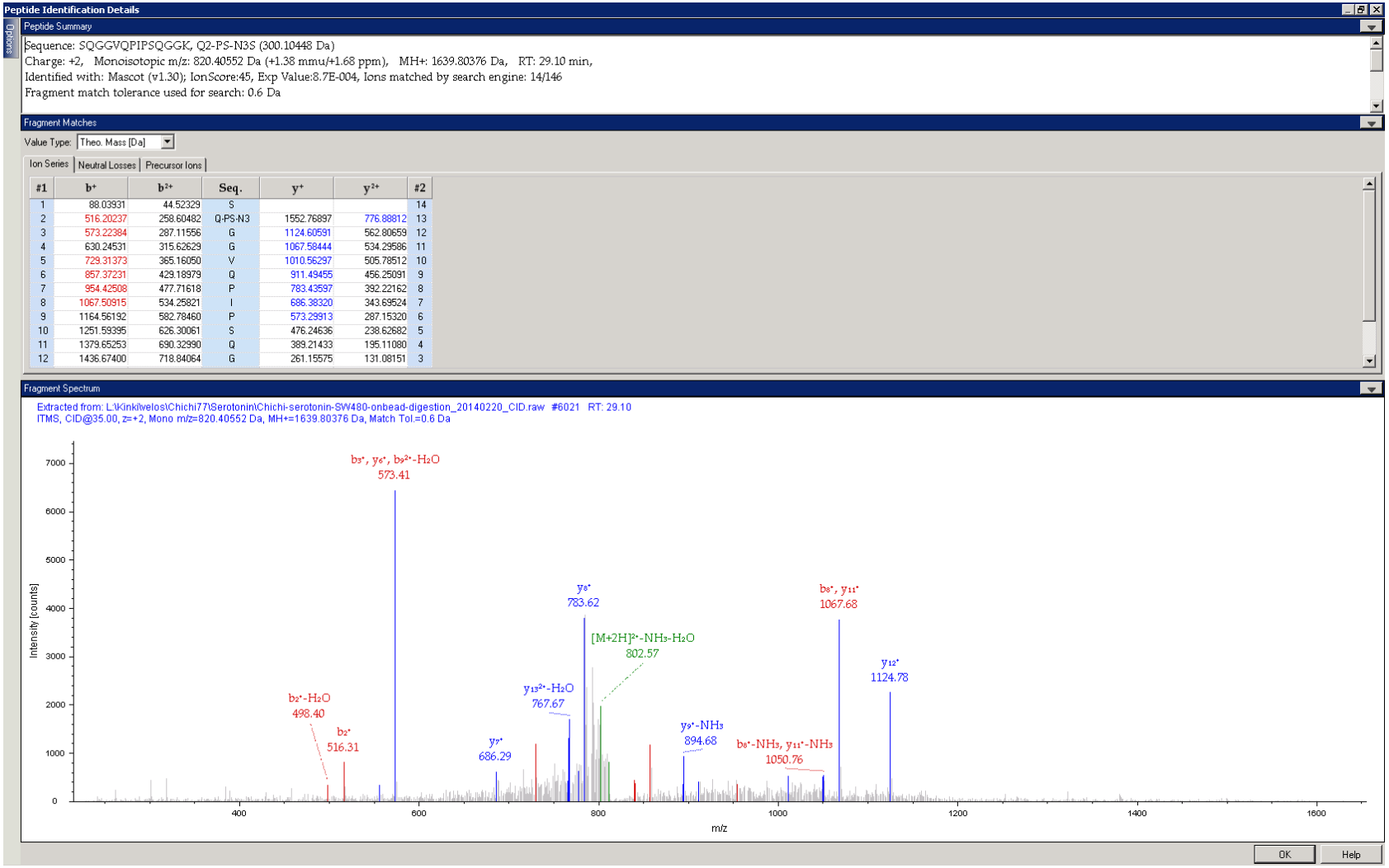


#1	b+	b2+	Seq.	y+	y2+	#2
1	72.04440	36.52584	A			13
2	185.12847	93.06787	L	1583.79993	792.40360	12
3	242.14994	121.57861	G	1470.71586	735.86157	11
4	670.31300	335.66014	Q-PS-N3	1413.69439	707.35083	10
5	784.35593	392.68160	N	985.53133	493.26930	9
6	881.40870	441.20799	P	871.48840	436.24784	8
7	982.45638	491.73183	T	774.43563	387.72145	7
8	1096.49931	548.75329	N	673.38795	337.19761	6
9	1167.53643	584.27185	A	559.34502	280.17615	5
10	1296.57903	648.79315	E	488.30790	244.65759	4
11	1395.64745	698.32736	V	359.26530	180.13629	3
12	1508.73152	754.86940	L	260.19688	130.60208	2

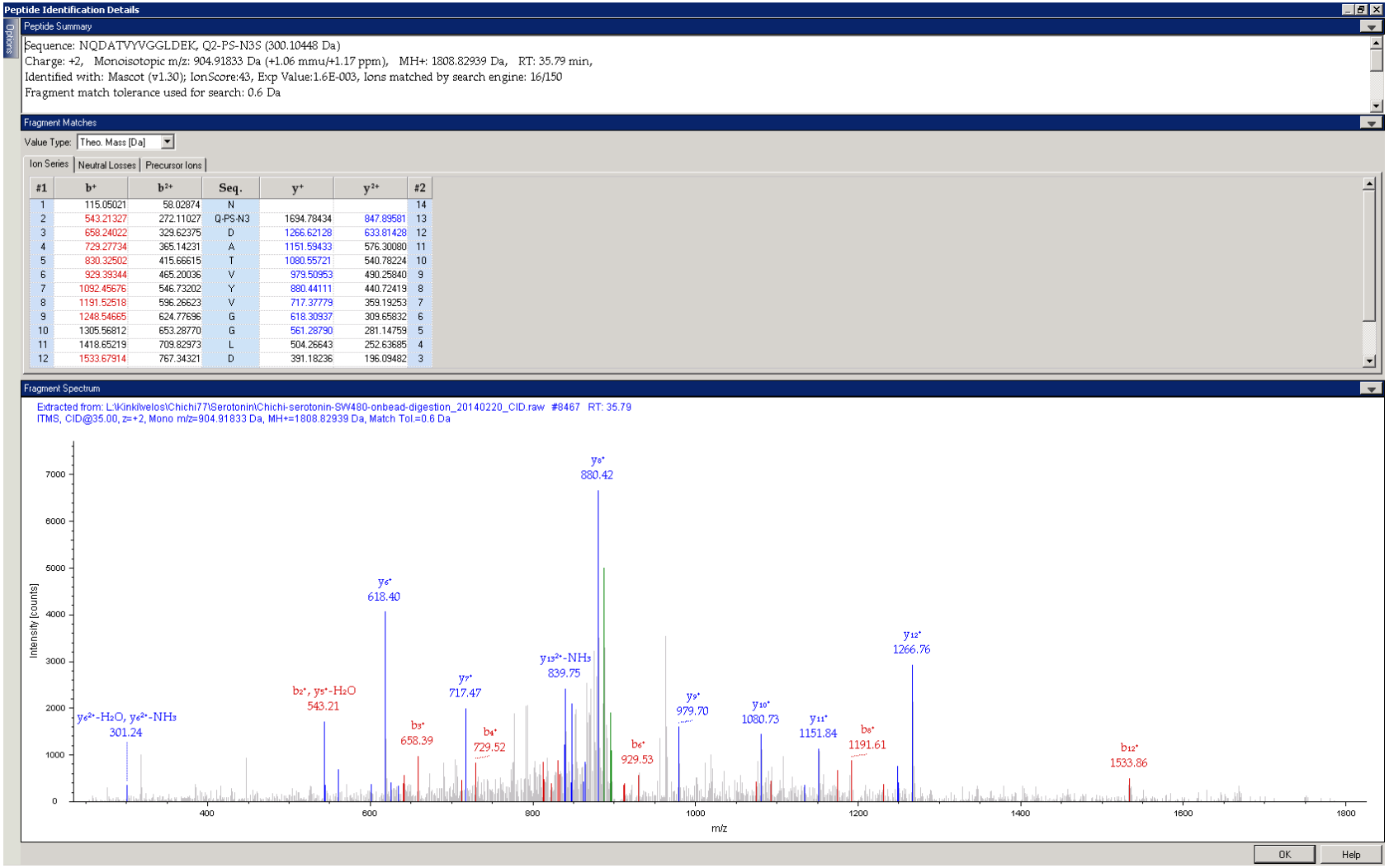
Protein G2_ ESAEQSSGPGPSLRPQ[202]NSTTWR_thiol product ion



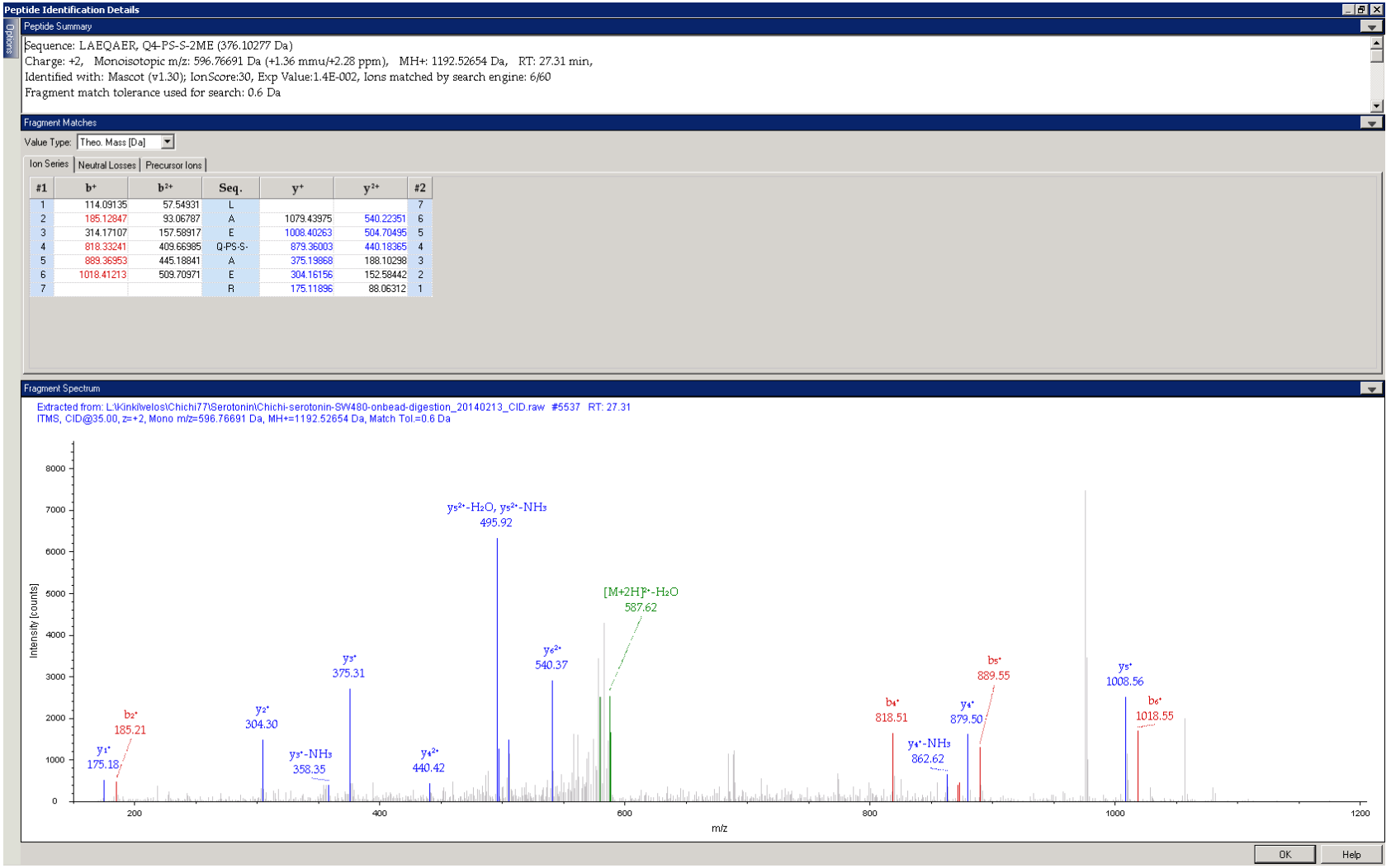
Oxidative stress-associated Src activator_ SQ^{934}GGVQPIPSQGGK_thiol product ion



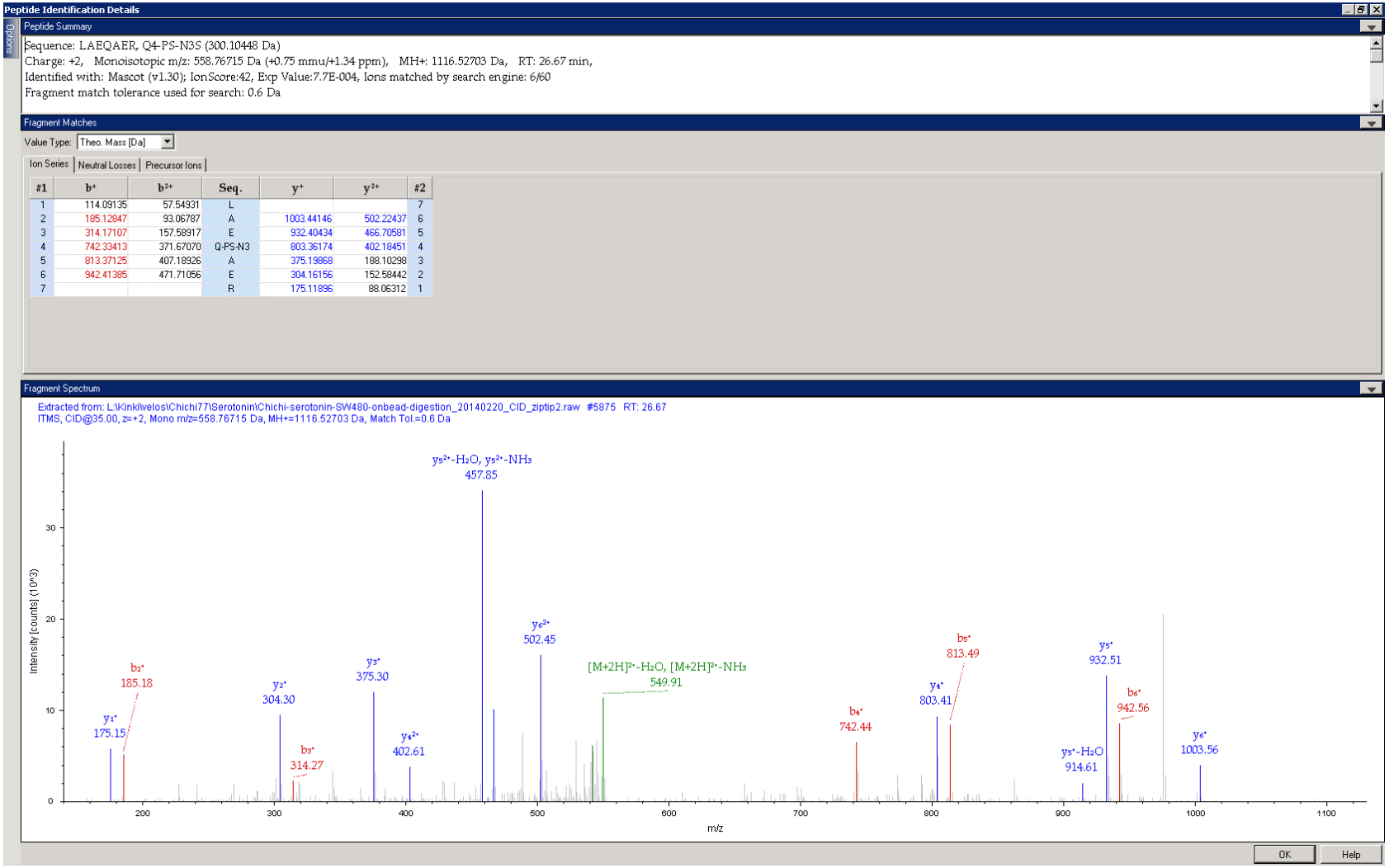
Splicing factor 3B subunit 4_ NQ[11]DATVYVGGLDEK_thiol product ion



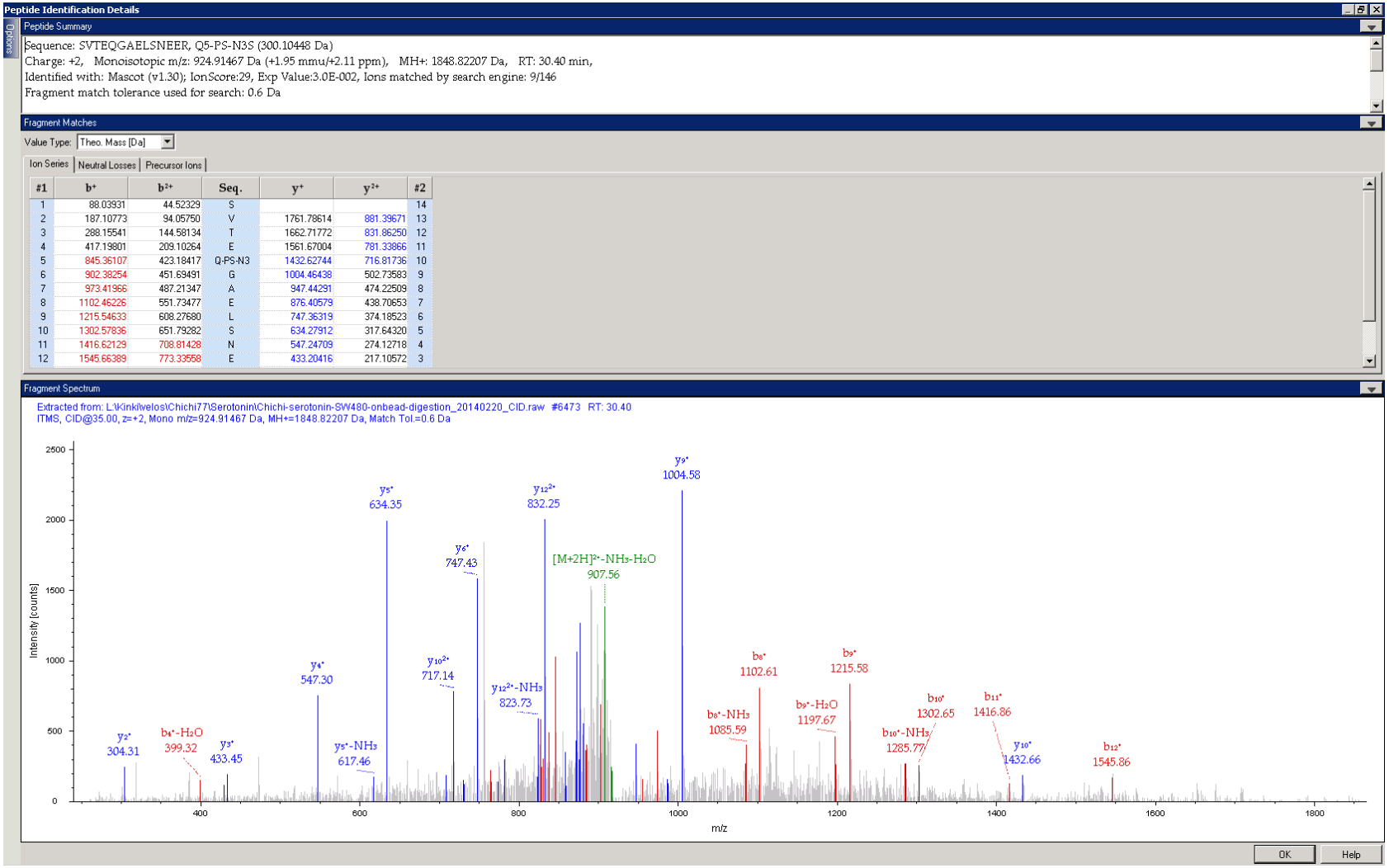
14-3-3 protein zeta/delta_ LAEQ[15]AER_2-ME-thiolated product ion



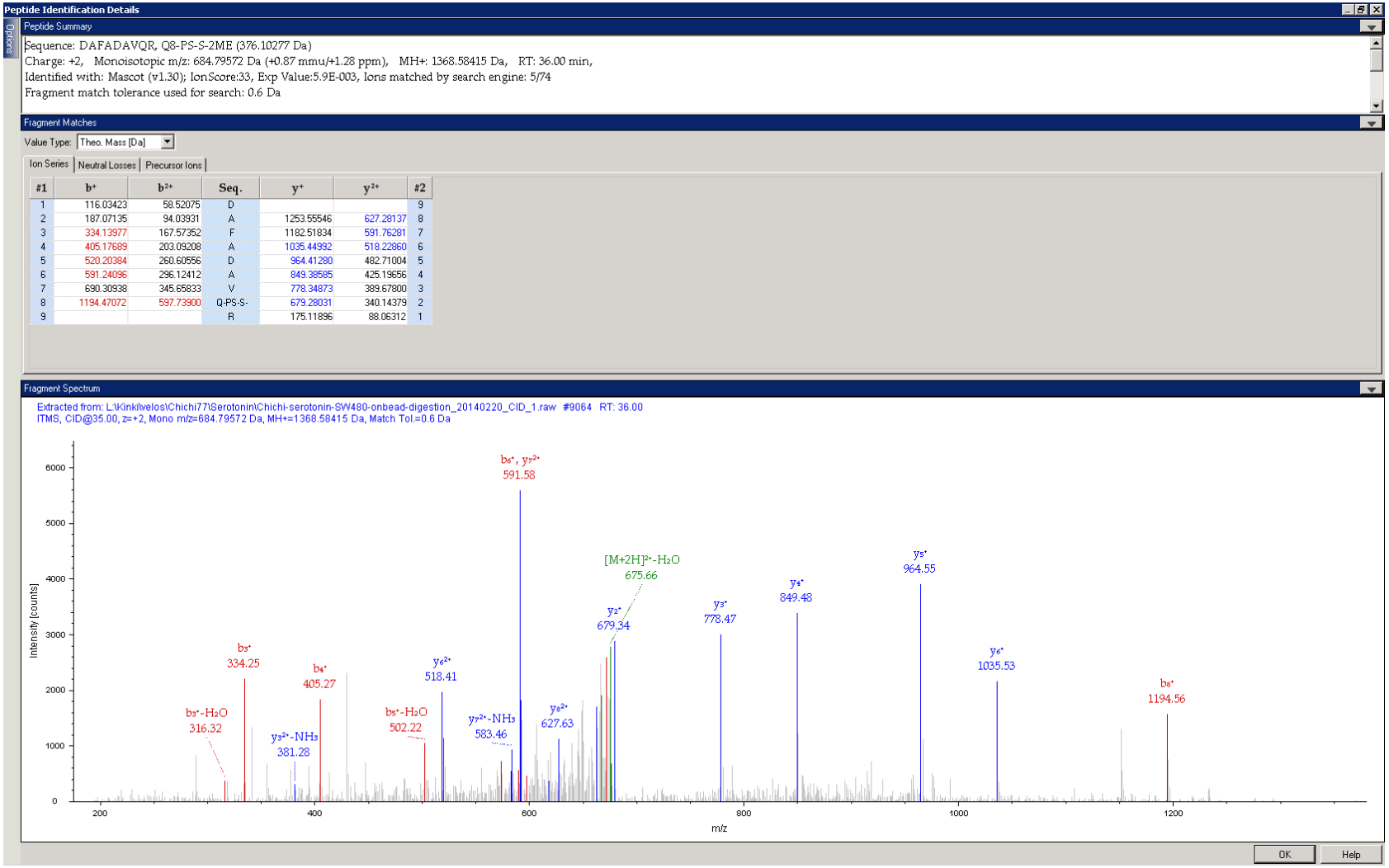
14-3-3 protein zeta/delta_ LAEQ[15]AER_thiol product ion



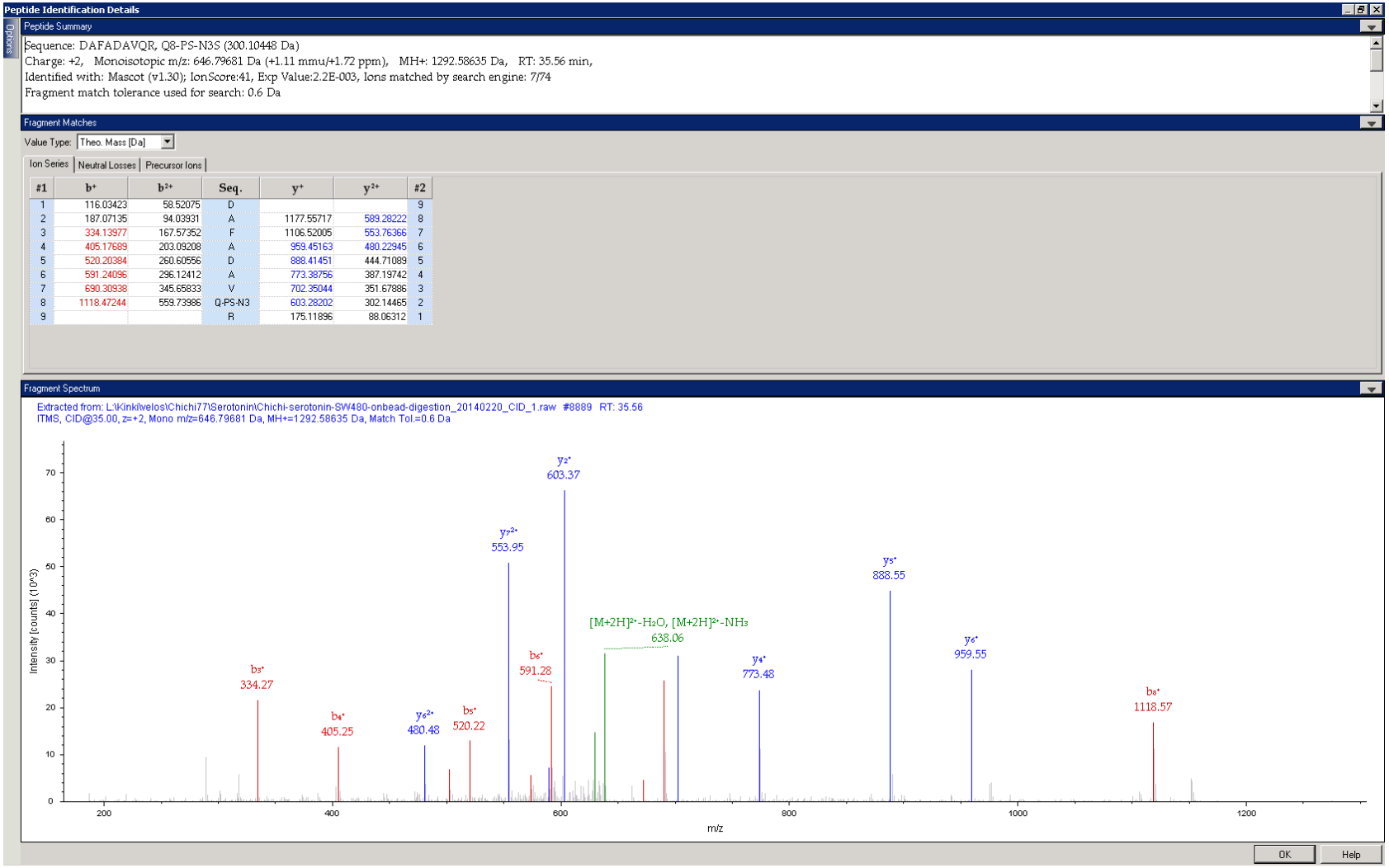
14-3-3 protein zeta/delta_ SVTEQ[32]GAELSNEER_thiol product ion



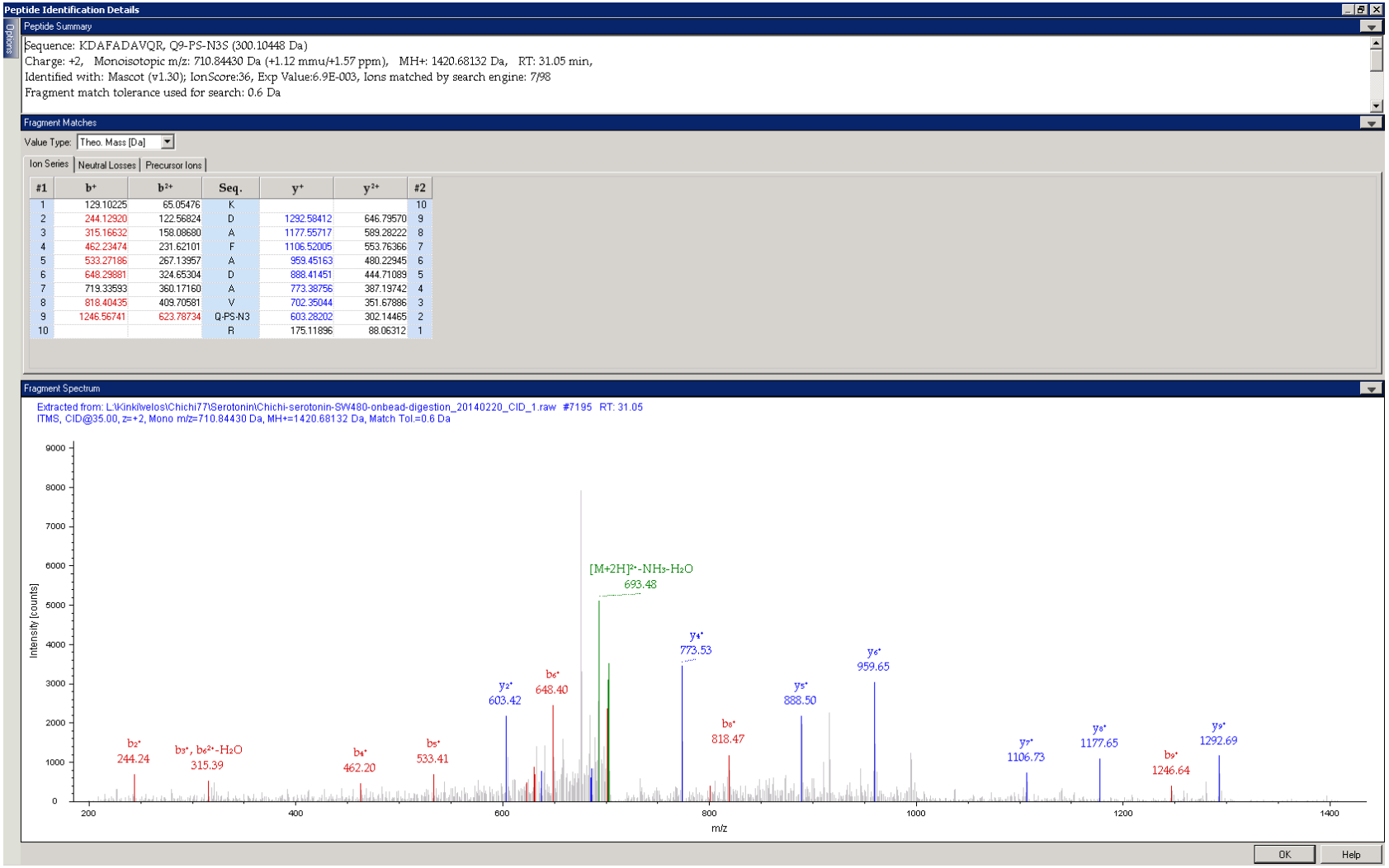
Far upstream element (FUSE)-binding protein 2_DAFADAVQ[79]R_2-ME-thiolated product ion



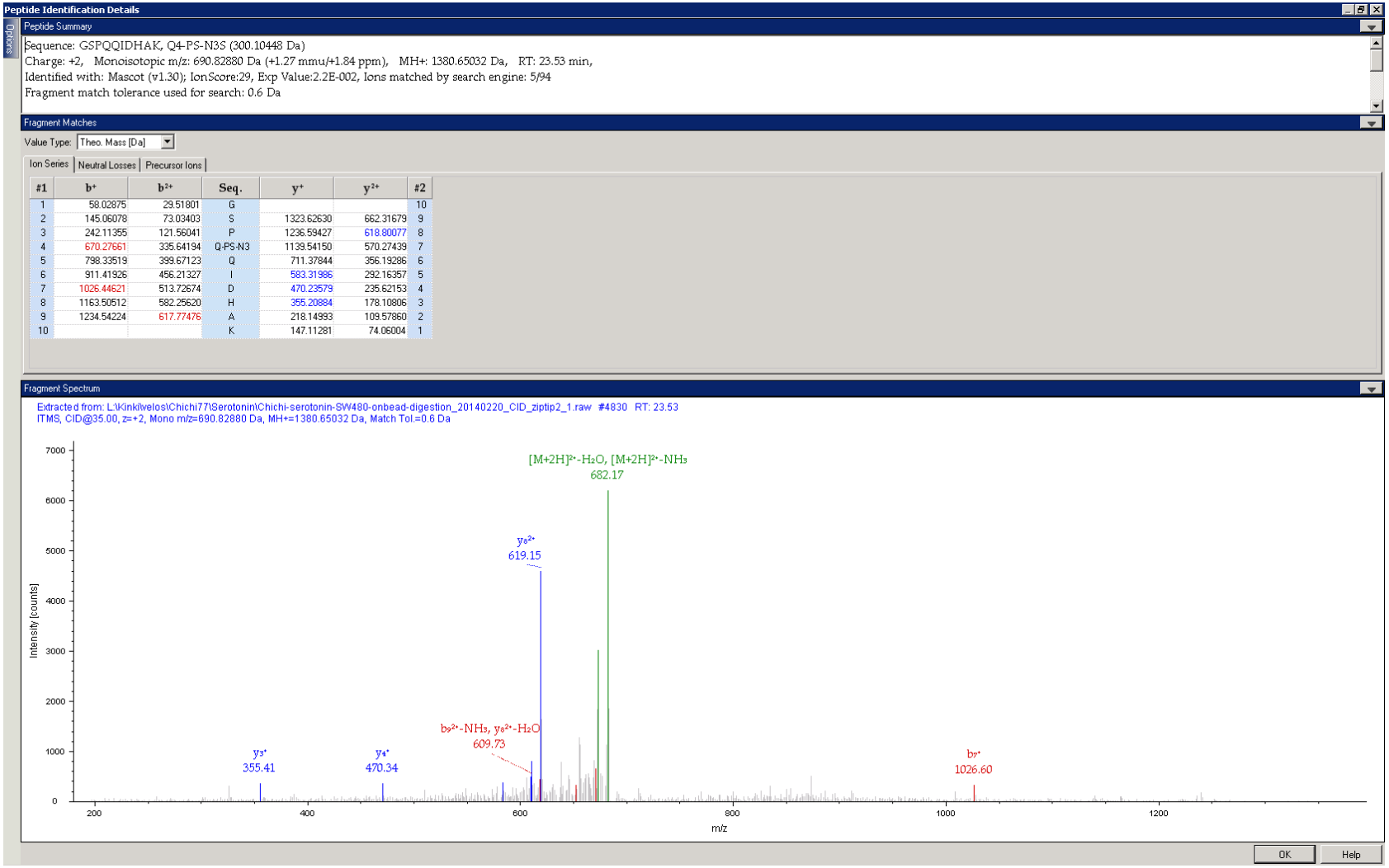
Far upstream element (FUSE)-binding protein 2_ DAFADAVQ[79]R_thiol product ion



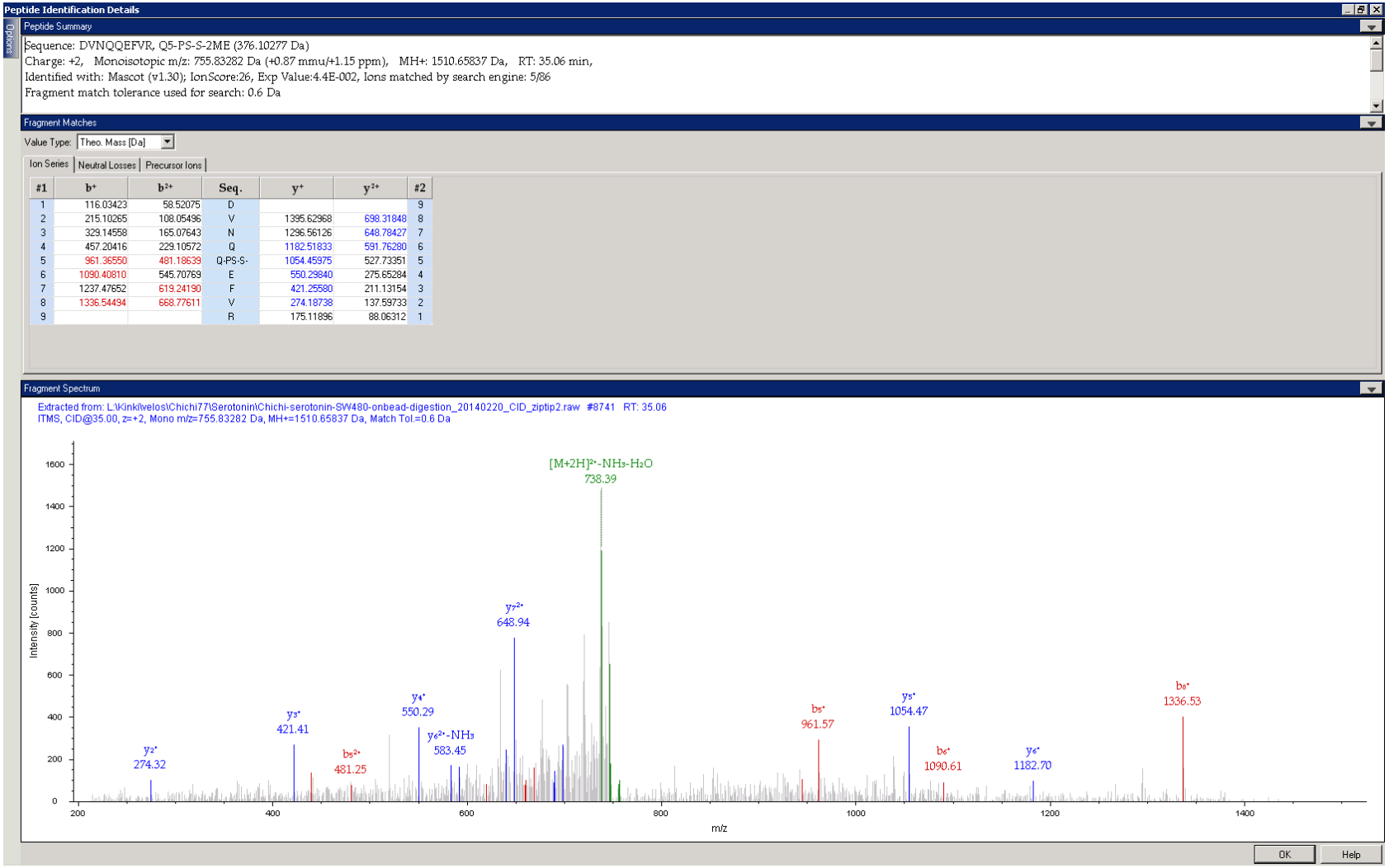
Far upstream element (FUSE)-binding protein 2_ KDAFADAVQ[79]R_thiol product ion



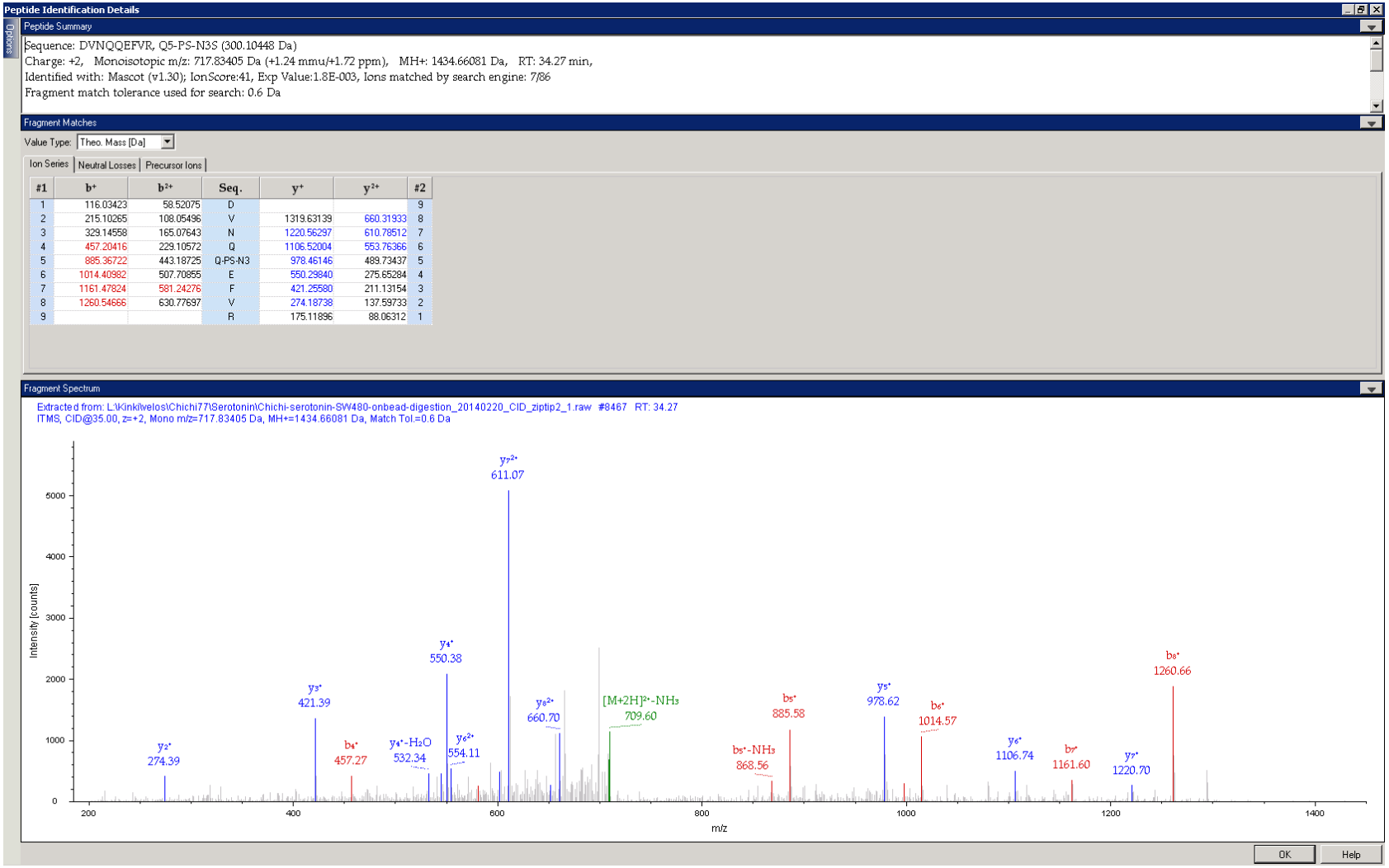
Far upstream element (FUSE)-binding protein 2_GSPQ[482]QIDHAK_thiol product ion



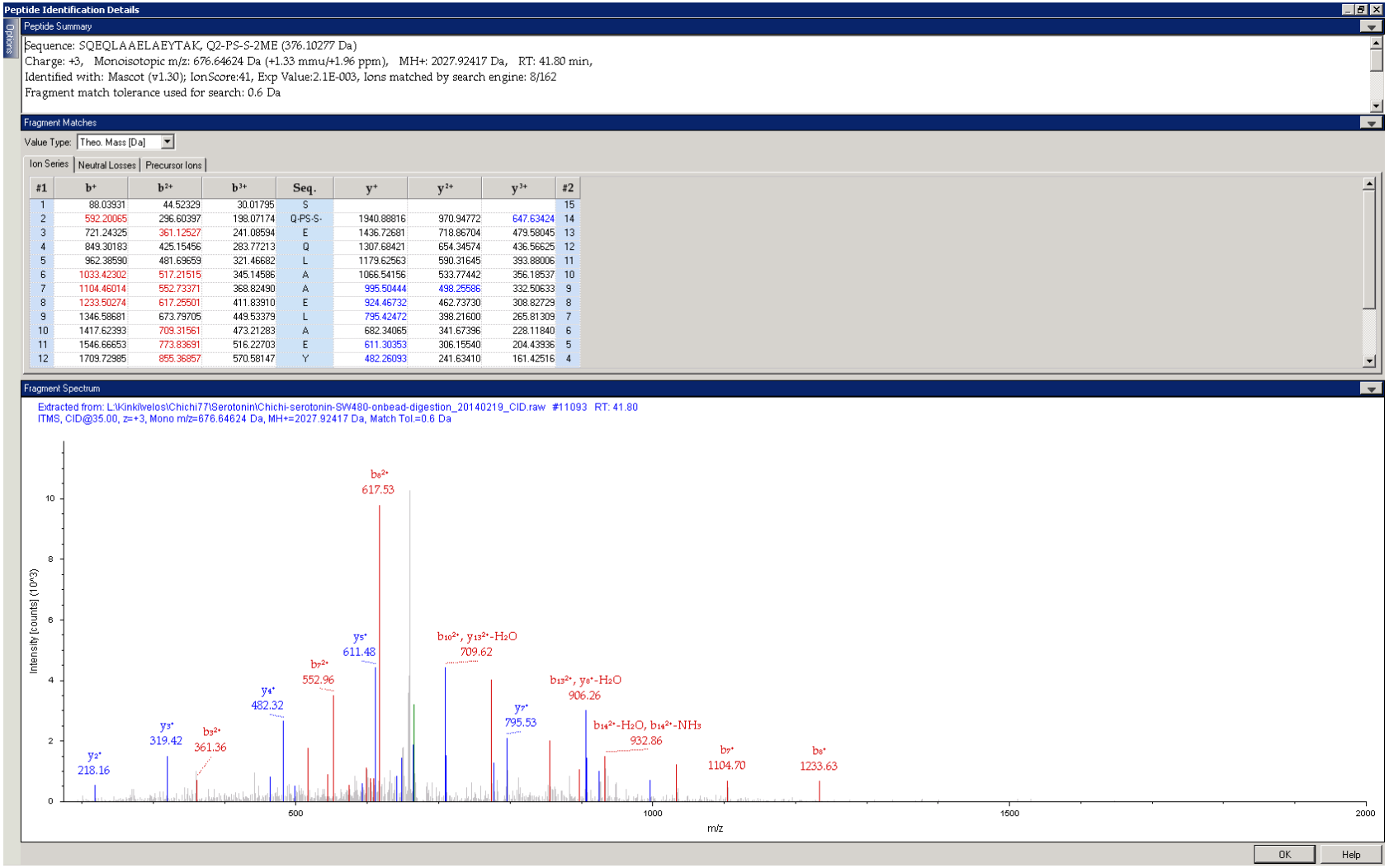
40S ribosomal protein S19_ DVNQQ[12]EFVR_2-ME-thiolated product ion



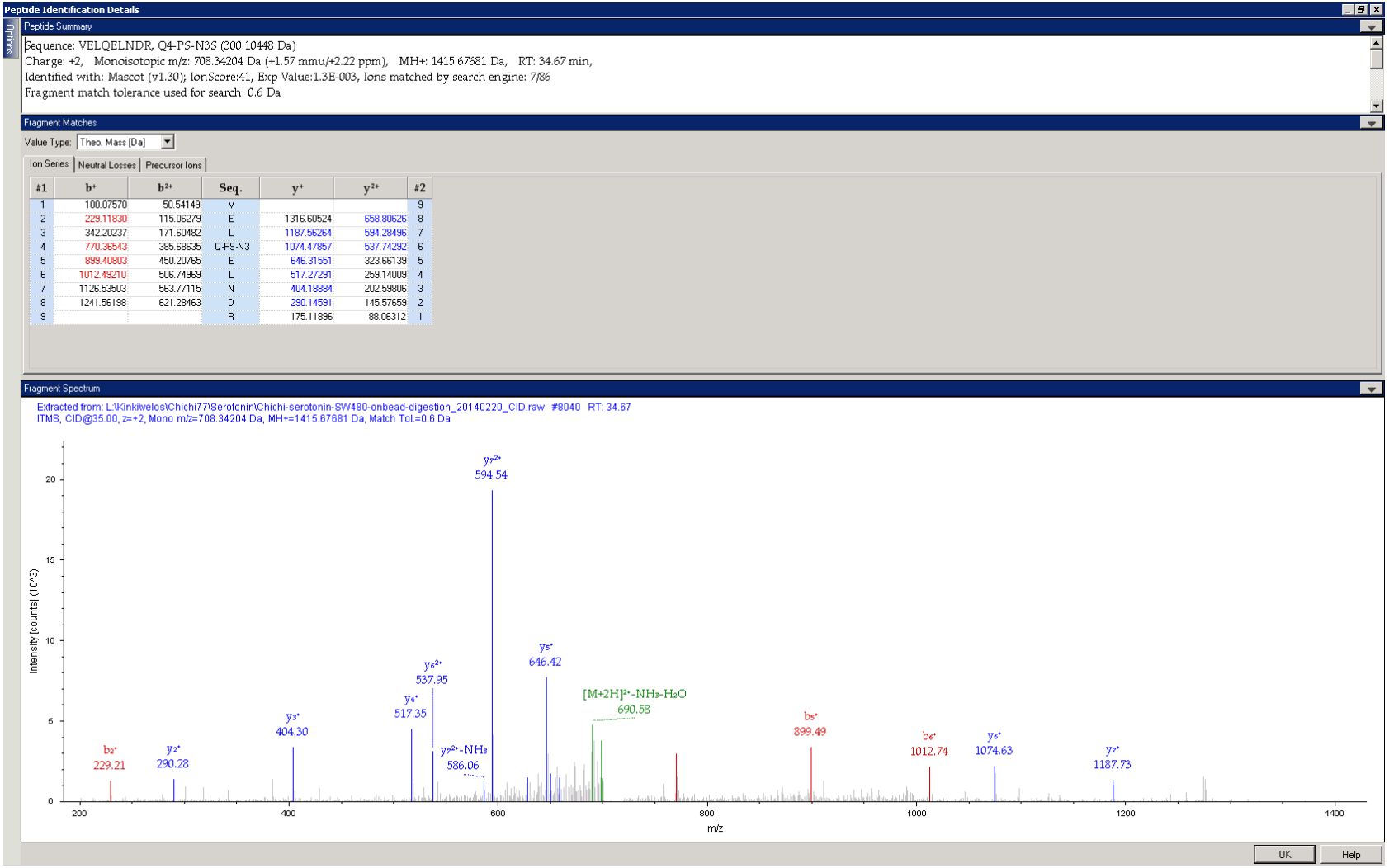
40S ribosomal protein S19_ DVNQQ[12]EFVR_thiol product ion



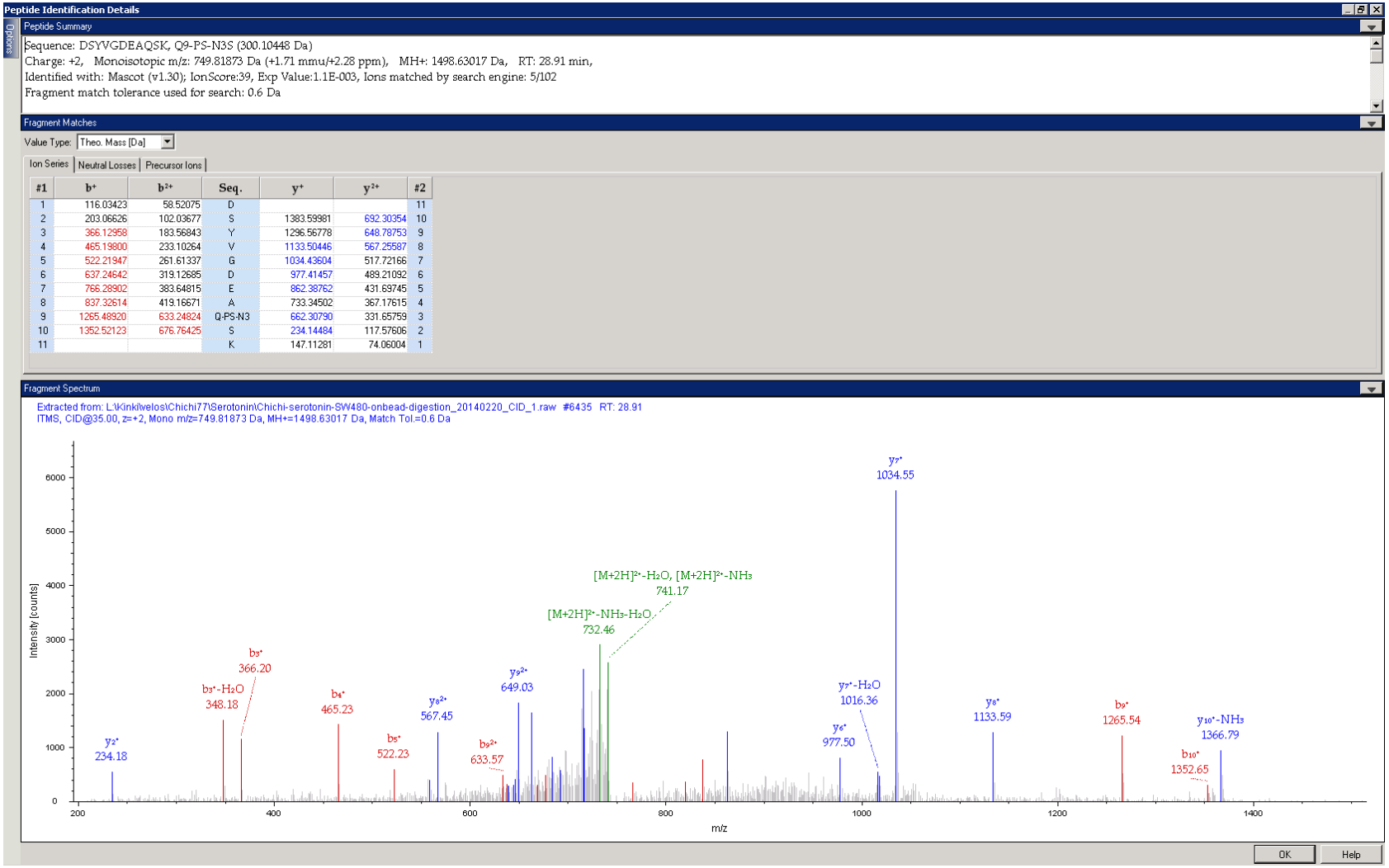
Ezrin_ SQEQ[416]LAAELAEYTAK_2-ME-thiolated product ion



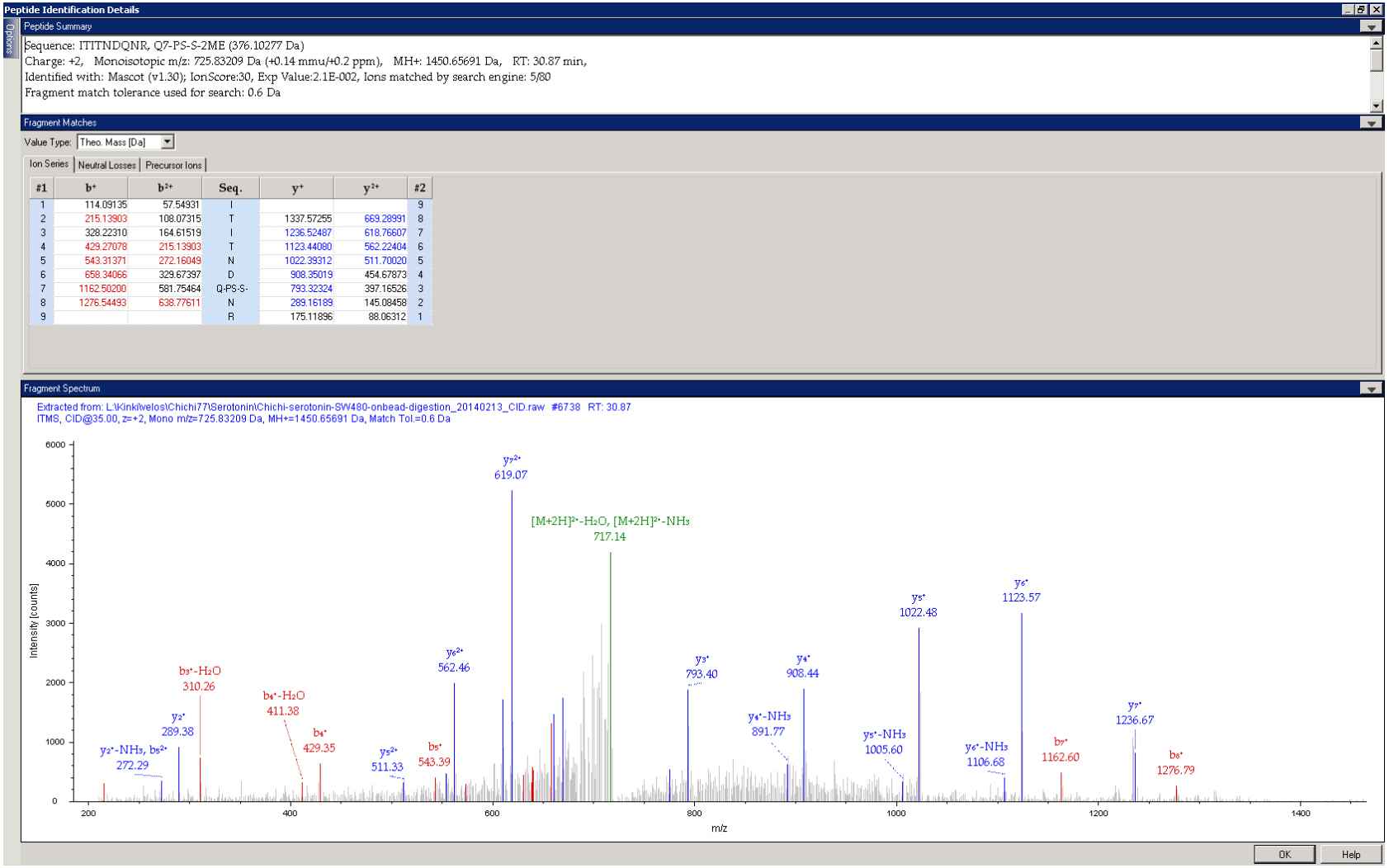
Vimentin_ VELQ[108]ELNDR_thiol product ion



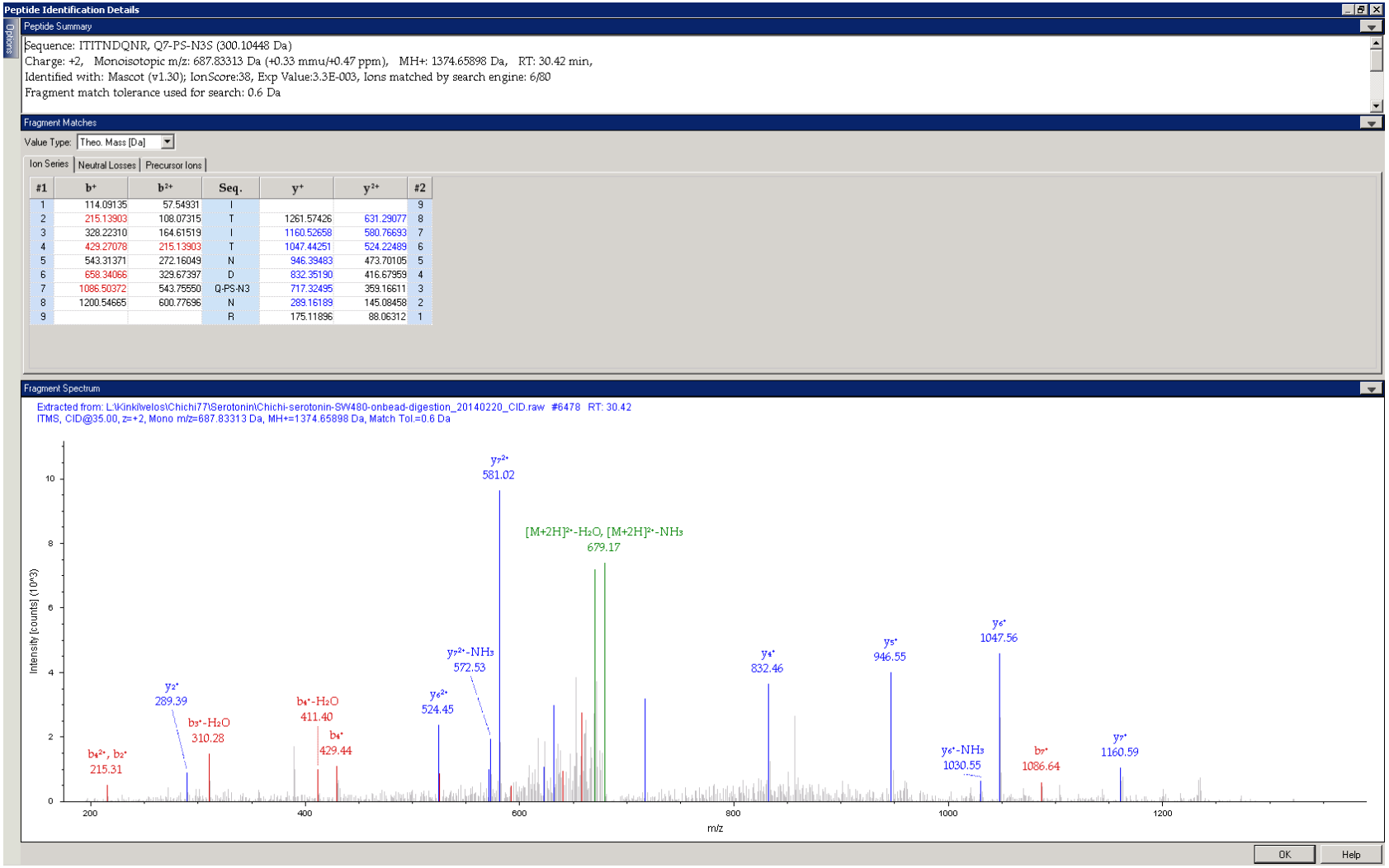
Actin_ DSYVGDEAQ[59]SK_thiol product ion



Heat shock protein A5_ITITNDQ[530]NR_2-ME-thiolated product ion

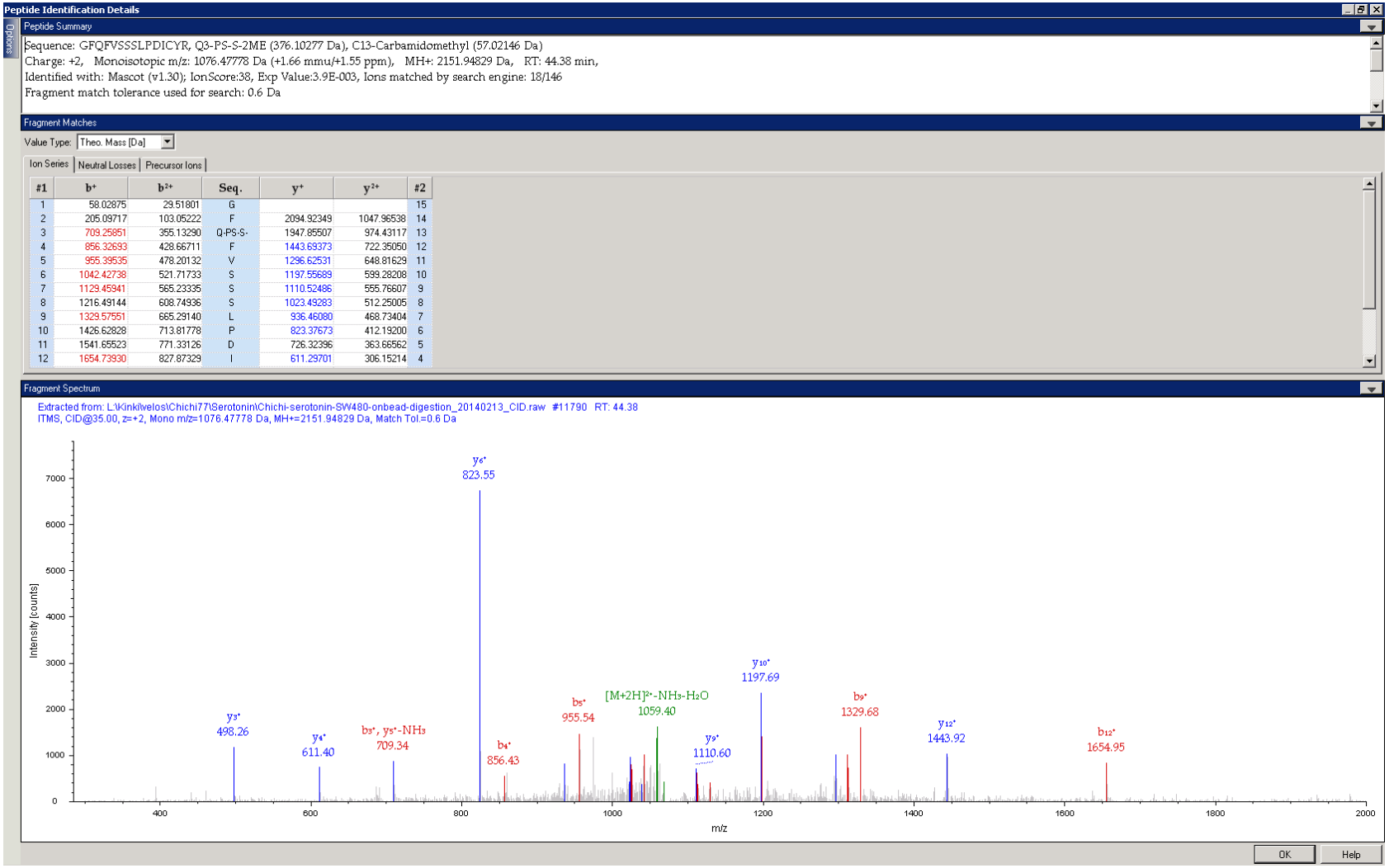


Heat shock protein A5_ ITITNDQ^{530}NR_thiol product ion

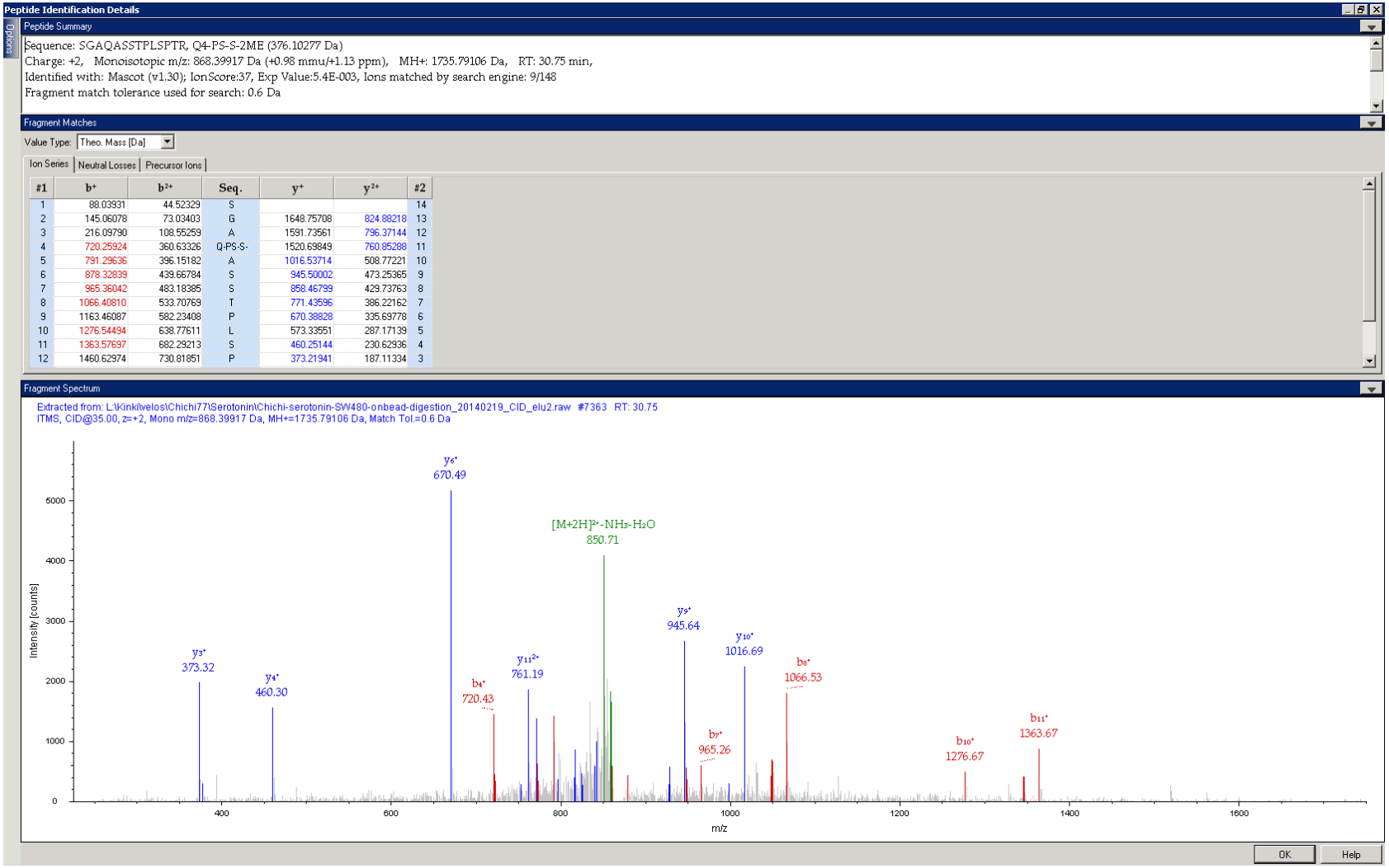


Mitochondrial 39S ribosomal protein L12_ LVESLPQ^{170}EIK_thiol product ion

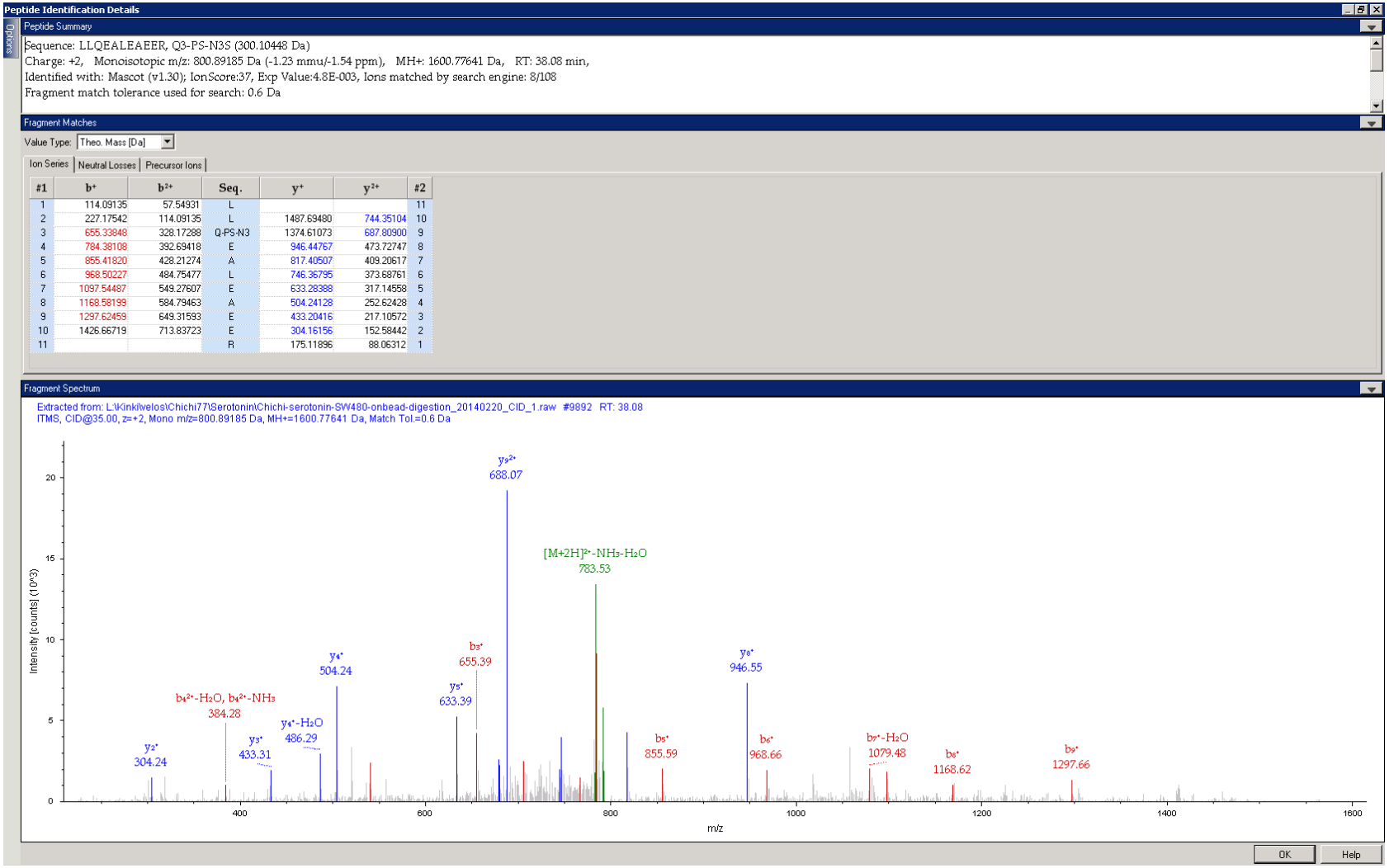
Zinc finger protein 9_ GFQ[44]FVSSSLPDICYR_2-ME-thiolated product ion



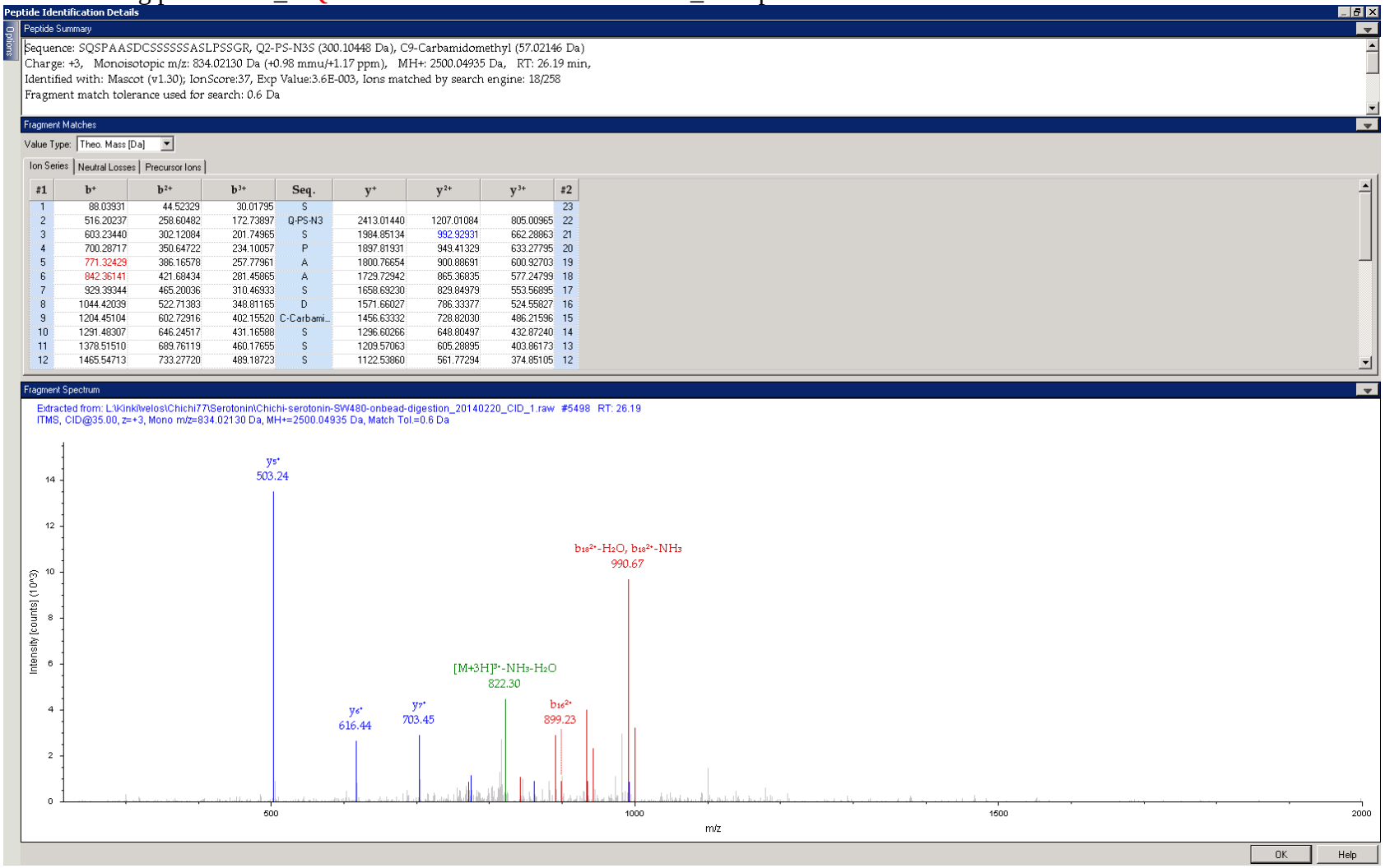
Prelamin-A/C_ SGAQ[15]ASSTPLSPTR_2-ME-thiolated product ion



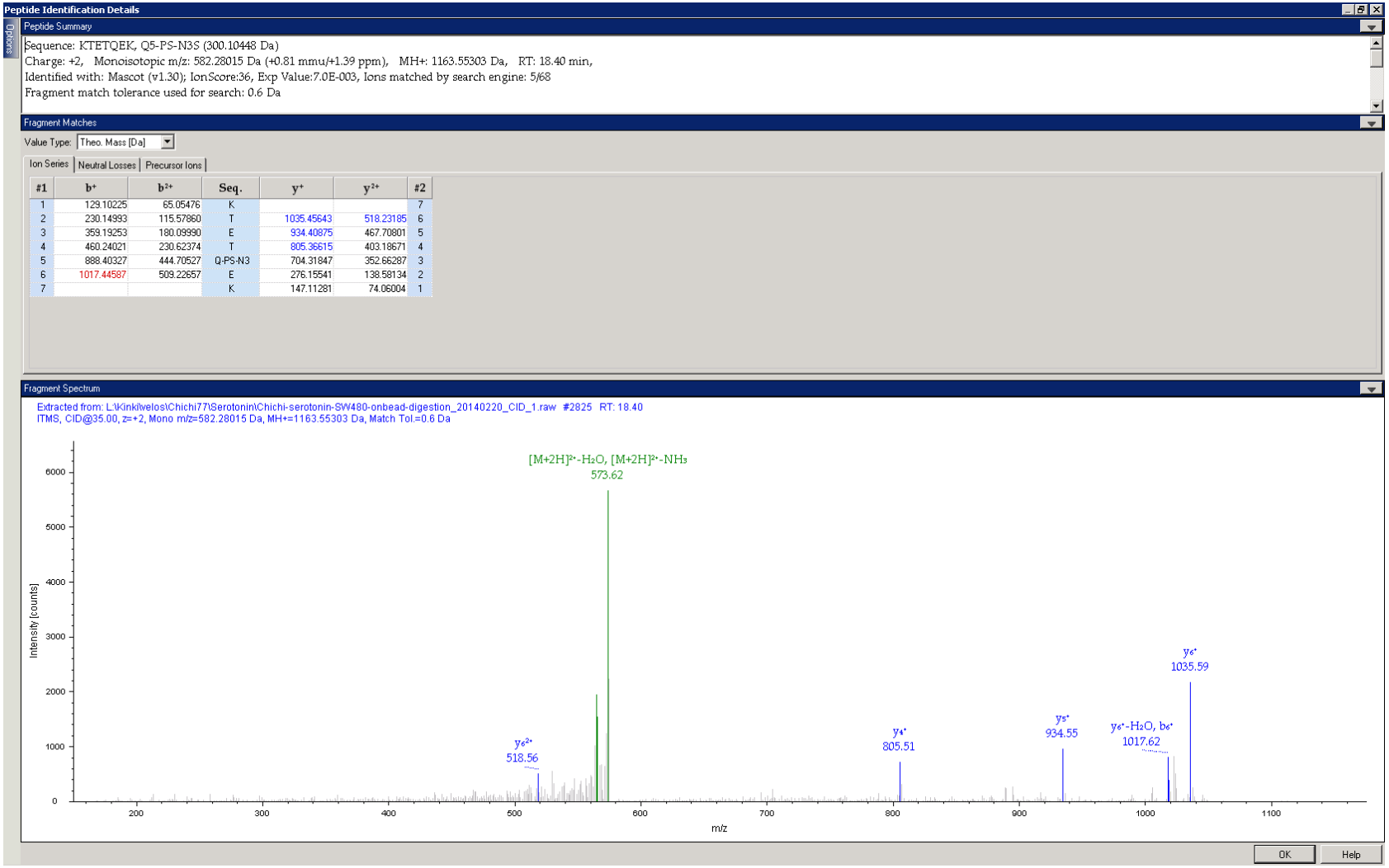
PDZ and LIM domain protein 2_ LLQ[246]EALEAEER_thiol product ion



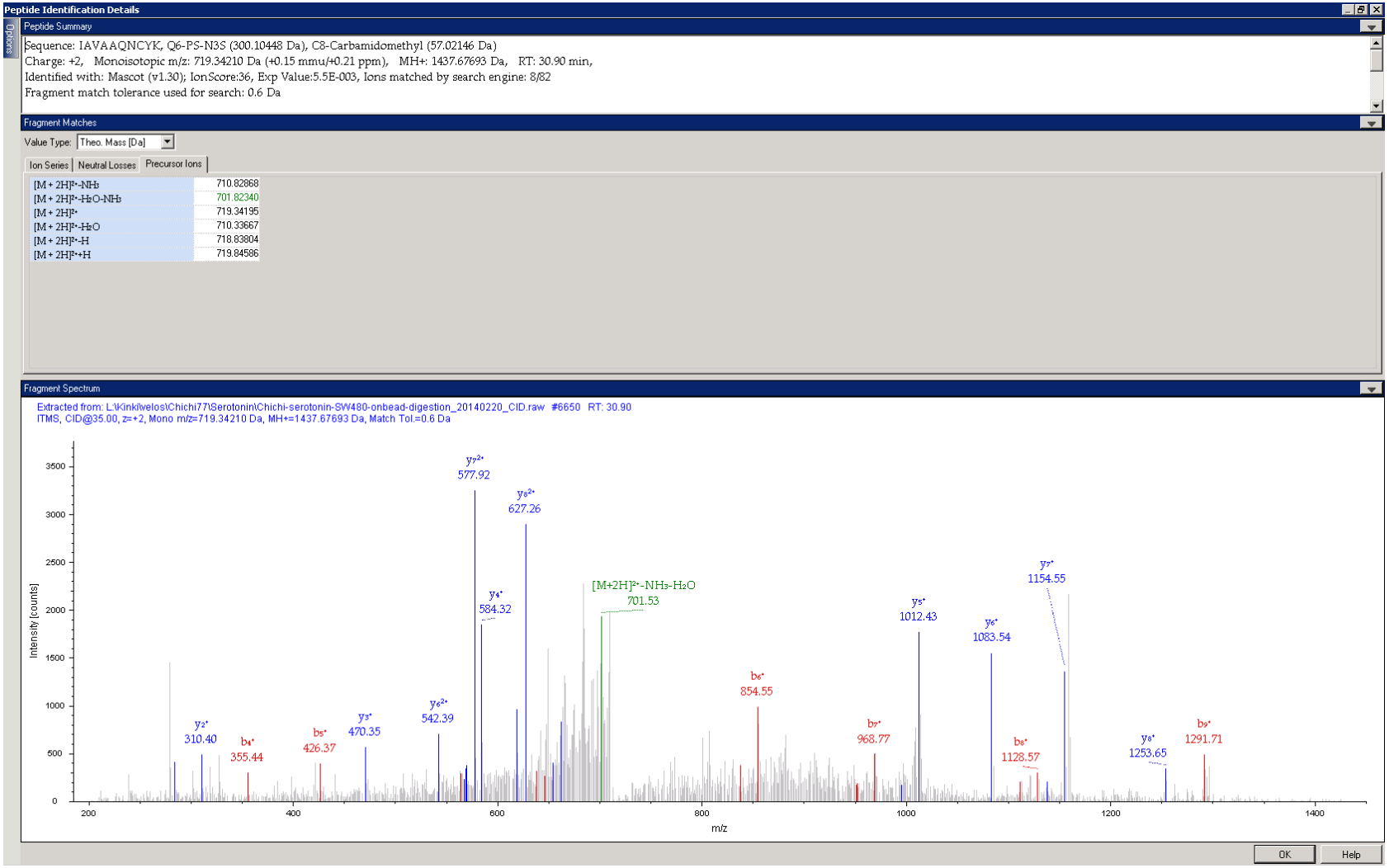
Bcl-2-binding protein Bis_ SQ[172]SPAASDCSSSSSSASLPSSGR_thiol product ion



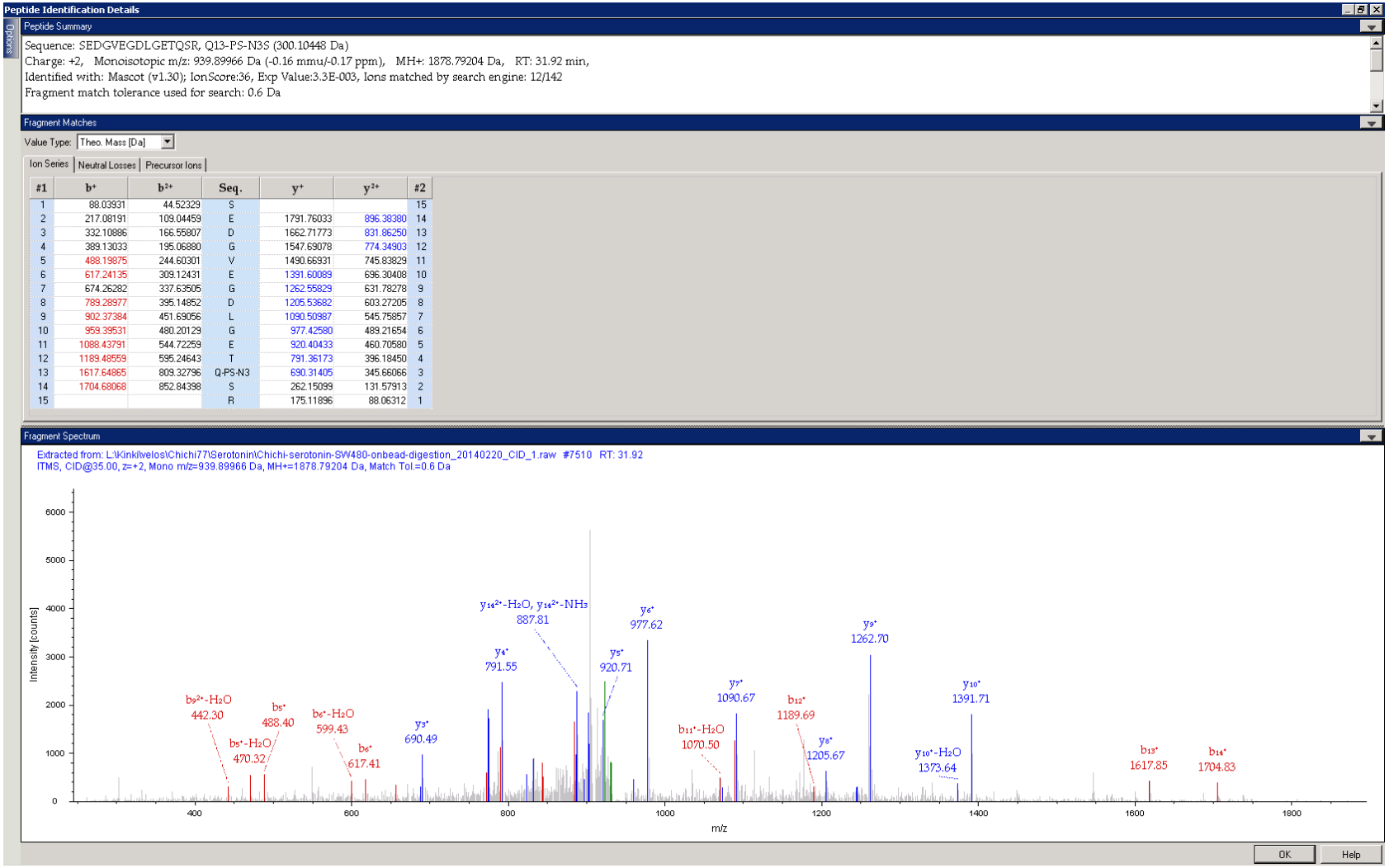
Thymosin beta-4_ KTETQ^{24}EK_thiol product ion



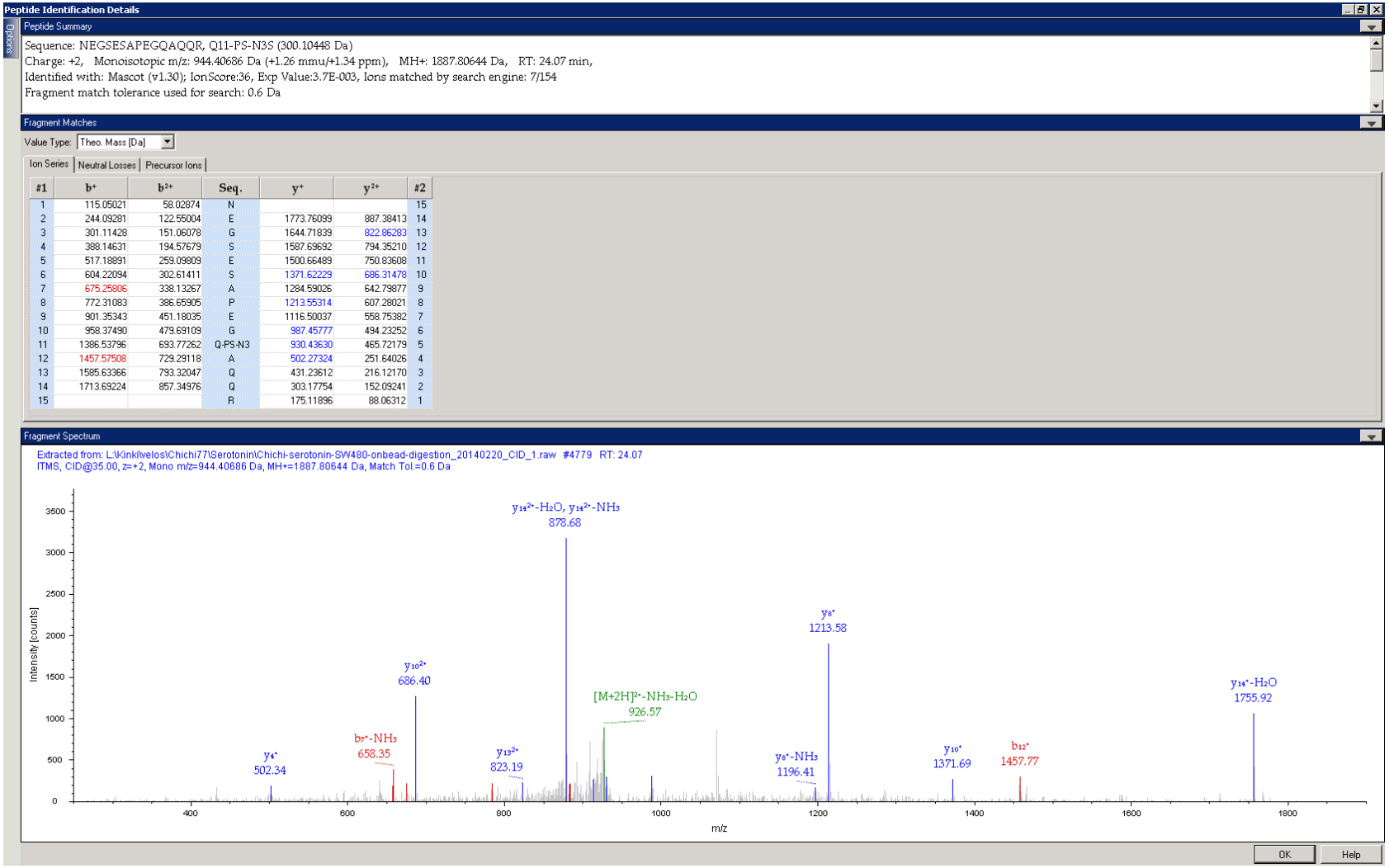
Triosephosphate isomerase_ IAVAAQ[102]NCYK_thiol product ion



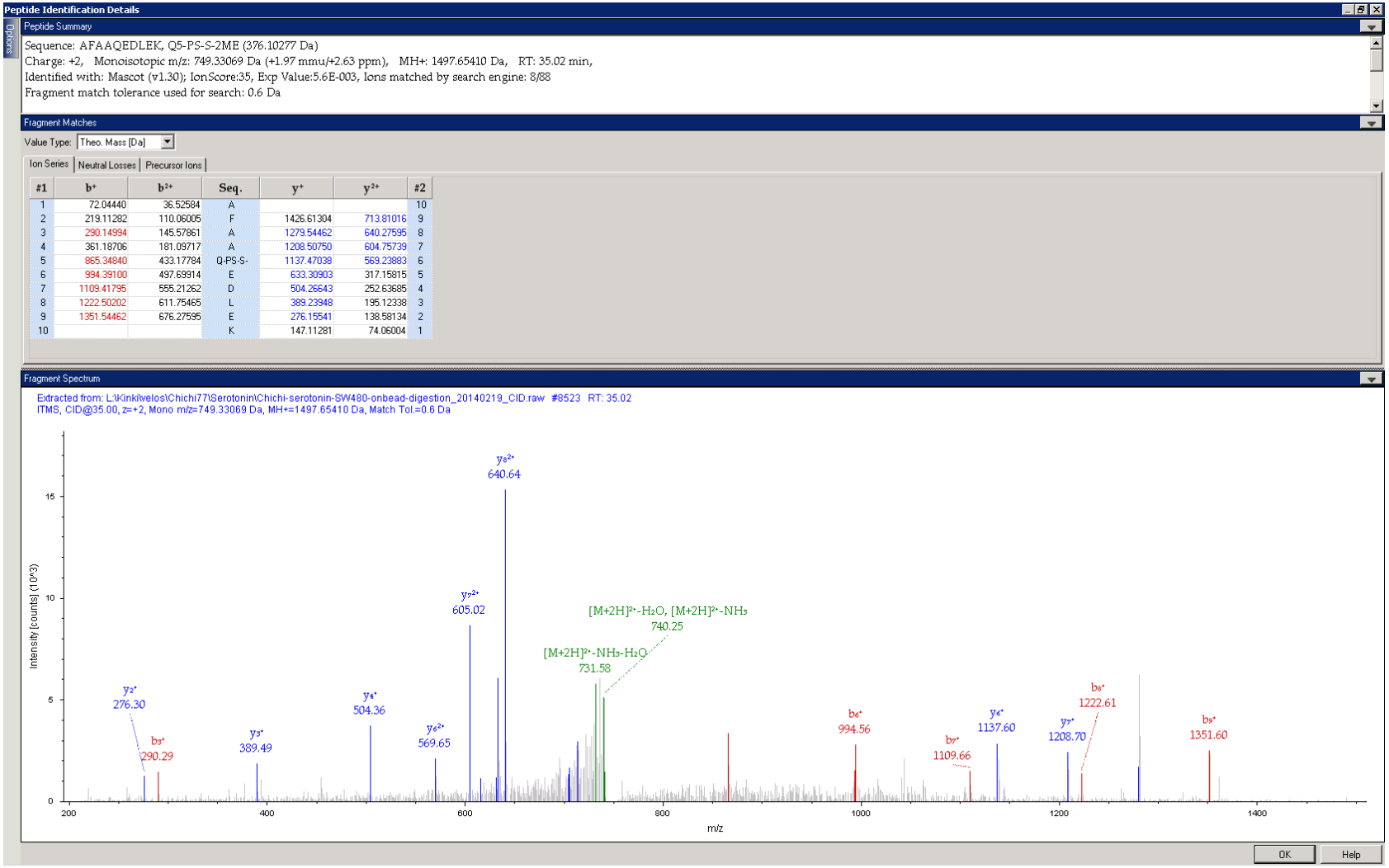
Desmoyokin_ SEDGVEGDLGETQ[147]SR_thiol product ion



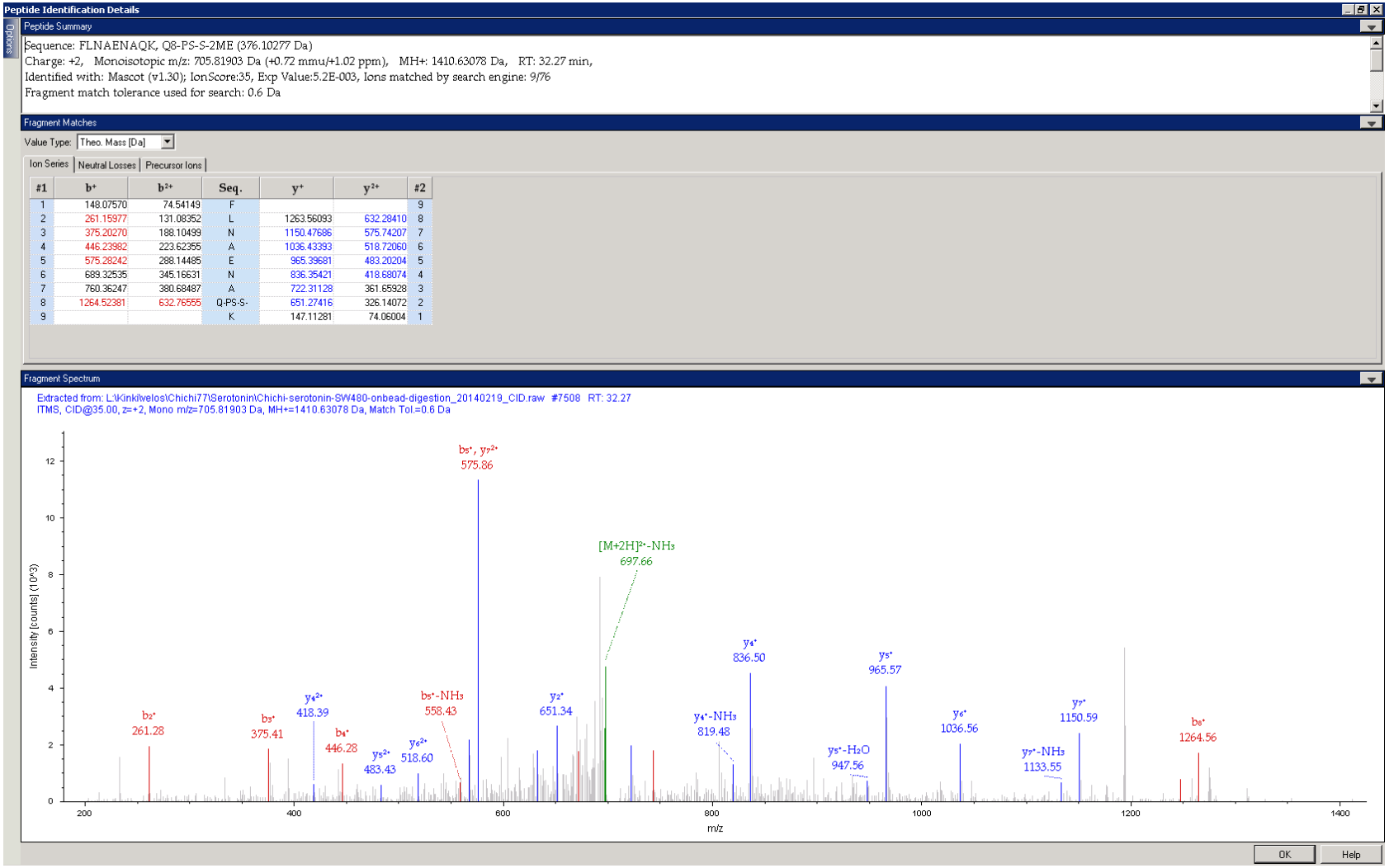
Y-box transcription factor_ NEGSESAPEGQ[181]AQQR_thiol product ion



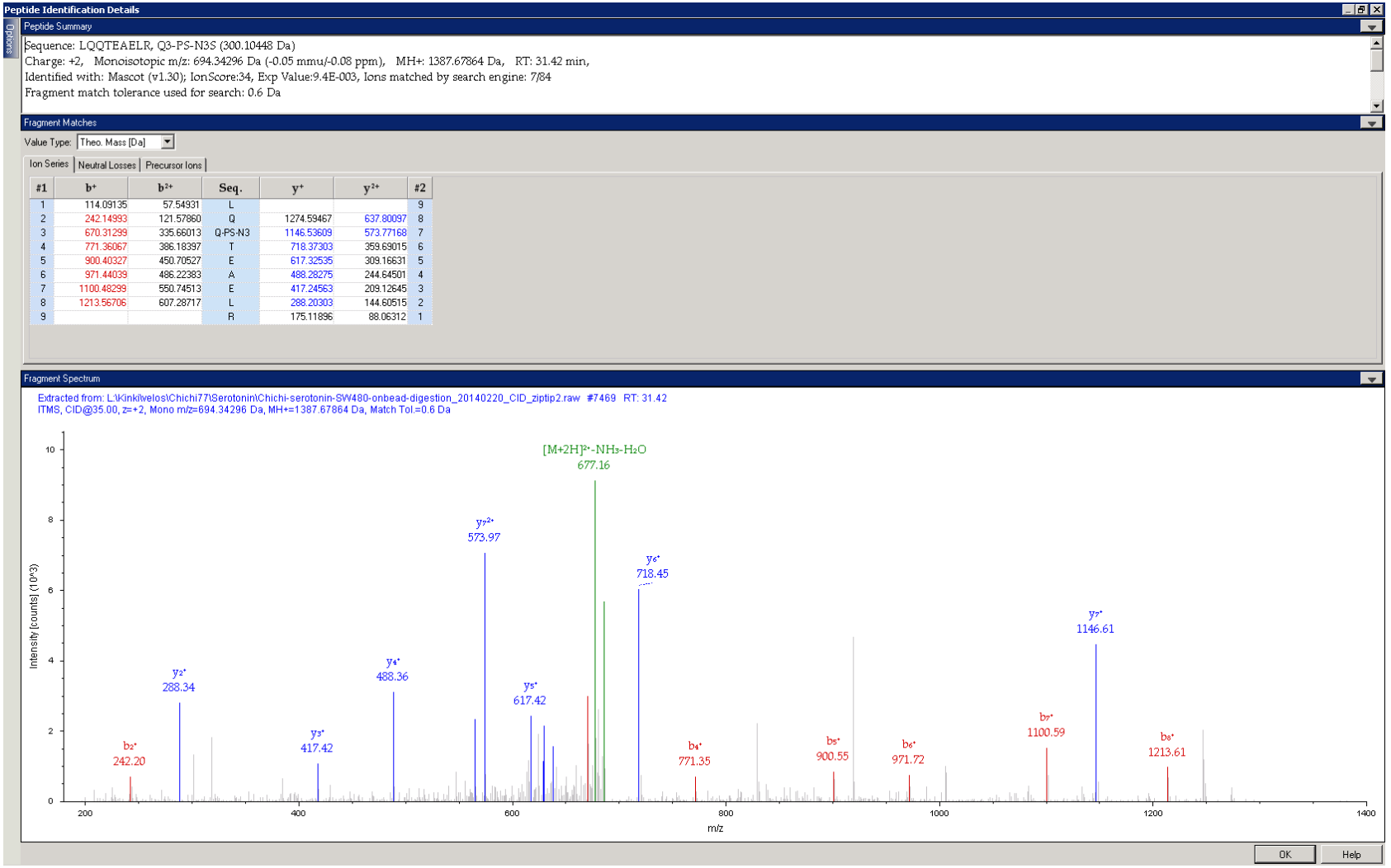
Radixin_ AFAAQ[453]EDLEK_2-ME-thiolated product ion



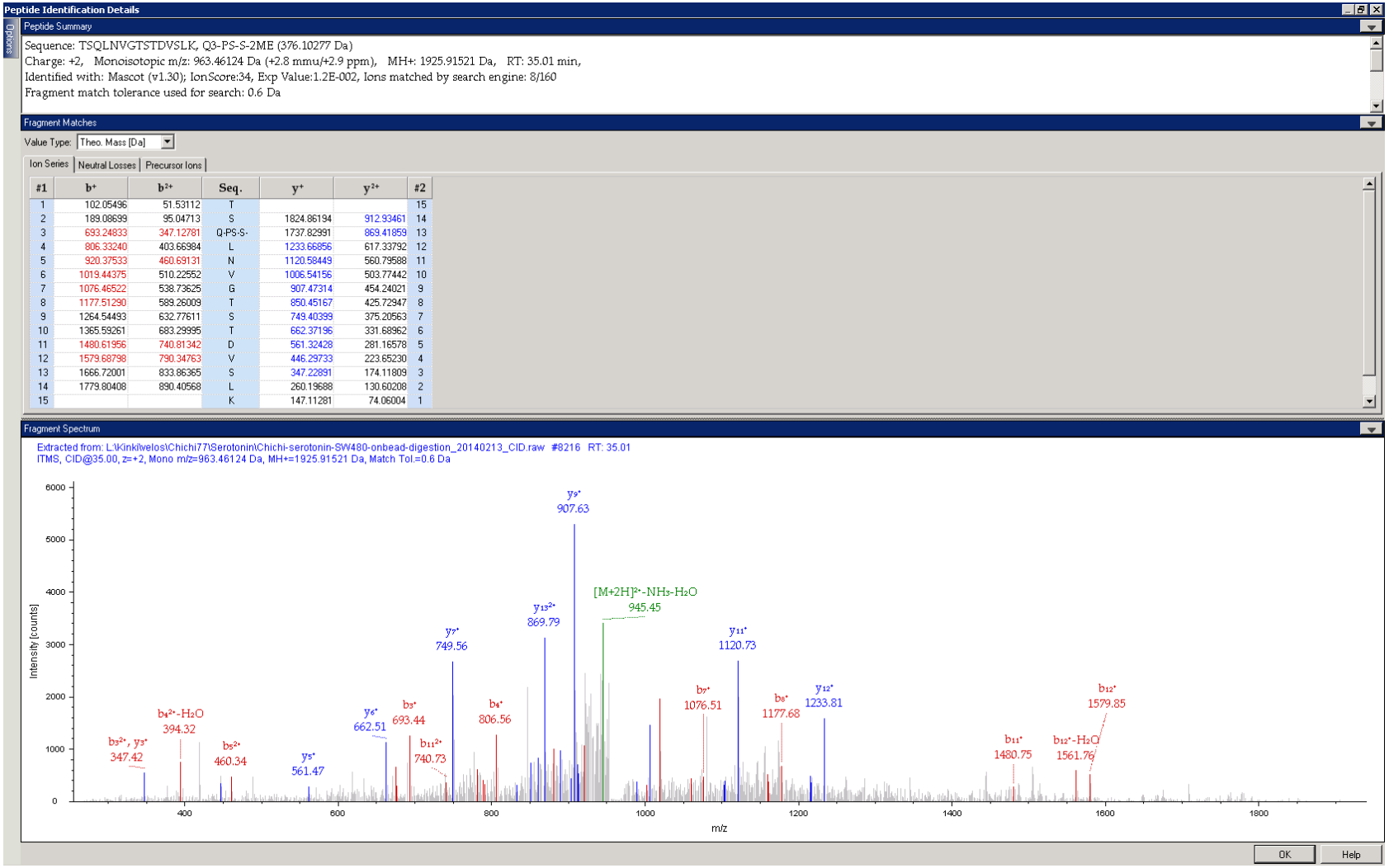
Ran-specific GTPase-activating protein (Ran-binding protein 1)_FLNAENAQ^{149}K_2-ME-thiolated product ion



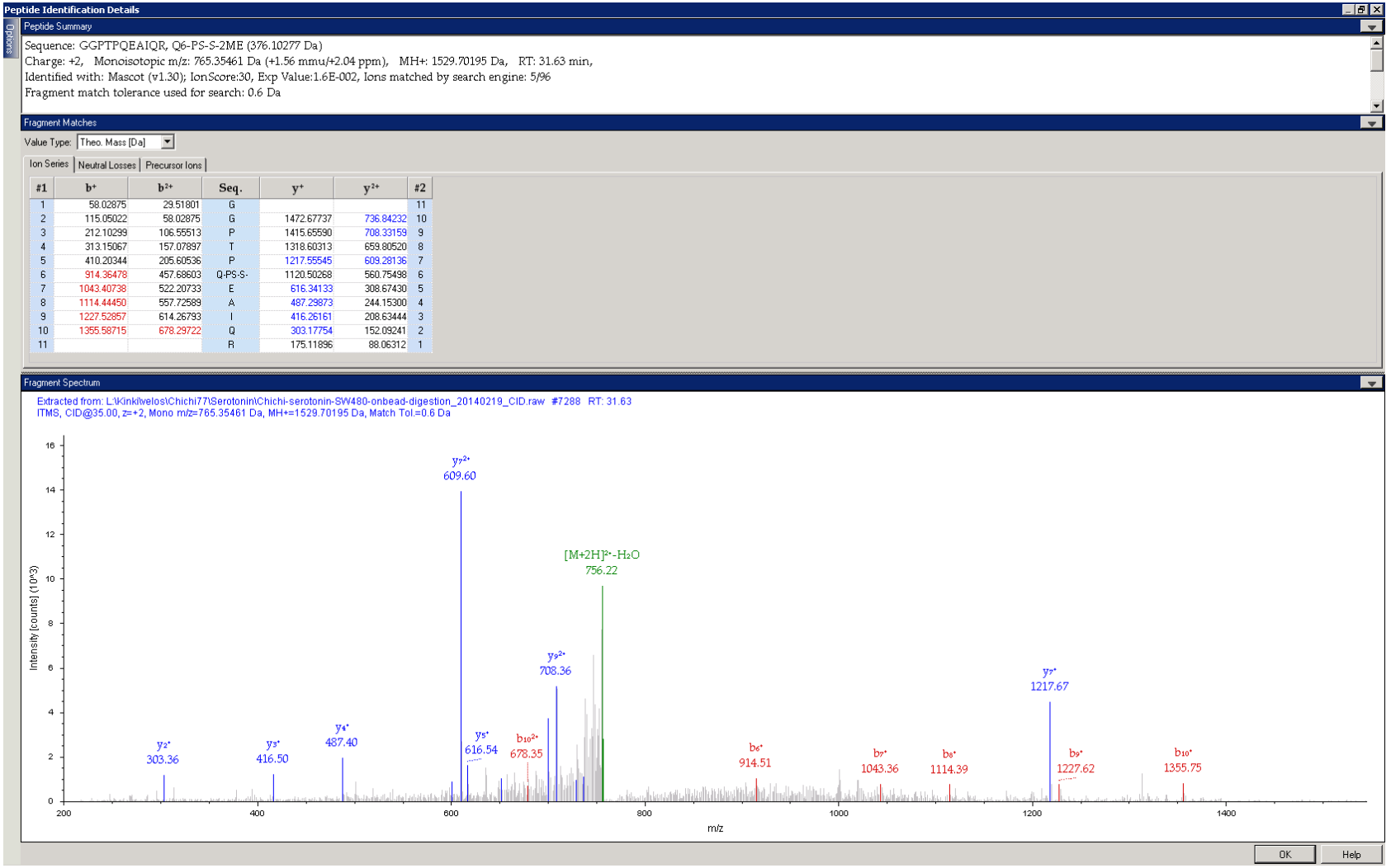
Valyl-tRNA synthetase_ LQQ[1245]TEAELR_thiol product ion



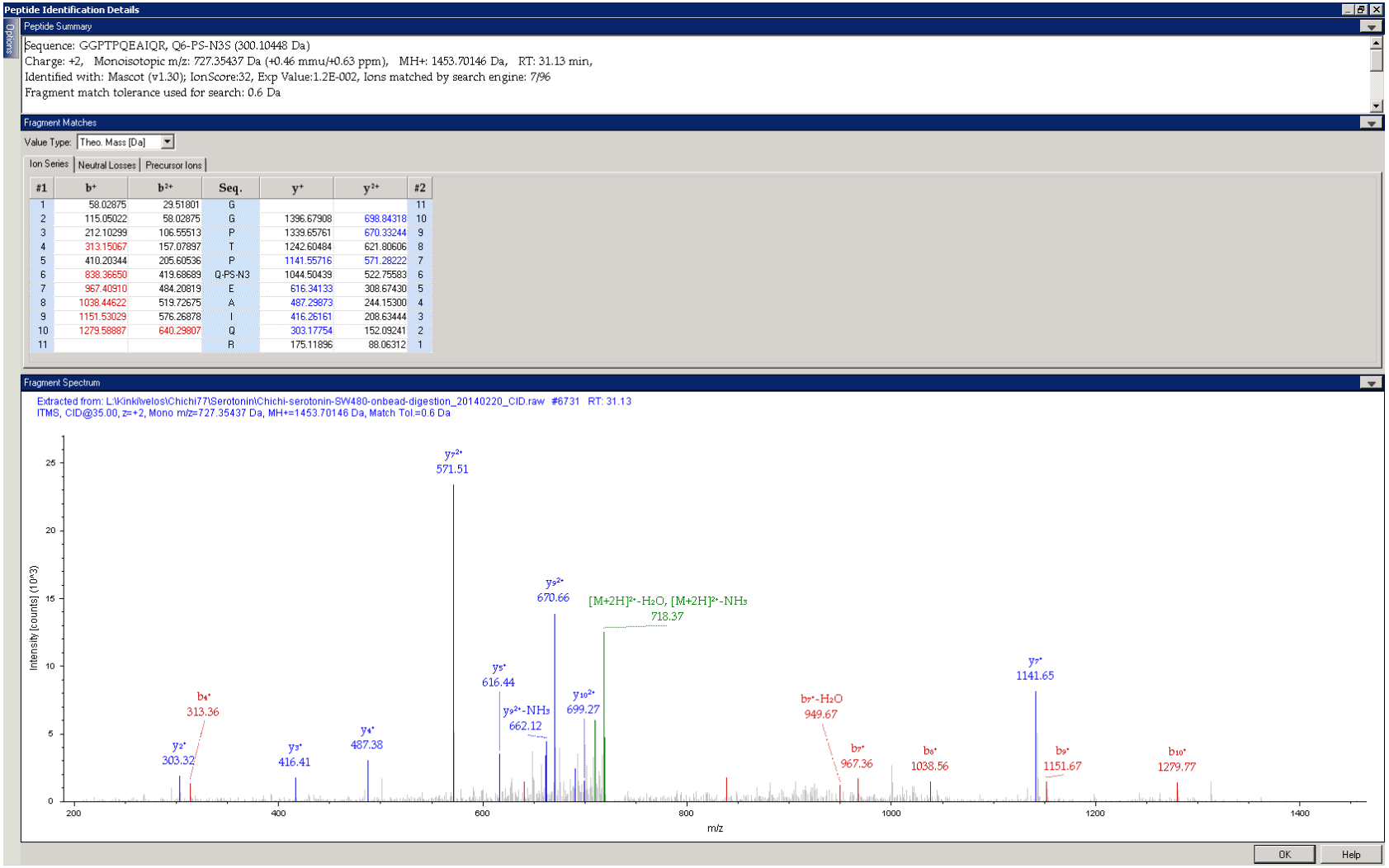
Filamin-C_ TSQ[1956]LNVGTSTDVSLK_2-ME-thiolated product ion



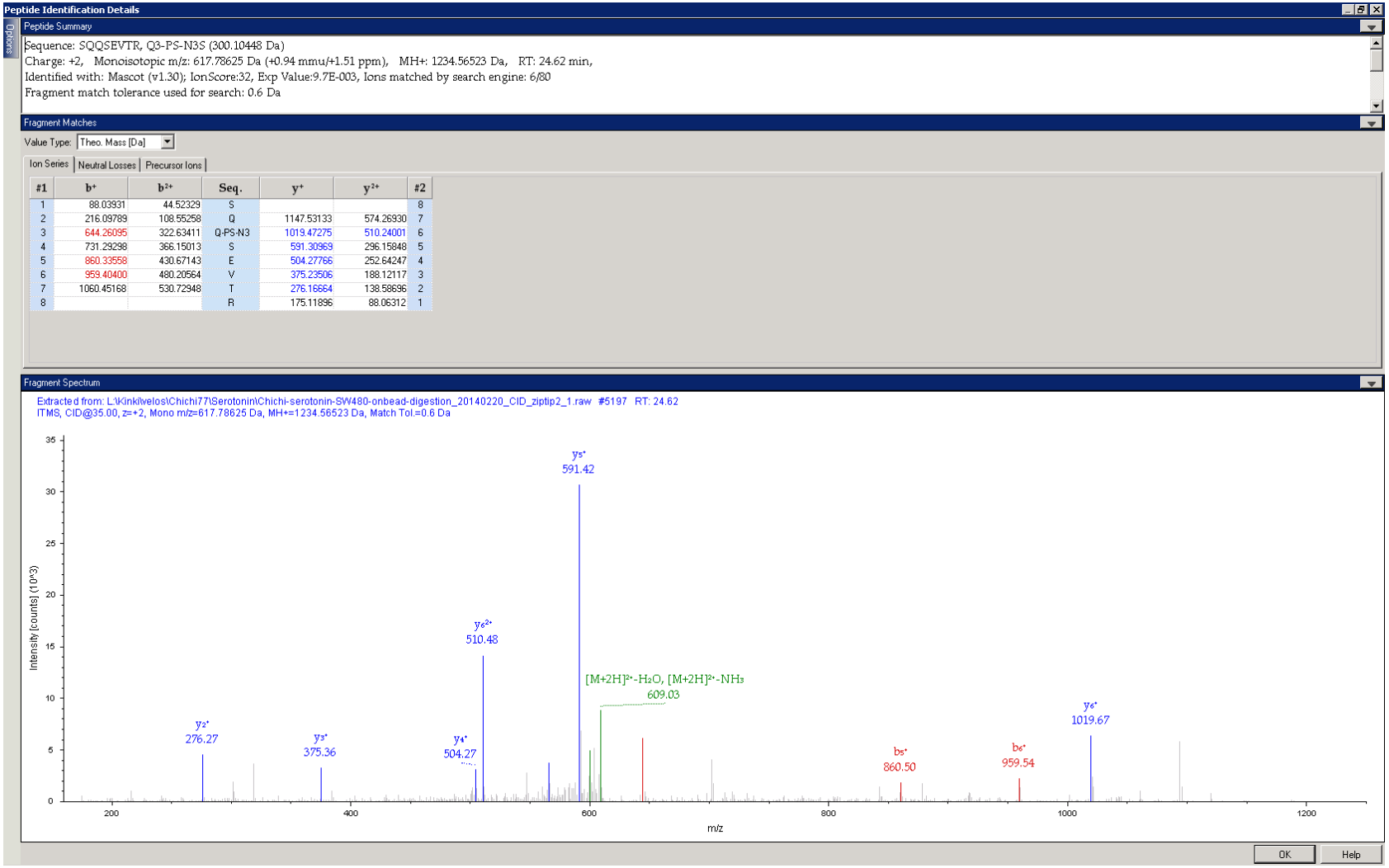
Chromatin-modifying protein 4b_ GGPTPQ[23]EAIQR_2-ME-thiolated product ion



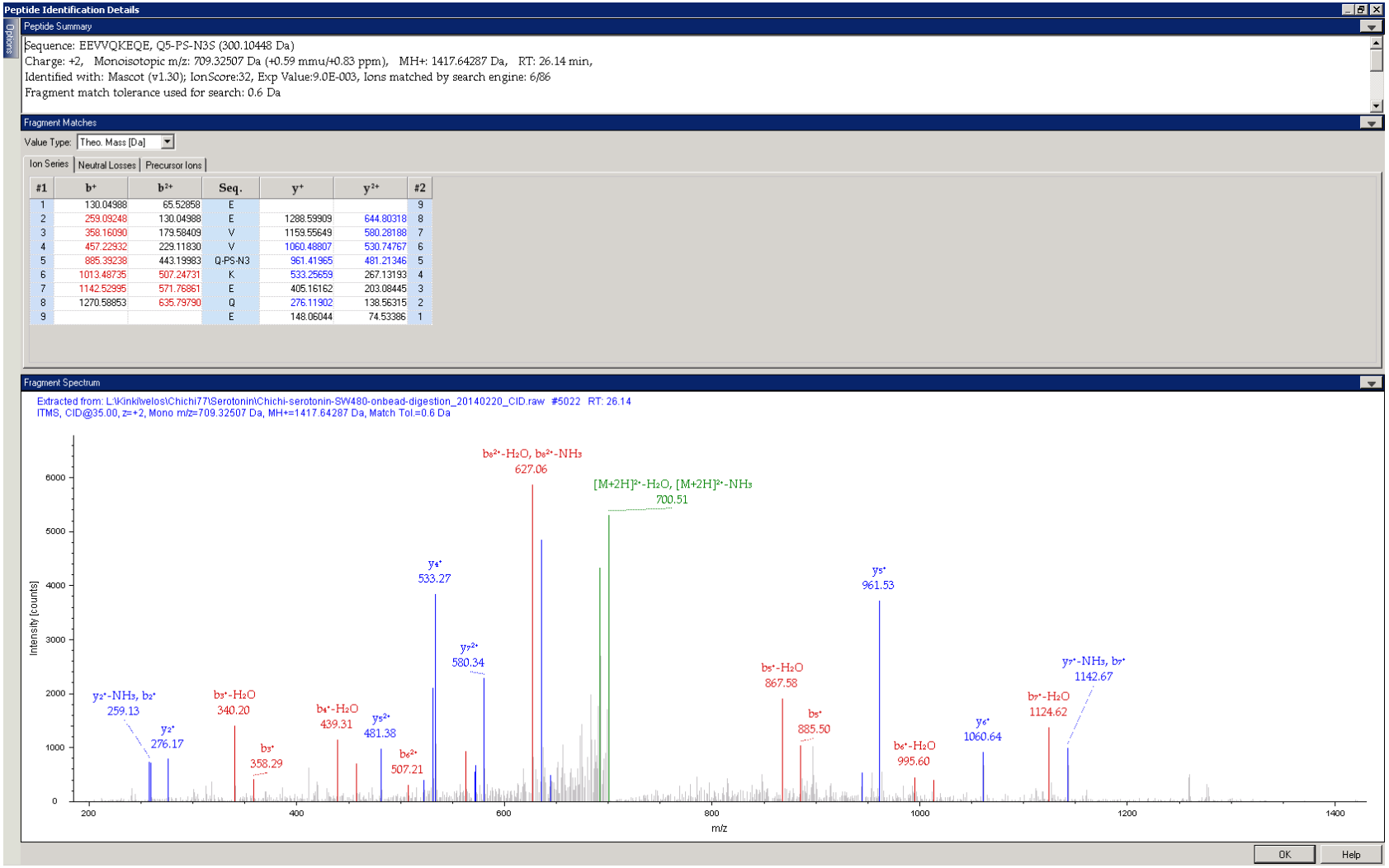
Chromatin-modifying protein 4b_ GGPTPQ[23]EAIQR_thiol product ion



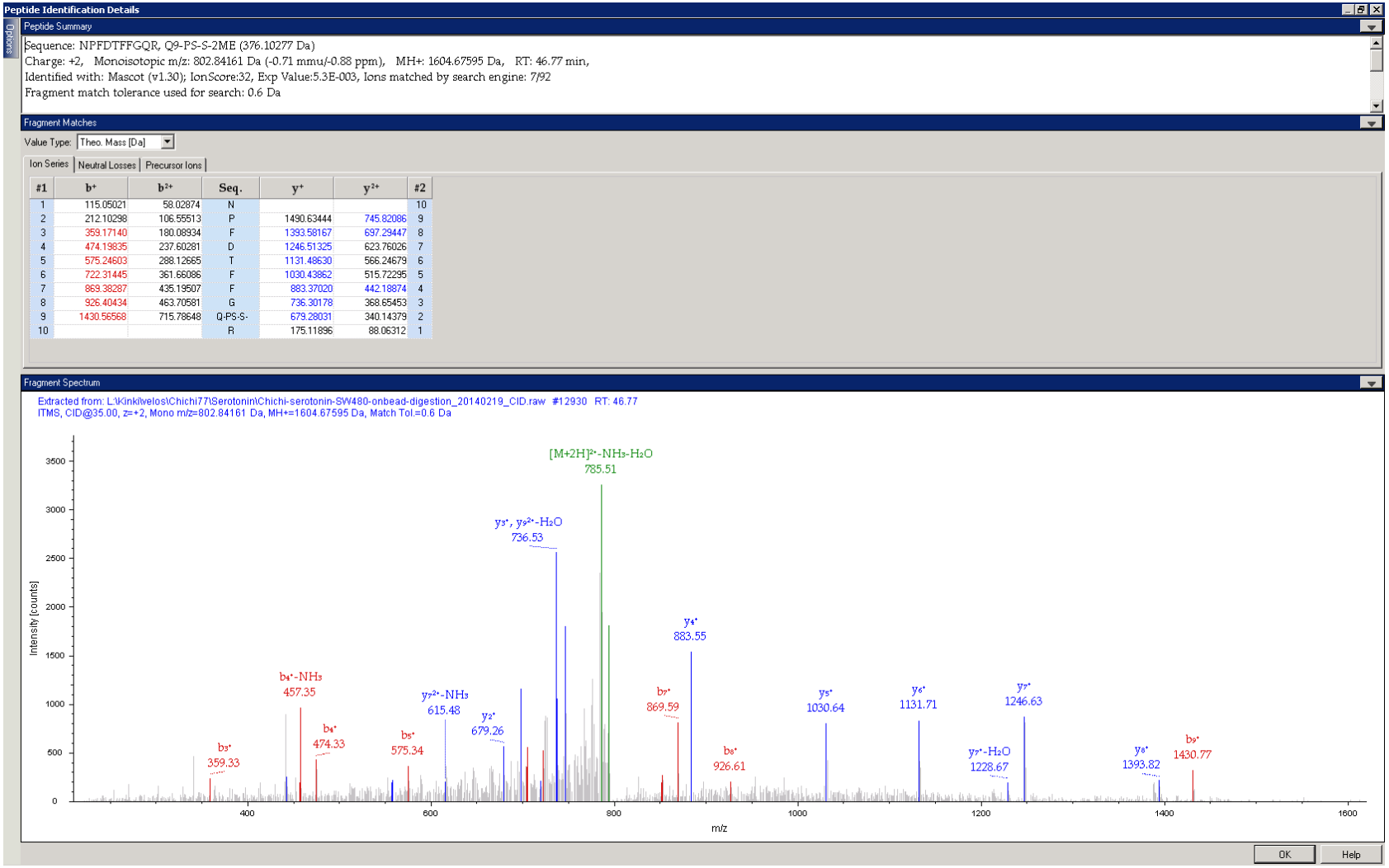
SH3 domain-binding protein 1_ SQQ[21]SEVTR_thiol product ion



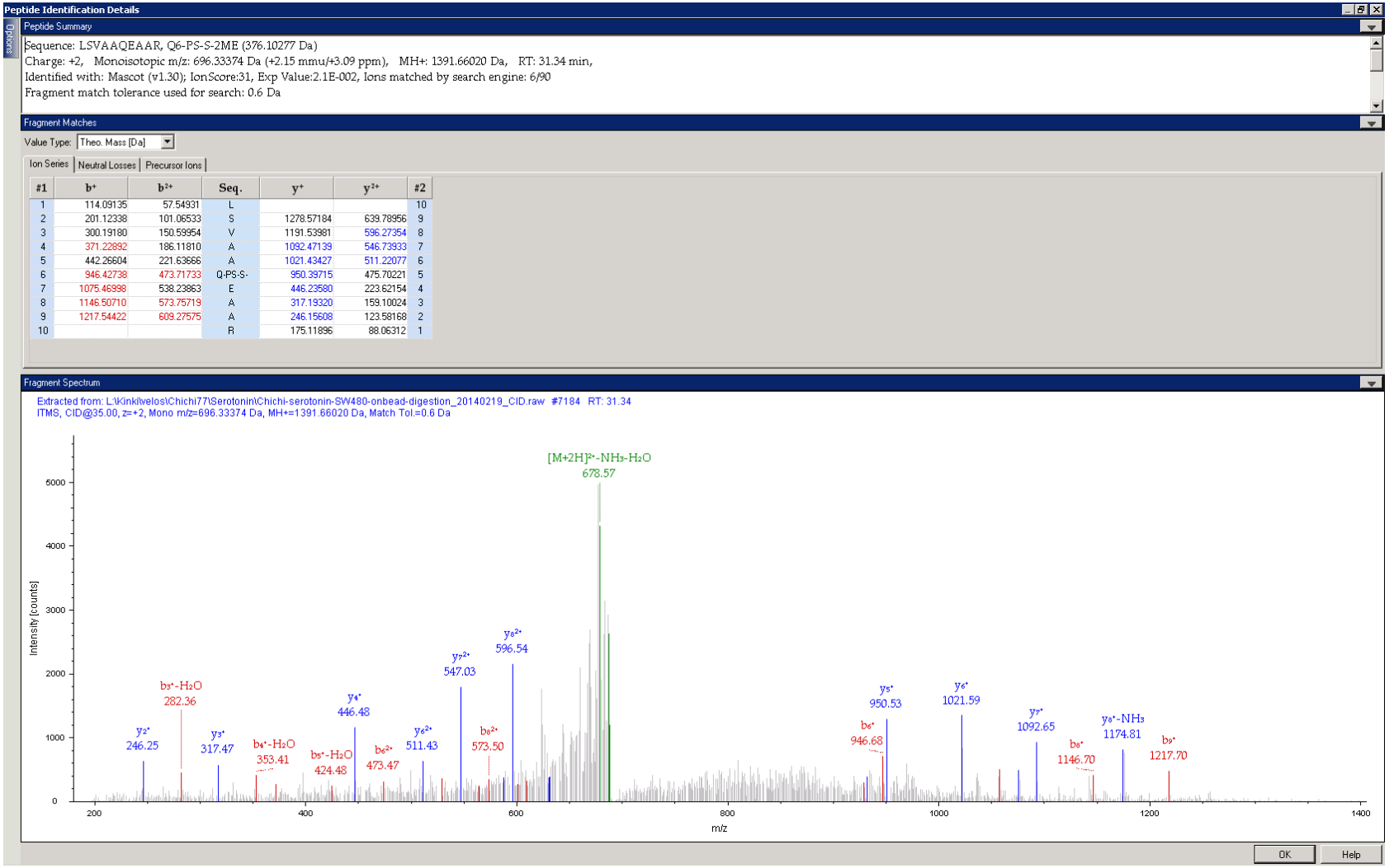
Eukaryotic translation initiation factor 4H_ EEVVQ^{244}KEQE_thiol product ion



Heat shock protein 40_ NPFDTFFGQ[118]R_2-ME-thiolated product ion

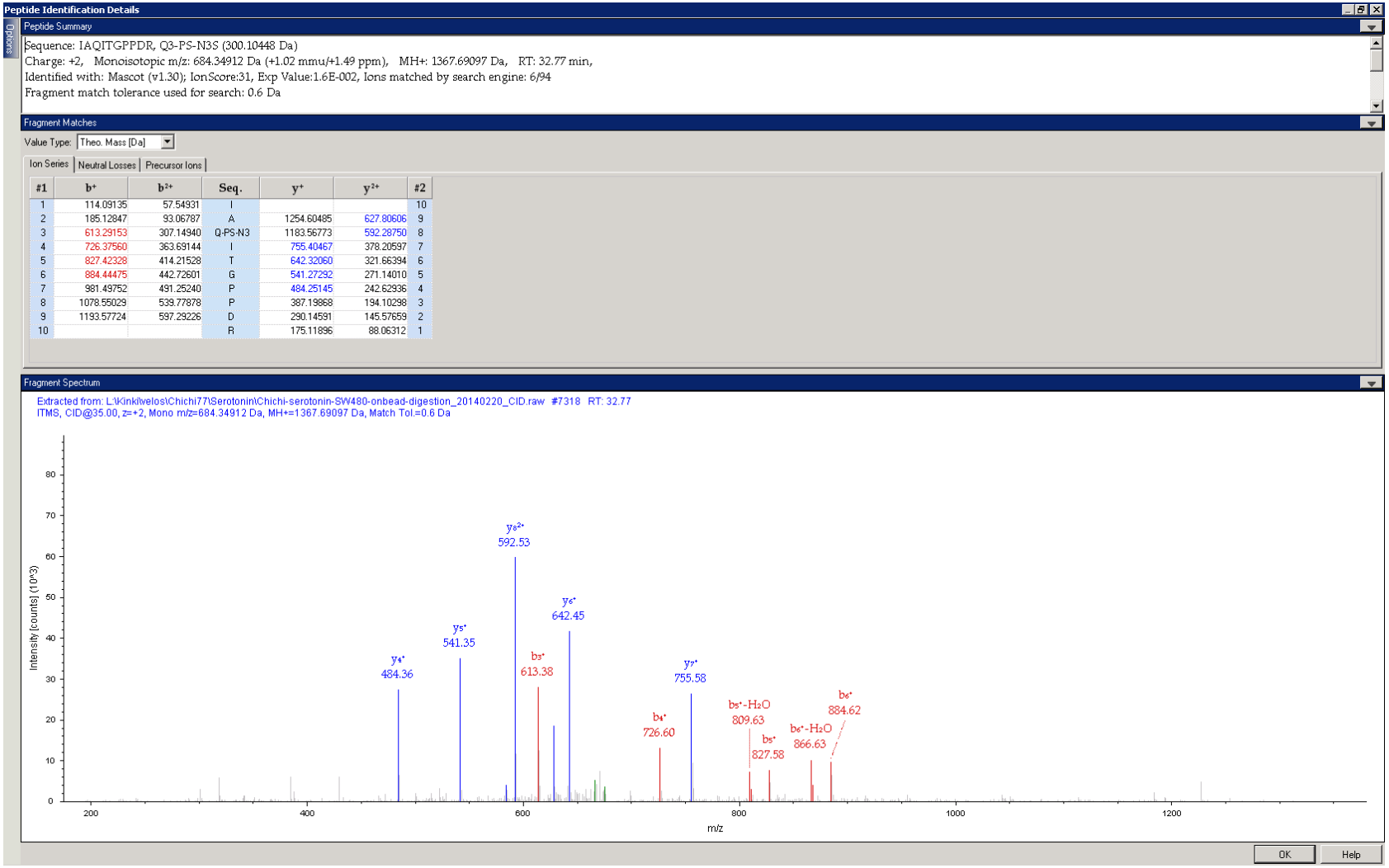


Plectin_ LSVAAQ[2425]EAAR_2-ME-thiolated product ion

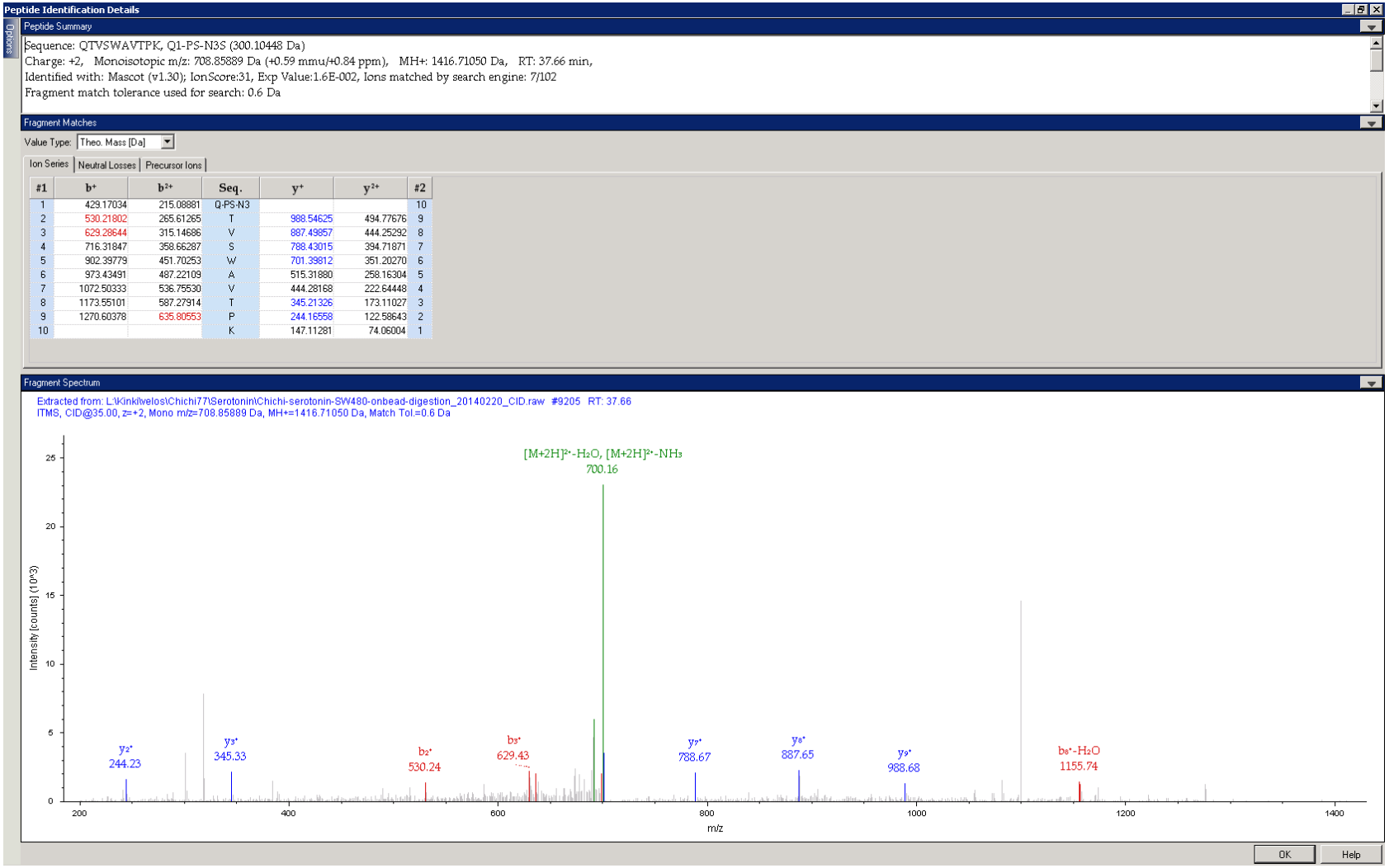


Mitochondrial ATP synthase subunit delta_ AEIQ^{154}IR_thiol product ion

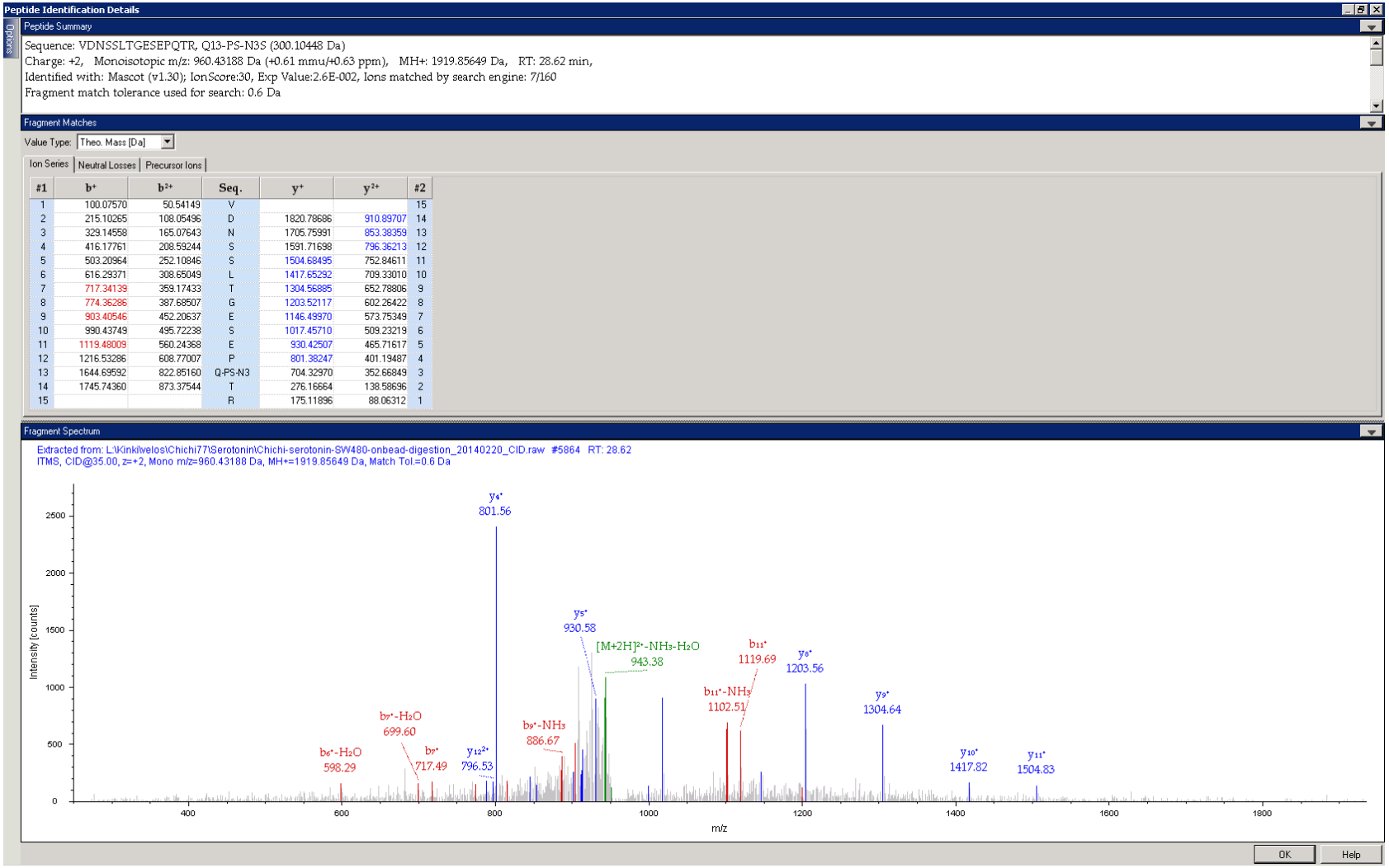
DNA helicase V_ IAQ^{324}ITGPPDR_thiol product ion



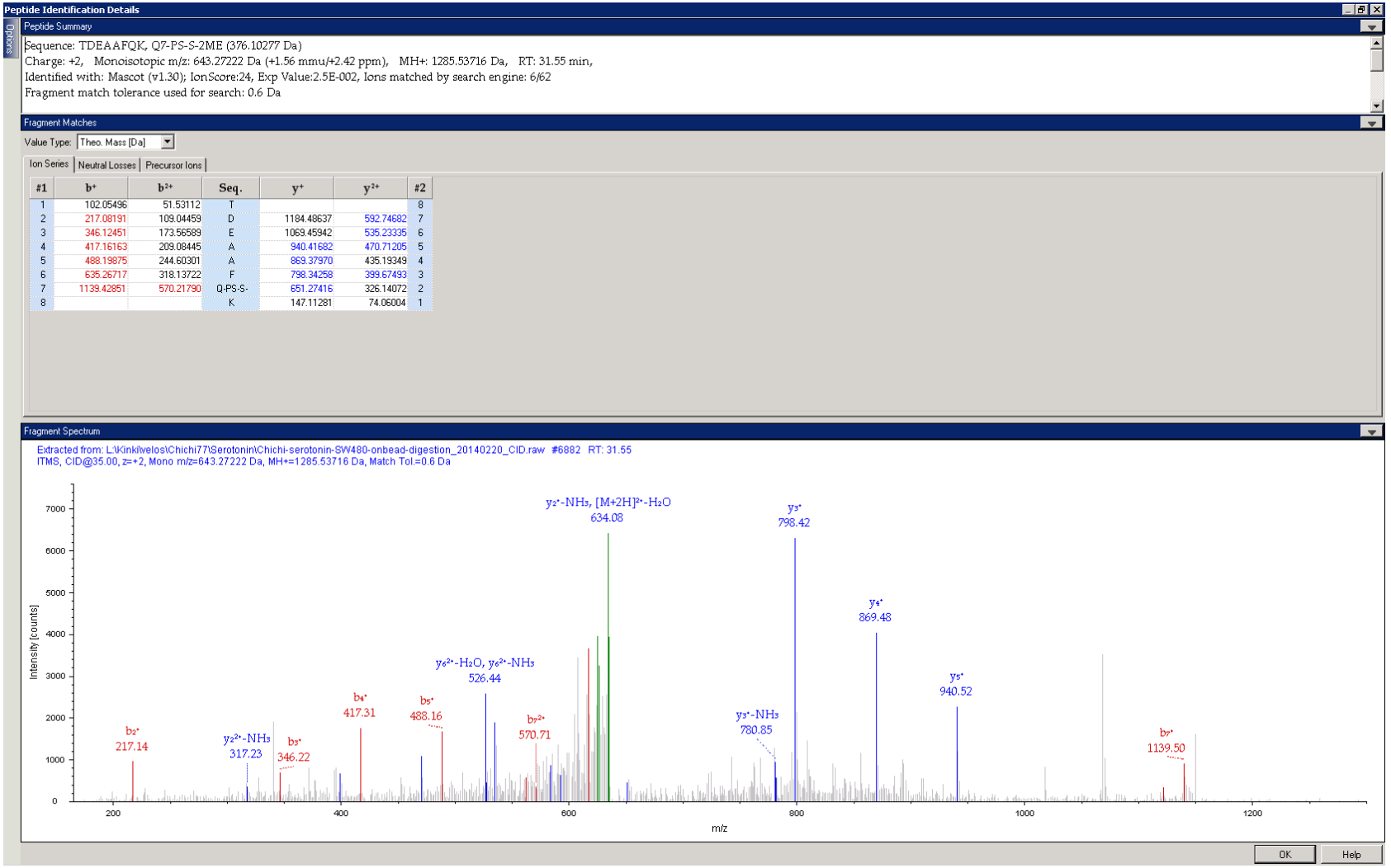
Alpha-2-macroglobulin_ Q[854]TVSWAVTPK_thiol product ion



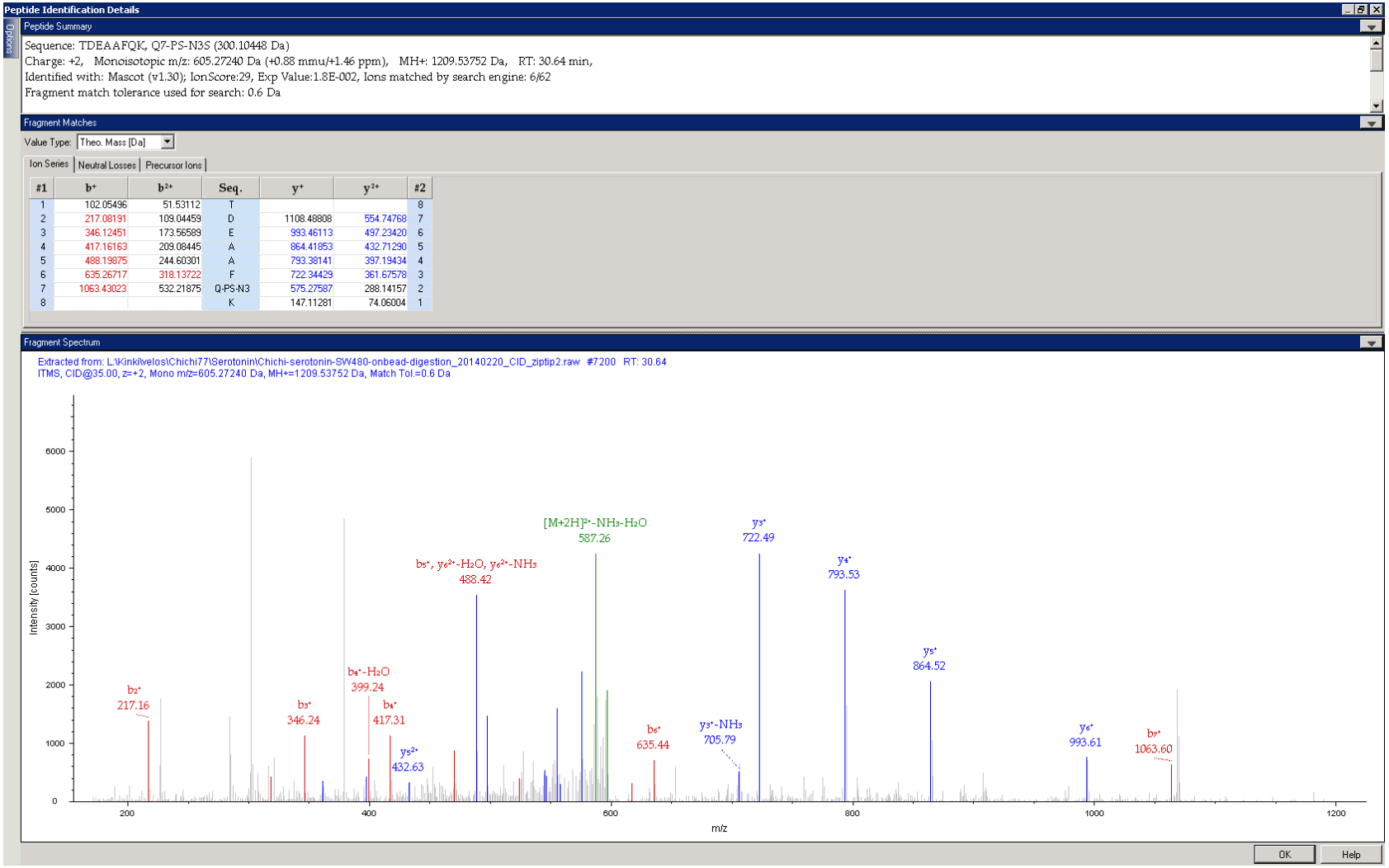
Na(+)/K(+) ATPase alpha(III) subunit_ VDNSSLTGESEPQ[215]TR_thiol product ion



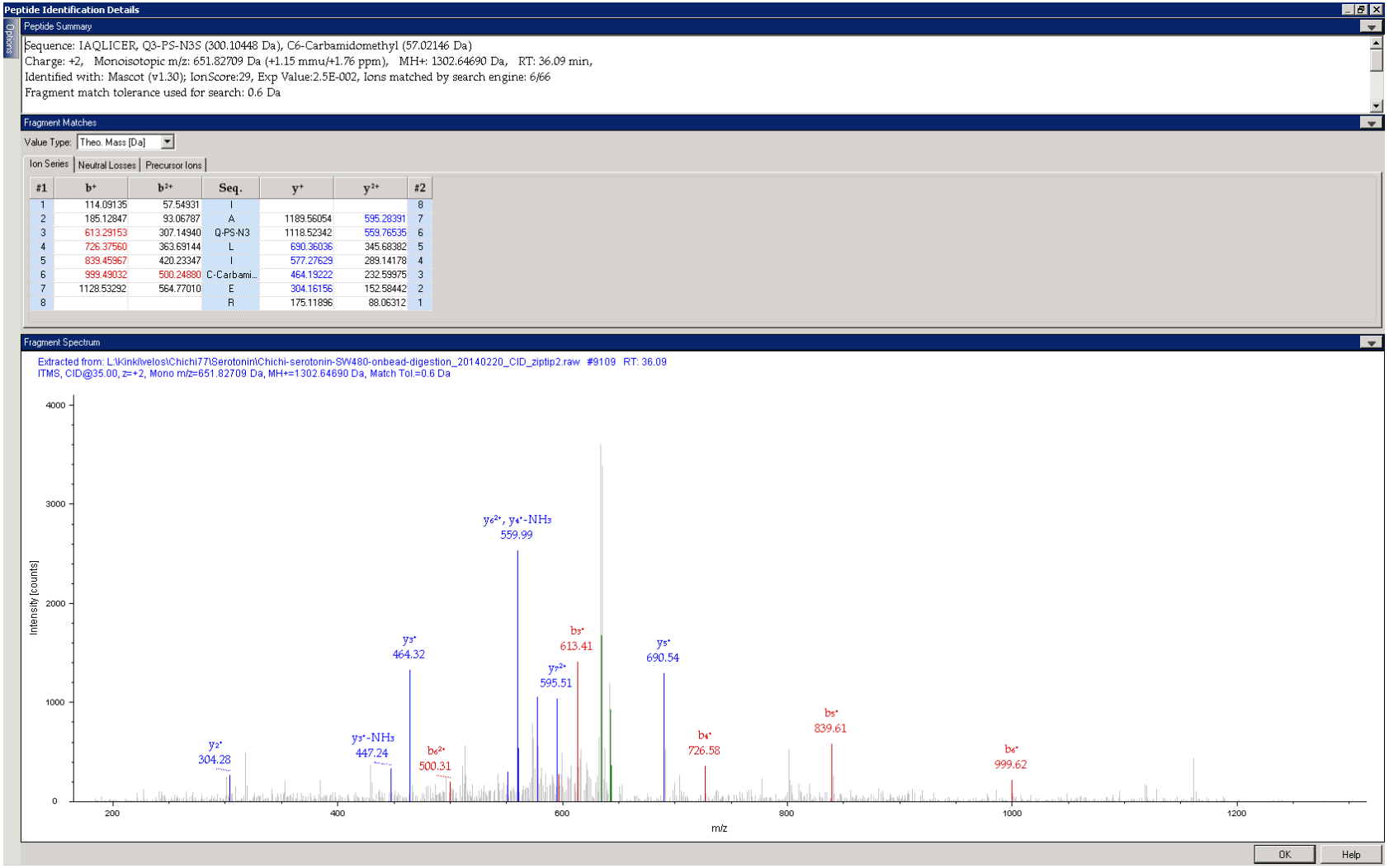
Calvasculin_ TDEAAFQ[56]K_2-ME-thiolated product ion



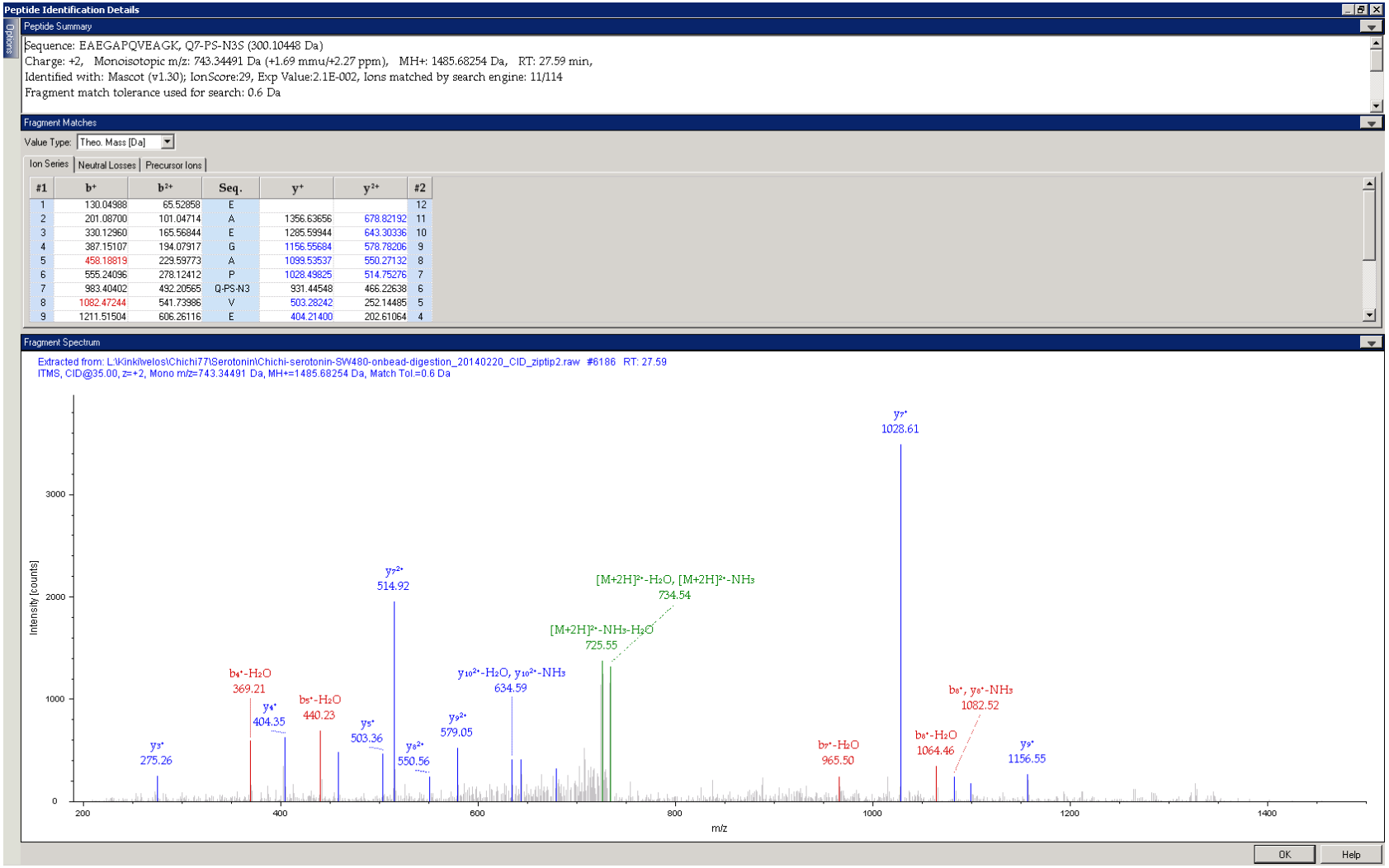
Calvasculin_ TDEAAFQ[56]K_thiol product ion



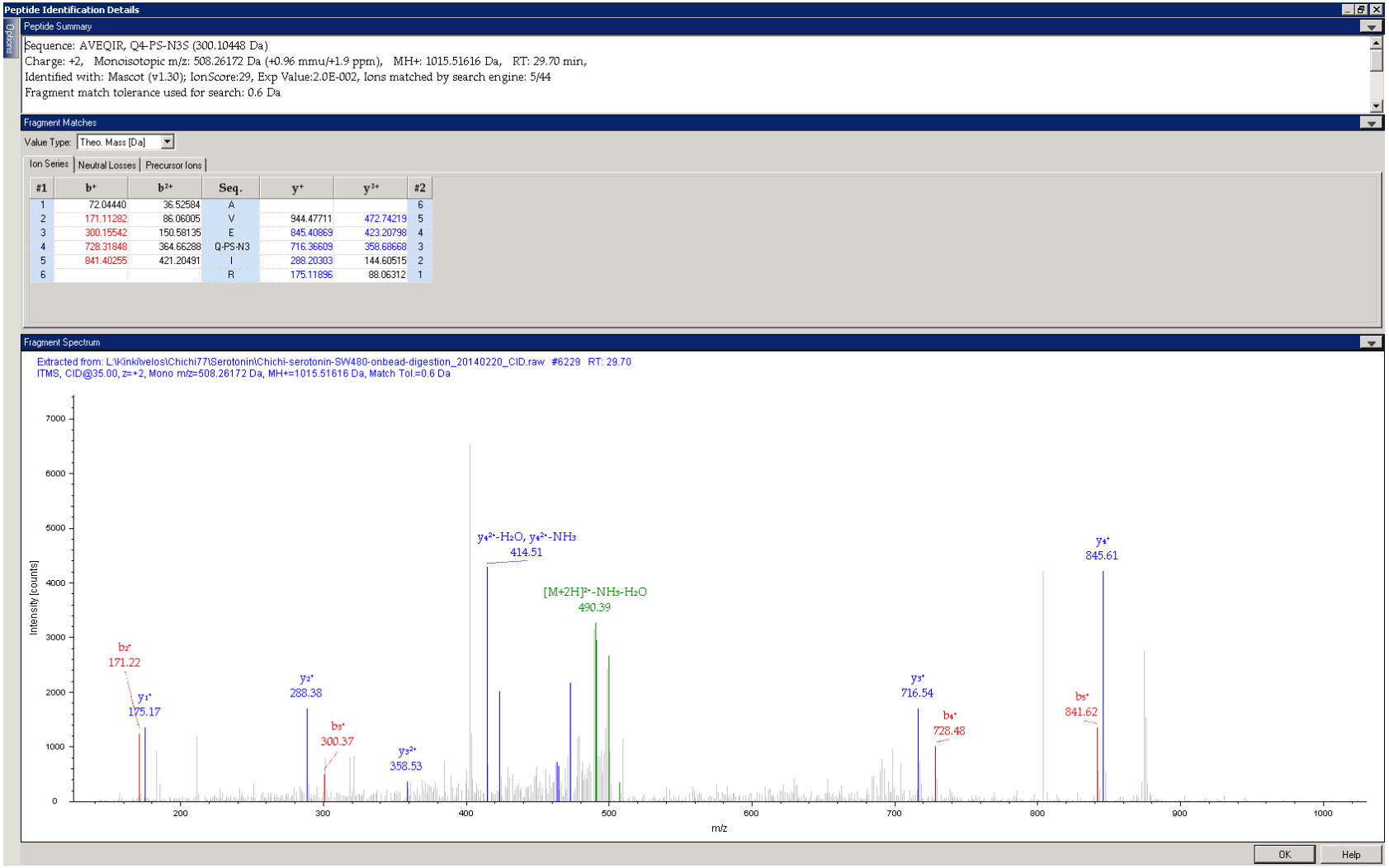
dUTP pyrophosphatase_ IAQ[219]LICER_thiol product ion



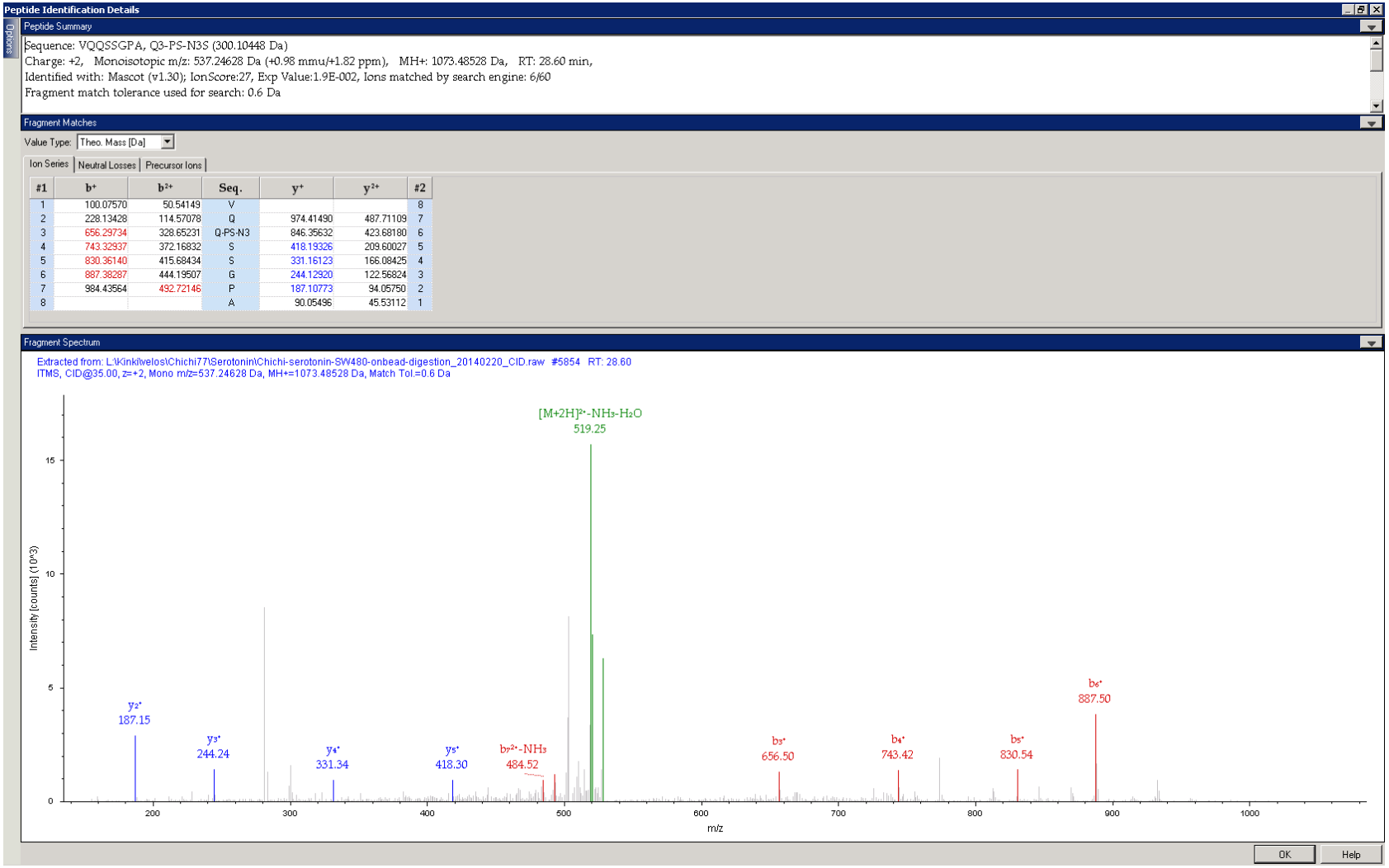
Caldesmon_ EAEGAPQ[529]VEAGK_thiol product ion



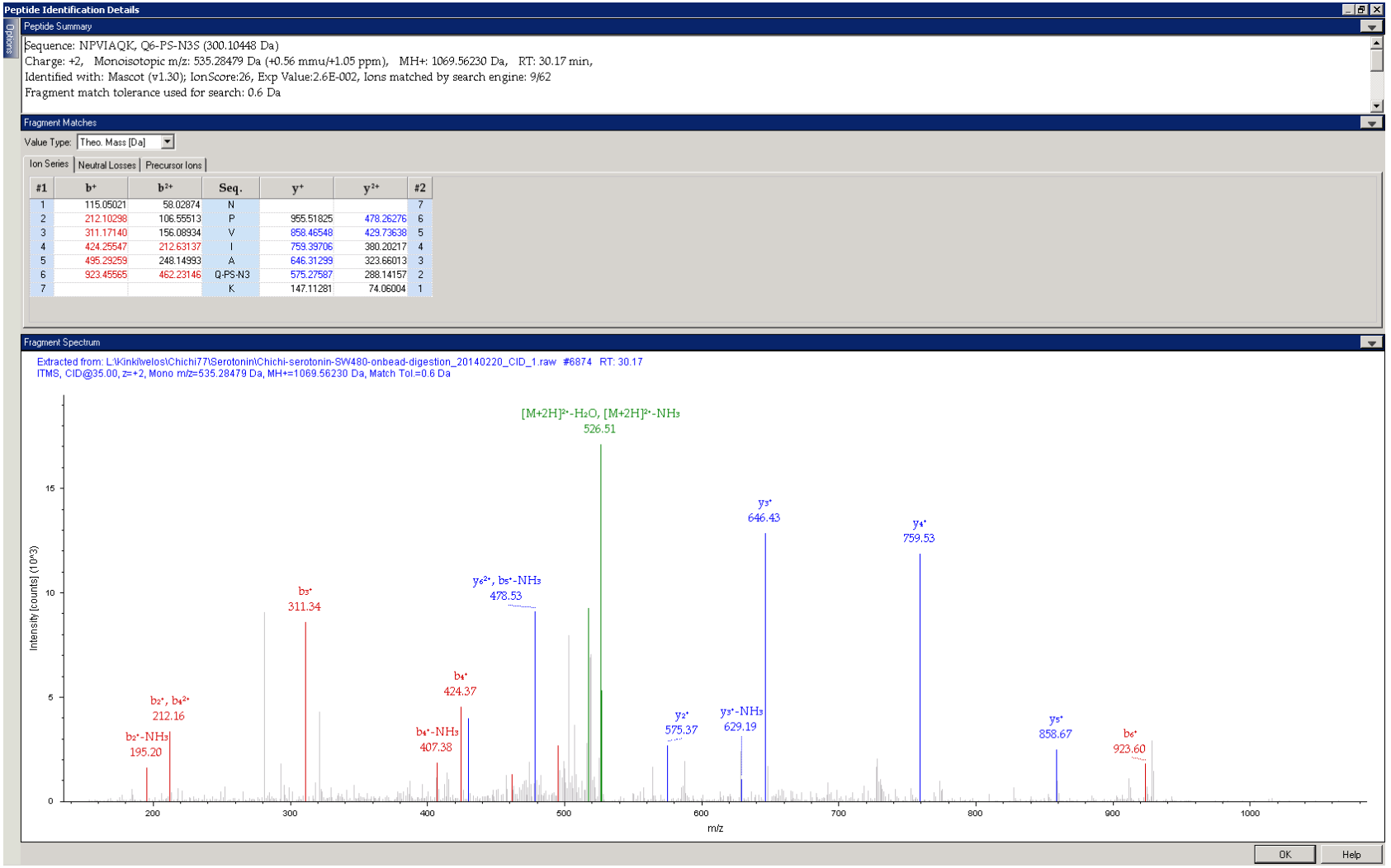
Zinc finger protein 162_ AVEQ[221]LR_thiol product ion



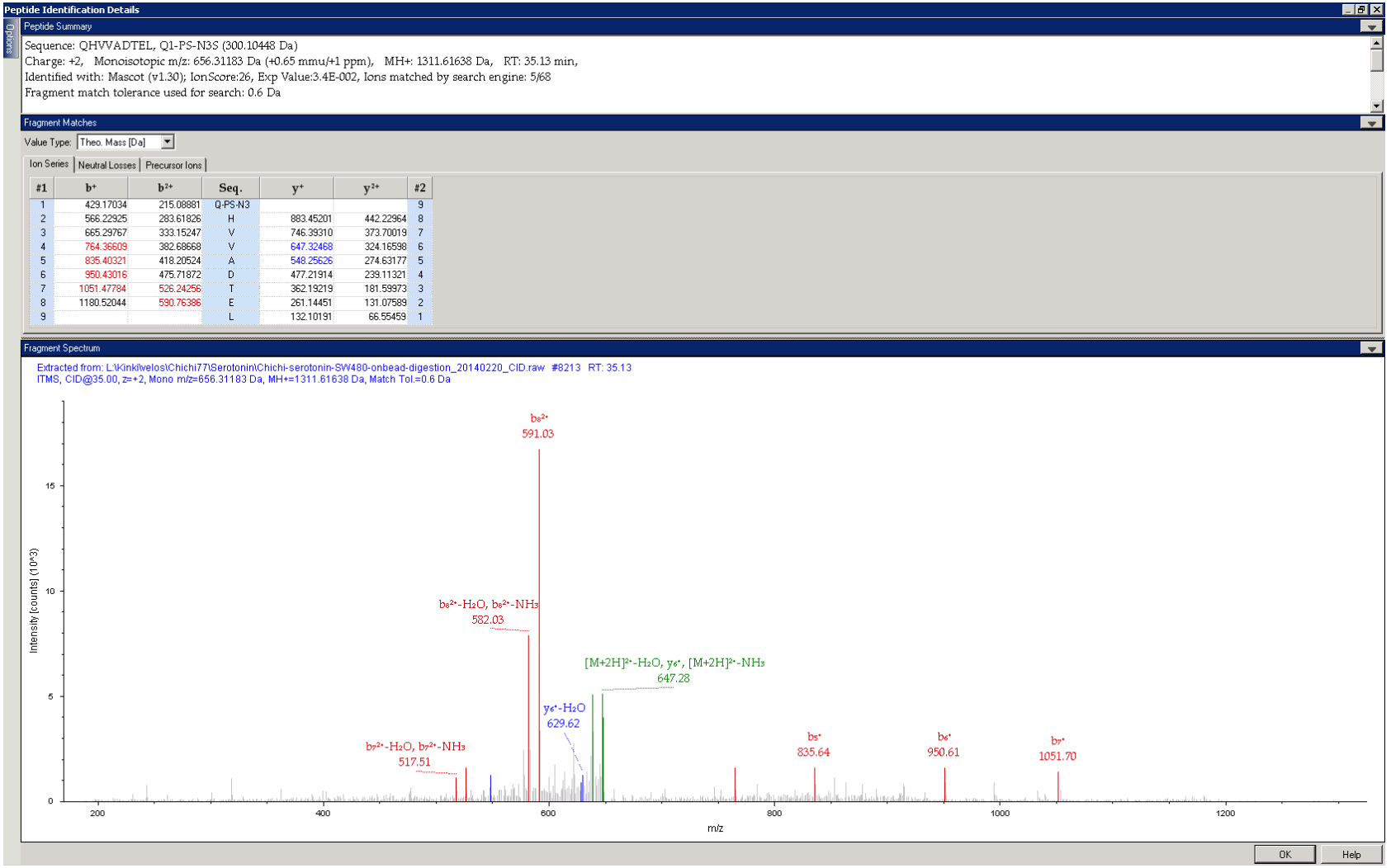
Mitochondrial import inner membrane translocase subunit Tim10_ VQQ[85]SSGPA_thiol product ion



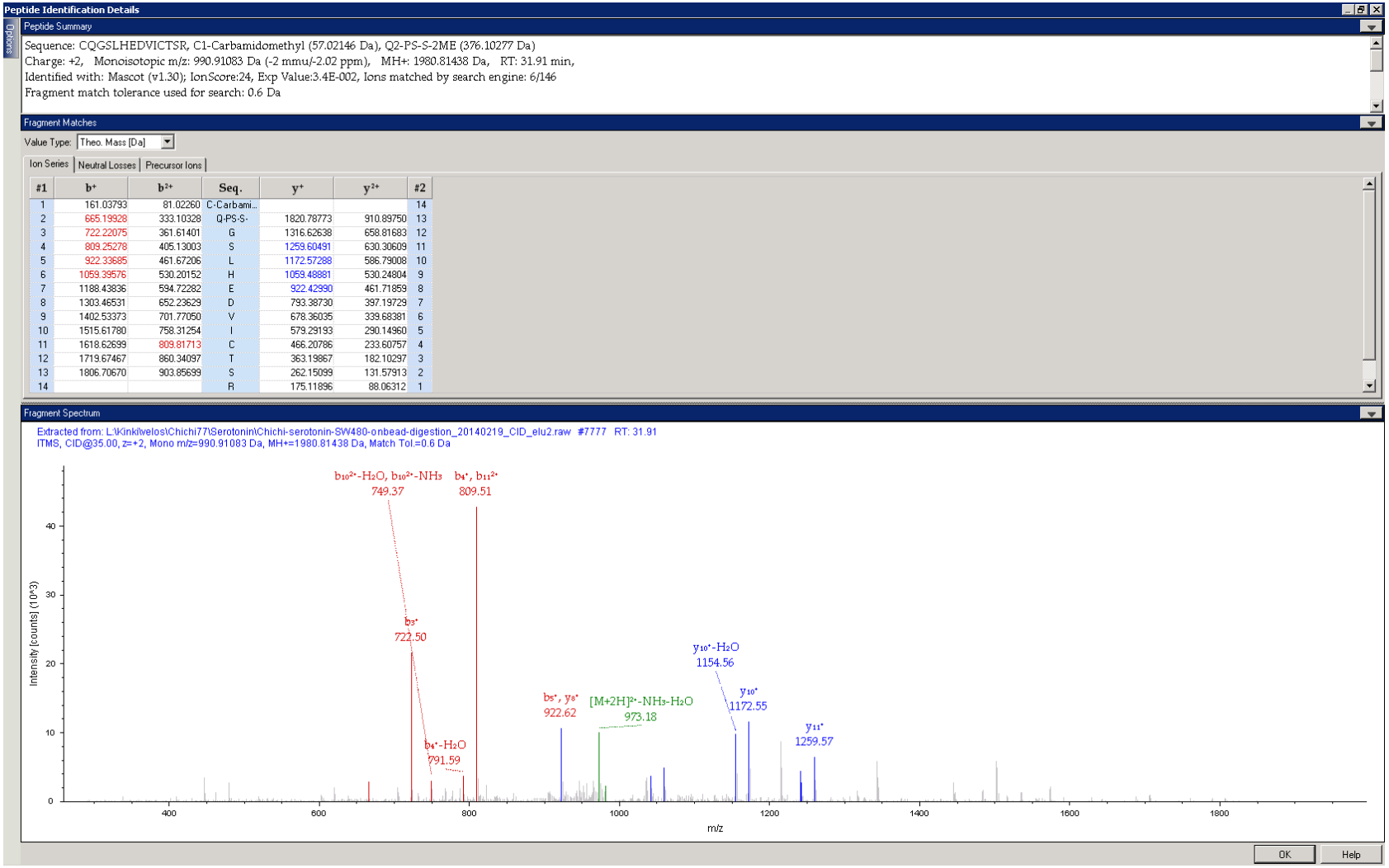
Stress-induced-phosphoprotein 1_NPVIAQ^{529}K_thiol product ion



CD34_Q[377]HVVADTEL_thiol product ion



DNA polymerase delta catalytic subunit_C[Q1062]GSLHEDVICTSR_2-ME-thiolated product ion



JKT41-binding protein_NQQ[145]DDGK_thiol product ion

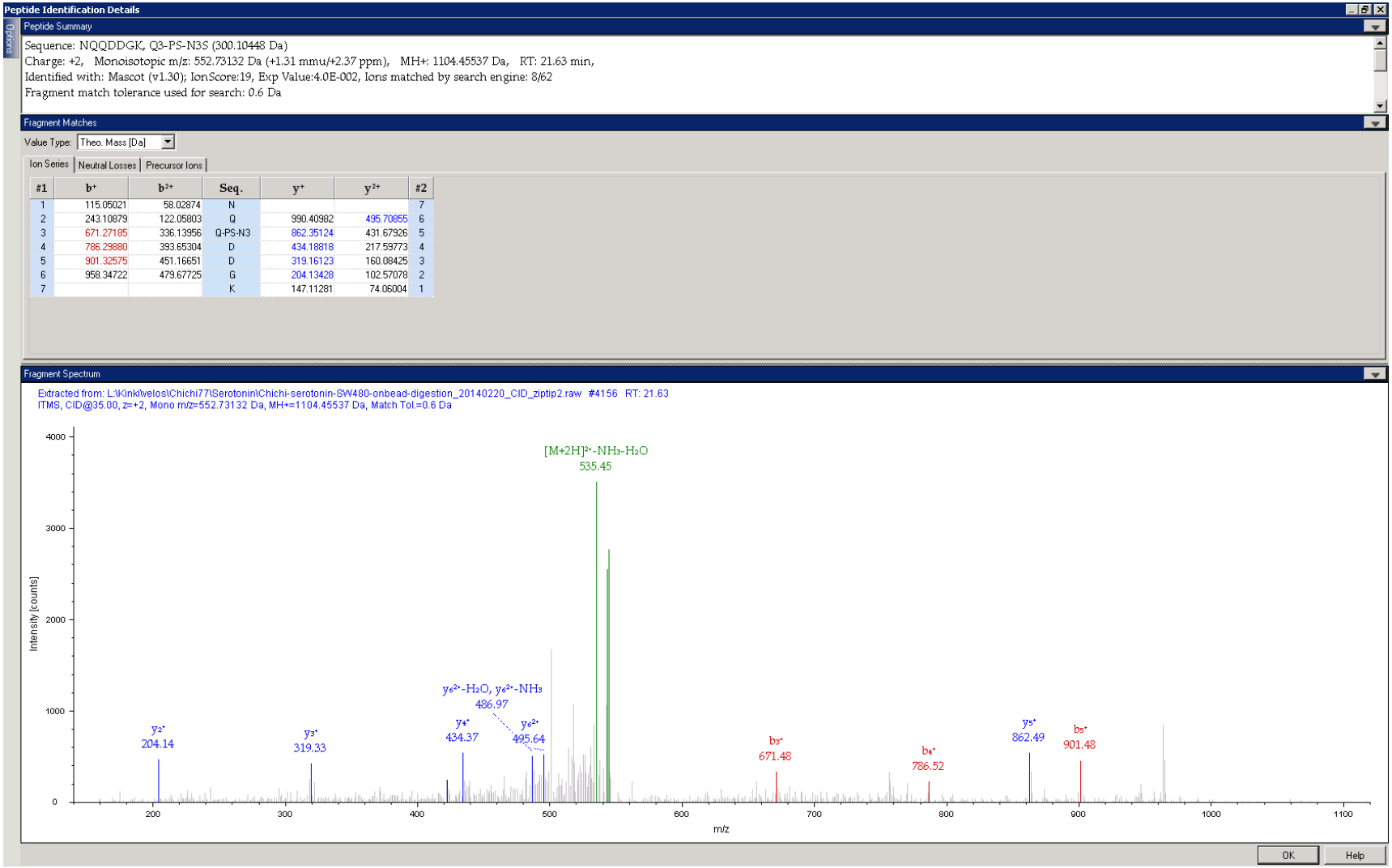


Table S2. A Comparison of CID vs HCD of 5-PT-modified Standard Peptide. *# PSMs* denotes the total number of identified peptides sequences (PSMs) for the protein, including those with redundant identifications.

CID:

Accession	Score	Coverage	# PSMs*	MW [kDa]	calc. pI
HRas57-68	5175.69	100.00	331	1.4	4.23

Sequence	# PSMs*	Modifications	IonScore	Exp Value	Charge	MH+ [Da]	ΔM [ppm]	m/z [Da]	RT [min]
DTAGqEEYSAMR	79	Q5(PS-S-2ME)	57	4.18752E-05	2	1733.67278	0.62	867.34003	40.91
DTAGqEEYSAMR	94	Q5(PS-N3SH)	53	9.86181E-05	2	1657.67290	-0.31	829.34009	39.90
DTAGqEEYSAmR	40	Q5(PS-N3SH); M11(Oxidation)	43	0.0009976	2	1673.66863	0.18	837.33795	33.94
DTAGqEEYSAmR	43	Q5(PS-S-2ME); M11(Oxidation)	41	0.001721696	2	1749.66692	0.17	875.33710	38.94

HCD:

Accession	Score	Coverage	# PSMs*	MW [kDa]	calc. pI
HRas57-68	6091.69	100.00	188	1.4	4.23

Sequence	# PSMs*	Modifications	IonScore	Exp Value	Charge	MH+ [Da]	ΔM [ppm]	m/z [Da]	RT [min]
DTAGqEEYSAMR	55	Q5(PS-S-2ME)	77	3.58886E-07	2	1733.67632	2.66	867.34180	50.17
DTAGqEEYSAMR	38	Q5(PS-N3SH)	70	2.18251E-06	2	1657.67461	0.72	829.34094	41.17

DTAGqEEYSAmR	28	Q5(PS-N3SH); M11(Oxidation)	60	1.94517E-05	2	1673.66875	0.25	837.33801	33.63
DTAGqEEYSAmR	35	Q5(PS-S-2ME); M11(Oxidation)	60	2.13775E-05	2	1749.66570	-0.52	875.33649	34.99

Table S3. A Comparison of CID vs HCD of 5-PT-modified Peptides from SW480 Lysate. *# PSMs* denotes the total number of identified peptides sequences (PSMs) for the protein, including those with redundant identifications.

CID: 40 5-PT-modified peptides

Sequence	# PSMs	Protein Group Accessions	Modifications	IonScore	Exp Value	Charge	MH+ [Da]	ΔM [ppm]	m/z [Da]
SqEQLAAELAEYTAK	4	P15311	Q2(PS-S-2ME)	66	7.36491E-06	2	2027.91838	-0.89	1014.46283
IPSAVGYQPTLATDMGTMqER	2	P06576	Q19(PS-S-2ME)	60	3.61465E-05	3	2642.18058	-2.47	881.39838
INPDGSqSVVEVPYAR	3	Q9Y2B0	Q7(PS-S-2ME)	56	8.56167E-05	2	2106.97075	-1.37	1053.98901
TGASWTDNIMAqK	2	Q7Z6K5	Q12(PS-S-2ME)	49	0.000168319	2	1798.76567	-2.98	899.88647
GVGqADWTPDLGLR	2	Q9C0C2	Q4(PS-S-2ME)	48	0.00040711	2	1860.84844	-1.95	930.92786
ASGqAFELILSPR	3	P16949	Q4(PS-S-2ME)	45	0.00100357	2	1764.85430	-1.01	882.93079
GASqAGMTGYGMPR	2	P37802	Q4(PS-S-2ME)	45	0.000248207	2	1759.71404	-1.81	880.36066
EqQIVIQSSGGLSK	2	P38646	Q2(PS-S-2ME)	45	0.001168097	2	1849.89141	-1.17	925.44934
LAqQQAALLMQQEER	3	Q13435	Q3(PS-S-2ME)	44	0.001389821	3	2133.00528	0.65	711.67328
TVIIEqSWGSPK	2	P10809	Q6(PS-S-2ME)	41	0.002858925	2	1720.81597	-1.54	860.91162
QAASSLQqASLK	4	P38646	Q8(PS-S-2ME)	40	0.002423812	2	1607.76555	-0.84	804.38641
EVYQqQQYGSGGR	12	Q99729	Q5(PS-S-2ME)	40	0.001136126	2	1875.78813	-1.07	938.39771

LLDqQNPDEDFS	8	P46108	Q4(PS-S-2ME)	40	0.000771362	2	1796.72222	-1.83	898.86475
TLQGTVSQAqER	2	Q9H5N1	Q10(PS-S-2ME)	39	0.003701404	2	1693.77629	-1.33	847.39178
LTESPcALVASqYGWSGNMER	2	P14625	C6(Carbamidomethyl); Q12(PS-S-2ME)	38	0.003540184	3	2732.16574	-2.48	911.39343
IASLPqEVQDVSLLEK	1	Q9NQG5	Q6(PS-S-2ME)	37	0.007659523	2	2145.07207	0.05	1073.03967
AFAAqEDLEK	2	P35241	Q5(PS-S-2ME)	36	0.003397941	2	1497.64568	-2.99	749.32648
GGPTPqEAIQR	3	Q9H444	Q6(PS-S-2ME)	36	0.004062487	2	1529.69744	-0.92	765.35236
LPPAQqDEIIDR	2	Q9H425	Q6(PS-S-2ME)	35	0.008114493	2	1770.82463	-3.18	885.91595
SGqQIVGPPR	2	Q9UNZ2	Q3(PS-S-2ME)	35	0.00539725	2	1414.66850	-2.39	707.83789
LLqEALEAEER	1	Q96JY6	Q3(PS-S-2ME)	35	0.008492138	2	1676.77532	-1.10	838.89130
AGGEESqFEMDI	4	Q13541	Q7(PS-S-2ME)	34	0.000809436	2	1688.63579	-1.91	844.82153
FGQGGAGPVGGqGPR	1	P23246	Q12(PS-S-2ME)	34	0.007674204	2	1717.76750	-0.68	859.38739
qQSELQSQVR	2	Q14847	Q1(PS-S-2ME)	33	0.012118752	2	1578.71416	-0.66	789.86072
ALGqNPTNAEVLK	2	P60660	Q4(PS-S-2ME)	32	0.01742149	2	1730.83391	-0.83	865.92059
QEMQEVqSSR	2	P22626	Q7(PS-S-2ME)	31	0.006396567	2	1597.65557	-0.05	799.33142
GQVSEGGqLR	1	Q96L92	Q8(PS-S-2ME)	30	0.020791938	2	1406.62956	-0.61	703.81842
ALPATPqLPSR	1	O43516	Q7(PS-S-2ME)	29	0.023600438	2	1526.75920	-0.99	763.88324
qEFLDIEDP	4	P16035	Q1(PS-S-2ME)	29	0.008674246	2	1481.60417	-2.32	741.30573
AqEPSAAIPK	1	Q15459	Q2(PS-S-2ME)	29	0.018981551	2	1387.64409	-4.08	694.32568
ITITNDqNR	1	P11021	Q7(PS-S-2ME)	29	0.022039328	2	1450.65471	-1.32	725.83099
WNQDTMEqK	2	Q15637	Q8(PS-S-2ME)	29	0.006640428	2	1555.61247	-0.16	778.30988

EPAAPVSIqR	1	Q14847	Q9(PS-S-2ME)	27	0.03229969	2	1443.68694	-0.19	722.34711
RAGGEESqFEMDI	2	Q13541	Q8(PS-S-2ME)	27	0.010360811	2	1844.73564	-2.44	922.87146
qVSPTEMVAK	1	Q5T5X7	Q1(PS-S-2ME)	26	0.041799562	2	1465.66179	-1.30	733.33453
cqGSLHEDVICTSR	2	P28340	C1(Carbamidomethyl); Q2(PS-S-2ME)	26	0.023411061	3	1980.81626	-1.07	660.94360
FNTPqQPK	1	Q9NTK5	Q5(PS-S-2ME)	26	0.052054922	2	1335.59636	-0.71	668.30182
qQSEISAAVER	2	Q9Y520	Q1(PS-S-2ME)	25	0.054929005	2	1593.71257	-1.45	797.35992
EEAqAEIEQYR	1	O75348	Q4(PS-S-2ME)	23	0.047199821	2	1741.72722	-2.12	871.36725
DNLTLWTSDMQGDGEEqNK	1	P62258	Q17(PS-S-2ME)	23	0.053182866	2	2557.03740	-2.17	1279.02234

HCD: 27 5-PT-modified peptides

Sequence	# PSMs	Protein Group Accessions	Modifications	IonScore	Exp Value	Charge	MH+ [Da]	ΔM [ppm]	m/z [Da]
IPSAVGYQPTLATDMGTMqER	4	P06576	Q19(PS-S-2ME)	80	3.79242E-07	3	2642.18149	-2.13	881.39868
FGQGGAGPVGGqGPR	2	P23246	Q12(PS-S-2ME)	60	1.73149E-05	2	1717.76079	-4.59	859.38403
SIqEIQELDKDDESLR	1	P52565	Q3(PS-S-2ME)	60	3.4792E-05	3	2294.03153	-4.93	765.34869
QAASSLQqASLK	4	P38646	Q8(PS-S-2ME)	59	3.7283E-05	2	1607.76286	-2.51	804.38507
GASqAGMTGYGMPR	2	P37802	Q4(PS-S-2ME)	54	3.06104E-05	2	1759.71111	-3.48	880.35919
ASGqAFELILSPR	3	P16949	Q4(PS-S-2ME)	54	0.000142303	2	1764.84978	-3.57	882.92853
ALPATPqLPSR	2	O43516	Q7(PS-S-2ME)	52	0.000134792	2	1526.75640	-2.82	763.88184
TVIIEqSWGSPK	1	P10809	Q6(PS-S-2ME)	51	0.000292366	2	1720.81389	-2.75	860.91058
VPFDAATLHTSTAMAAqHGMDDDGTGQK	3	P06396	Q17(PS-S-2ME)	51	0.000214481	4	3249.36977	-4.93	813.09790

HqGVMVGMGQK	3	P60709	Q2(PS-S-2ME)	50	0.000117271	2	1547.66899	-3.17	774.33813
INPDGSqSVVEVPYAR	4	Q9Y2B0	Q7(PS-S-2ME)	49	0.000373437	2	2106.96294	-5.07	1053.98511
GGPTPqEAIQR	2	Q9H444	Q6(PS-S-2ME)	49	0.000188913	2	1529.69560	-2.11	765.35144
EPAAPVSIqR	2	Q14847	Q9(PS-S-2ME)	48	0.000299591	2	1443.67974	-5.18	722.34351
EVYQqQQYGSGGR	8	Q99729	Q5(PS-S-2ME)	44	0.000463906	2	1875.78703	-1.66	938.39716
LLqEALEAEER	1	Q96JY6	Q3(PS-S-2ME)	43	0.00120027	2	1676.77446	-1.61	838.89087
QqSEISAAVER	2	Q9Y520	Q2(PS-S-2ME)	43	0.000980748	2	1593.71147	-2.14	797.35938
qEMQEVQSSR	3	P22626	Q1(PS-S-2ME)	40	0.000713277	2	1597.65178	-2.42	799.32953
GQVSEGGqLR	2	Q96L92	Q8(PS-S-2ME)	40	0.001902292	2	1406.62493	-3.90	703.81610
ITITNDqNR	2	P11021	Q7(PS-S-2ME)	39	0.001993097	2	1450.65312	-2.41	725.83020
SQEqLAAELAEYTAK	2	P15311	Q4(PS-S-2ME)	38	0.004563518	3	2027.91190	-4.09	676.64215
LTSEPQPqR	3	P61106	Q8(PS-S-2ME)	33	0.007858739	2	1431.64568	-3.58	716.32648
qQSELQSQVR	4	Q14847	Q1(PS-S-2ME)	31	0.015353099	2	1578.71074	-2.82	789.85901
SGqQIVGPPR	2	Q9UNZ2	Q3(PS-S-2ME)	29	0.020901263	2	1414.66680	-3.60	707.83704
AFAAqEDLEK	2	P35241	Q5(PS-S-2ME)	28	0.022918504	2	1497.64800	-1.44	749.32764
DAFADAVqR	1	Q92945	Q8(PS-S-2ME)	28	0.015697232	2	1368.57805	-3.18	684.79266
GNSLAEqR	1	Q6ZVM7	Q7(PS-S-2ME)	27	0.022720007	2	1250.53752	-2.40	625.77240
QqYESVAAK	1	P08670	Q2(PS-S-2ME)	26	0.026632634	2	1399.60723	-4.37	700.30725