Supporting Information for

Multi-site SUMOylation restrains DNA Polymerase η interactions with DNA damage sites

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Table S1.
Results of high-throughput microscopy screen for regulators of Pol η interaction with DNA damage sites
U2OS/GFP-Pol η cells were transfected with an siRNA library targeting ubiquitin and UBL signaling factors (1251 genes, two siRNAs per gene). After 72 h, cells were treated with 30 μM Cisplatin for 6 h and subsequently grown overnight in fresh media. Cells were then pre-extracted, fixed and stained with DAPI. GFP-Pol η foci counts were determined using QIBC. Table shows modified Z-scores for all siRNAs in the library.

Table S2.
List of validated Ub/UBL signaling factors whose knockdown leads to enhanced formation of DNA damage-induced Pol η foci

Table S3.
Overview of endogenous Pol η SUMOylation sites identified by mass spectrometry

Figure S1.
SUMO-dependent regulation of Pol η association with DNA damage sites.
A. Immunoblot analysis of whole-cell extracts (WCE) of U2OS cells transfected with non-targeting control (CTRL) siRNA or siRNAs targeting indicated SUMO enzymes. Expression levels of SAE1, UBA2 and UBC9 (normalized to MCM6 expression) were quantified using ImageJ. **B**. Results of validation screen. The sum of GFP-Pol η foci intensity in U2OS/GFP-Pol η cells transfected with indicated siRNAs, exposed to cisplatin for 6 h and fixed 16 h later was quantified using QIBC analysis (mean±SD; ≥294 cells quantified per condition). **C**. Results of validation screen. The sum of GFP-Pol η foci intensity at different time points after exposure to cisplatin in cells treated as in (B) was quantified using QIBC analysis (mean±SD; ≥3,000 cells quantified per condition). **D**. U2OS/GFP-Pol η cells were treated or not with SUMO E1 inhibitor (SUMOi) for 30 min, exposed to UV and fixed 6 h later. GFP-Pol η foci count per nucleus was quantified by QIBC analysis (mean±SEM; *n*=3 independent experiments; ≥7,482 cells quantified per condition).

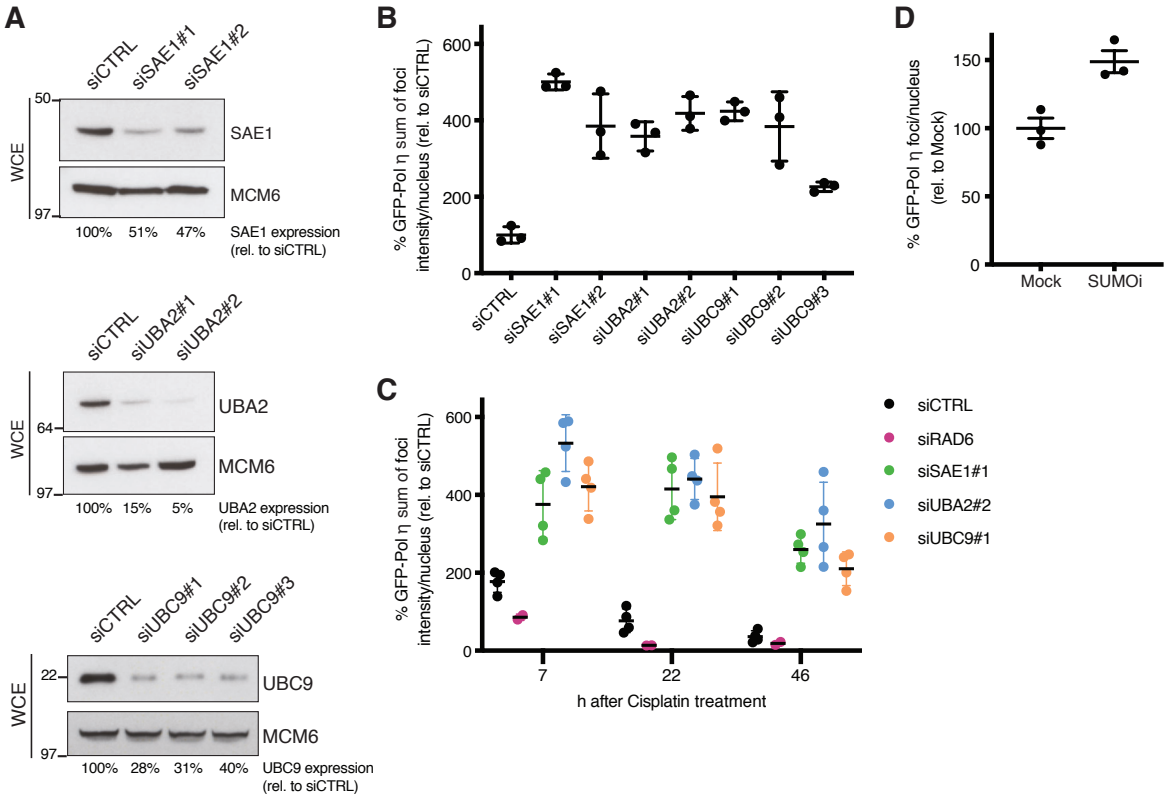
A
siCTRL
siSAE1#1
siSAE1#2
WCE
50
SAE1
MCM6
97
100% 51% 47%
SAE1 expression
(rel. to siCTRL)
siCTRL
siUBA2#1
siUBA2#2
WCE
64
UBA2
MCM6
97
100% 15% 5%
UBA2 expression
(rel. to siCTRL)
siCTRL
siUBC9#1
siUBC9#2
siUBC9#3
WCE
22
UBC9
MCM6
97
100% 28% 31% 40%
UBC9 expression
(rel. to siCTRL)
B
% GFP-Pol η sum of foci
intensity/nucleus (rel. to siCTRL)
600
400
200
0
siCTRL
siSAE1#1
siSAE1#2
siUBA2#1
siUBA2#2
siUBC9#1
siUBC9#2
siUBC9#3
C
% GFP-Pol η sum of foci
intensity/nucleus (rel. to siCTRL)
600
400
200
0
7
22
46
h after Cisplatin treatment
siCTRL
siRAD6
siSAE1#1
siUBA2#2
siUBC9#1
D
% GFP-Pol η foci/nucleus
(rel. to Mock)
150
100
50
0
Mock
SUMOi

Figure S2.

Impact of removing SUMO modification components on cell cycle distribution, PCNA monoubiquitylation and Pol η SUMOylation

A. U2OS cells transfected with indicated siRNAs were pulse-labeled with EdU, fixed and processed for EdU labeling and staining of total DNA with DAPI. Cell cycle profiles were analyzed using QIBC (≥1869 cells quantified per condition). Percentage of cells in different cell cycle stages (G1 phase, blue; S phase, pink; G2/M phase, green) is indicated. **B**. Immunoblot analysis of chromatin-enriched fractions of U2OS cells transfected with indicated siRNAs and exposed to UV. **C**. U2OS left untreated or transfected with plasmids encoding the indicated GFP-Pol η alleles and exposed to UV were lysed and subjected to GFP immunoprecipitation (IP) under denaturing conditions, followed by immunoblotting with SUMO2/3, GFP and MCM6 (loading control) antibodies. **D**. Quantification of GFP-Pol η foci formation in U2OS/GFP-Pol η collected at indicated times after exposure to UV (mean±SEM; *n*=2 independent experiments; ≥508 cells quantified per condition). **E**. U2OS/GFP-Pol η cells transfected with indicated siRNAs and exposed to UV were lysed and subjected to GFP immunoprecipitation (IP) under denaturing conditions, followed by immunoblotting with SUMO2/3, GFP and MCM6 (loading control) antibodies. **F**. Cell cycle profiles of cells transfected with indicated PIAS1 siRNAs or non-targeting control (CTRL) siRNA were determined as in (A) (≥2936 cells quantified per condition).

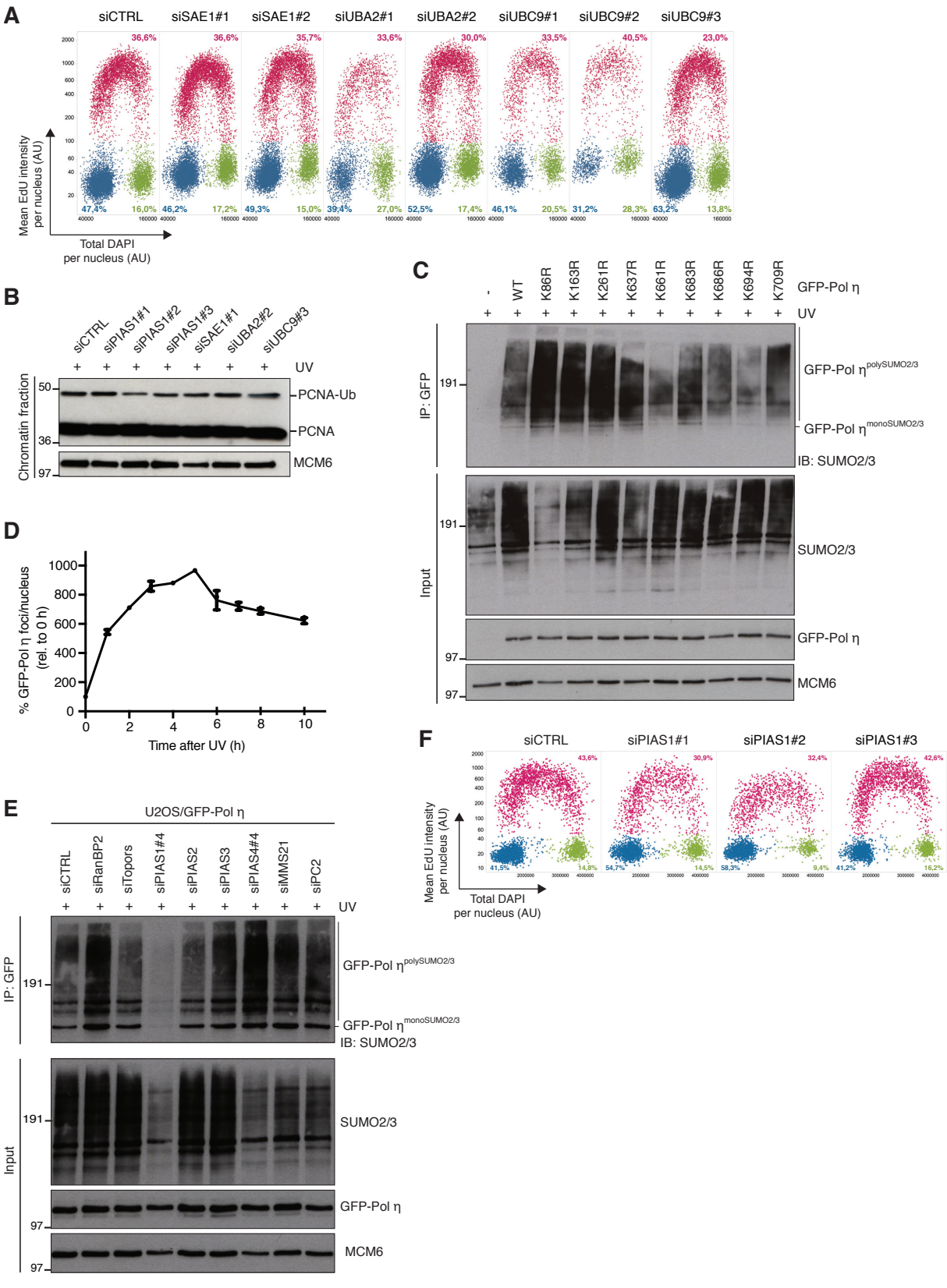
A
siCTRL
siSAE1#1
siSAE1#2
siUBA2#1
siUBA2#2
siUBC9#1
siUBC9#2
siUBC9#3
Mean EdU intensity per nucleus (AU)
Total DAPI per nucleus (AU)
B
siCTRL
siPIAS1#1
siPIAS1#2
siPIAS1#3
siSAE1#1
siUBA2#2
siUBC9#3
UV
Chromatin fraction
PCNA-Ub
PCNA
MCM6
C
WT
K86R
K163R
K261R
K637R
K661R
K683R
K686R
K694R
K709R
GFP-Pol η
UV
IP: GFP
GFP-Pol η polySUMO2/3
GFP-Pol η monoSUMO2/3
IB: SUMO2/3
Input
SUMO2/3
GFP-Pol η
MCM6
D
% GFP-Pol η foci/nucleus (rel. to 0 h)
Time after UV (h)
E
U2OS/GFP-Pol η
siCTRL
siRanBP2
siTopors
siPIAS1#4
siPIAS2
siPIAS3
siPIAS4#4
siMMS21
siPC2
UV
IP: GFP
GFP-Pol η polySUMO2/3
GFP-Pol η monoSUMO2/3
IB: SUMO2/3
Input
SUMO2/3
GFP-Pol η
MCM6
F
siCTRL
siPIAS1#1
siPIAS1#2
siPIAS1#3
Mean EdU intensity per nucleus (AU)
Total DAPI per nucleus (AU)

Figure S3.
Impact of impaired SUMO-dependent processing of Pol η on its association with DNA damage sites and TLS functionality

A. Quantification of the sum of GFP-Pol η foci intensity using QIBC analysis in U2OS/GFP-Pol η cells transfected with indicated siRNAs, exposed to UV and fixed 6 h later (mean±SEM; *n*=2 independent experiments; ≥1991 cells quantified per condition). **B**. Immunoblot analysis of U2OS cells transfected with non-targeting control (CTRL) or RNF4 siRNAs using indicated antibodies. **C**. qPCR analysis of RNF111 mRNA levels in U2OS cells transfected with indicated siRNAs relative to siCTRL. Primers to GAPDH were used as a normalization control (mean±SEM; *n*=2 independent experiments). **D**. Quantification of the sum of GFP-Pol η foci intensity using QIBC analysis in U2OS/GFP-Pol η cells transfected with indicated siRNAs and treated as in (A) (mean±SEM; *n*=4 independent experiments; ≥1254 cells quantified per condition). **E**. U2OS cell lines stably expressing GFP-Pol η WT or 20KR mutant (clone 1 (c1) and clone 2 (c2)) were transfected or not with siRNA targeting the 3'UTR of endogenous Pol η (siPol η#2), exposed to UV (5 J/m^2) and fixed 6 h later. Cells were then stained with antibody specific to cyclobutane pyrimidine dimers (CPDs) under native conditions, where only CPDs in single-stranded DNA (ssDNA) are exposed, and CPD intensity per nucleus was quantified by QIBC analysis (mean±SEM; *n*=2 independent experiments; ≥10,086 cells quantified per condition). Both GFP-Pol η WT or 20KR are able to rescue the increased occurrence of CPDs in ssDNA resulting from knockdown of endogenous Pol η, indicating that both alleles are proficient for TLS-mediated bypass of these lesions. **F**. Clonogenic survival of U2OS cells and derivative stable U2OS/NLS-GFP-Pol η WT and U2OS/NLS-GFP-Pol η 20KR mutant (c1) cell lines transfected with siCTRL or siPol η#2 and exposed to the indicated doses of UV. After UV radiation the medium was supplemented with 0,4 mM caffeine (mean±SEM; *n*=2 experiments).

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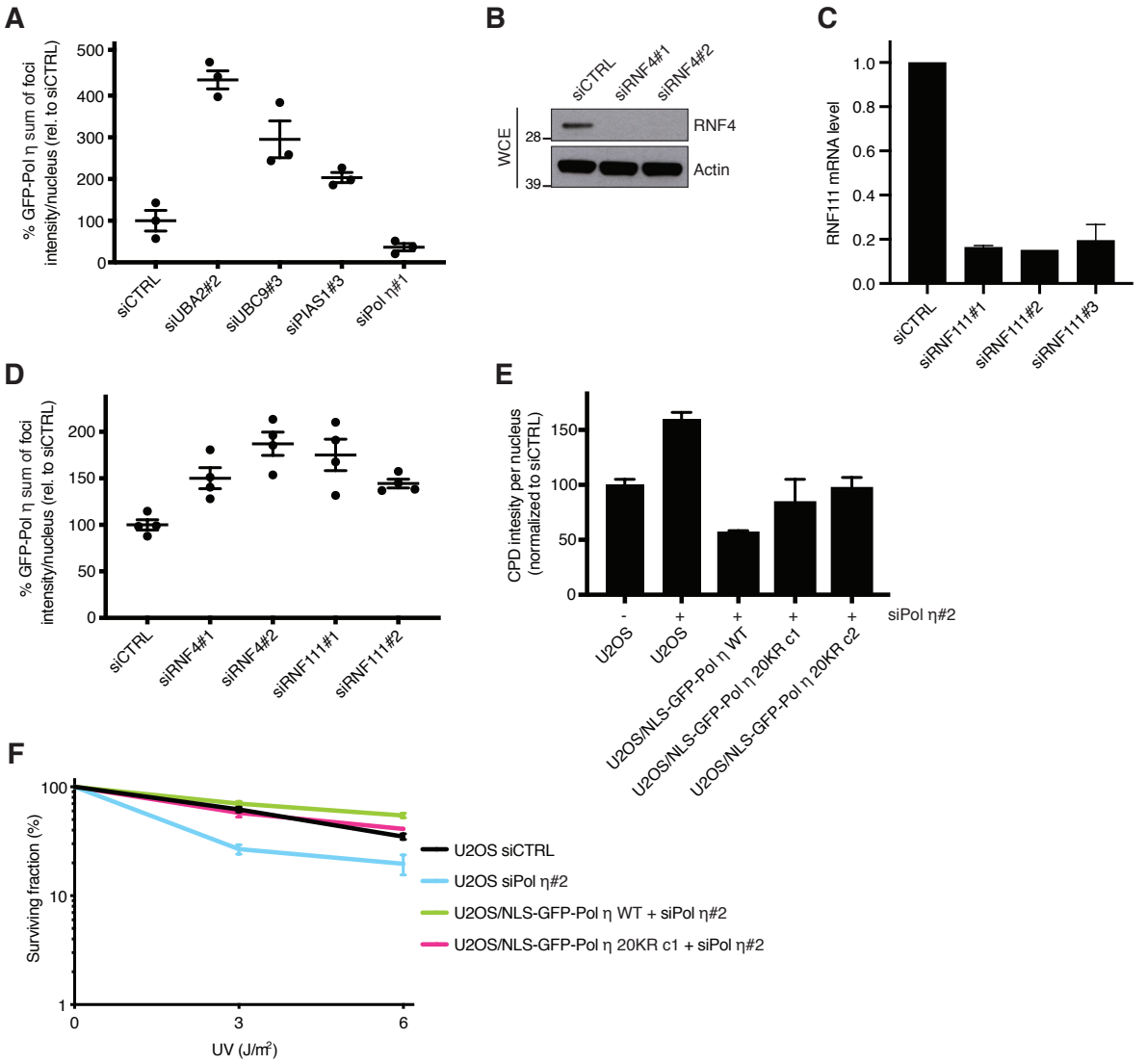
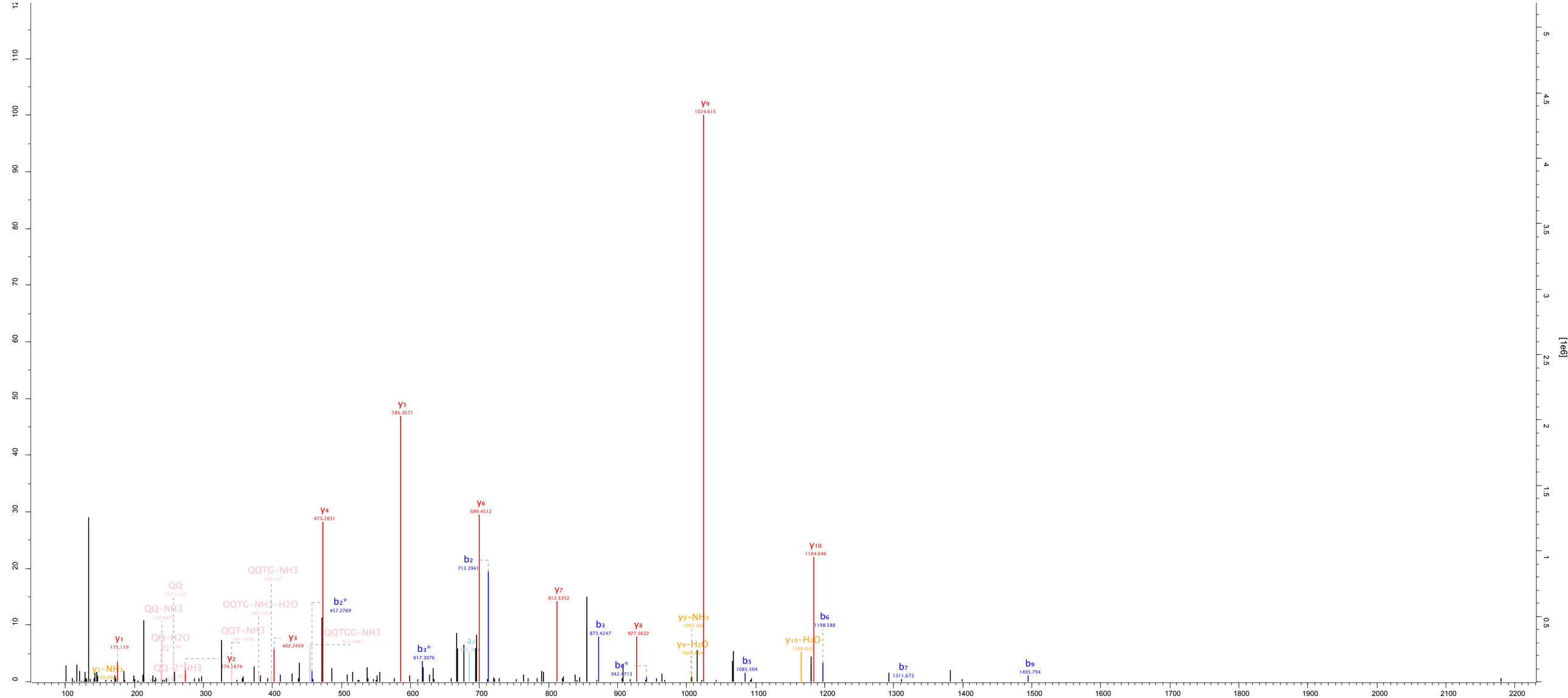


Figure S4.
MS spectra for identified SUMO sites in human Pol η
A collection of 19 fully annotated MS/MS spectra corresponding to all POLH SUMO sites reported in this work. Both "Standard" and "Advanced" annotations via the built-in MaxQuant Viewer software are available for each spectrum. Note that "qq" in the peptide sequence corresponds to attachment of the SUMO C-terminal mass remnant, QQTGG, covalently linked to the lysine residue via its C-terminal glycine residue.

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_3_F5	25646	FTMS; HCD	208.28	633.02	POLH

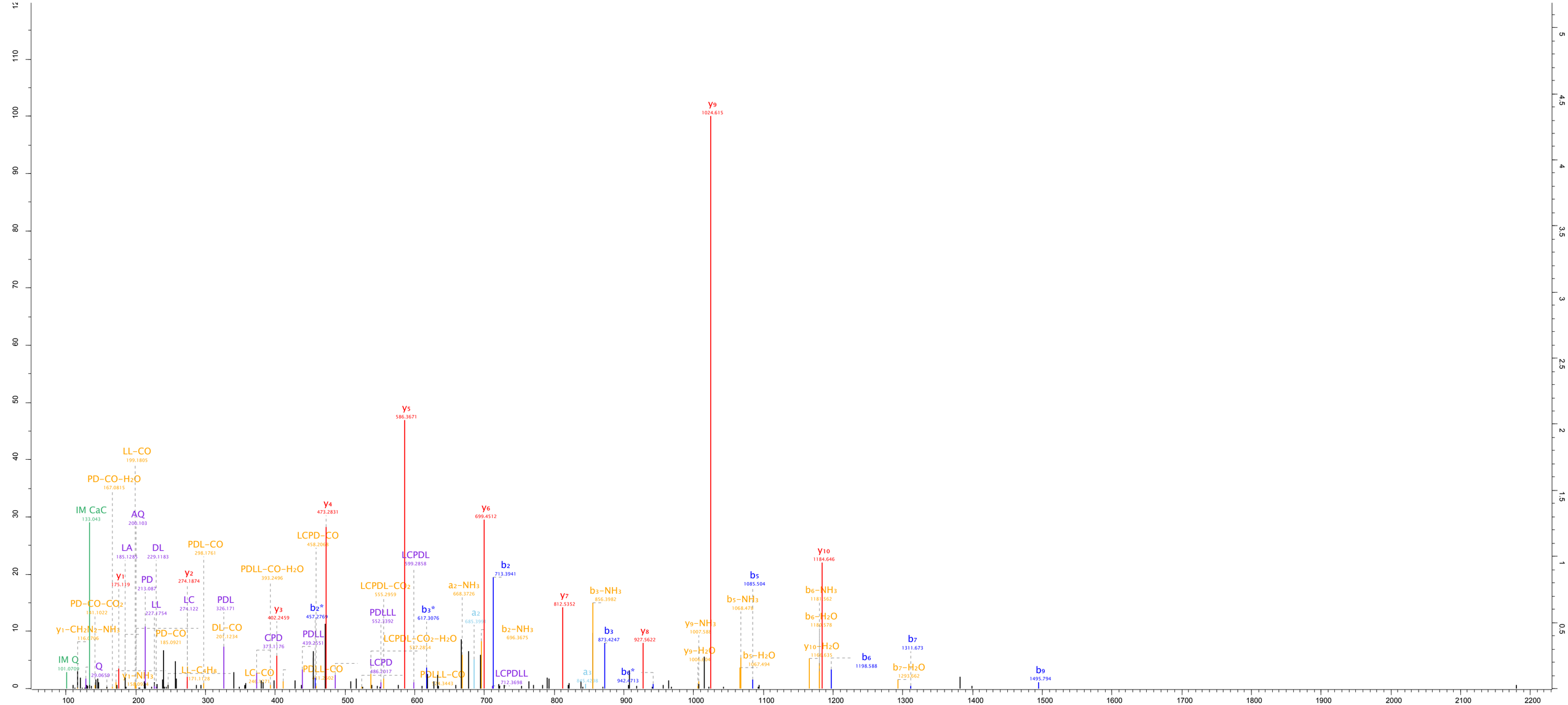


qq

-	K	L	C	P	D	L	L	L	A	Q	V	R	-
	b2	b3	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1	

Standard: POLH-K70-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_3_F5	25646	FTMS; HCD	208.28	633.02	POLH

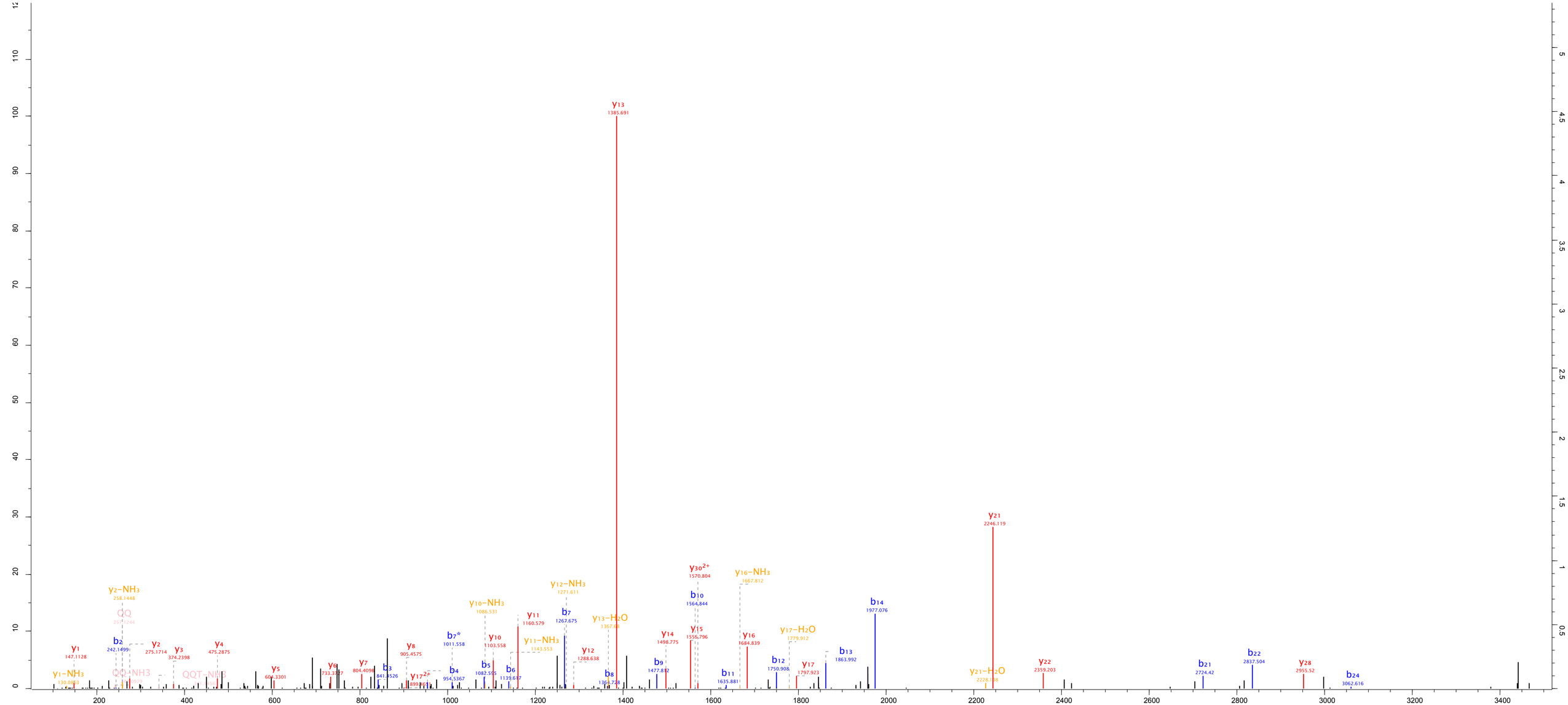


qq

-	K	L	C	P	D	L	L	L	A	Q	V	R	-
	b2	b3	y10	y9	b5	b6	b7	y6	b9	y8	y7	y5	y4	y3	y2	y1

Advanced: POLH-K70-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F5	21588	FTMS; HCD	237.01	1408.74	POLH



Standard: POLH-K131-SUMO

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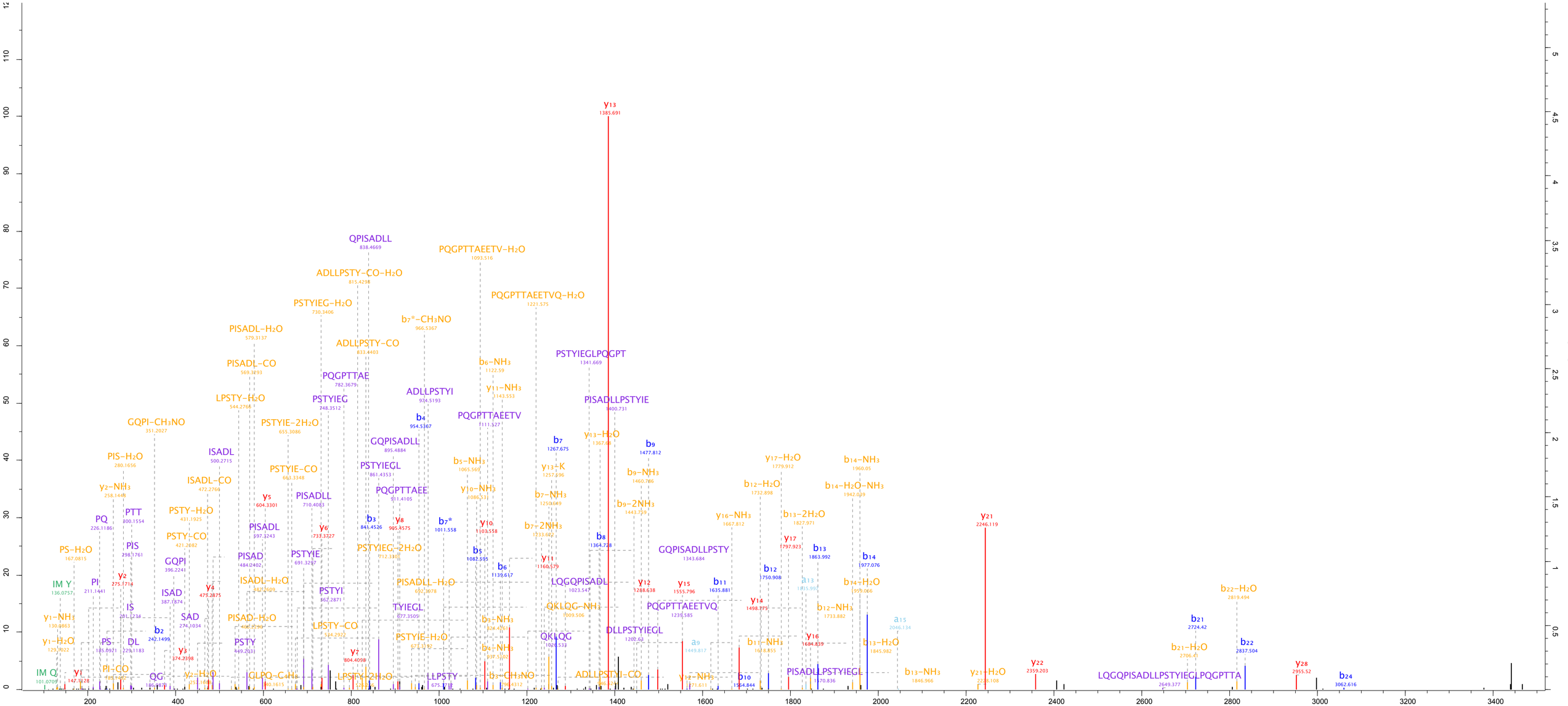
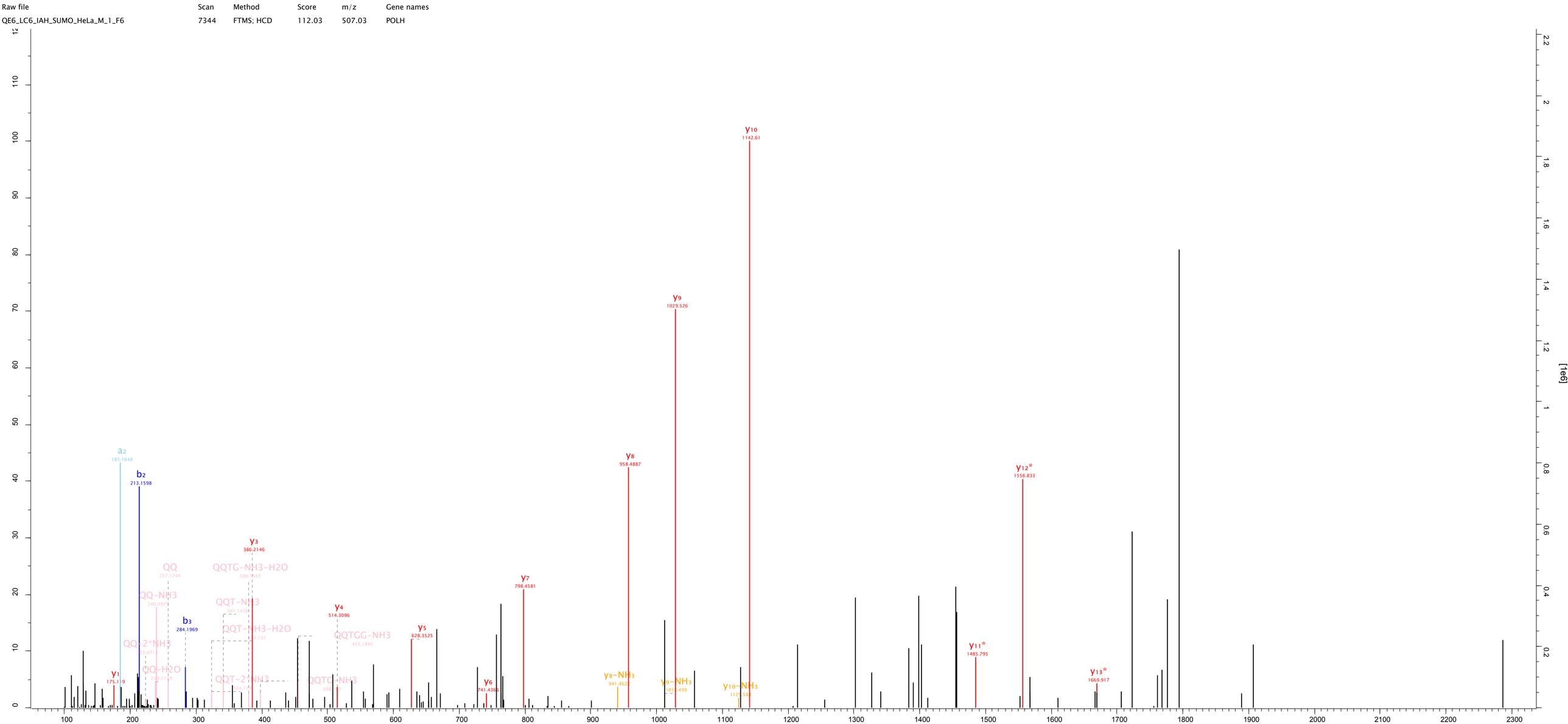
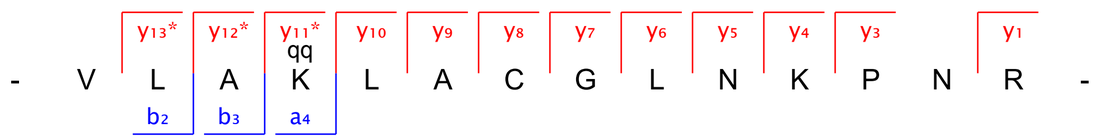


Advanced: POLH-K131-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	7344	FTMS; HCD	112.03	507.03	POLH





Standard: POLH-K224-SUMO

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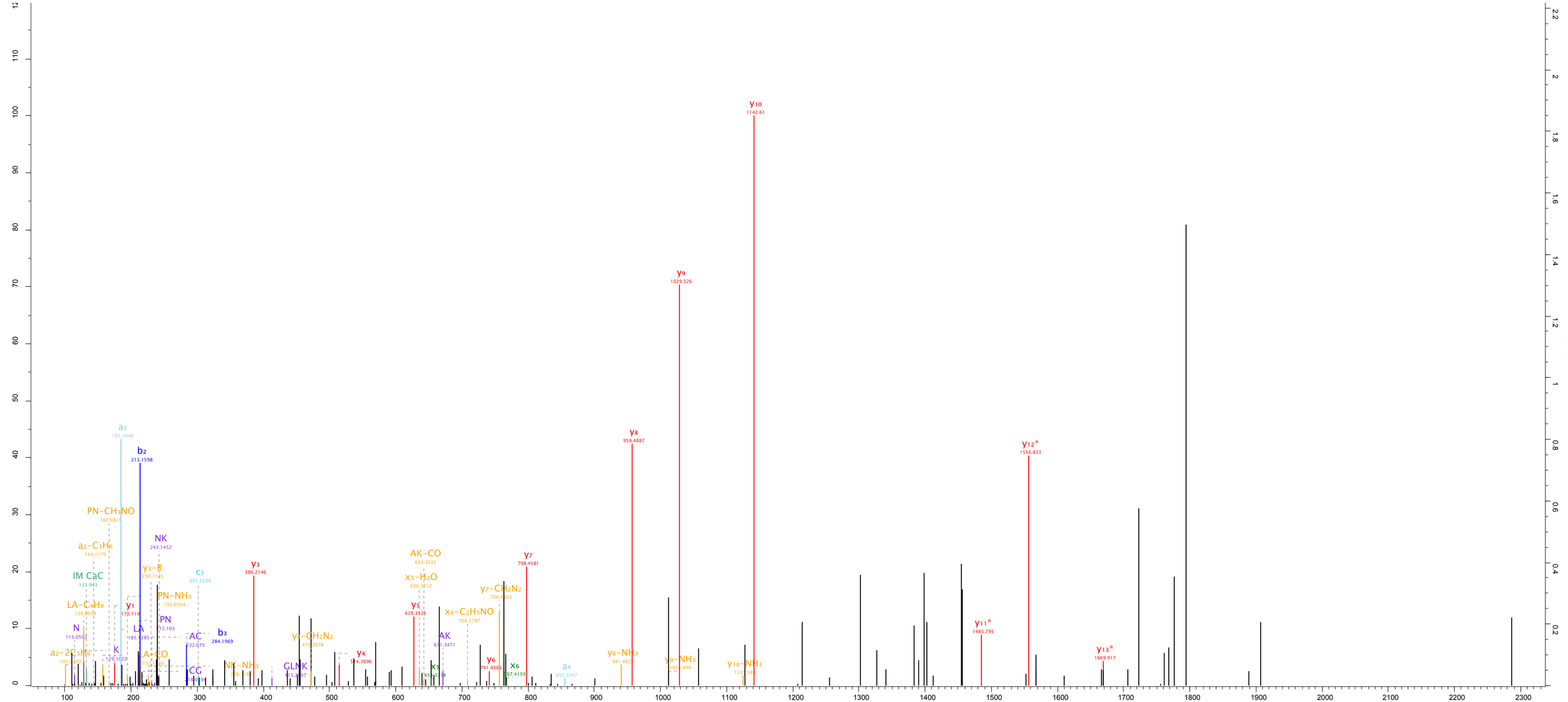
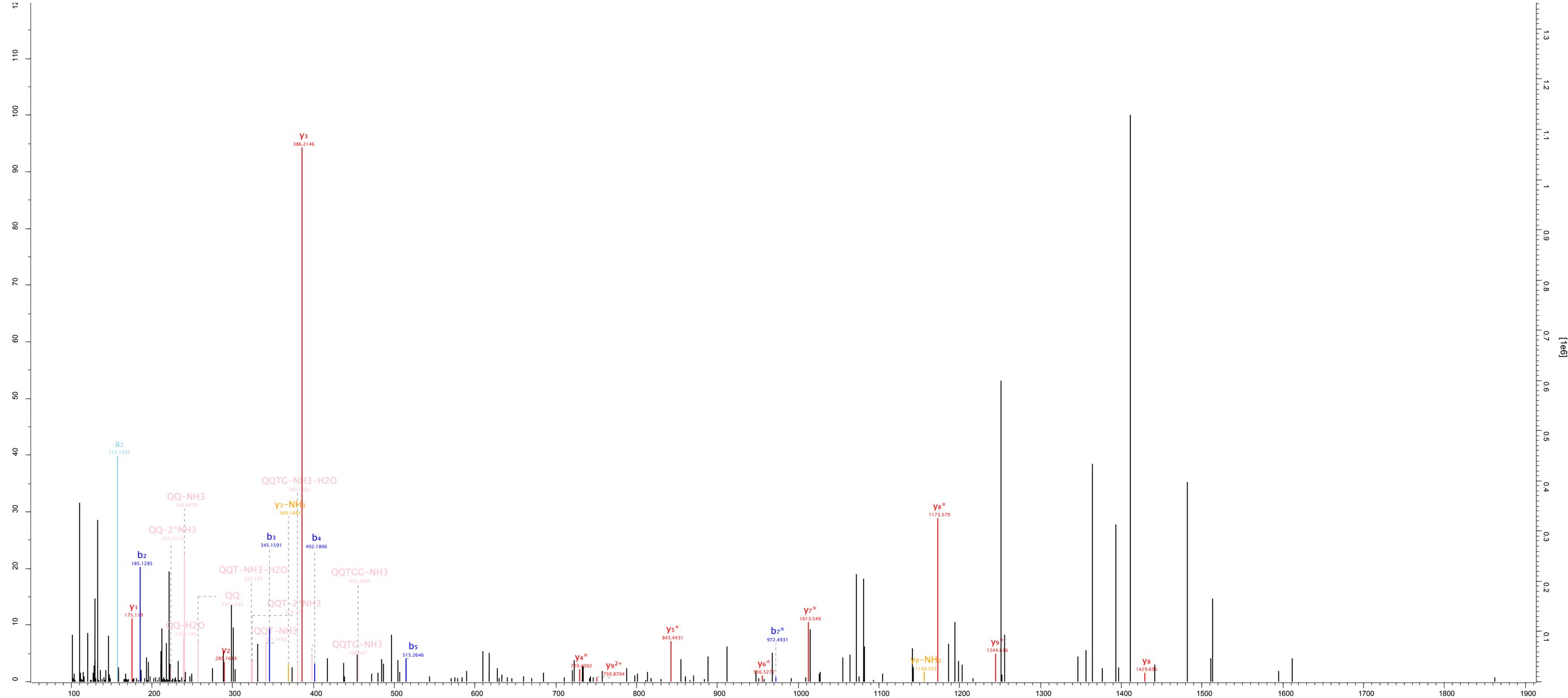
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	7344	FTMS; HCD	112.03	507.03	POLH



Advanced: POLH-K224-SUMO

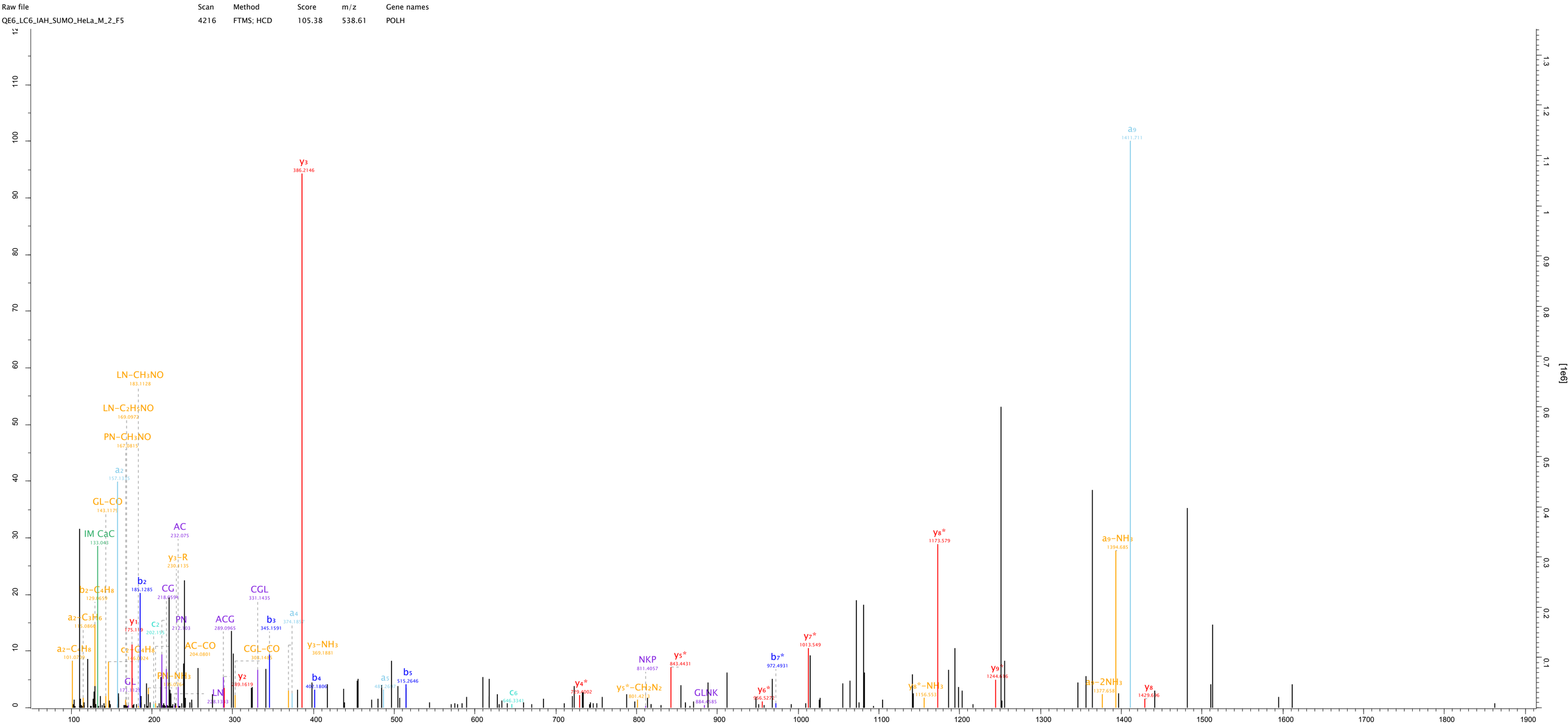
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F5	4216	FTMS; HCD	105.38	538.61	POLH



		y9*	y8	y7*	y6*	y5*	y4* qq	y3	y2	y1
-	L	A	C	G	L	N	K	P	N	R	-
		b2	b3	b4	b5		b7*			

Standard: POLH-K231-SUMO

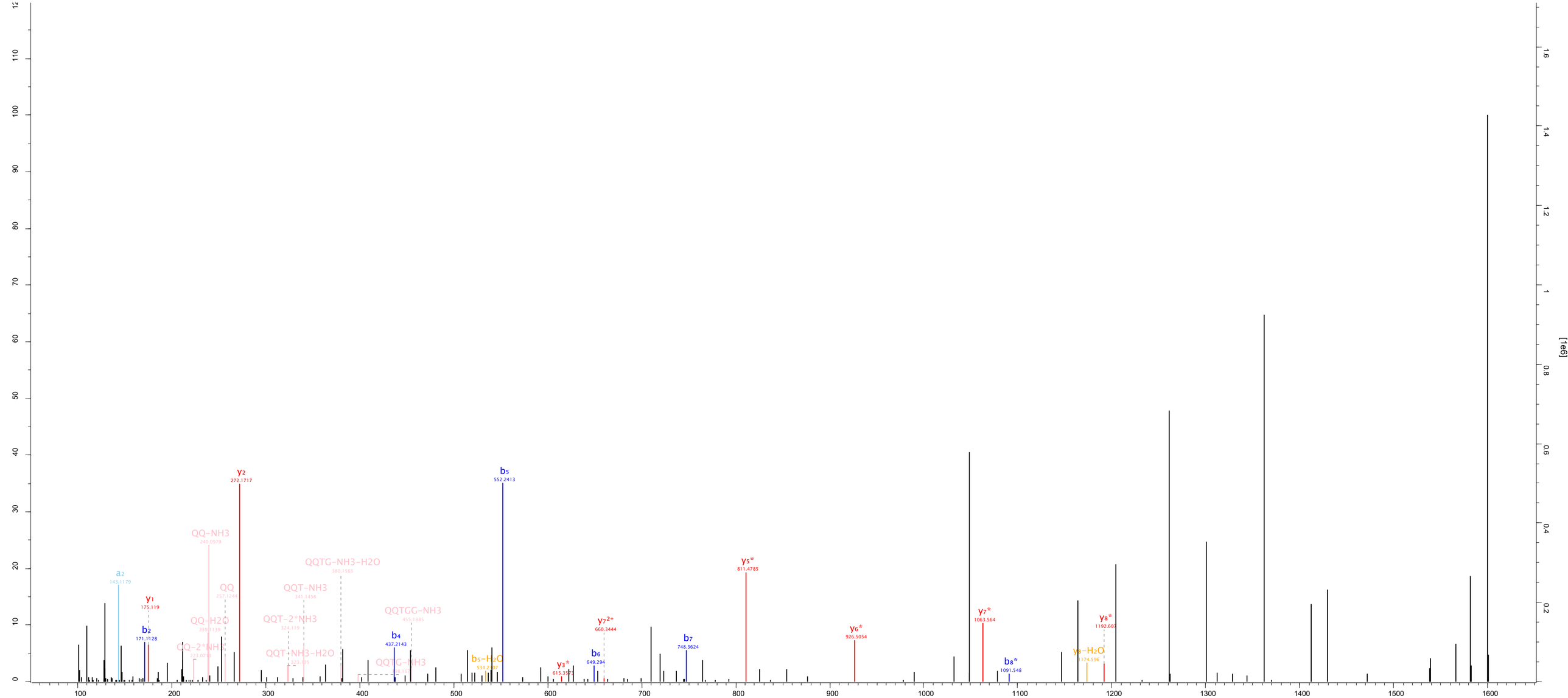
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F5	4216	FTMS; HCD	105.38	538.61	POLH

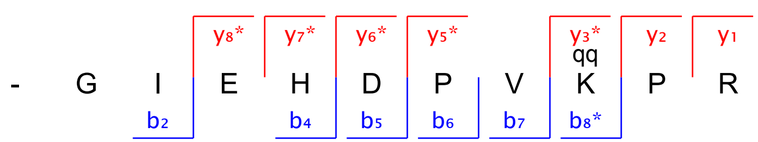


y9*	y8	y7*	y6*	y5*	y4* qq	y3	y2	y1
-	L	A	C	G	L	N	K	P	N	R	-
		b2	b3	b4	b5		b7*				

Advanced: POLH-K231-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_U2OS_M_2_F1	7391	FTMS; HCD	114.79	540.28	POLH





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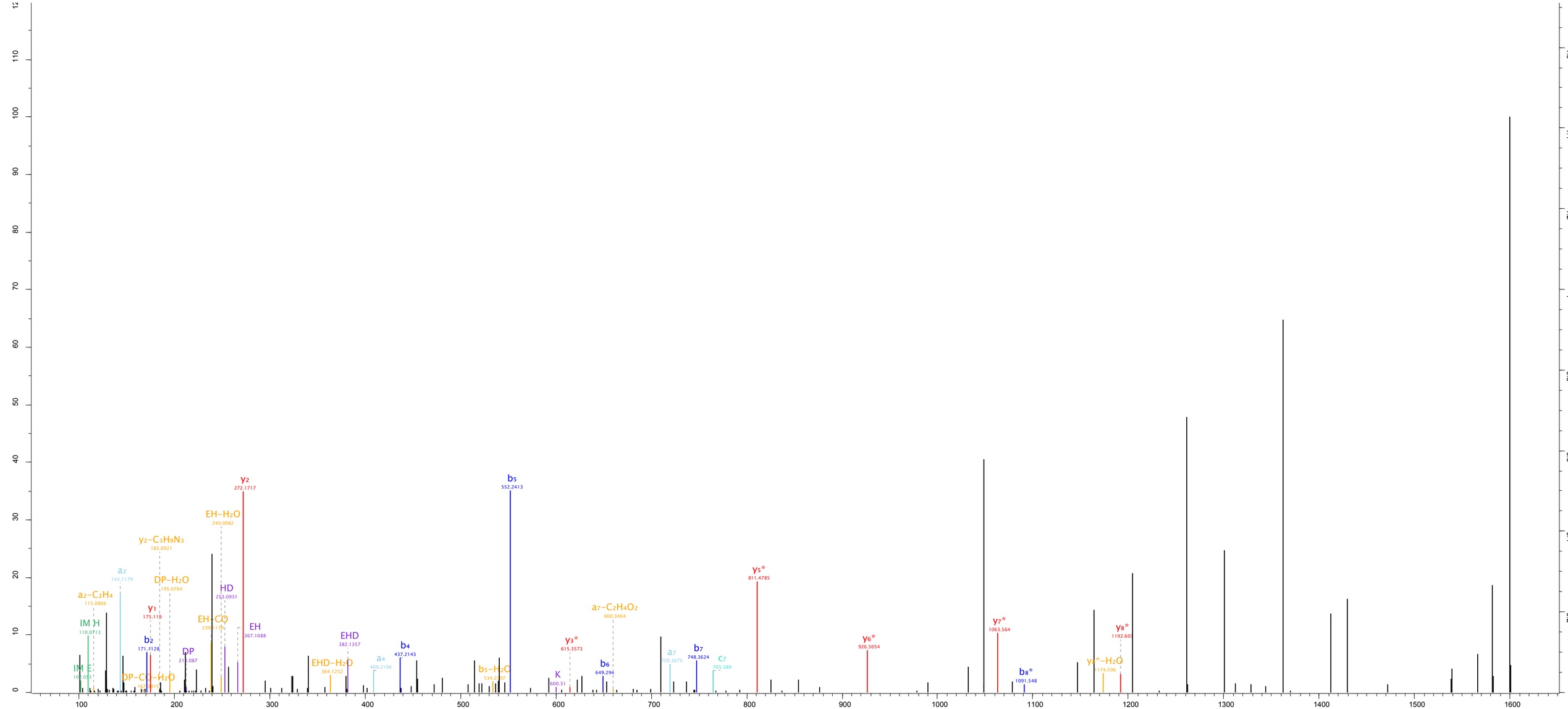

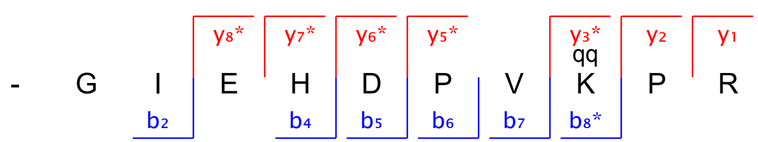

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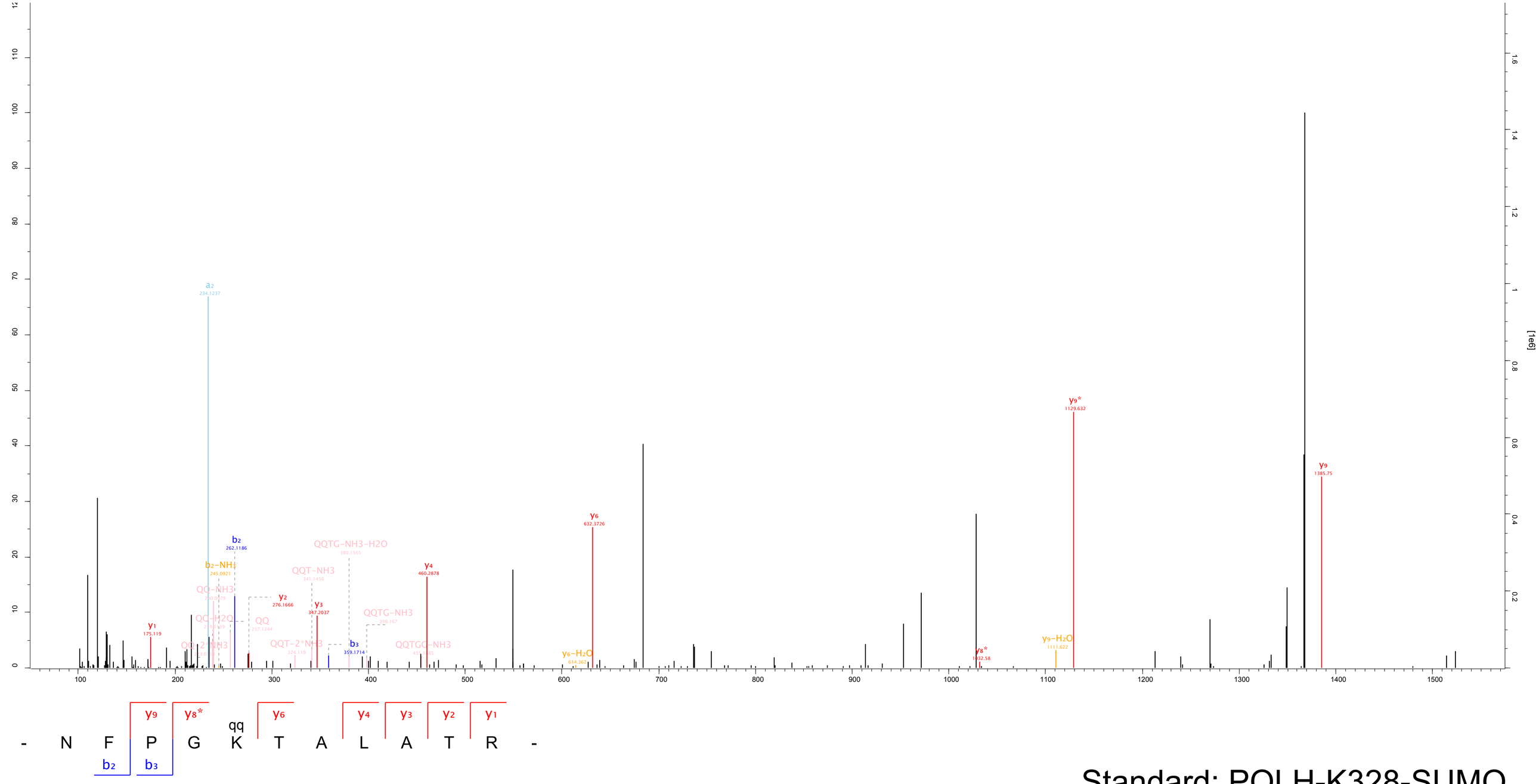

Standard: POLH-K328-SUMO

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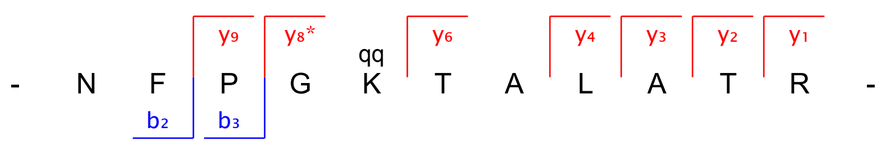
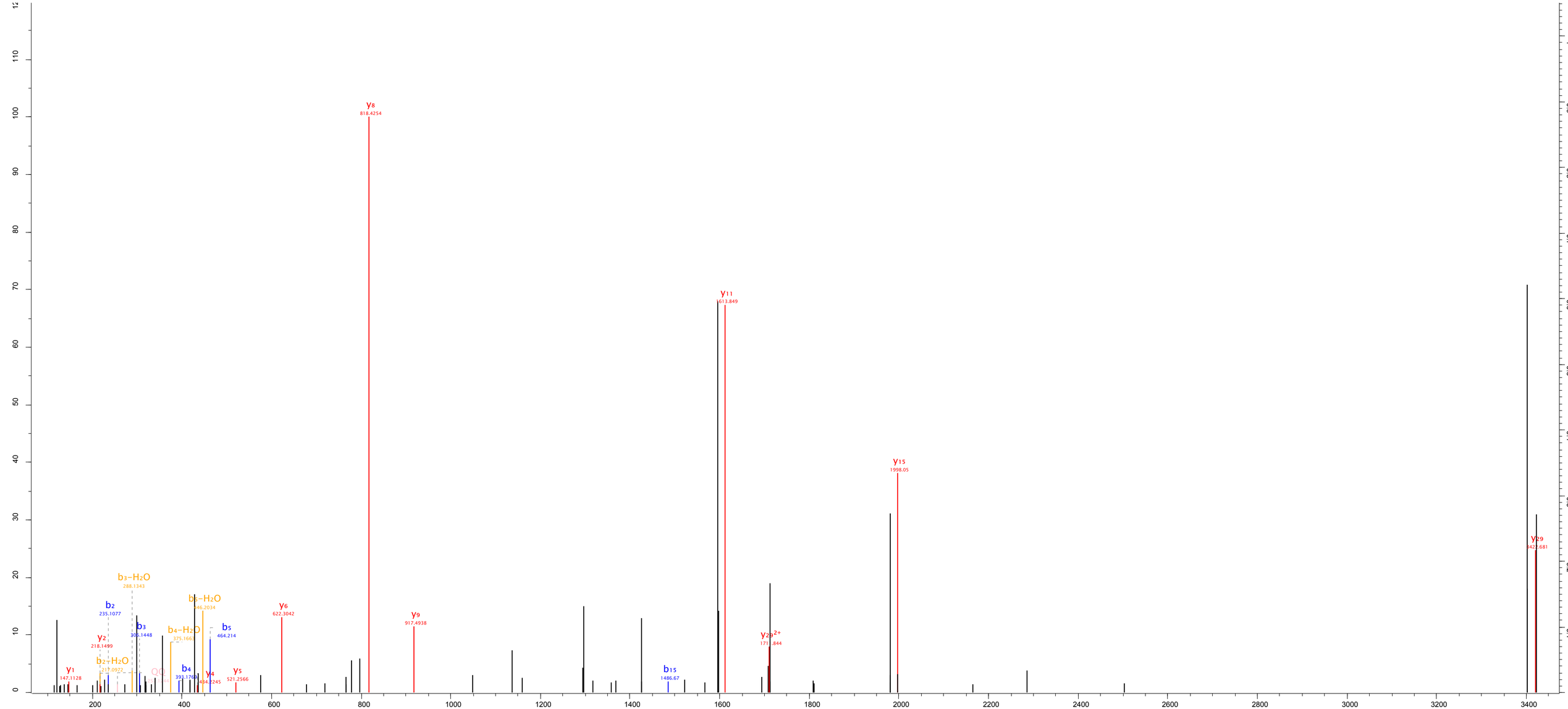


Advanced: POLH-K328-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_3_F5	31361	FTMS; HCD	47.99	1296.64	POLH



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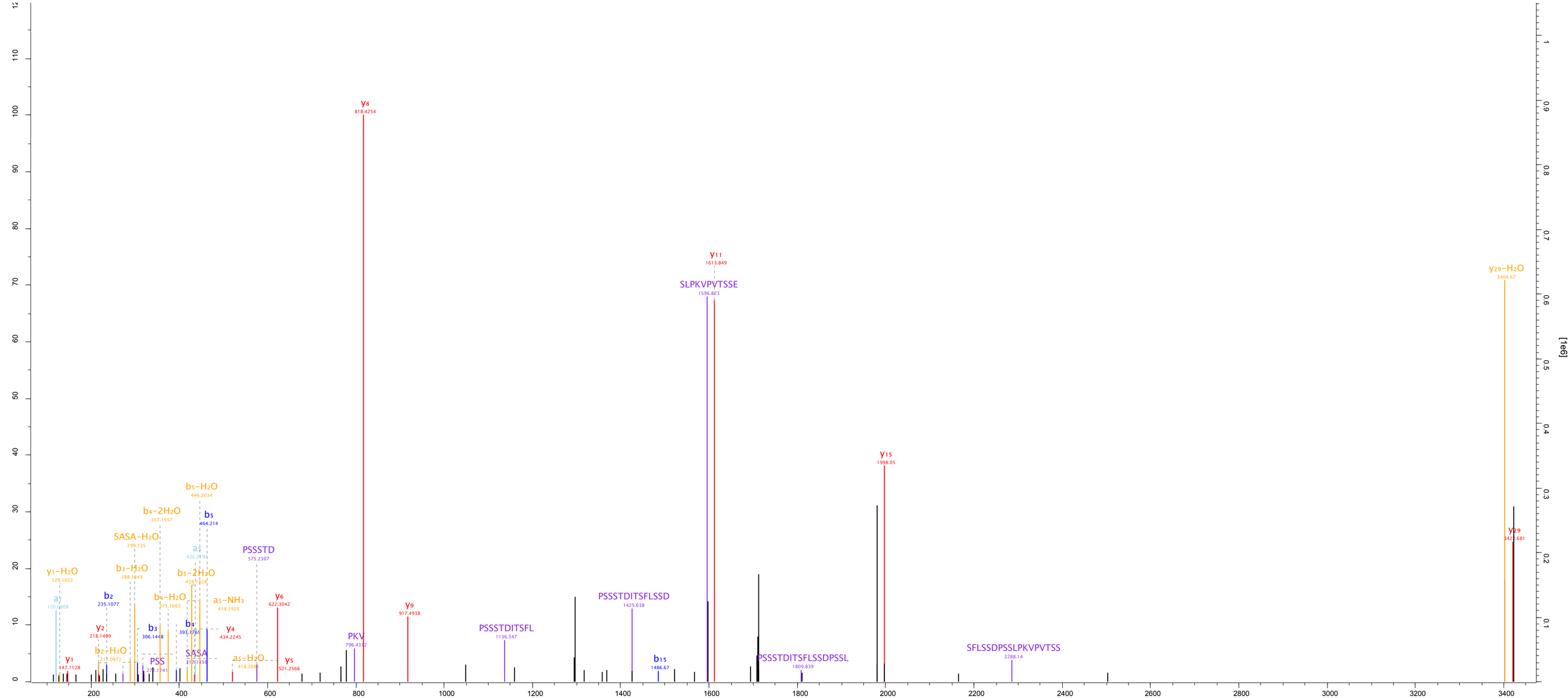

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y29 y15 y11 y9 y8 y6 y5 y4 y2 y1

a1 b2 b3 b4 b5 b15

Advanced: POLH-K453-SUMO

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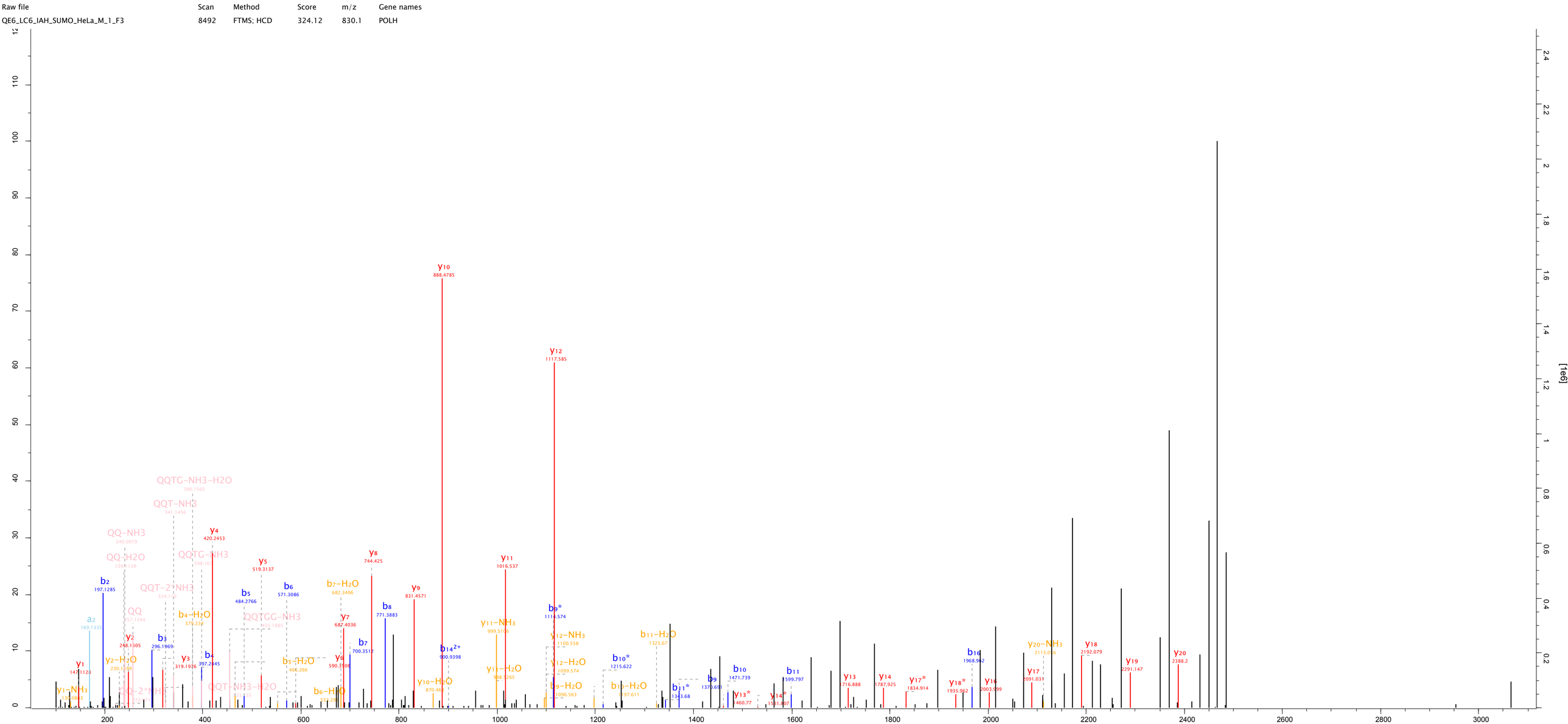

Standard: POLH-K462-SUMO

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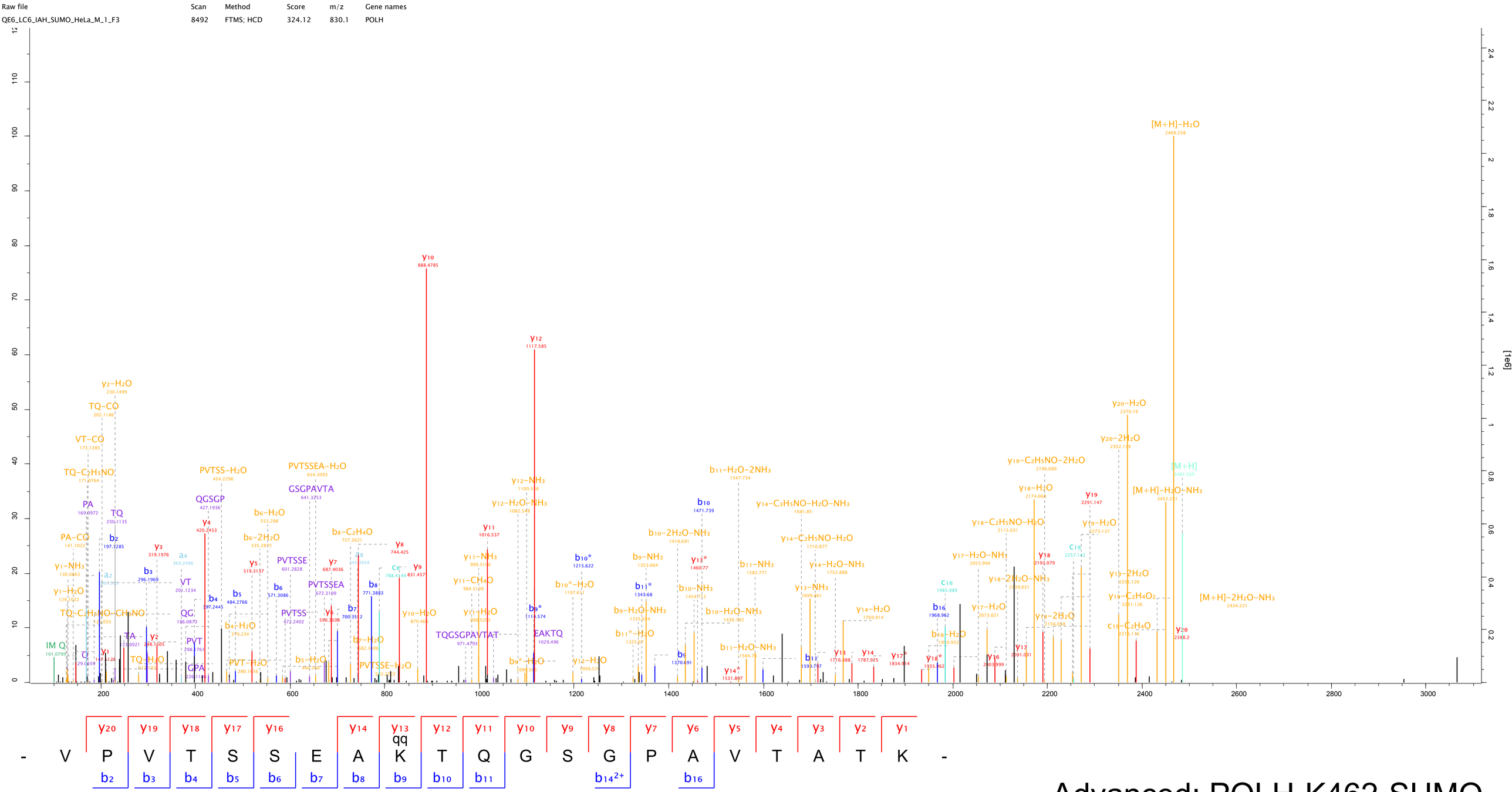

Raw file	Scan	Method	Score	m/z	Gene names
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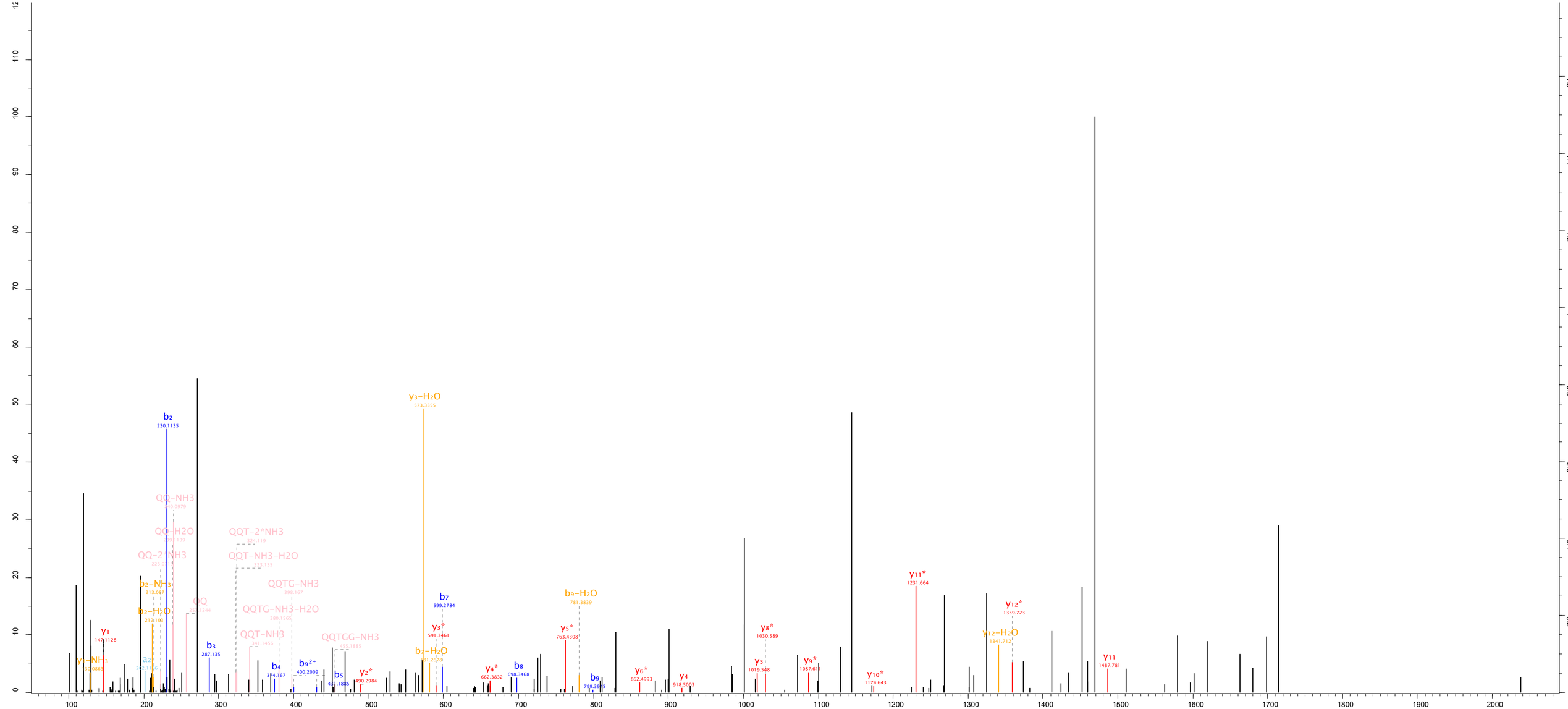

	y12*	y11	y10*	y9*	y8*		y6*	y5	y4	y3*	y2* qq	y1		
-	T	Q	G	S	G	P	A	V	T	A	T	K	K	-
	c1	b2	b3	b4	b5		b7	b8	b9					

Standard: POLH-K474-SUMO

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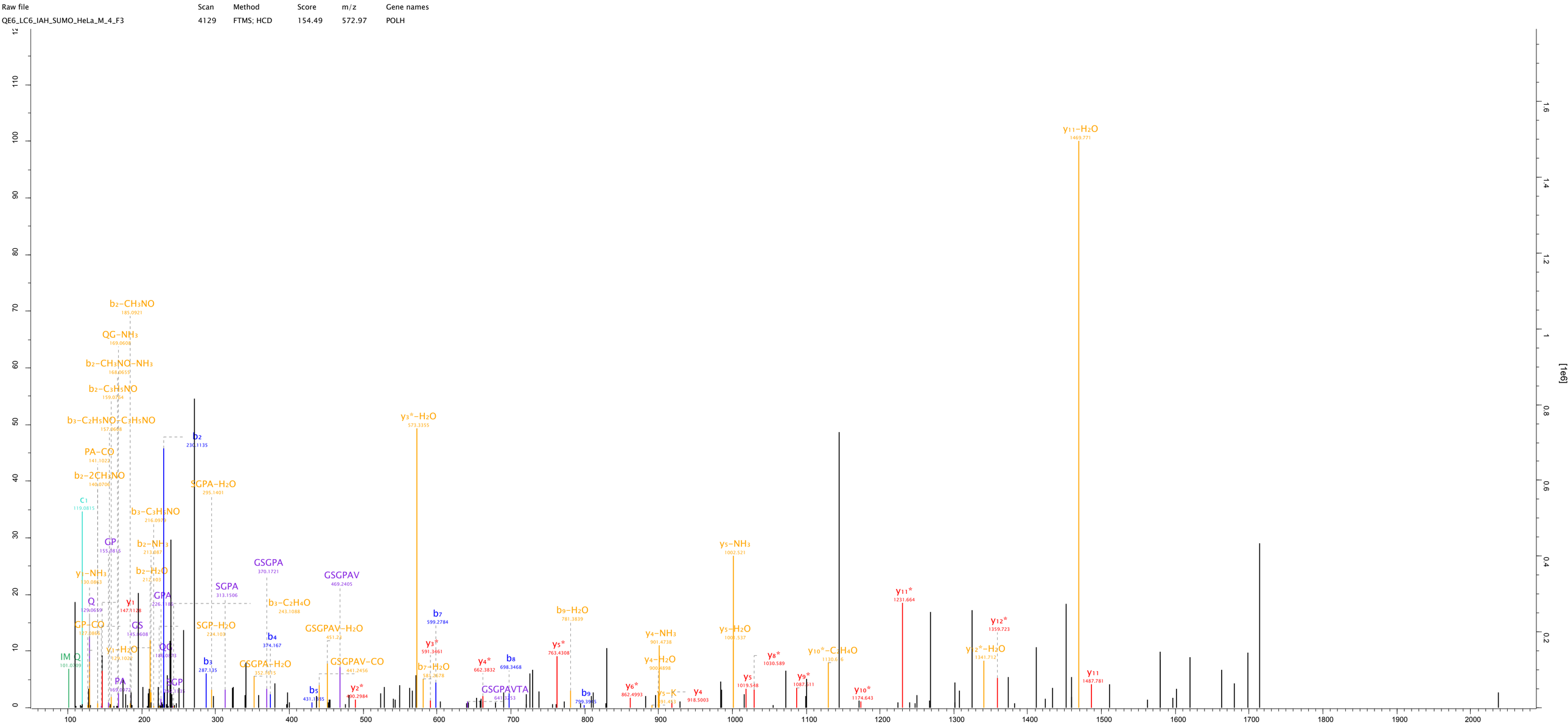

Advanced: POLH-K474-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
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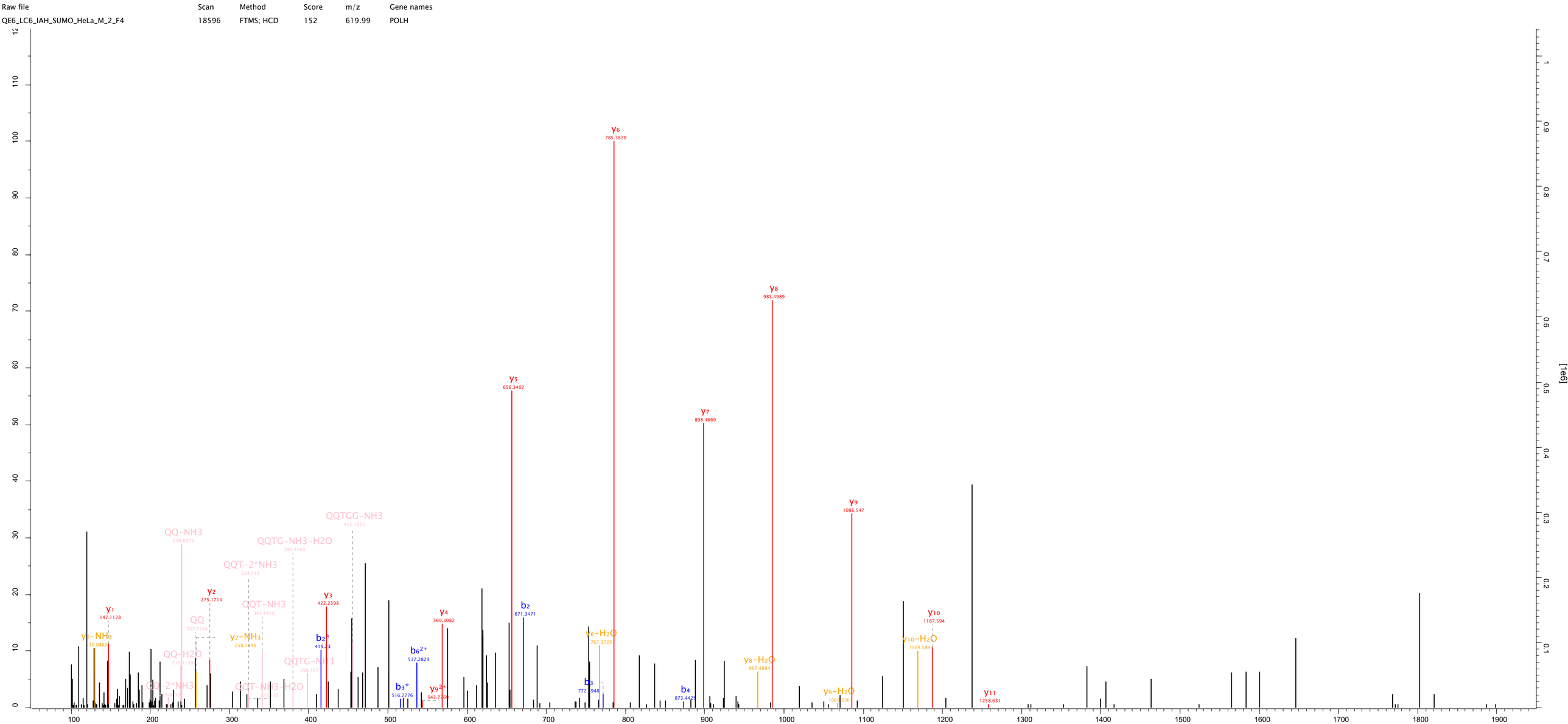

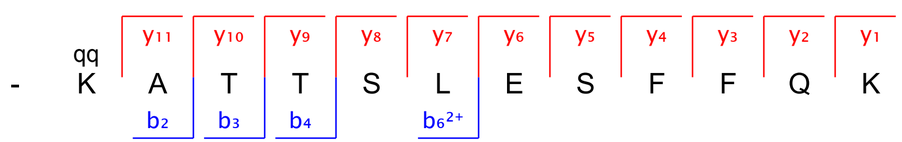

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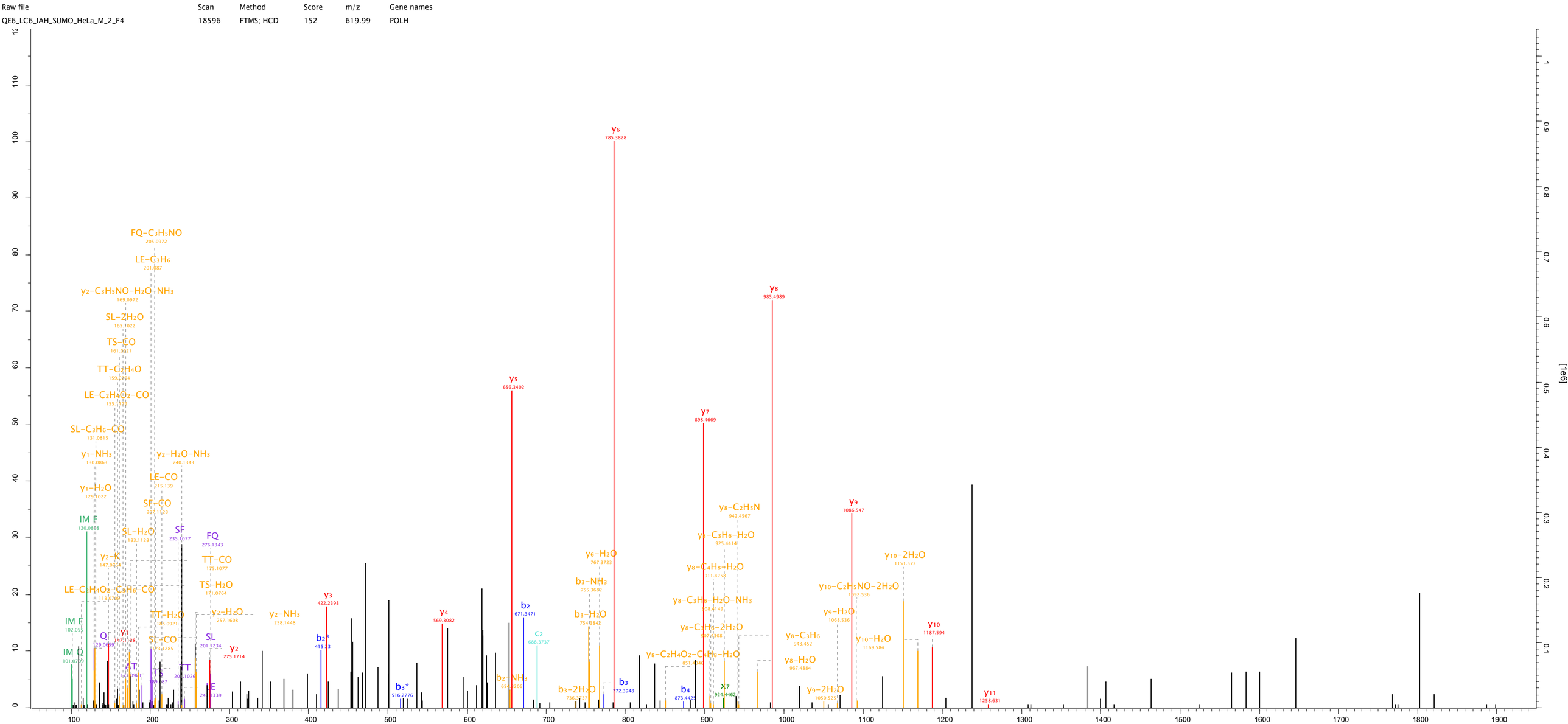
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F4	18596	FTMS; HCD	152	619.99	POLH

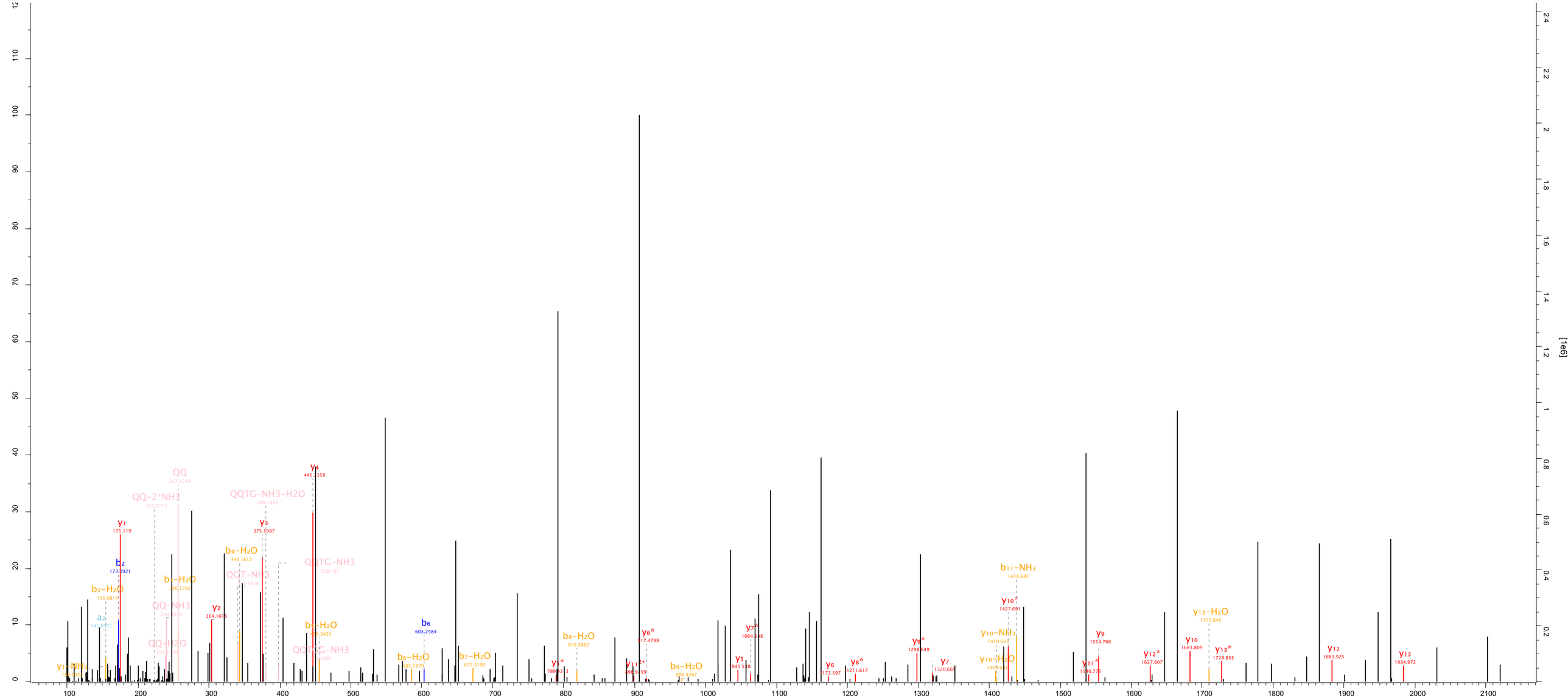


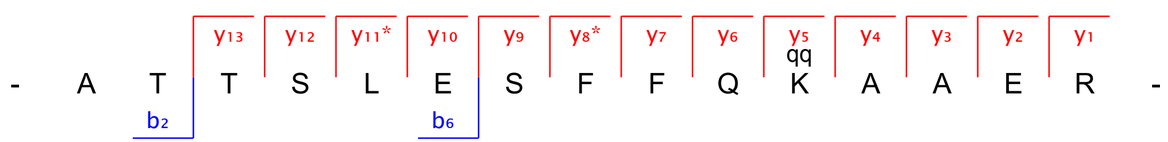
qq

y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1
- K	A	T	T	S	L	E	S	F	F	Q	K -
	b2	b3	b4		b6 2+						

Advanced: POLH-K475-SUMO

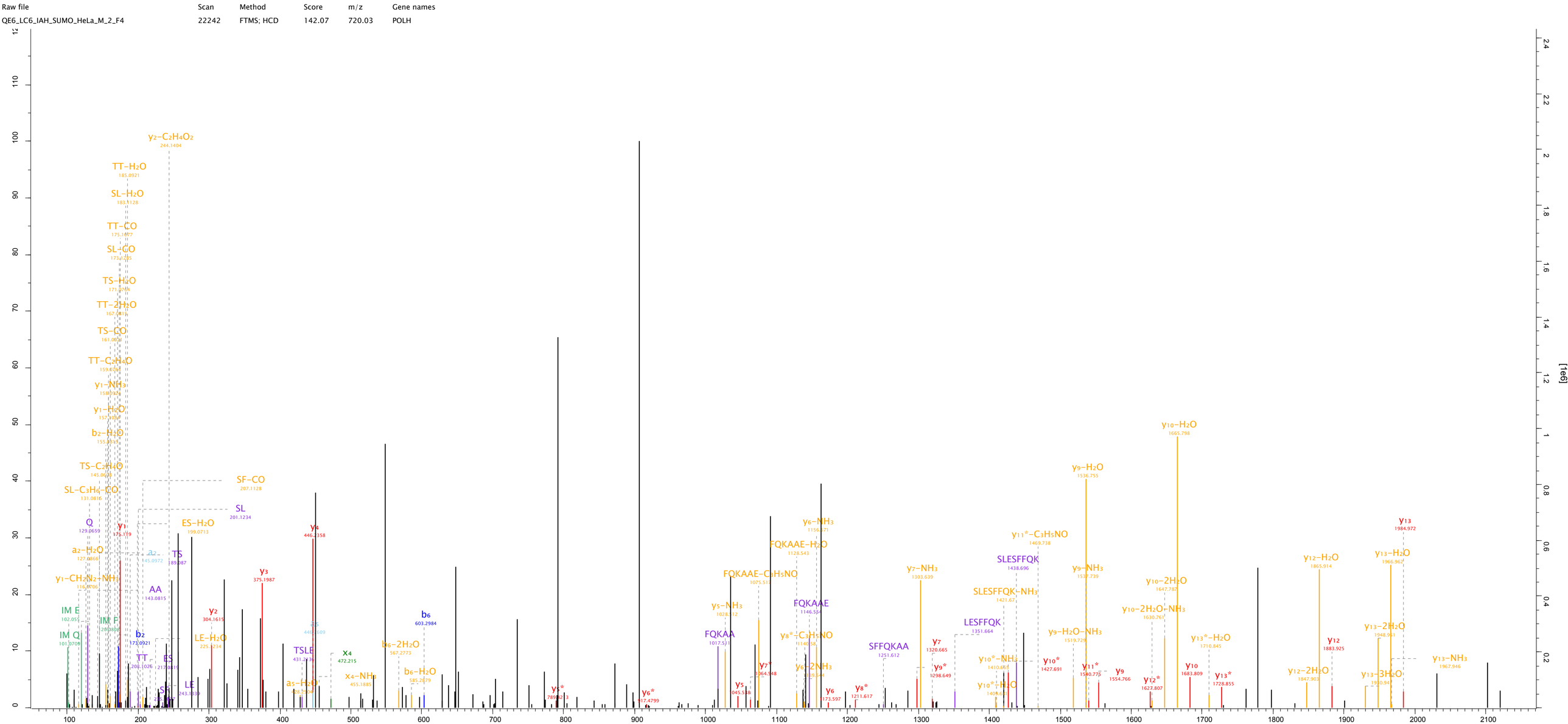
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F4	22242	FTMS; HCD	142.07	720.03	POLH





Standard: POLH-K486-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_2_F4	22242	FTMS; HCD	142.07	720.03	POLH



Advanced: POLH-K486-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F5	16043	FTMS; HCD	60.47	935.14	POLH

qq

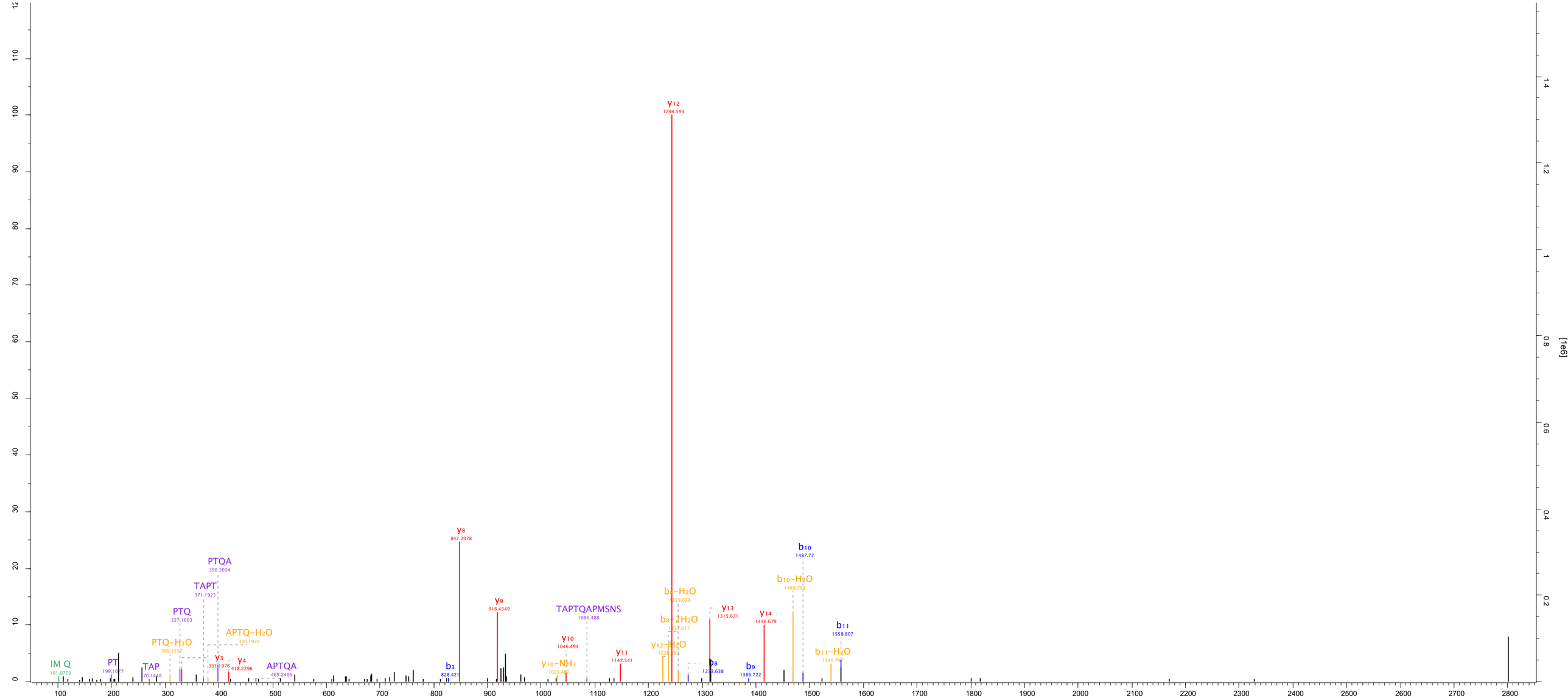
- V K E A S L S S L T A P T Q A P M S N S P S K -

b3 b8 b9 b10 b11

y14 y13 y12 y11 y10 y9 y8 y4 y3

Standard: POLH-K494-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F5	16043	FTMS; HCD	60.47	935.14	POLH

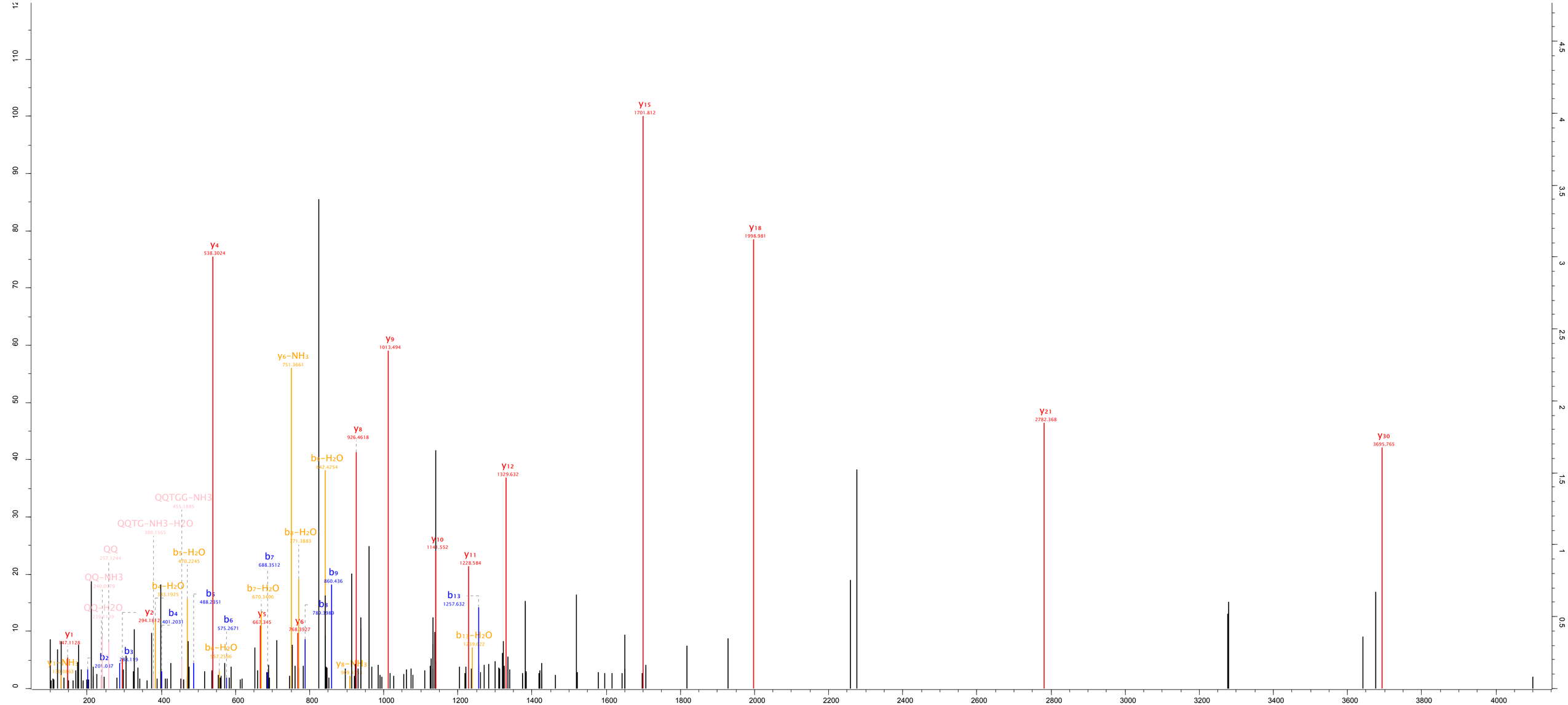


qq

-	V	K	E	A	S	L	S	S	L	T	A	P	T	Q	A	P	M	S	N	S	P	S	K	-
			b3					b8	b9	b10	b11									y4	y3			
										y14	y13	y12	y11	y10	y9	y8								

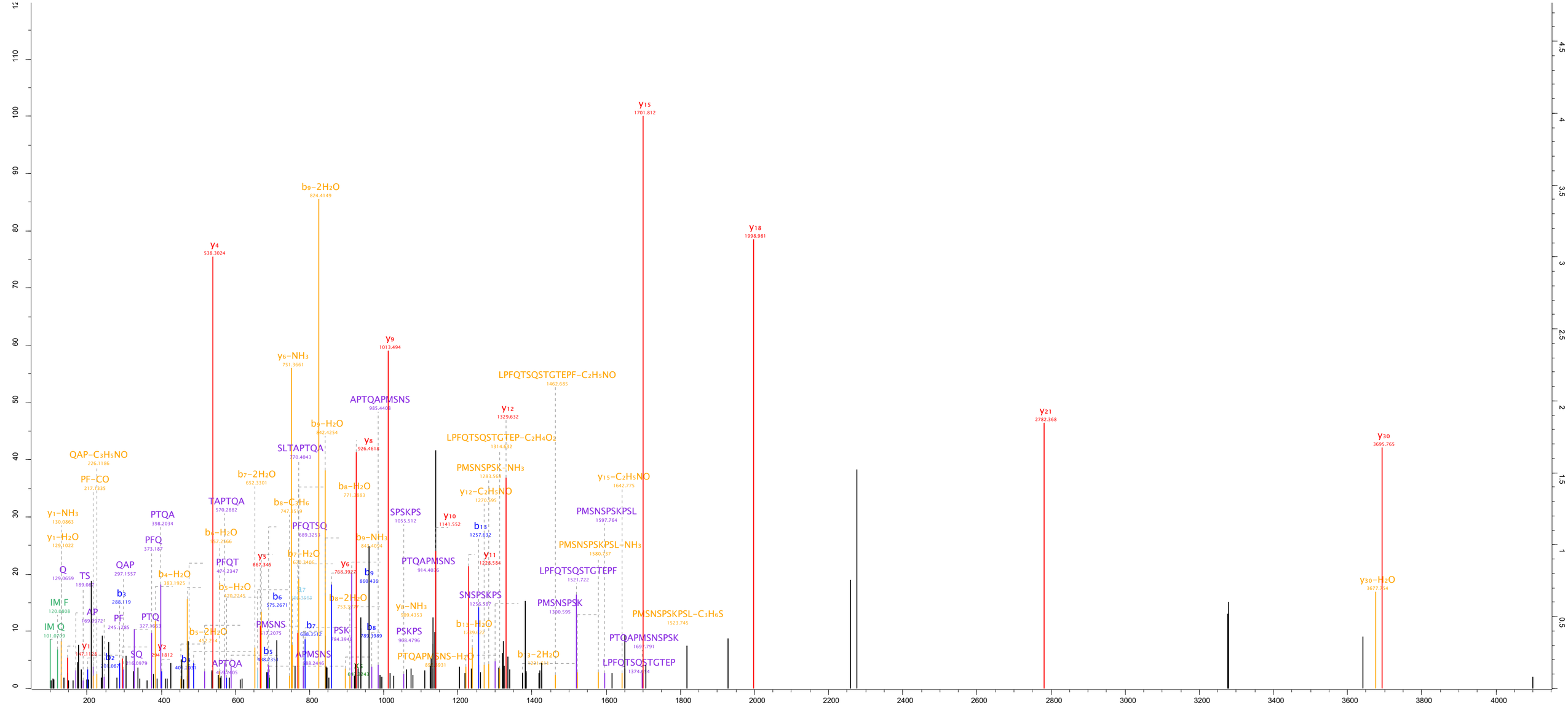
Advanced: POLH-K494-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F5	27746	FTMS; HCD	68.16	1140.06	POLH

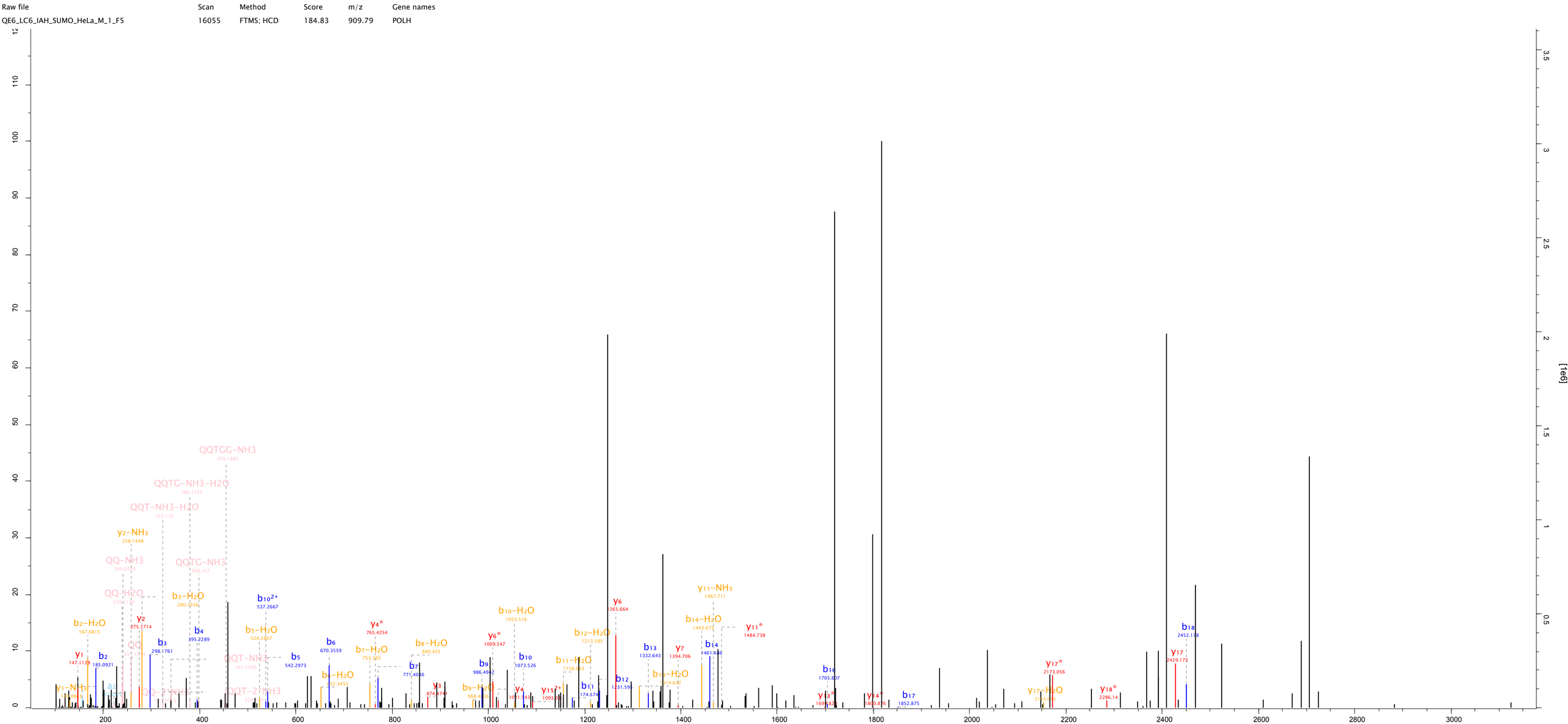


Standard: POLH-K515-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F5	27746	FTMS; HCD	68.16	1140.06	POLH

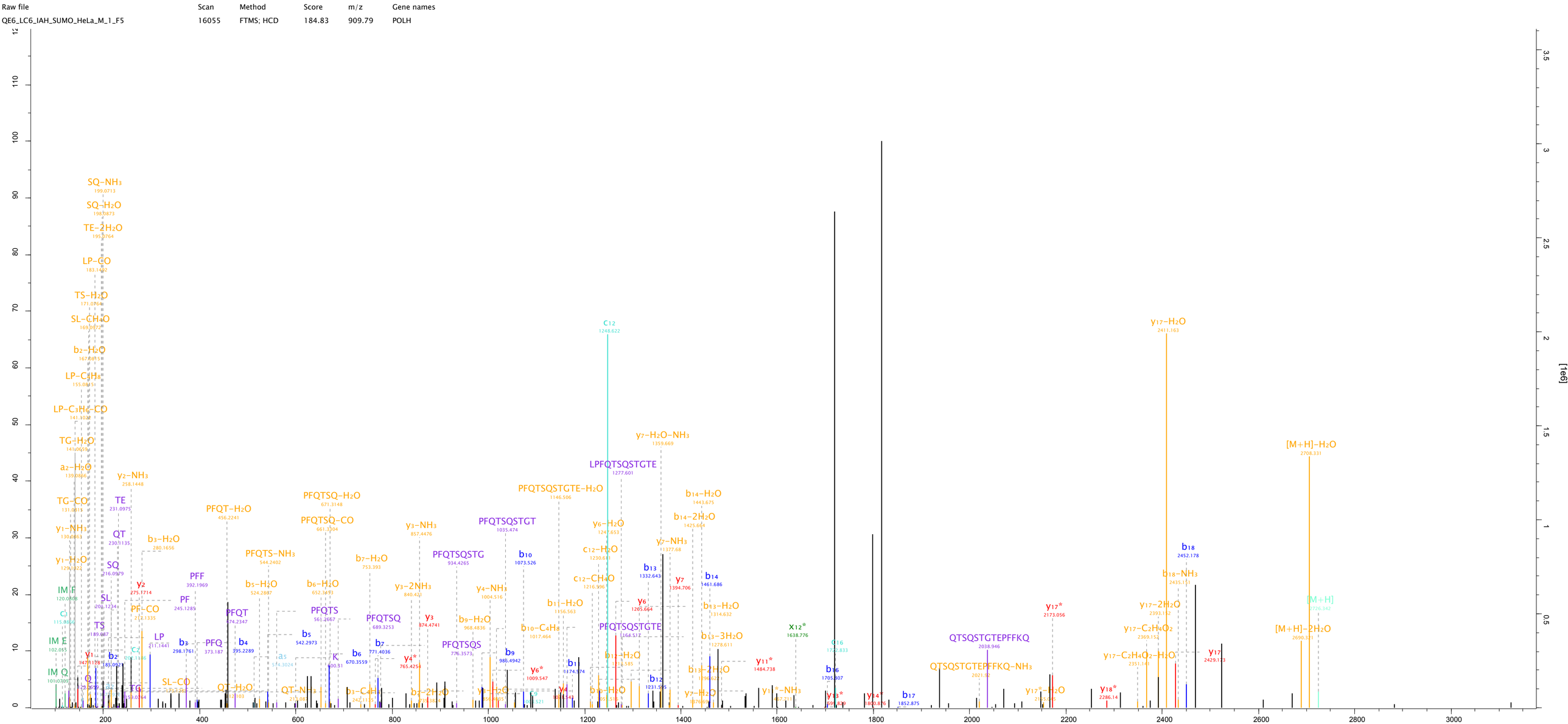


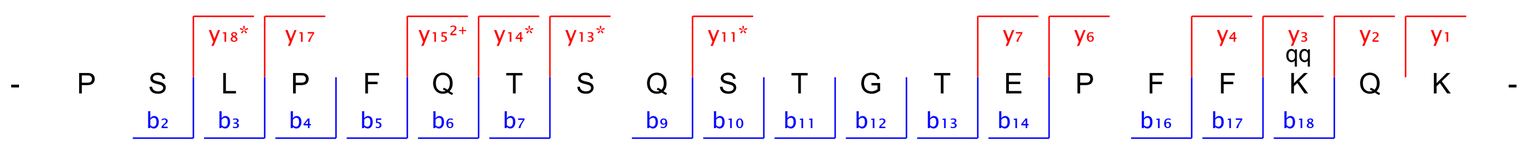
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F5	16055	FTMS; HCD	184.83	909.79	POLH



Standard: POLH-K533-SUMO

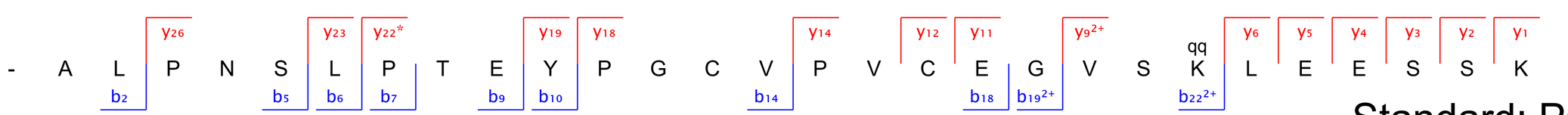
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F5	16055	FTMS; HCD	184.83	909.79	POLH





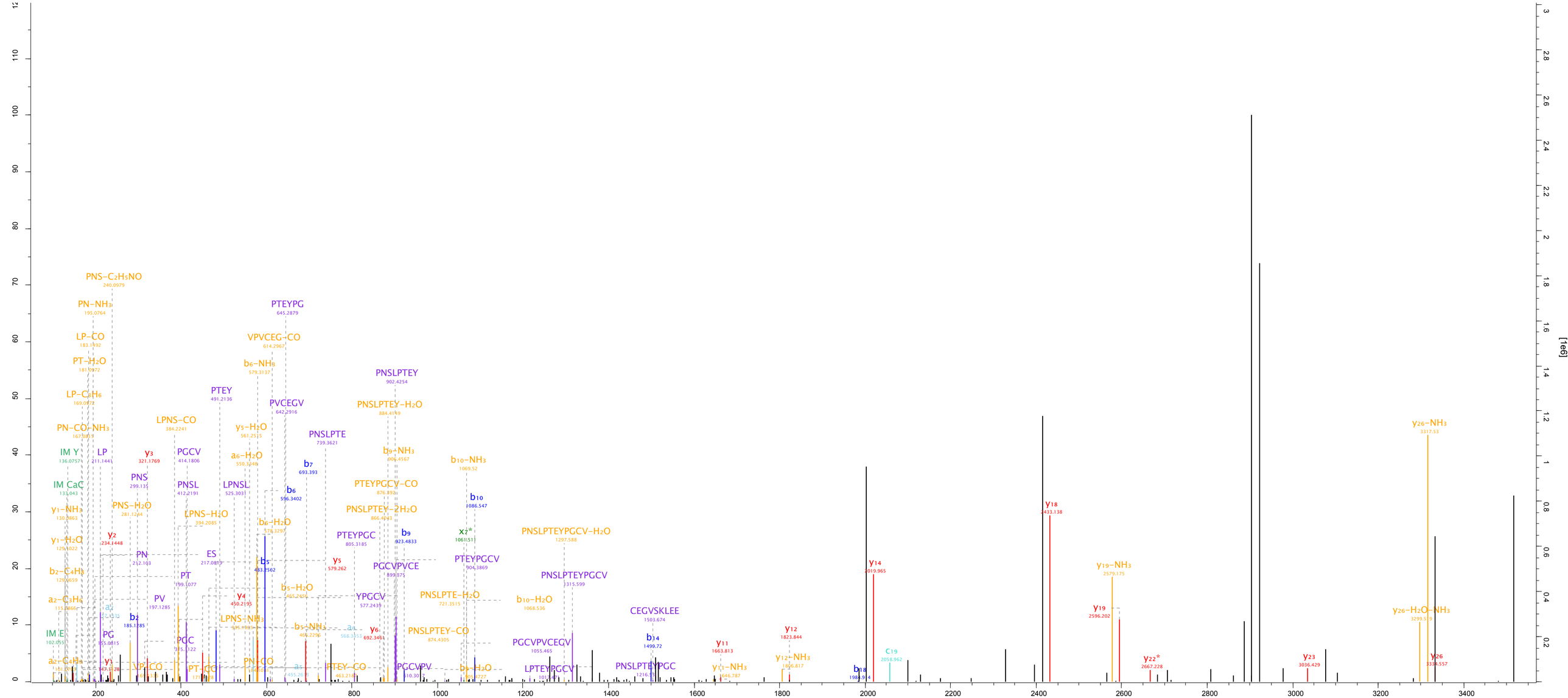
Advanced: POLH-K533-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F4	23233	FTMS; HCD	127.24	1174.57	POLH



Standard: POLH-K583-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F4	23233	FTMS; HCD	127.24	1174.57	POLH



- A L P N S L P T E Y P G C V P V C E G V S K L E E S S K -

y26 y23 y22* y19 y18 y14 y12 y11 y9 2+ qq y6 y5 y4 y3 y2 y1

b2 b5 b6 b7 b9 b10 b14 b18 b19 2+ b22 2+

Advanced: POLH-K583-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_3_F2	16707	FTMS; HCD	58.35	684.32	POLH

qq

y22	y21	y20	y19	y18	y17	y16		y13		y9		y6						y1
-	L	E	E	S	S	K	A	T	P	A	E	M	D	L	A	H	N	S	Q	S	M	H	A	S	S	A	S	K	-
		b2	b3	b4	c5																								

Standard: POLH-K589-SUMO

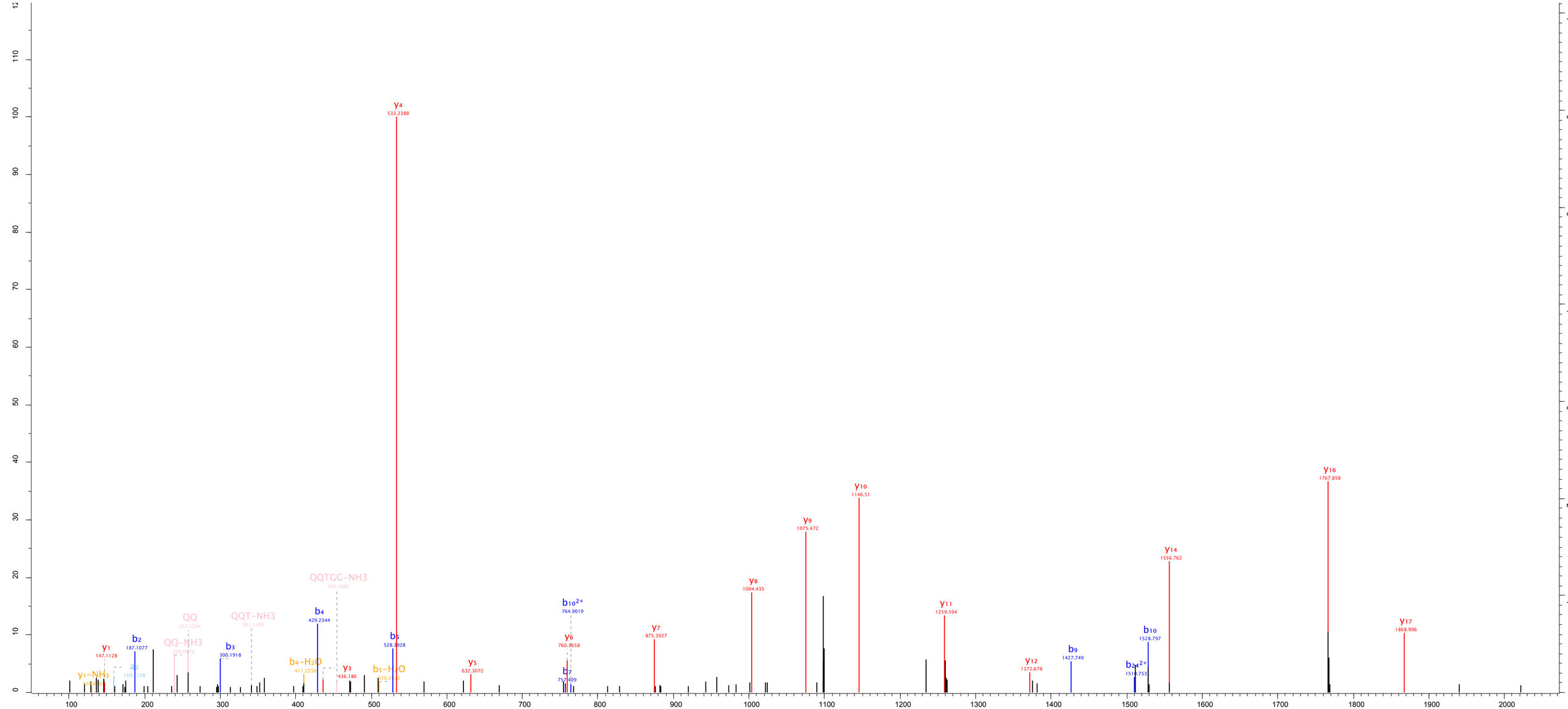
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_3_F2	16707	FTMS; HCD	58.35	684.32	POLH

qq

y22	y21	y20	y19	y18	y17	y16			y13			y9			y6					y1	
-	L	E	E	S	S	K	A	T	P	A	E	M	D	L	A	H	N	S	Q	S	M	H	A	S	S	A	S	K	-
		b2	b3	b4	c5																								

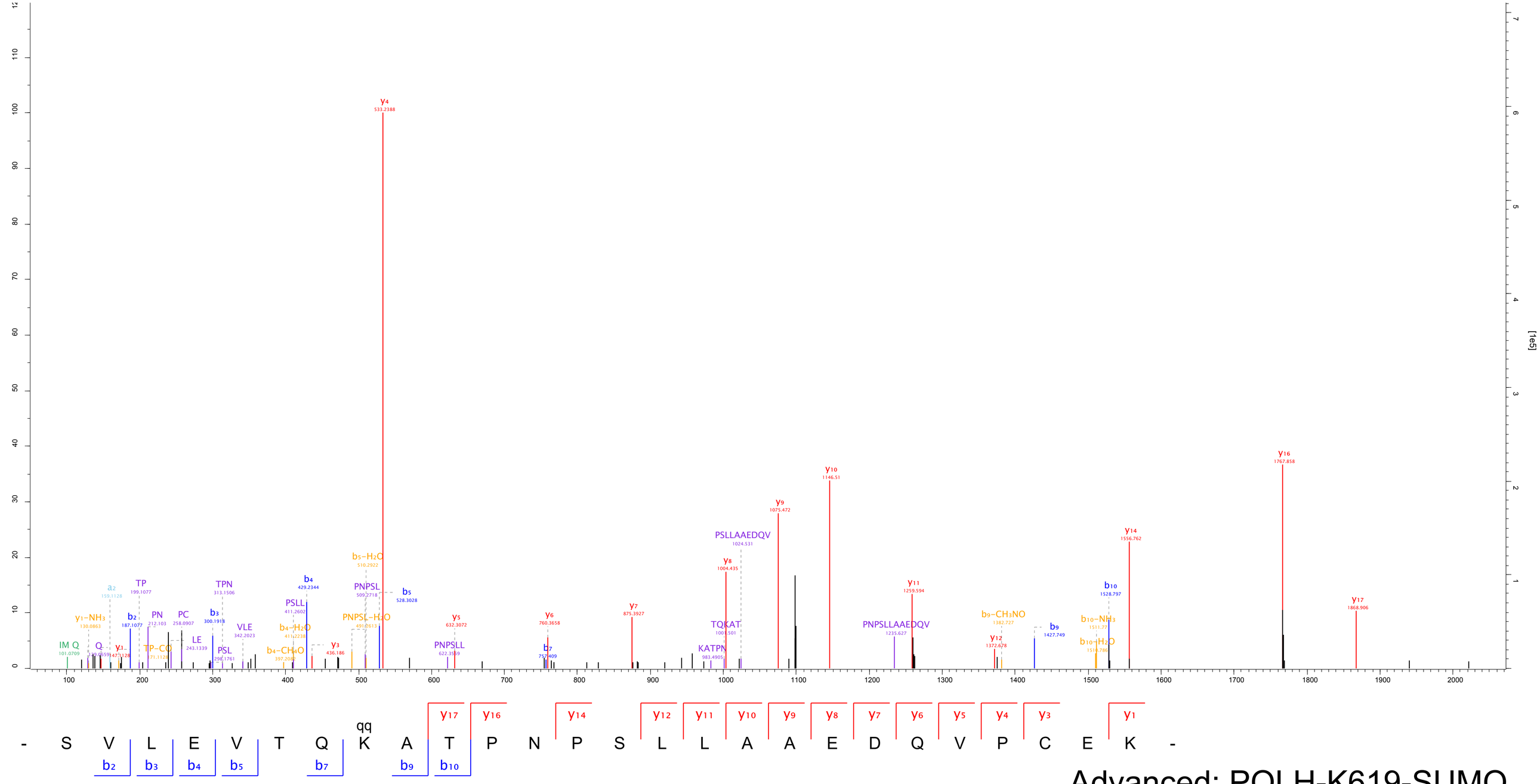
Advanced: POLH-K589-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F3	27256	FTMS; HCD	103.85	1099.56	POLH



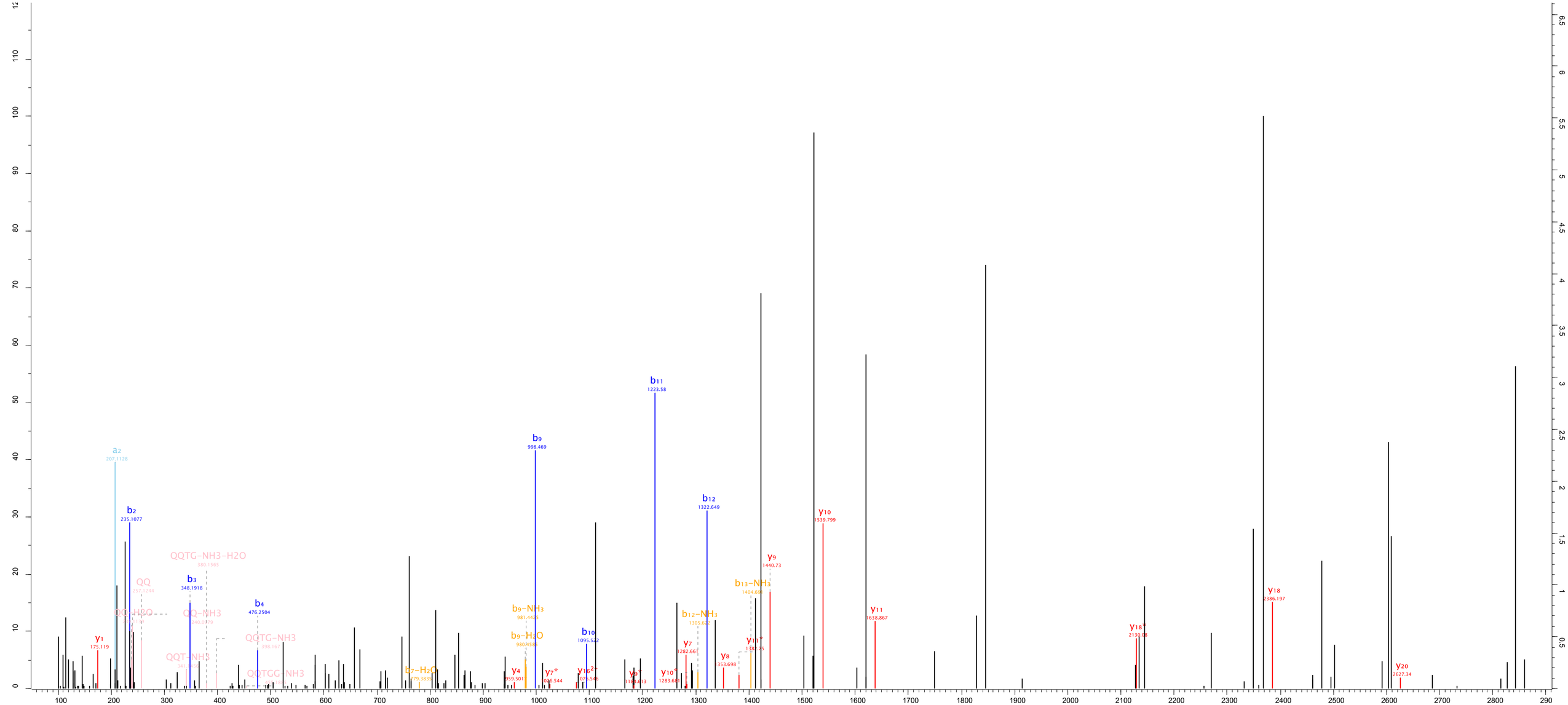
Standard: POLH-K619-SUMO

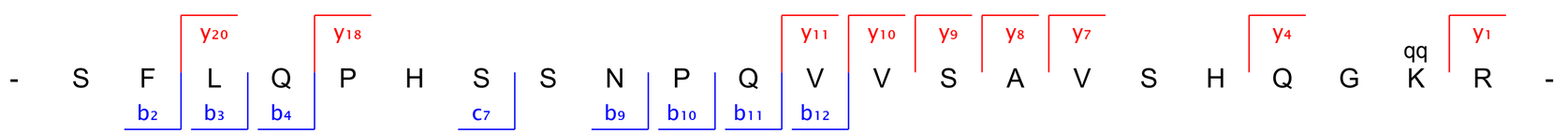
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_4_F3	27256	FTMS; HCD	103.85	1099.56	POLH



Advanced: POLH-K619-SUMO

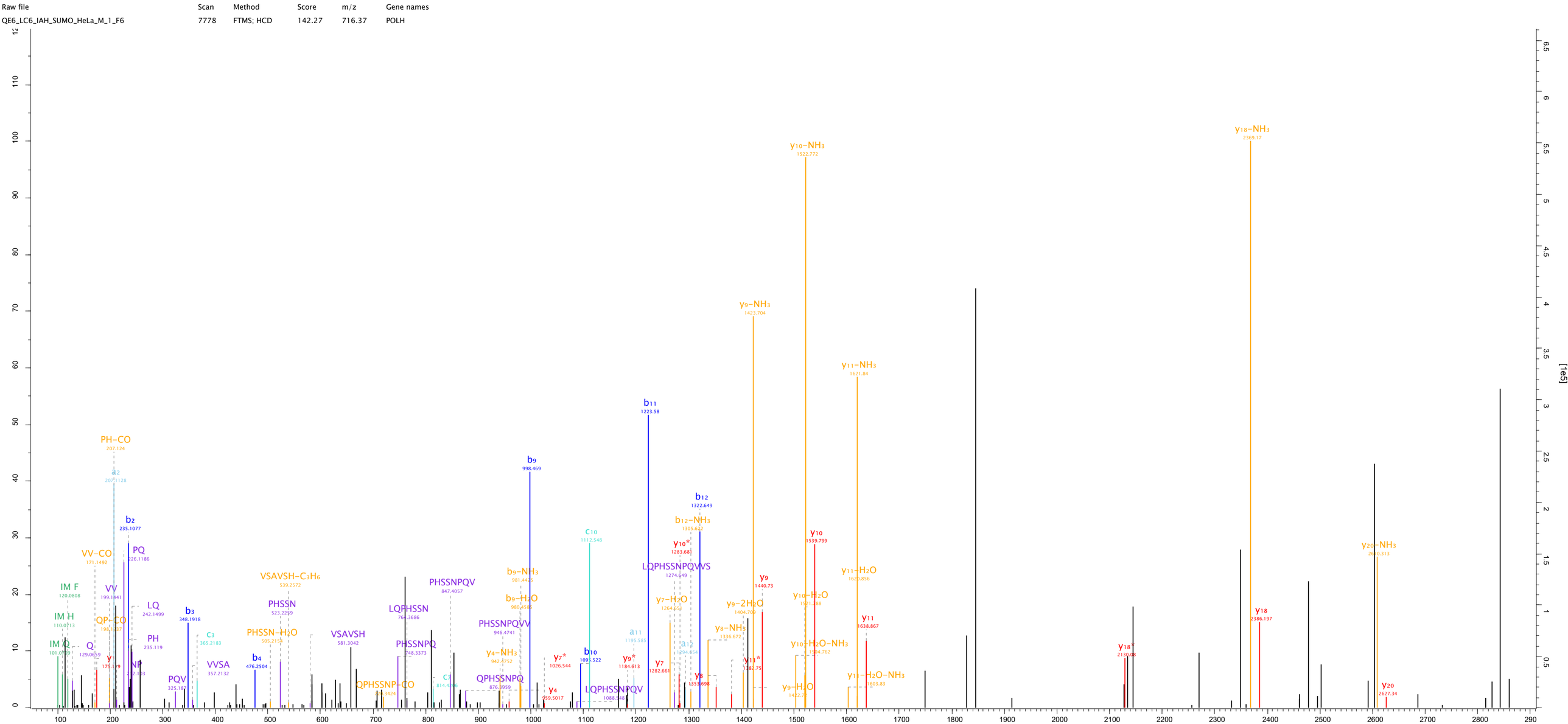
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	7778	FTMS; HCD	142.27	716.37	POLH

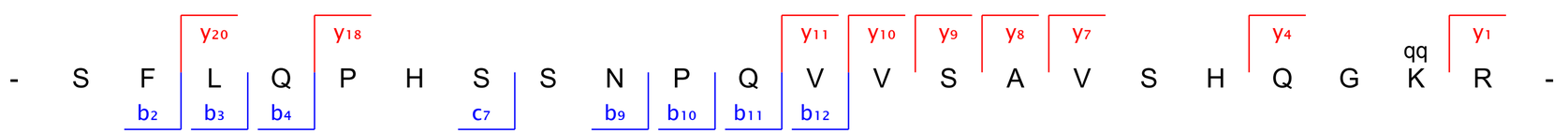




Standard: POLH-K682-SUMO

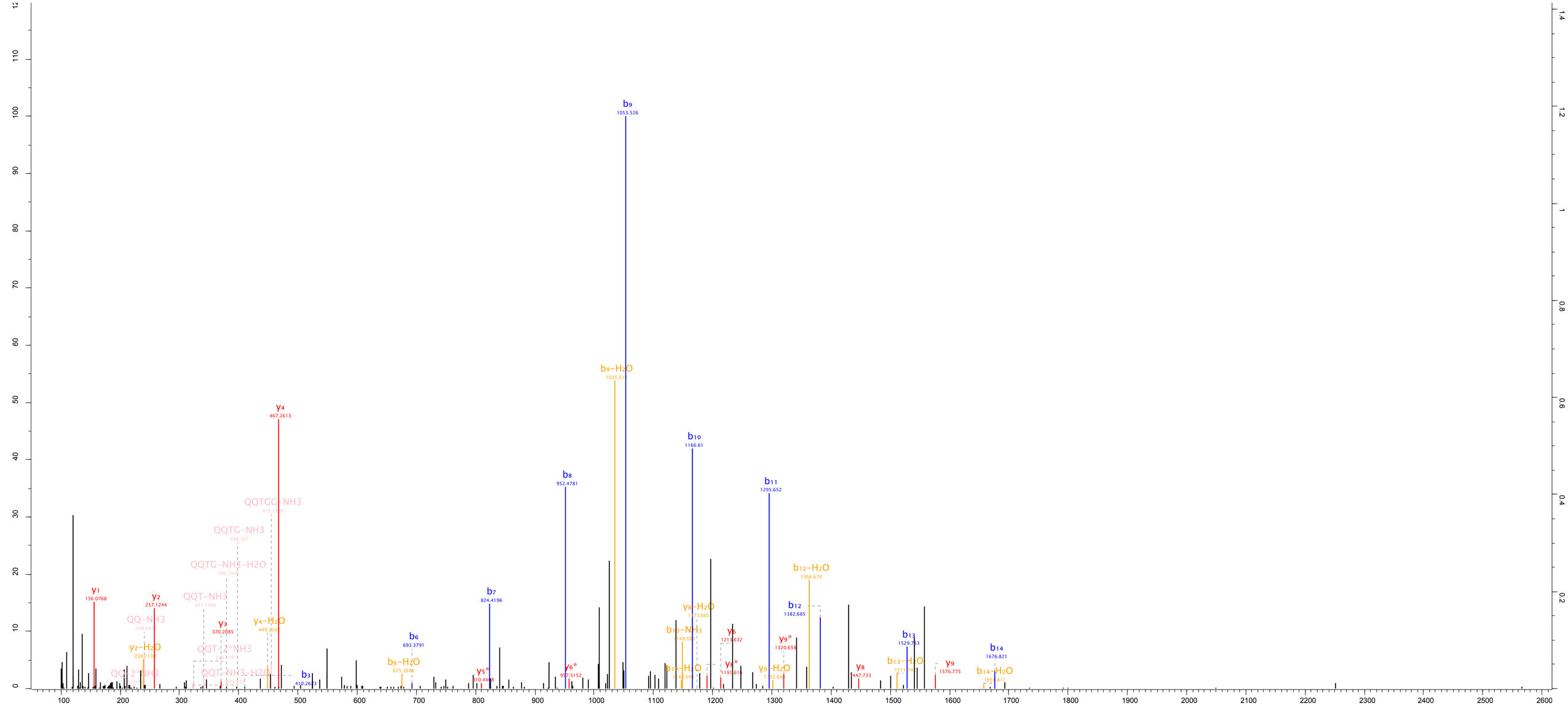
Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	7778	FTMS; HCD	142.27	716.37	POLH



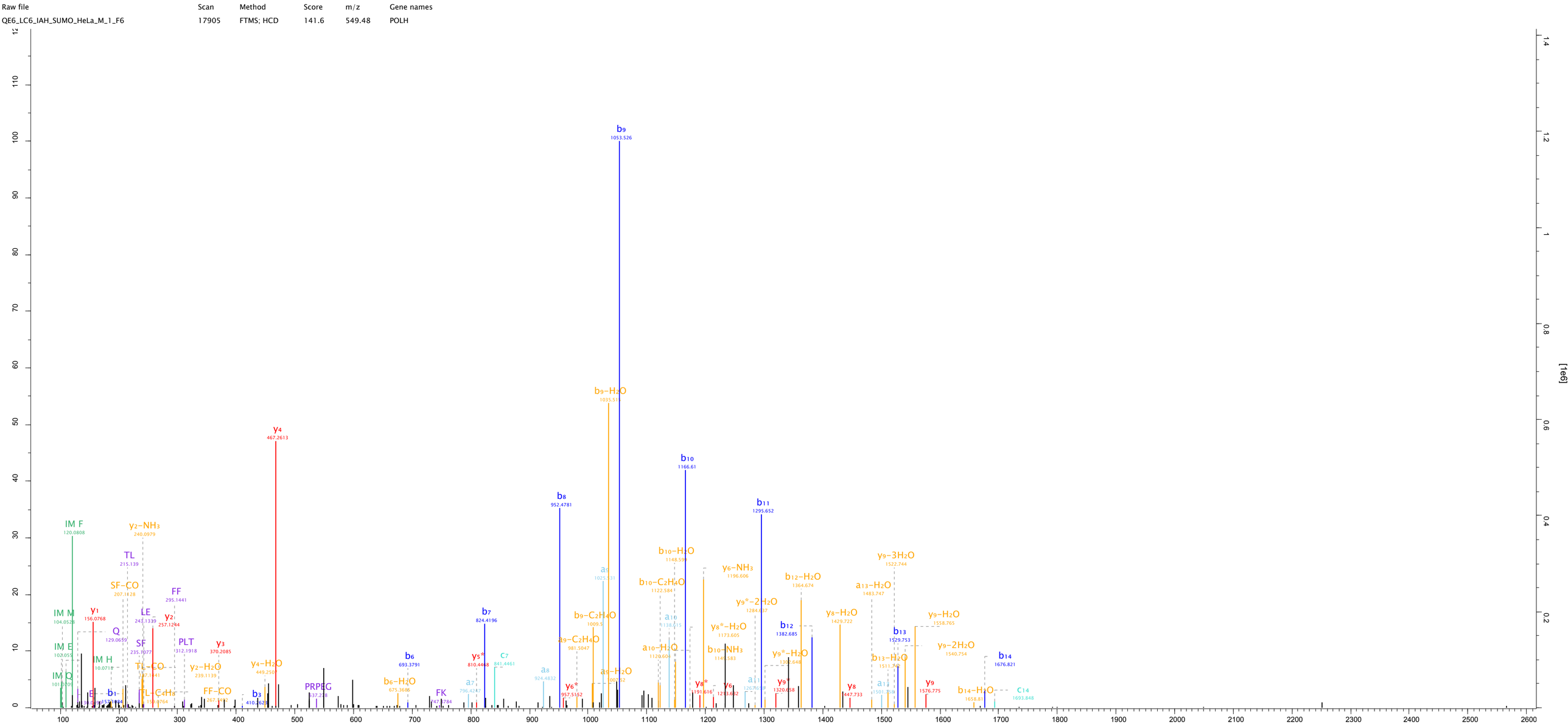


Advanced: POLH-K682-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	17905	FTMS; HCD	141.6	549.48	POLH



-	R	P	R	P	E	G	M	Q	T	L	E	S	F	F	K	P	L	T	H	-
	b1		b3			b6	b7	b8	b9	b10	b11	b12	b13	b14						
											y9	y8		y6	y5* qq	y4	y3	y2	y1	

Standard: POLH-K709-SUMO

Raw file	Scan	Method	Score	m/z	Gene names
QE6_LC6_IAH_SUMO_HeLa_M_1_F6	17905	FTMS; HCD	141.6	549.48	POLH