Cytoplasmic N-glycosyltransferase of *Actinobacillus pleuropneumoniae* is an inverting enzyme and recognizes the N-X-S/T consensus sequence

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Experimentally measured chemical shifts of the two glucosylated tamra-DANYTK peptides in comparison to observed chemical shifts for model compounds and calculated values.

glycan unit	nucleus	experimental tamra-DANYTK-Glc[a]	Glc-β-Asn (1) [b]	Glc-α-Asn (1) [b]	experimental tamra-DANYTK-Glc_3[a]	methyl α-isomalto-trioside (2) [c]	β-isomalto-triose (3)	Glcα1,6Glcα1,6Glcβ-Asn calculated by CASPER (4) [e]
Glc_A	C1 / H1	82.0 / 4.96	81.9	79.3	82.3 / 4.98	102.0 / 4.81	- / 4.66	82.3 / 5.03
	C2 / H2	74.5 / 3.42	74.6	72.1	74.4 / 3.44	74.0 / 3.56	- / 3.24	74.5 / 3.44
	C3 / H3	79.2 / 3.55	79.2	75.4	79.5 / 3.55	76.1 / 3.65	- / 3.46	79.4 / 3.57
	C4 / H4	72.0 / 3.43	72.0	72.1	71.9 / 3.55	72.2 / 3.50	- / 3.49	72.0 / 3.55
	C5 / H5	80.4 / 3.50	80.3	75.8	78.7 / 3.69	72.7 / 3.82	- / 3.63	79.0 / 3.70
	C6 / H6 & H6′	63.3 / 3.87 & 3.72	63.3	63.3	68.3 / 3.97 & 3.74	68.3 / 3.99 & 3.74	- / 3.95 & 3.75	68.5 / 4.08 & 3.64
Glc_B	C1 / H1				100.7 / 4.94	100.5 / 4.96	- / 4.95	100.6 / 4.97
	C2 / H2				74.2 /3.57	74.0 / 3.57	- / 3.56	74.1 / 3.59
	C3 / H3				76.1 / 3.71	76.0 / 3.71	- / 3.72	76.0 / 3.73
	C4 / H4				72.3 / 3.51	72.2 / 3.51	- / 3.50	72.3 / 3.54
	C5 / H5				73.0 / 3.90	73.0 / 3.90	- / 3.73[d]	73.0 / 3.90
	C6 / H6 & H6′				68.3 / 3.95 & 3.73	68.3 / 3.96 & 3.74	- / 3.96 & 3.89[d]	68.6 / 3.97 & 3.77
Glc_T	C1 / H1				100.5 / 4.95	100.5 / 4.95	- / 4.95	100.6 / 4.96
	C2 / H2				74.2 / 3.56	74.3 /3.54	- / 3.54	74.1 / 3.57
	C3 / H3				75.9 /3.72	75.8 / 3.71	- / 3.71	75.8 / 3.74
	C4 / H4				72.3 /3.43	72.2 / 3.42	- / 3.41	72.4 / 3.44
	C5 / H5				74.6 / 3.71	74.6 / 3.70	- / 3.73	74.5 / 3.73
	C6 / H6 & H6′				63.2 /3.84 & 3.76	63.2 / 3.83 & 3.75	- / 3.83 & 3.76	63.4 / 3.86 & 3.77

[a] Chemical shifts are referenced to DSS according to Markley *et al*. (5).

[b] ^{13}C chemical shifts are shifted by +1.9 ppm, original shifts were referenced to TMS via internal 1,4-dioxane.

[c] ^{1}H chemical shifts are shifted by +0.05 ppm, ^{13}C chemical shifts by 2.72 ppm (difference between neat TMS and DSS in H_2O)

[d] ^{1}H chemical shifts of H5 and H6′ are incorrectly assigned and need to be swapped

[e] ^{13}C chemical shifts are shifted by + 1.7 ppm, CASPER references to TMS

Supplementary Figure 1
Phylogenetic tree of representative homologs of the HMW1C protein of *H. influenzae* found among proteobacteria
Homologs were identified by BLAST search and the tree was constructed with Phylogeny.fr (6). Organisms indicated in black are gamma-proteobacteria, whereas *Burkholderia xenovorans* LB400 belongs to the group of beta-proteobacteria.

Supplementary Figure 2
X. campestris OGT does not modify tamra-labeled DANYTK peptide in presence of donor substrates
Reaction products were separated by Tricine-SDS-PAGE analysis and fluorescent signals were acquired by an image analyzer.

Supplementary Figure 3
Genomic region of *A. pleuropneumoniae* AP76 encoding for *ngt*
Arrows indicate open reading frames.

Supplementary Figure 4
NGT and α6GlcT are metal ion-independent glycosyltransferases
Tamra-labeled peptides were incubated with NGT and α6GlcT in presence or absence of EDTA. Reaction products were separated by Tricine-SDS-PAGE analysis and fluorescent signals were acquired by an image analyzer.

Supplementary Figure 5
MALDI-MS analysis of glucosylated products shows that the glucosyltransferase α6GlcT elongates the N-linked glucose with up to six units of glucose in presence of a 1:1000 acceptor:donor ratio.

Supplementary Figure 6
^{1}H-^{13}C HSQC spectra of tamra-DANYTK (A), tamra-DANYTK-glucose (B), and tamra-DANYTK-glucose$_3$ (C). In the last spectrum three different glucose species were identified: terminal glucose (Glc_T), bridging glucose (Glc_B) and the Asn-linked glucose (Glc_A). A star denotes an impurity from the initial peptide.

Supplementary Figure 7
MALDI-MS/MS spectrum of the precursor ion at *m/z* 1882.93 matches with fragmentation of SIVNPGGSN(Hex)LTYTIER glycopeptide

Supplementary Figure 8
MALDI-MS/MS spectra of precursor ions corresponding to glucosylated peptides indicated in Figure 4B
A) Fragmentation of ion at *m/z*=2610.20 matches with fragmentation of YN(Hex)YSNPGFSESTGHFTQVVWK. B) Fragmentation of ion at *m/z*=2579.20 matches with fragmentation of LN(Hex)STFEDAVIPLIFSEYGCNK. C) Fragmentation of ion at *m/z*=2229.10 matches with fragmentation of LLN(Hex)SSQTATISLADGTEAFK. D) Fragmentation of ion at *m/z*=1702.78 matches with fragmentation of FHN(Hex)YTLDWAMDK. E) Fragmentation of ion at *m/z*=2966.57 matches with fragmentation of LSN(Hex)YTGQFSGALSFLNDDYEFFIR. F) Fragmentation of ion at *m/z*=2848.37 matches with fragmentation of FSSN(Hex)ETLAIVYSHNAPLNQVVNLR. G) Fragmentation of ion at *m/z*=2188.072666.15 matches with fragmentation of SFAN(Hex)TTAFALSPPVDGFVGK. H) Fragmentation of ion at *m/z*=1999.07 matches with fragmentation of TLGYN(Hex)TSLTLLDNHFK. I) Fragmentation of ion

at *m/z*=1869.87 matches with fragmentation of IDADFN(Hex)ATFYSMANK. J) Fragmentation of ion at *m/z*=1813.85 matches with fragmentation of SDAGFN(Hex)ISLSDLWAR. K) Fragmentation of ion at *m/z*=2659.32 matches with fragmentation of ILGIDPN(Hex)VTQYTGYLDVEDEDK. L) Fragmentation of ion at *m/z*=2252.16 matches with fragmentation of LEYLDIN(Hex)STSTTVDLYDK. M) Fragmentation of ion at *m/z*=2024.86 matches with fragmentation of GGANFDSSSSN(Hex)FSCNALK. N) Fragmentation of ion at *m/z*=2630.37 matches with fragmentation of LAPTYQELADTYAN(Hex)ATSDVLIAK. O) Fragmentation of ion at *m/z*=2842.44 matches with fragmentation of FAWWAAEEEGLLGSNFYAYN(Hex)LTK.

Supplementary Figure 9

LC-ESI-MS/MS analysis of the tryptic products of glucosylated AcrA

A) Spectrum from fragmentation of the doubly charged precursor ion at *m/z*=700.31 corresponds to the peptide ATFENASKDFNR. B) Spectrum from fragmentation of the doubly charged ion at *m/z*=862.39 matches with the glycopeptide ATFEN(Hex)ASKDFN(Hex)R. C) Spectrum from fragmentation of the doubly charged precursor ion at *m/z*=1378.21 corresponds to the peptide AVFDNNNSTLLPGAFATITSEGFIQK. D) Spectrum from fragmentation of the doubly charged ion at *m/z*=1459.24 matches with the glycopeptide AVFDNNN(Hex)STLLPGAFATITSEGFIQK. The exact location of the hexose could not be identified.

Supplementary References

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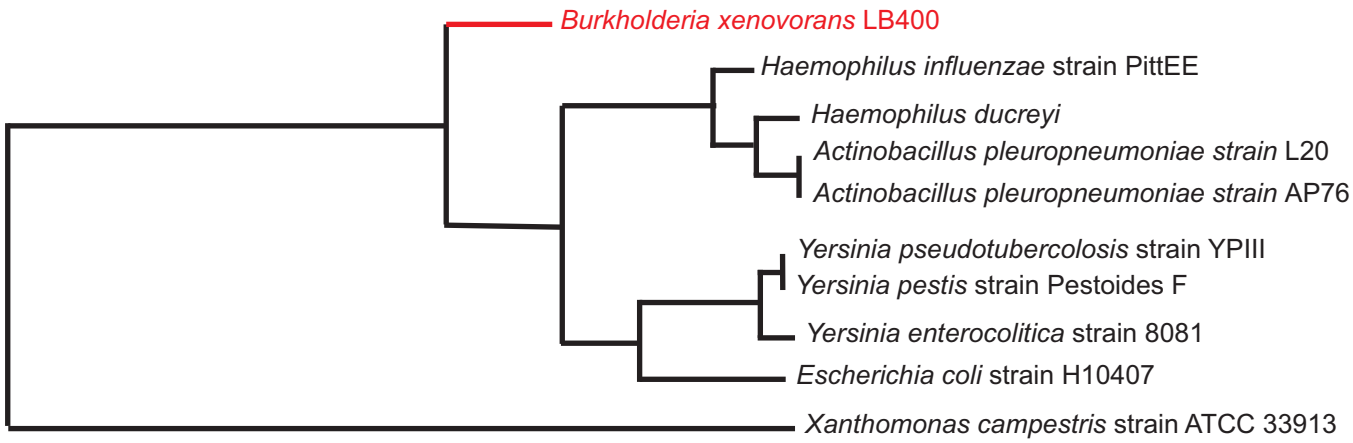
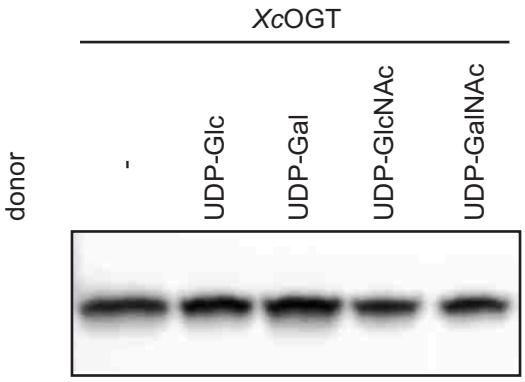
Supplementary Figure 1



Supplementary Figure 2



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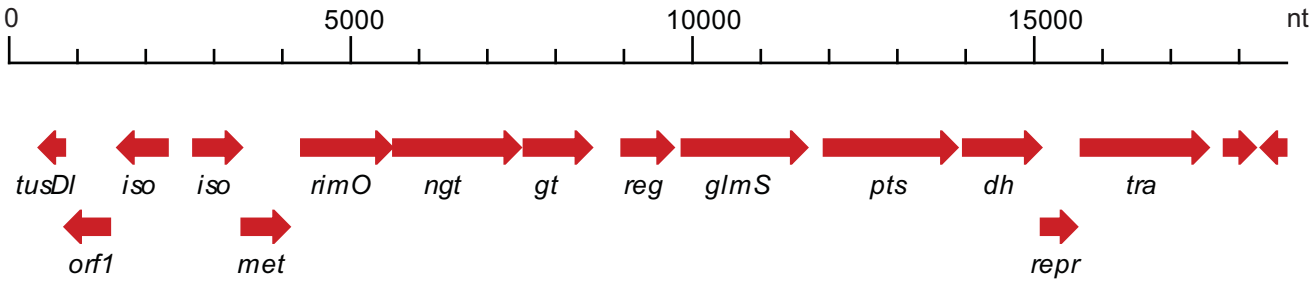
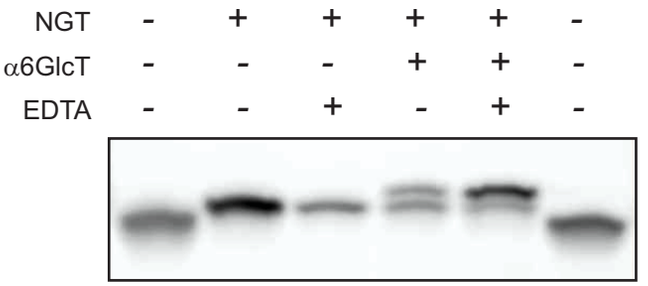


tusDl	Sulfurtransferase TusD-like protein
orf1	Putative uncharacterized protein
iso	Peptidyl-prolyl cis-trans isomerase
iso	Thiol-disulfide isomerase and thioredoxin
met	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase
rimO	Ribosomal protein S12 methylthiotransferase
ngt	N-Glycosyltransferase
gt	Putative glycosyltransferase
reg	Predicted transcriptional regulator of sugar metabolism
glmS	Glucosamine-fructose-6-phosphate aminotransferase
pts	PTS System mannitol-specific EIICBA component
dh	Mannitol-1-phosphate dehydrogenase
repr	Mannitol operon repressor
tra	Di- and tricarboxylate transporter

Supplementary Figure 4



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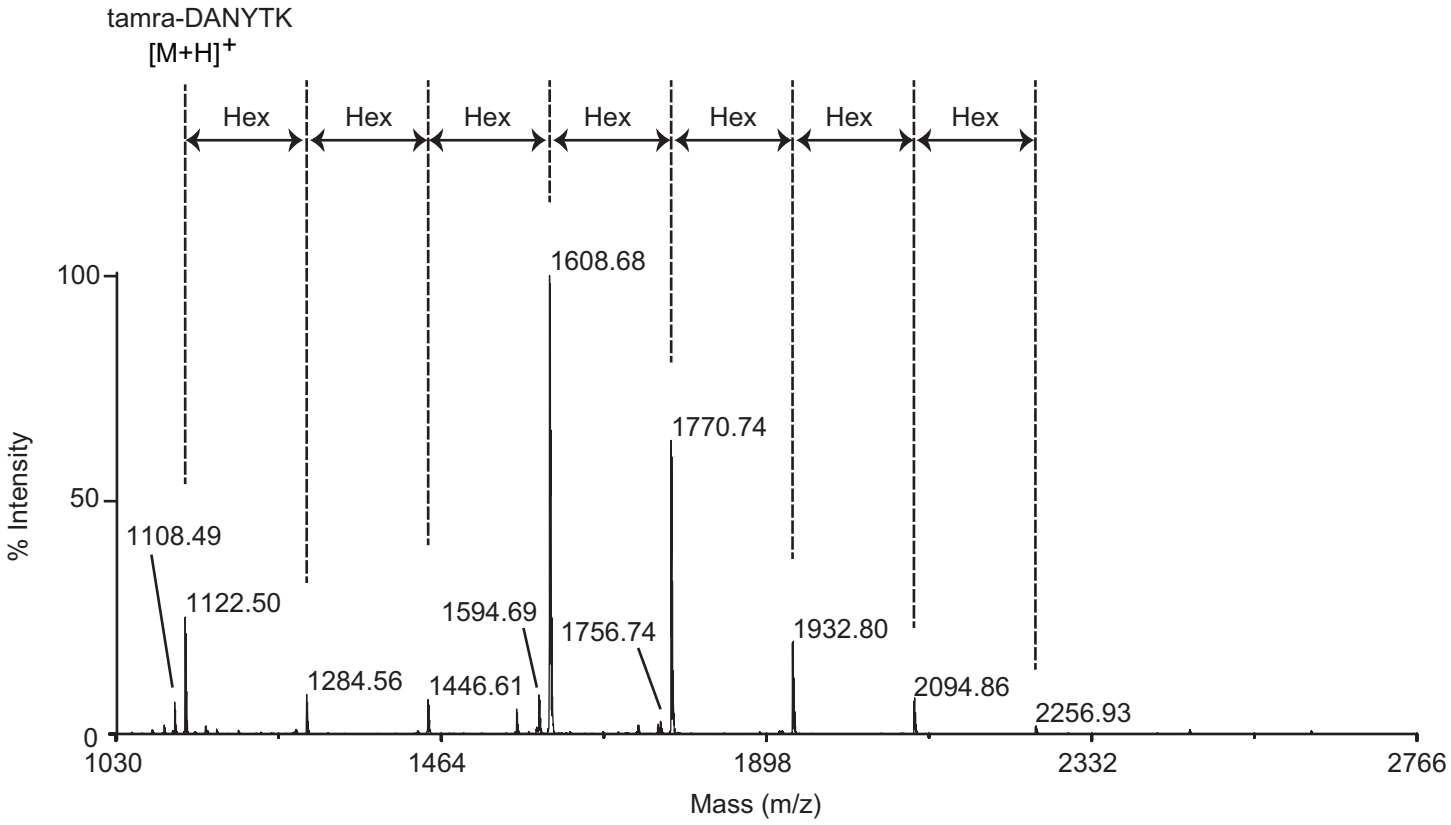

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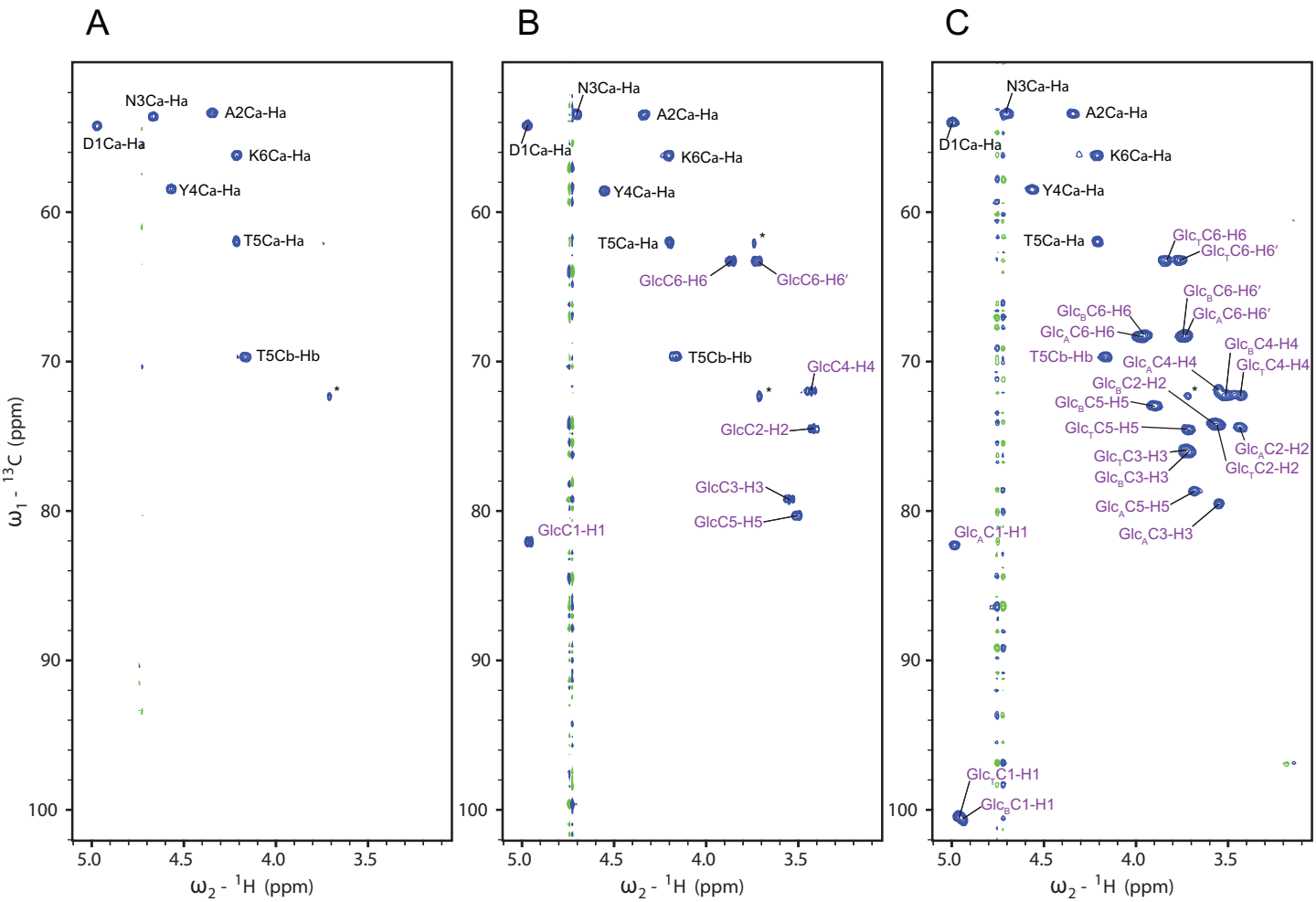

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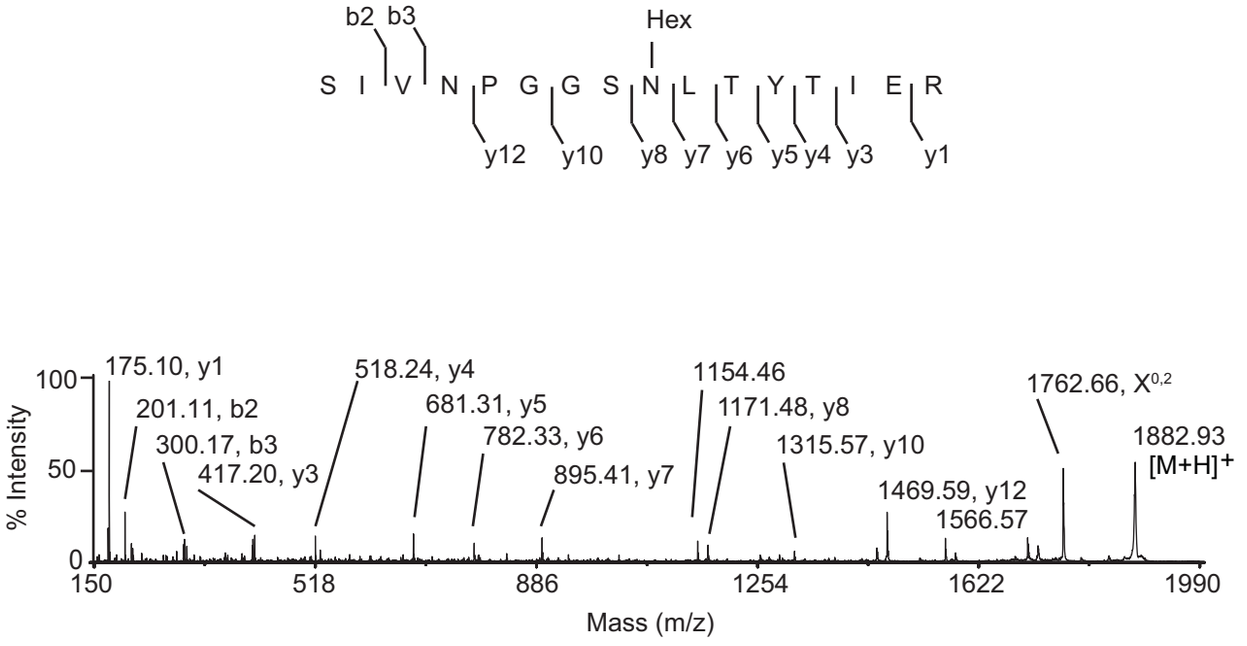

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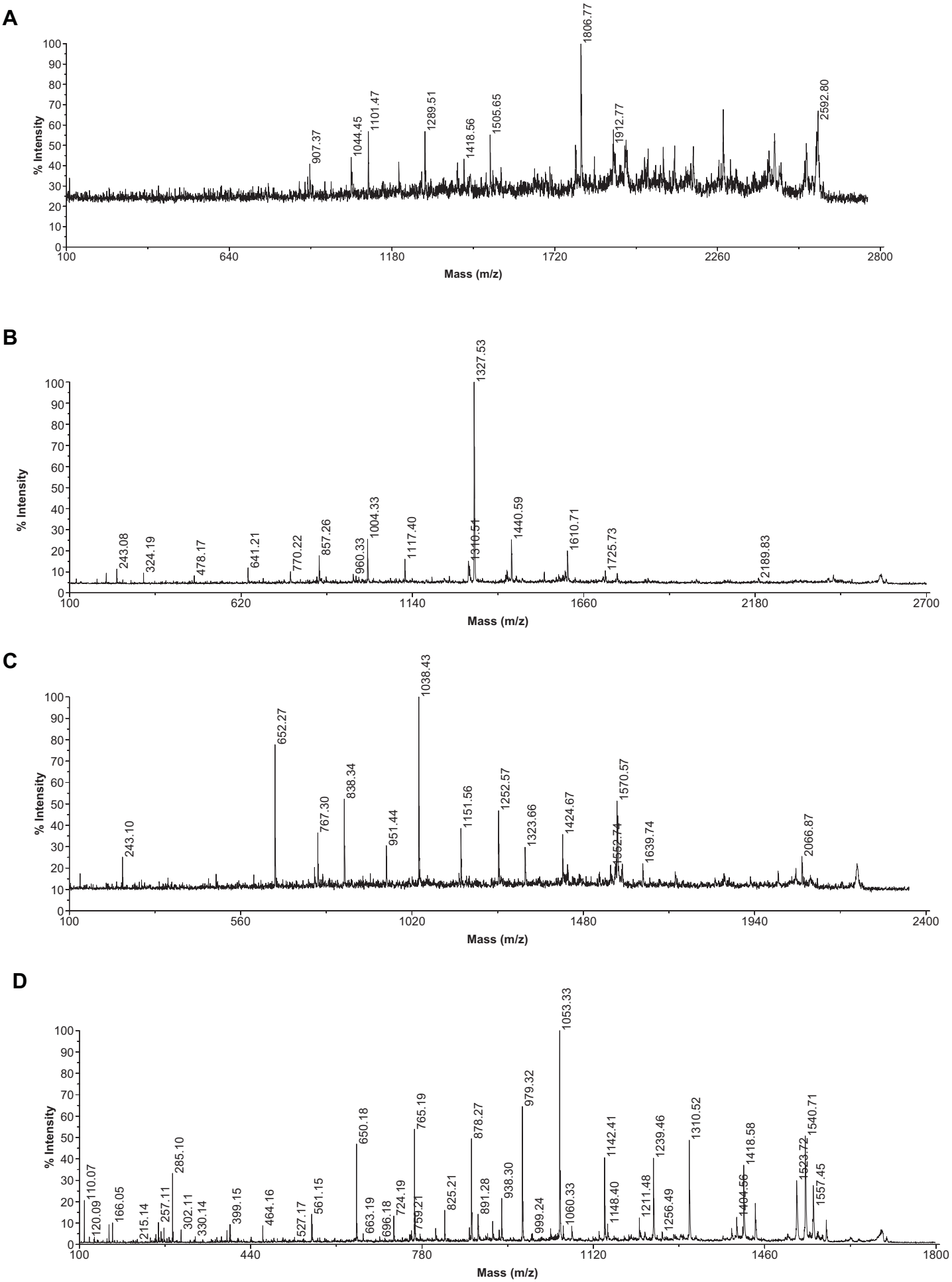

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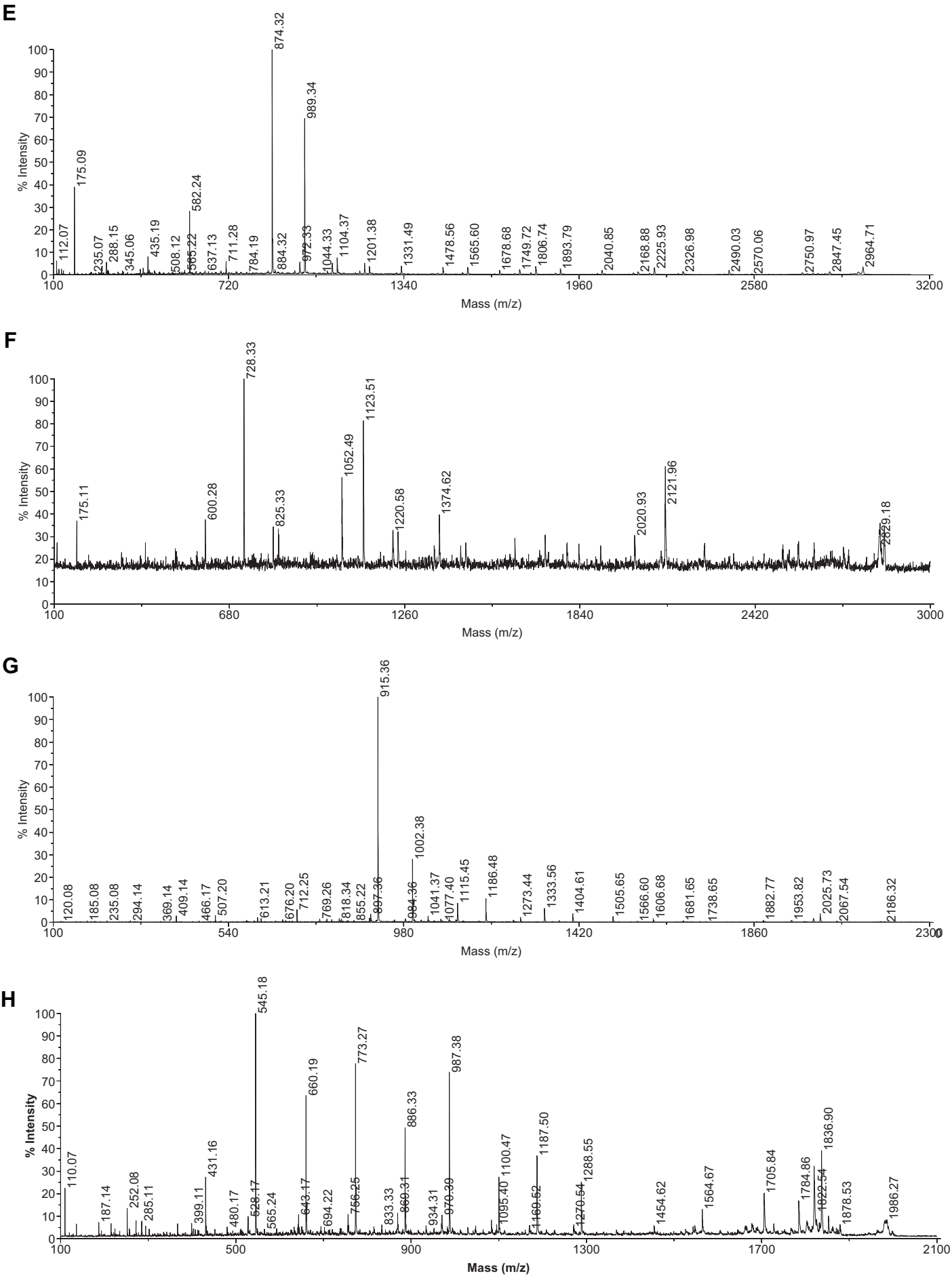

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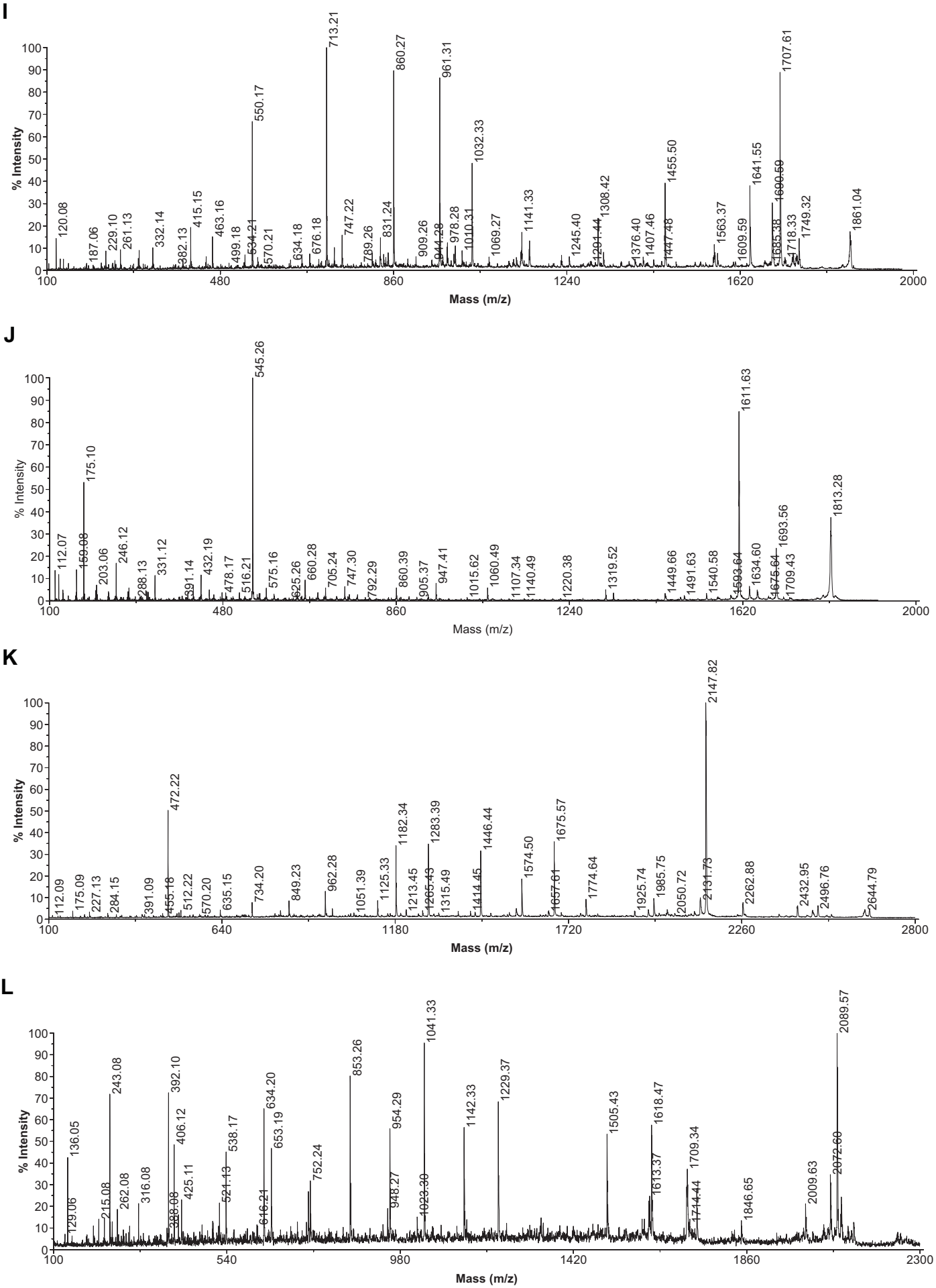

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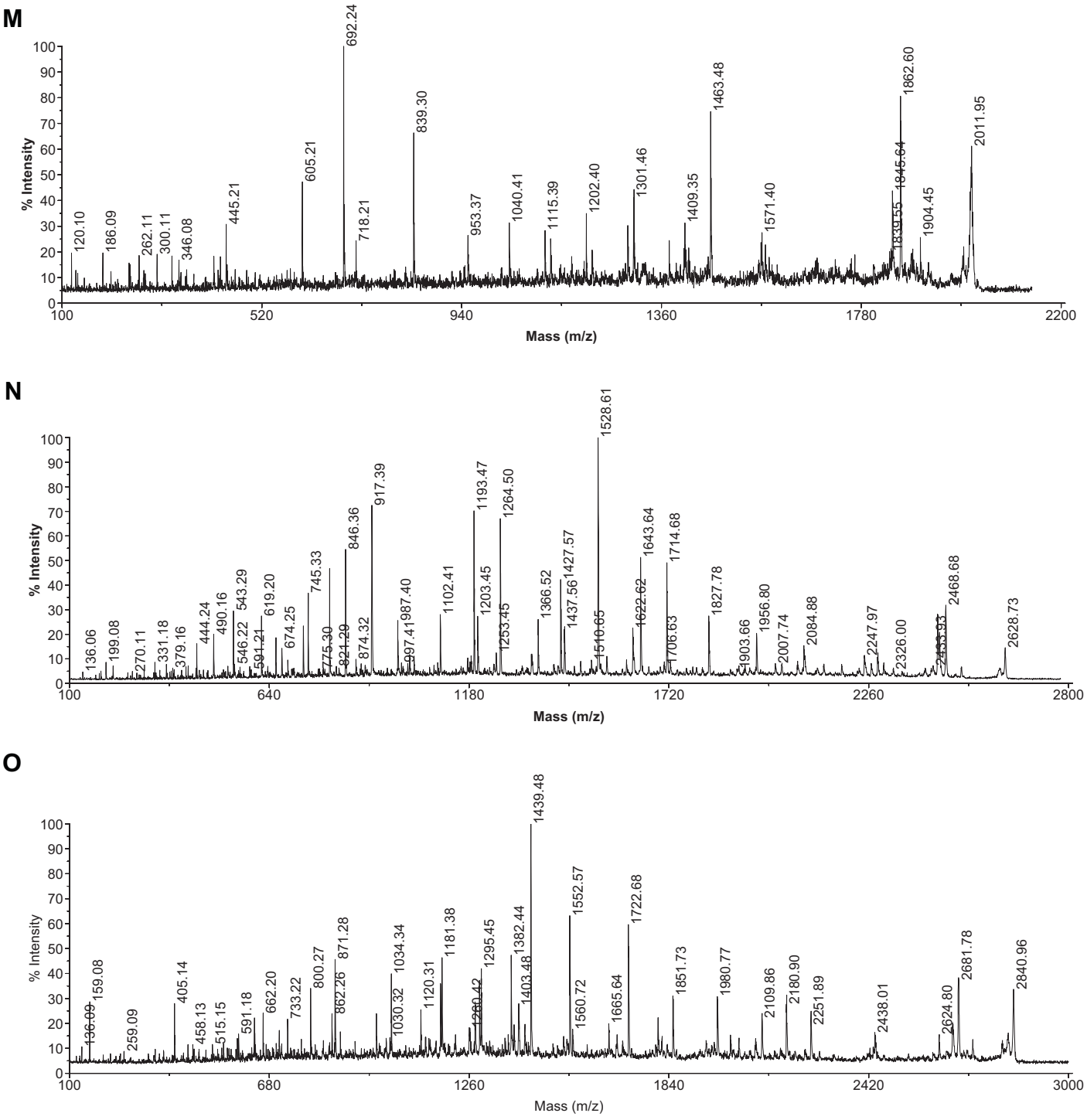

Supplementary Figure 9