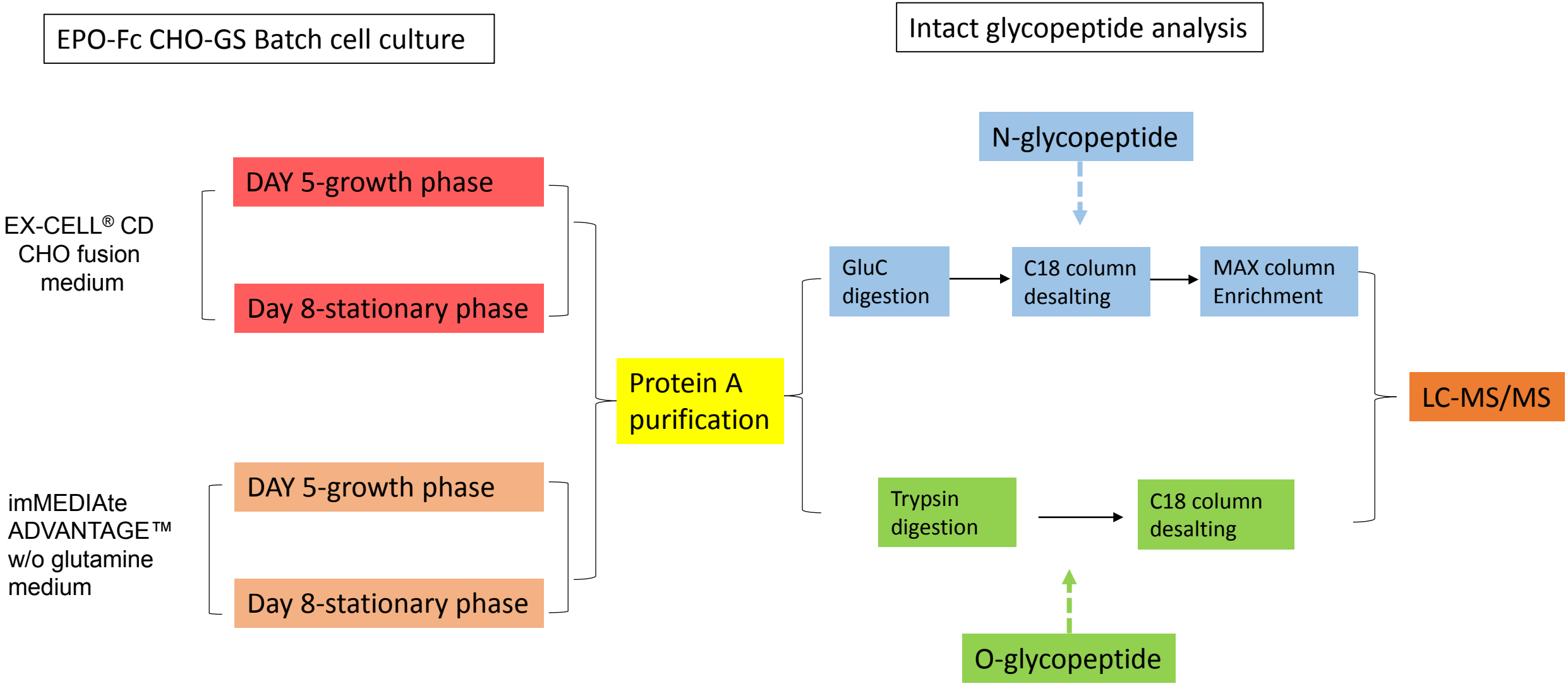
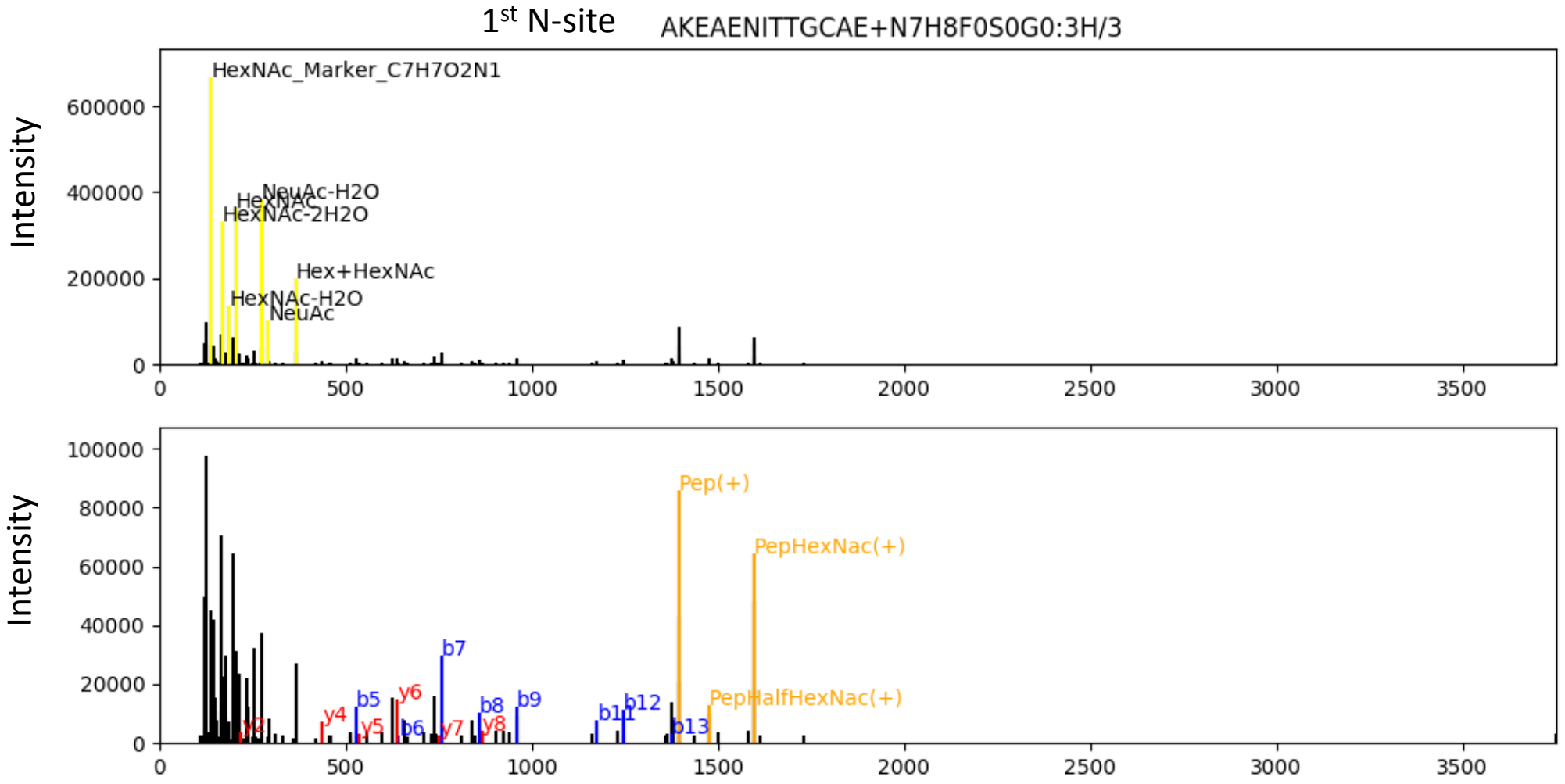
Supplemental Data

Supplemental Figure 1



Supplemental Figure 1. The workflow of experiments in this work.

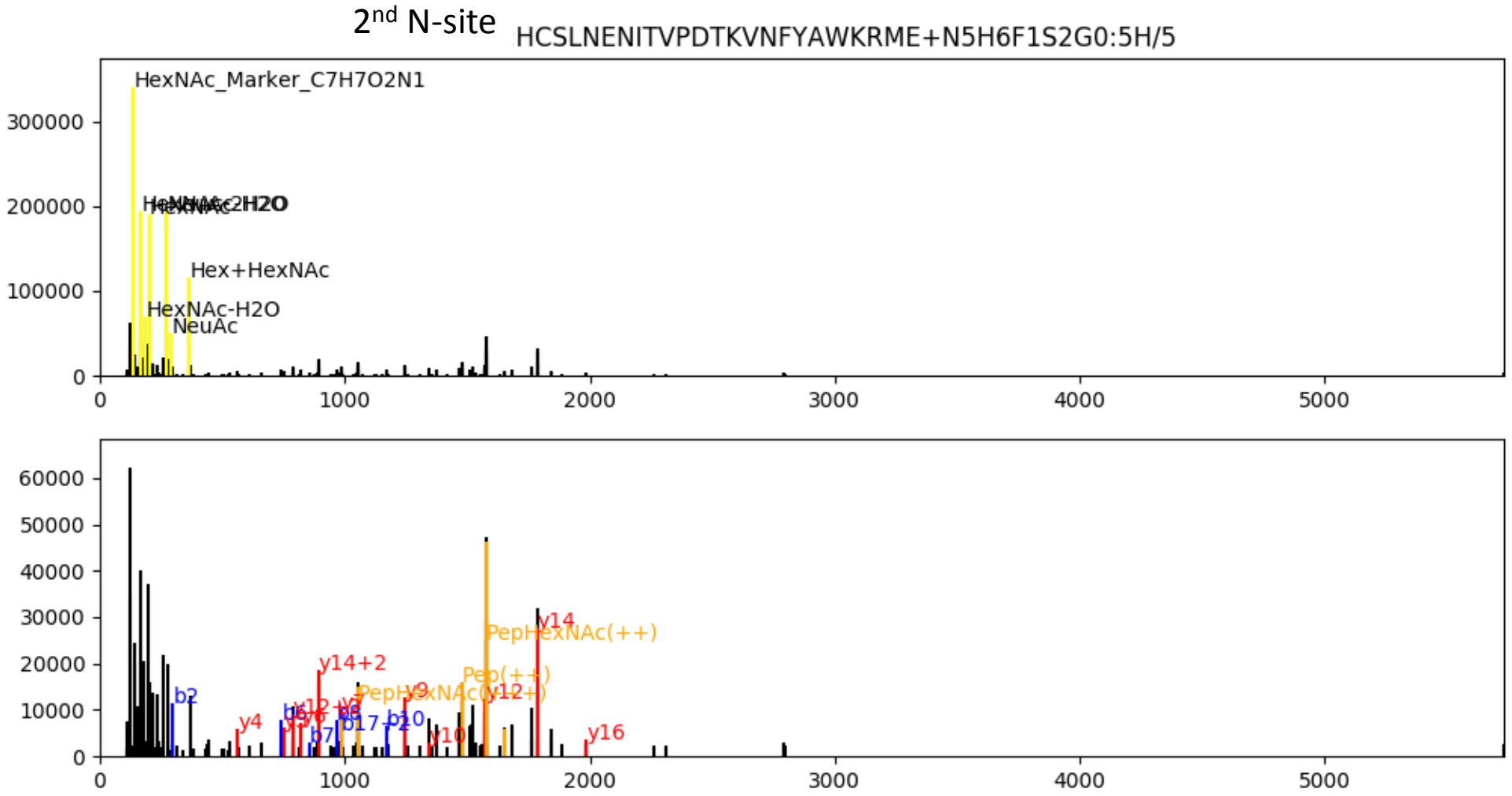
Supplemental Figure 2



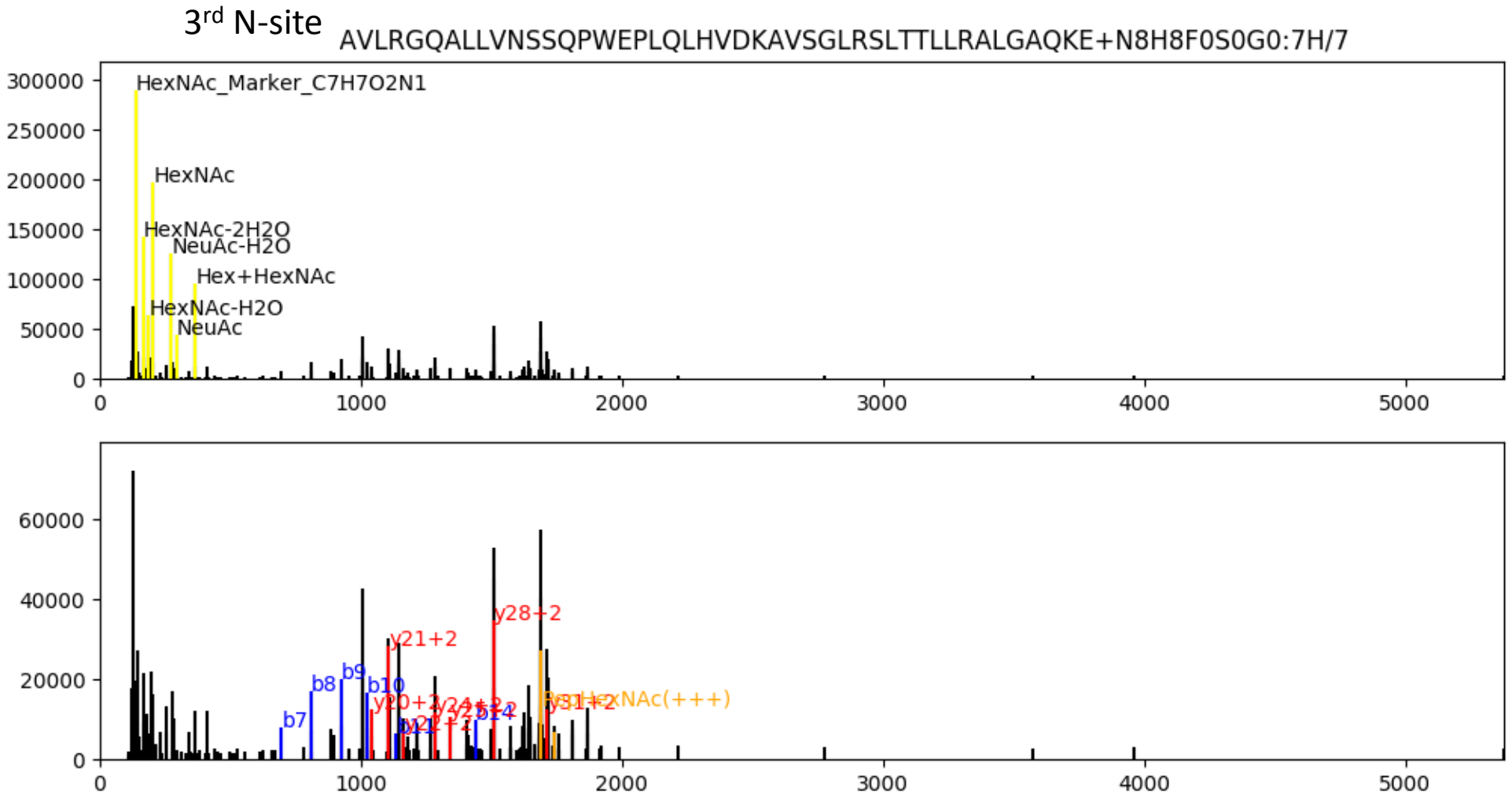
Supplemental Figure 2. Representative MS/MS spectra of the identified N- and O-glycopetides in EPO-Fc protein. For example, in Figure 2A, Spectral title: AKEAENITTGCAE + N4H5F3S0G0/3. Glycopeptide sequence: AKEAENITTGCAE; N-glycan: N4H5F3S0G0 (N: HexNAc, H: Hex, F: Fuc, S: Neu5Ac, G: Neu5Gc); Charge: 3. The same labeling works for For (2B)-(2E). Upper figure: highlight only oxonium ions in yellow; Lower figure: after removing oxonium ions, highlghts b-, y-, peptide- and peptide-glyan(Y1) fragment ions.

Supplemental Figure 2

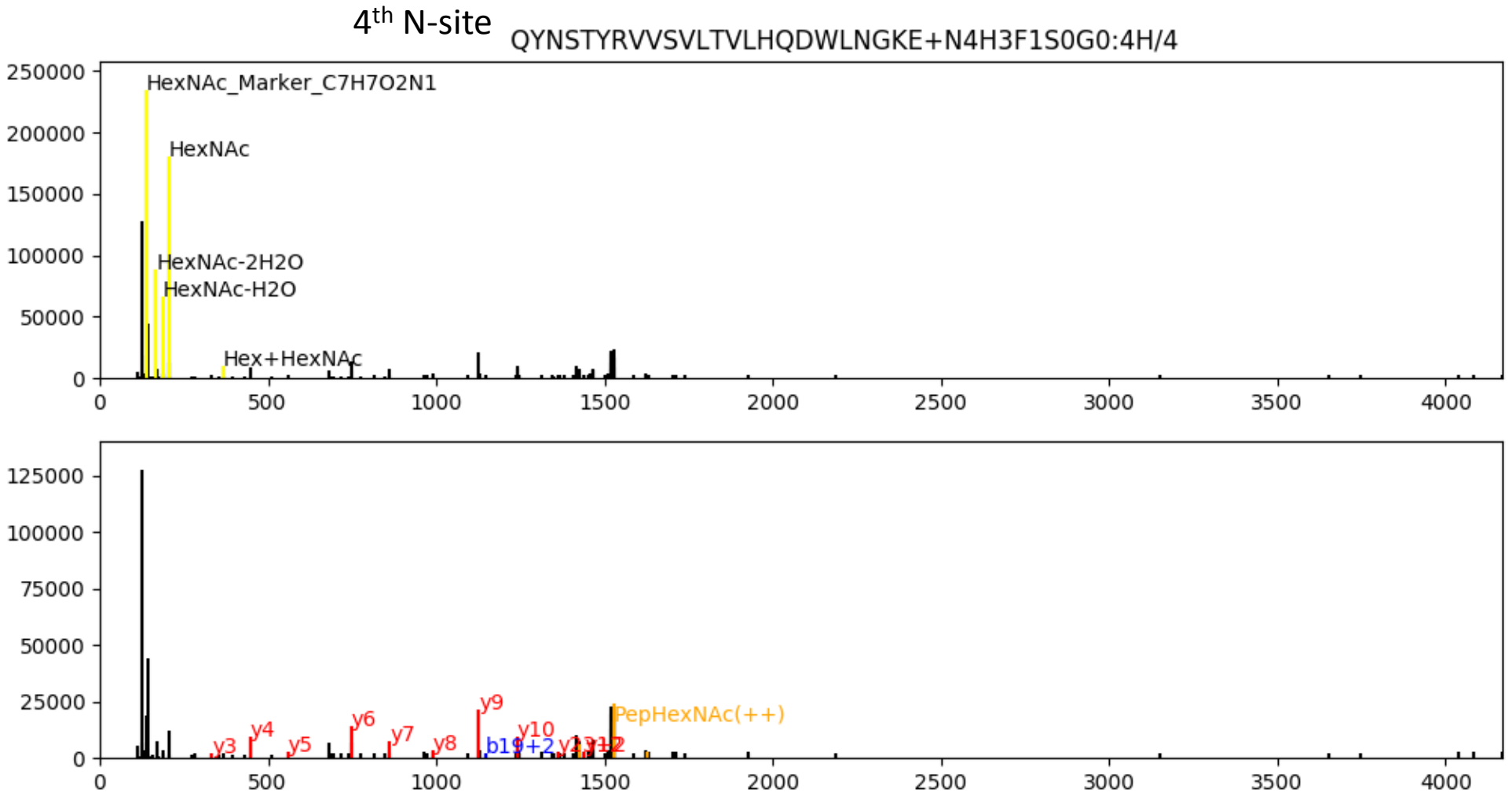
2B



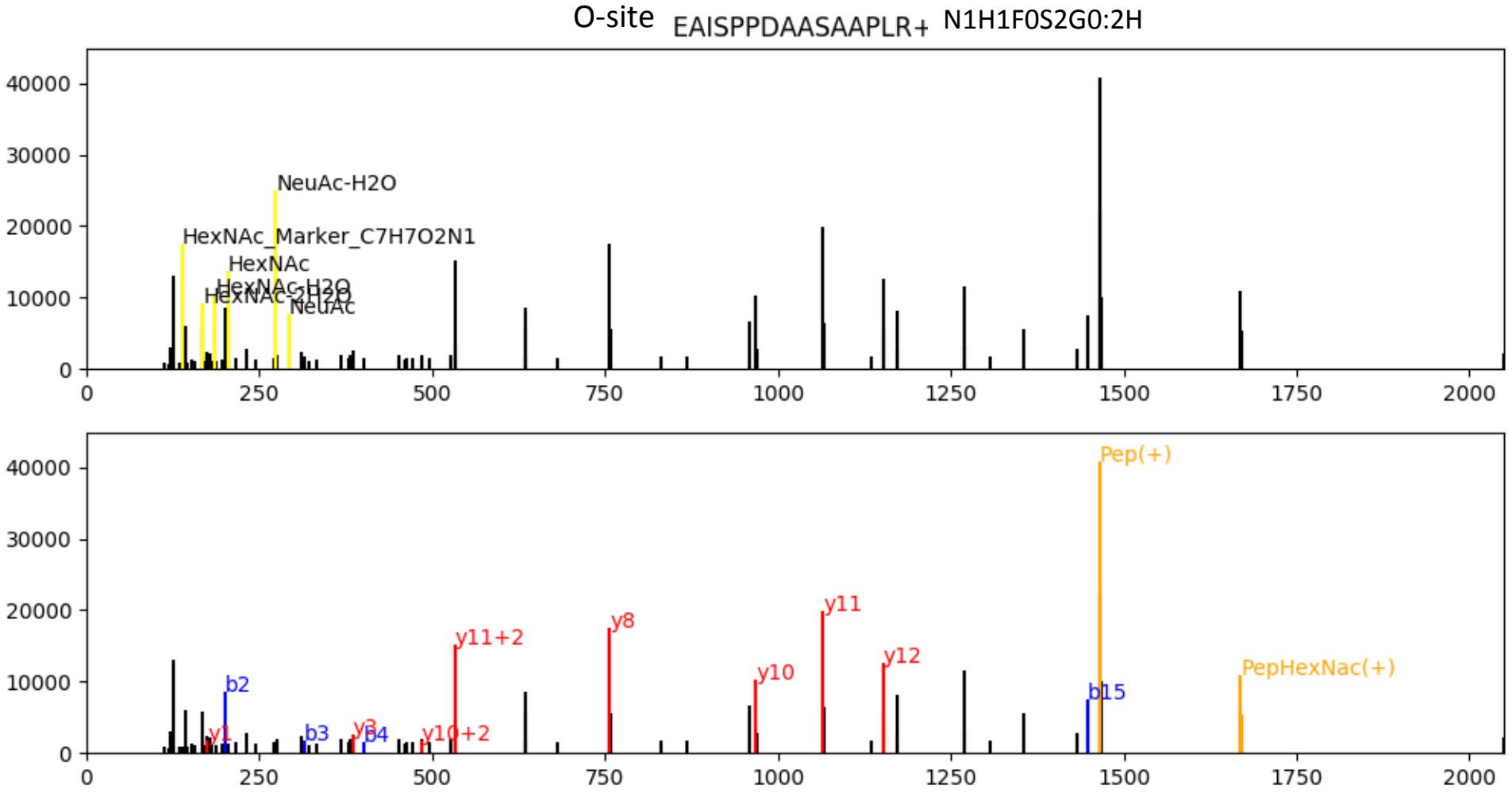
2C


3rd N-site
AVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKE+N8H8F0S0G0:7H/7
HexNAc_Marker_C7H7O2N1
HexNAc
HexNAc-2H2O
NeuAc-H2O
Hex+HexNAc
HexNAc-H2O
NeuAc
300000
250000
200000
150000
100000
50000
0
0
1000
2000
3000
4000
5000
60000
40000
20000
y28+2
y21+2
b9
b8
b10
b7
y31+2
0
1000
2000
3000
4000
5000

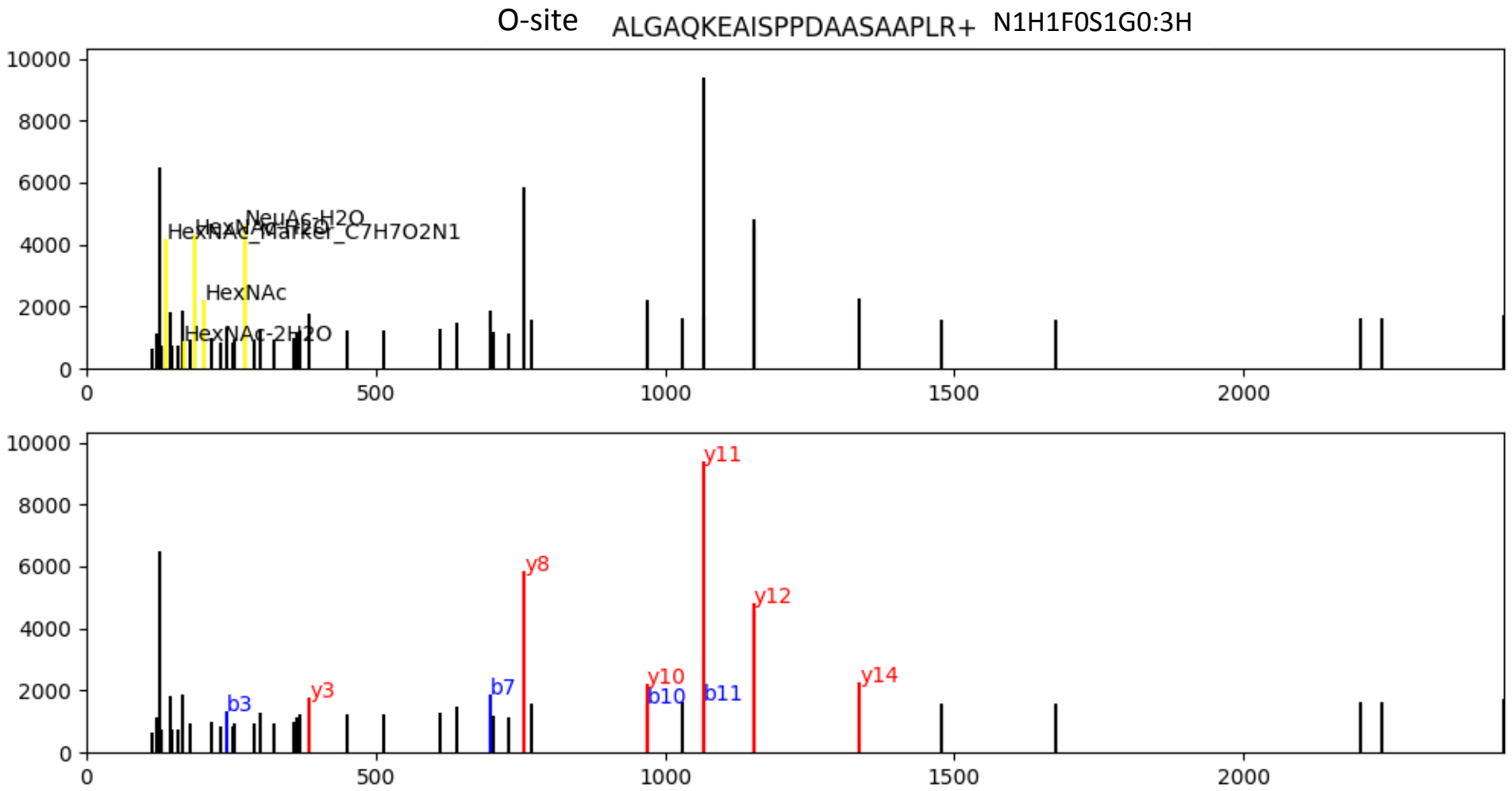
2D


4th N-site
QYNSTYRVVSVLTVLHQDWLNGKE+N4H3F1S0G0:4H/4
HexNAc_Marker_C7H7O2N1
HexNAc
HexNAc-2H2O
HexNAc-H2O
Hex+HexNAc
PepHexNAc(++)
y3
y4
y5
y6
y7
y8
y9
y10

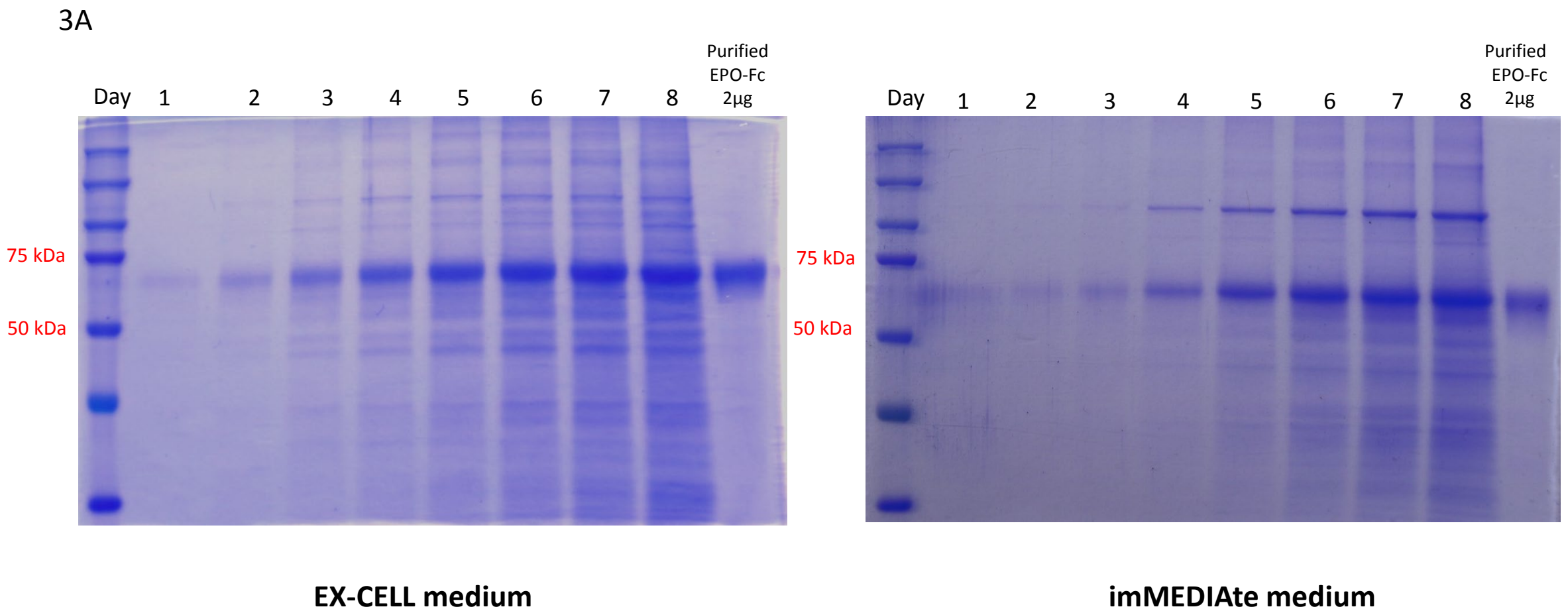
2E


O-site EAISPPDAASAAPLR+ N1H1F0S2G0:2H
NeuAc-H2O
HexNAc_Marker_C7H7O2N1
HexNAc
HexNAc-H2O
HexNAc-2H2O
NeuAc
Pep(+)
y11
y8
y11+2
y12
y10
PepHexNac(+)
b15
b2
y1
b3
y3
b4
y10+2
0
10000
20000
30000
40000
0
250
500
750
1000
1250
1500
1750
2000

2F


O-site ALGAQKEAISPPDAASAAPLR+ N1H1F0S1G0:3H
HexNAc_Marker_C7H7O2N1
NeuAc-H2O
HexNAc
HexNAc-2H2O
y11
y8
y12
y10
b10
b11
y14
b7
y3
b3

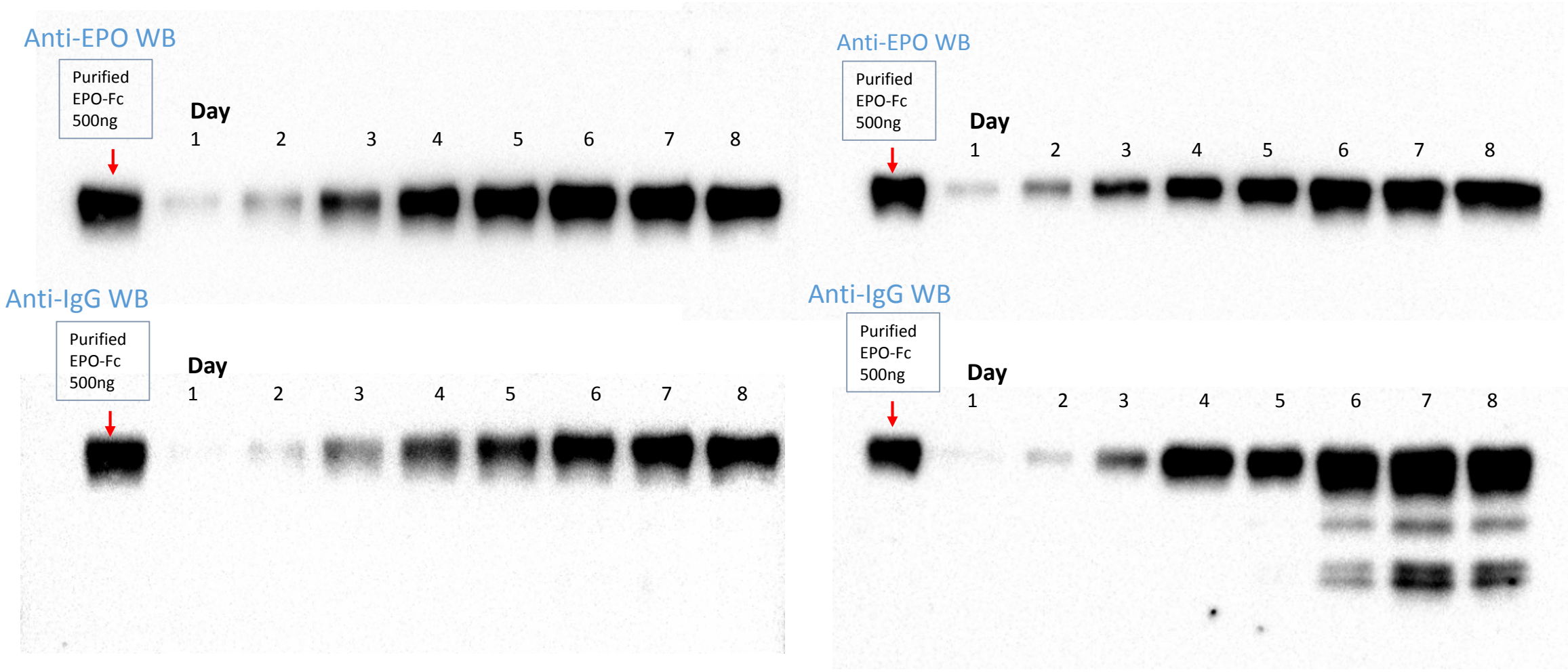
Supplemental Figure 3



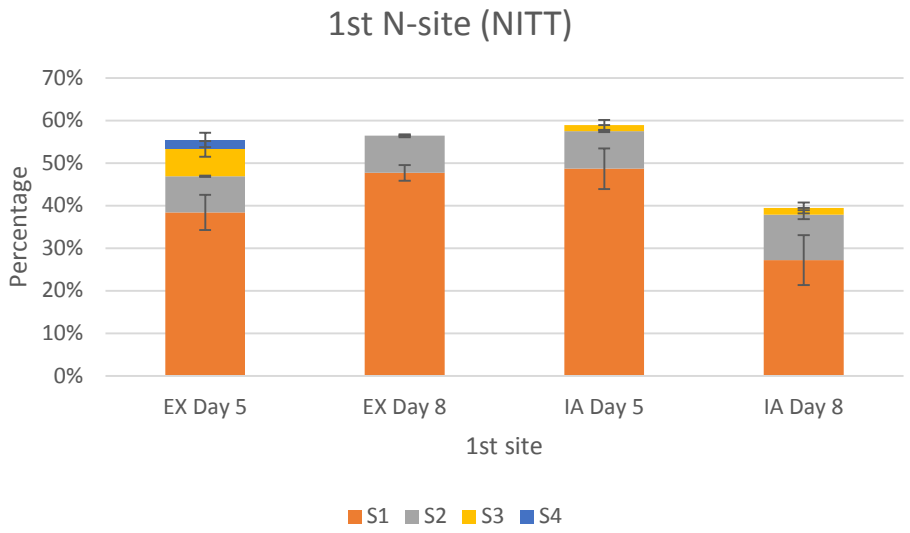
Supplemental Figure 3. (3A) The abundance of EPO-Fc protein in the supernatant per day in the growth study. 40µl supernatant sample was loaded each well. 2µg purified EPO-Fc protein from EX-CELL medium on Day 6 cell culture were collected and used as positive control. (3B) Anti-EPO and Anti-IgG immunoblots for detection of EPO-Fc protein in the daily supernatant in the EX-CELL and imMEDIAte media growth studies individually. 15µl supernatant was loaded each well. 500 ng purified EPO-Fc protein from EX-CELL medium on Day 6 cell culture were collected and used as positive control.

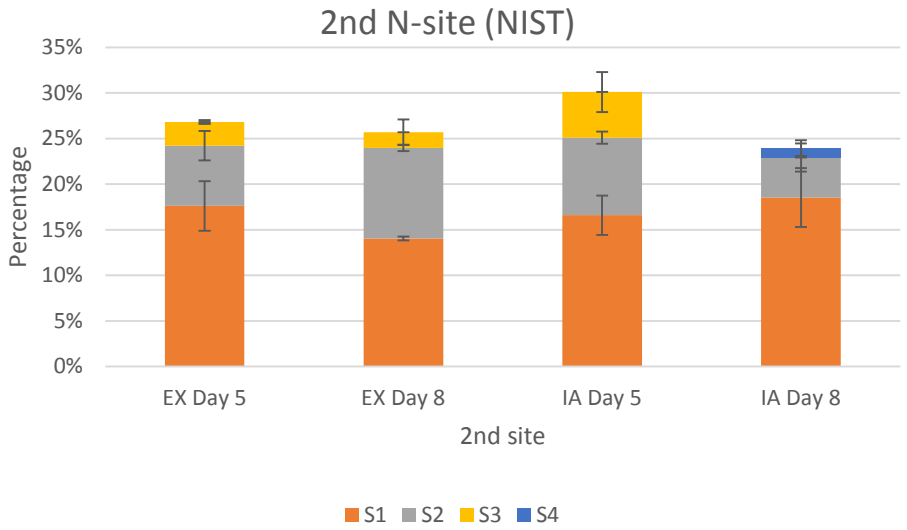
Supplemental Figure 3

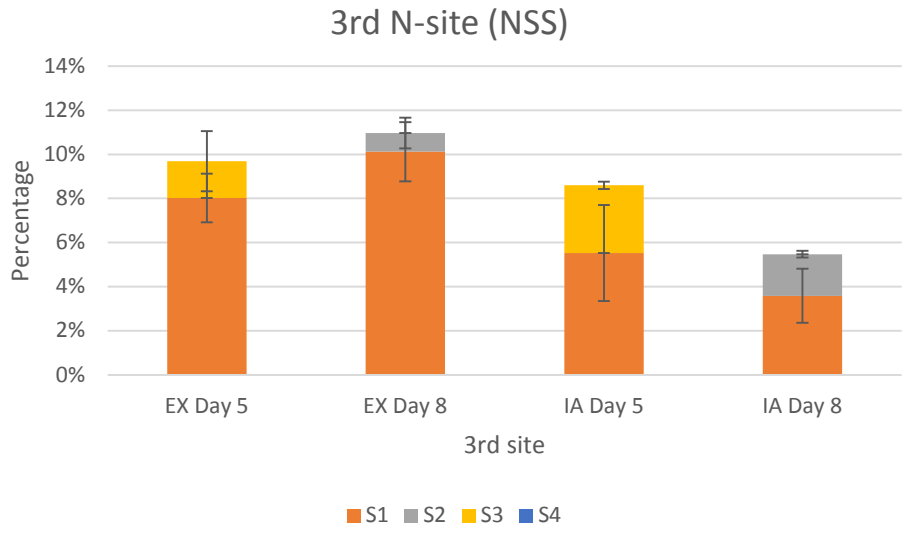
3B



Supplemental Figure 4



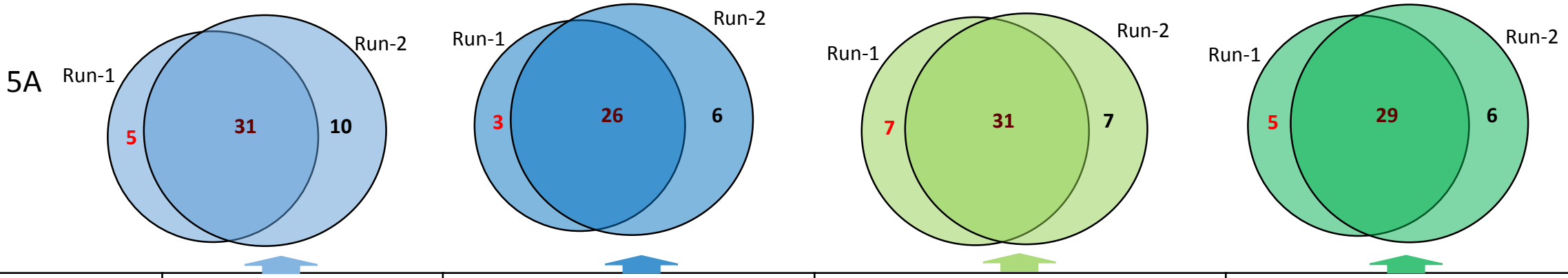




Supplemental Figure S4, The site-specific Neu5Ac sialylation distribution of EPO-Fc protein. S1: glycans with one sialic acid, S2: glycans with two sialic acids, S3: glycans with three sialic acids, S4: glycans with 4 sialic acids. NITT, NIST and NSS are the amino acids at 1st, 2nd, and 3rd N-glycan sites. At the 4th N-glycan site (Fc N-site), no sialylation has been detected.

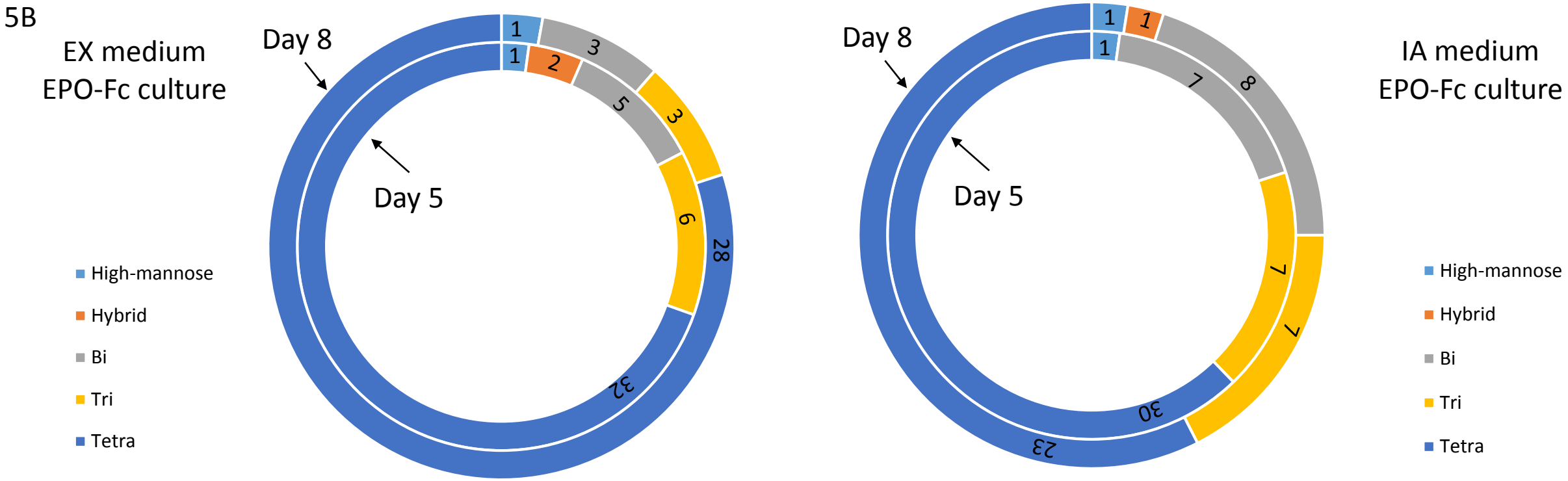
Supplemental Figure 5

5A



	EX medium Day 5	EX medium Day 5	IA medium Day 5	IA medium Day 8
Total glycan types	46	35	45	40

5B



Supplemental Figure S5, The N-glycan types analysis of EPO-Fc protein using intact glycopeptide characterization. (S5A). The number of N-glycan types identified from each sample. The total glycan types were summarized from two biological runs. (S5B) The branch distribution of glycan types of EPO-Fc protein cultured in EX and IA media harvested at growth (Day 5) and stationary (Day 8) phases.