Poster Reprint

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Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using InstantPC and 2-AB with LC-FLD-MS

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Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process. The structure of N-linked glycans can influence the function of glycosylated biotherapeutics[1], frequently making glycosylation a critical quality attribute (CQA). N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD) and mass spectrometry (MS). Many of the frequently used fluorescent tags such as 2-AB[2] are limited with regard to MS sensitivity compared with recently introduced dyes such as InstantPC, and pre-existing N-glycan sample preparation workflows can be time-consuming.[3]

Here we present streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans coupled with analysis using Agilent LC/FLD/MS instrumentation.

Experimental

Sample Preparation

Gly-*X* with InstantPC and 2-AB workflows (Figure 1) were used to prepare labeled N-glycans from monoclonal antibody rituximab (Rituxan, lot # M190170) and Fc fusion protein etanercept (Enbrel, lot # 1092537), 40 µg protein per preparation.

InstantPC labeled samples were prepared using a developmental protocol on an Agilent Bravo liquid handling system with an adapted Gly-*X* in-solution deglycosylation protocol followed by instant glycosylamine labeling of released N-glycans (Figure 2), followed by vacuum-driven cleanup of free dye using HILIC SPE.

2-AB labeled samples were prepared per standard Gly-*X* 2-AB Express manual method with reductive amination chemistry. Released N-glycans were desolvated by vacuum filtration on a solid-state matrix followed by an on-matrix 2-AB labeling step, eliminating the need for glycan drying prior to the labeling step, thereby reducing total sample preparation time. Four replicates of each sample were analyzed with fluorescence/MS detection and relative percent peak areas calculated.

N-Glycan Analysis

InstantPC and 2-AB labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent AdvanceBio Glycan Mapping column along with an Agilent 1290 Infinity II UHPLC system with in-line fluorescence detection (Table 1) coupled to an Agilent 6545 LC/Q-TOF mass spectrometer (Table 2).

Experimental

All HILIC separations were conducted under the conditions described in Table 1. A fixed flow splitter was utilized post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. MassHunter BioConfirm software was used for data processing, with a personal compound database (PCD).

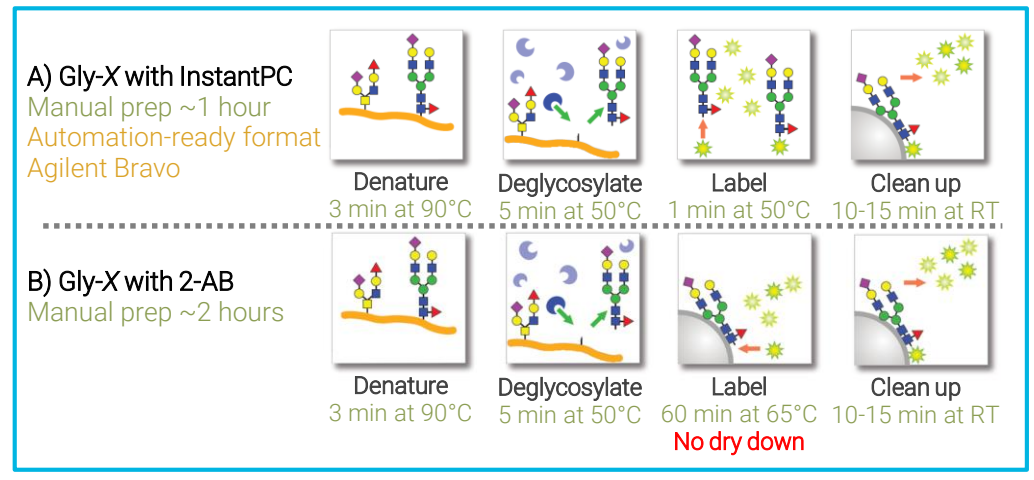


Figure 1. Gly-X N-glycan sample prep. A) InstantPC workflow with in solution deglycosylation and labeling followed by on-matrix cleanup; B) 2-AB workflow with deglycosylation in solution, followed by on-matrix labeling and cleanup.

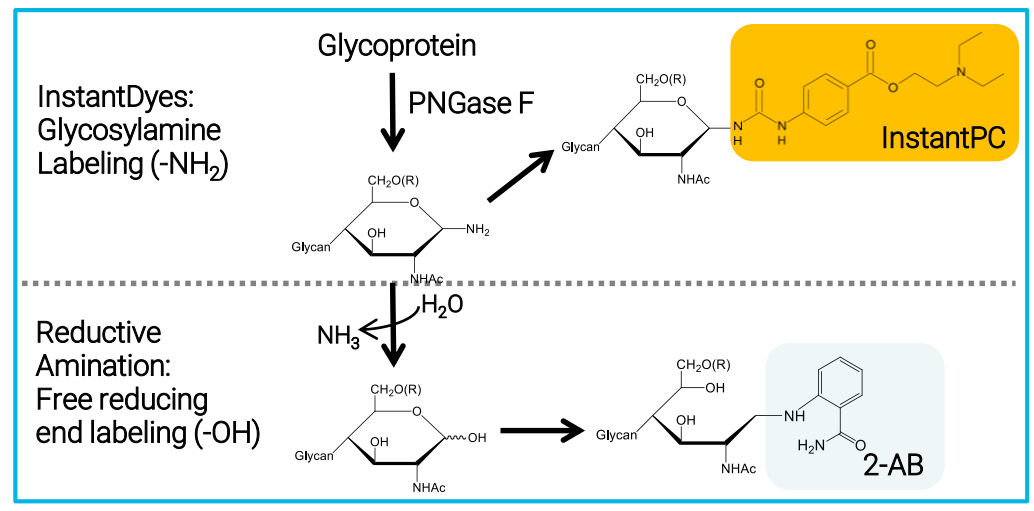


Figure 2. Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

Table 1. UHPLC HILIC/FLD conditions (Agilent 1290).

Parameter	Value
Column	Agilent AdvanceBio Glycan Mapping, 2.1 x 150 mm, 1.8 µm (p/n 859700-913)
Column Temp	40 °C
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile
Gradient Program	**InstantPC** Time (min) / %B / Flow rate (mL/min): 0 / 80 / 0.5 2 / 75 / 0.5 48 / 62 / 0.5 49 / 40 / 0.5 51.5 / 80 / 0.5 52 / 80 / 0.5 60 / 80 / 0.5 **2-AB** Time (min) / %B / Flow rate (mL/min): 0 / 82 / 0.4 2 / 82 / 0.4 2.5 / 77 / 0.4 48 / 62 / 0.4 49 / 40 / 0.4 51.5 / 40 / 0.4 52 / 82 / 0.4 54 / 82 / 0.4 58 / 82 / 0.6 58.5 / 82 / 0.6 60 / 82 / 0.4
Injection Volume	1 µL (equivalent to glycans from 0.4 µg protein)
Detection	Agilent 1260 Infinity II FLD InstantPC: λ_{Ex} 285 nm, λ_{Em} 345 nm 2-AB: λ_{Ex} 260 nm, λ_{Em} 430 nm

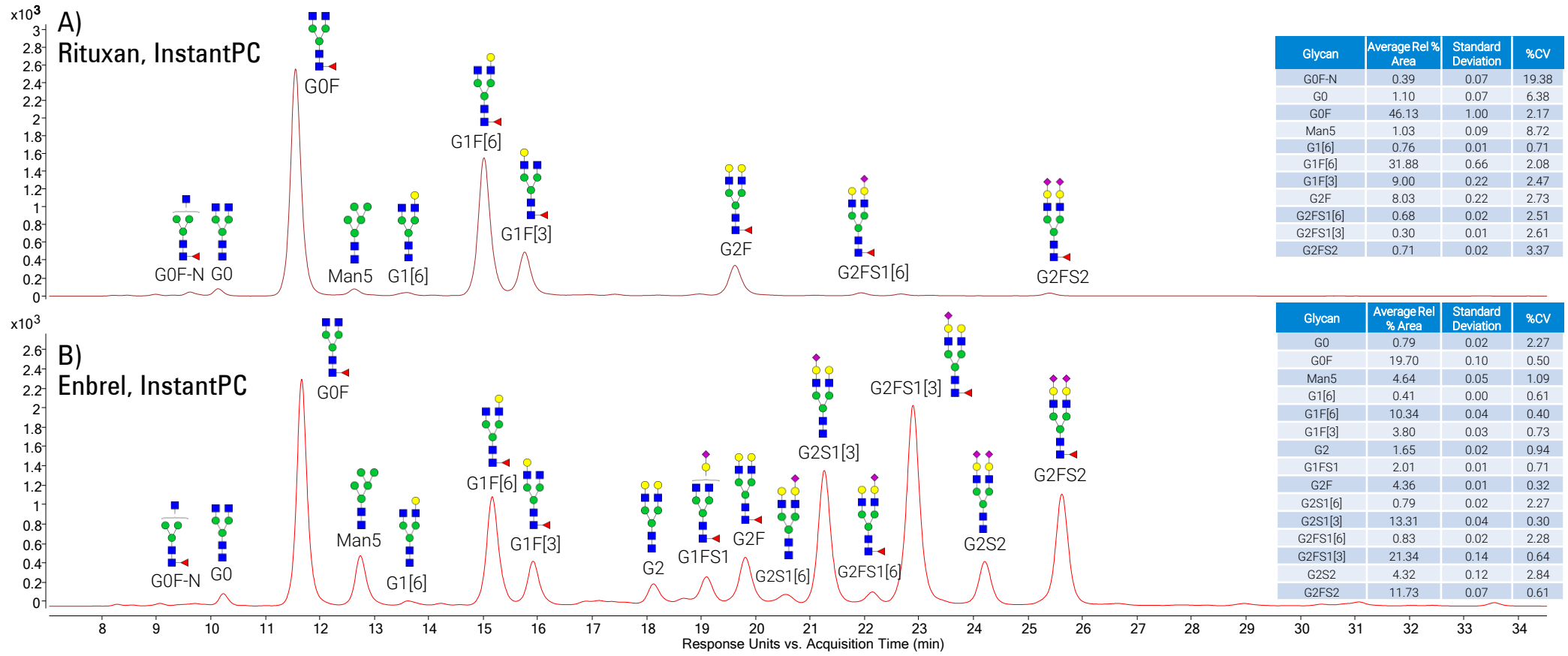
Table 2. 6545XT Q-TOF

6545XT Q-TOF	
Source	Dual AJS ESI
Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Vcap	3000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	m/z 600-3000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

Results and Discussion

HILIC Separation of InstantPC and 2-AB N-Glycans

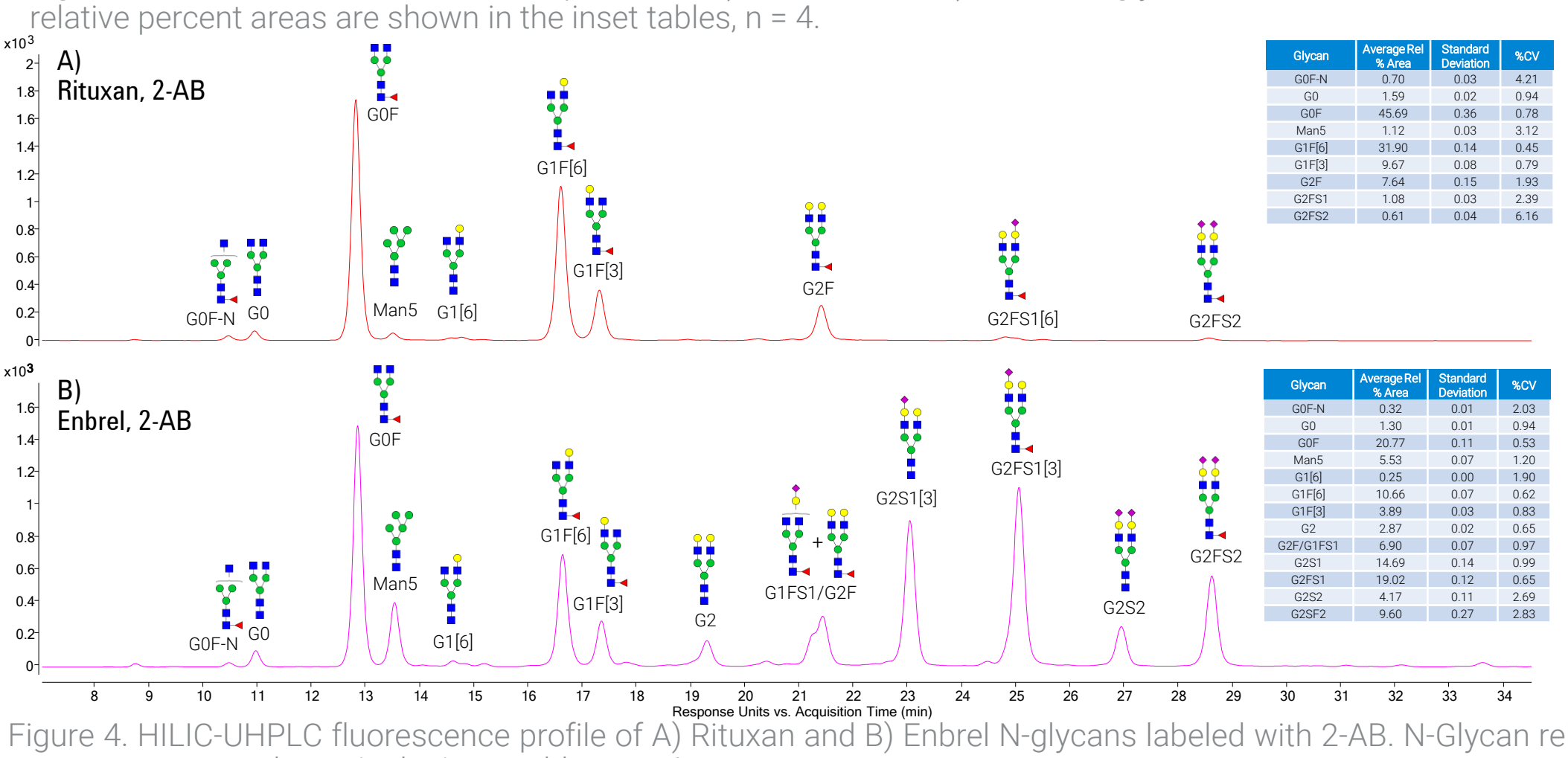
HILIC separation with fluorescence detection of InstantPC and 2-AB labeled N-glycans from Rituxan and Enbrel results in well resolved peaks for major glycan species from Rituxan and Enbrel (Figures 3 & 4). The HILIC retention time of 2-AB N-glycans is shorter than InstantPC N-glycans, although elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and G1F[6]/[3], which are often monitored during the development process of biotherapeutics are well separated with both InstantPC and 2-AB labels, lending to confident determination of relative percentage composition determination. An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from Enbrel (Figure 3) compared to 2-AB (Figure 4) using above described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics Rituxan and Enbrel results in comparable relative percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2 and G2FS2.



Glycan	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.39	0.07	19.38
G0	1.10	0.07	6.38
G0F	46.13	1.00	2.17
Man5	1.03	0.09	8.72
G1[6]	0.76	0.01	0.71
G1F[6]	31.88	0.66	2.08
G1F[3]	9.00	0.22	2.47
G2F	8.03	0.22	2.73
G2FS1[6]	0.68	0.02	2.51
G2FS1[3]	0.30	0.01	2.61
G2FS2	0.71	0.02	3.37

Glycan	Average Rel % Area	Standard Deviation	%CV
G0	0.79	0.02	2.27
G0F	19.70	0.10	0.50
Man5	4.64	0.05	1.09
G1[6]	0.41	0.00	0.61
G1F[6]	10.34	0.04	0.40
G1F[3]	3.80	0.03	0.73
G2	1.65	0.02	0.94
G1FS1	2.01	0.01	0.71
G2F	4.36	0.01	0.32
G2S1[6]	0.79	0.02	2.27
G2S1[3]	13.31	0.04	0.30
G2FS1[6]	0.83	0.02	2.28
G2FS1[3]	21.34	0.14	0.64
G2S2	4.32	0.12	2.84
G2FS2	11.73	0.07	0.61

Figure 3. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in the inset tables, n = 4.



Glycan	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.70	0.03	4.21
G0	1.59	0.02	0.94
G0F	45.69	0.36	0.78
Man5	1.12	0.03	3.12
G1F[6]	31.90	0.14	0.45
G1F[3]	9.67	0.08	0.79
G2F	7.64	0.15	1.93
G2FS1	1.08	0.03	2.39
G2FS2	0.61	0.04	6.16

Glycan	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.01	2.03
G0	1.30	0.01	0.94
G0F	20.77	0.11	0.53
Man5	5.53	0.07	1.20
G1[6]	0.25	0.00	1.90
G1F[6]	10.66	0.07	0.62
G1F[3]	3.89	0.03	0.83
G2	2.87	0.02	0.65
G2F/G1FS1	6.90	0.07	0.97
G2S1	14.69	0.14	0.99
G2FS1	19.02	0.12	0.65
G2S2	4.17	0.11	2.69
G2SF2	9.60	0.27	2.83

Figure 4. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in the inset tables, n = 4.

FLD and MS detection of InstantPC and 2-AB N-Glycans

InstantPC displays higher fluorescence and MS signal compared to 2-AB (Figure 5). Individual spectra for InstantPC and 2-AB labeled Man5 also show higher MS signal of InstantPC (Figure 6).

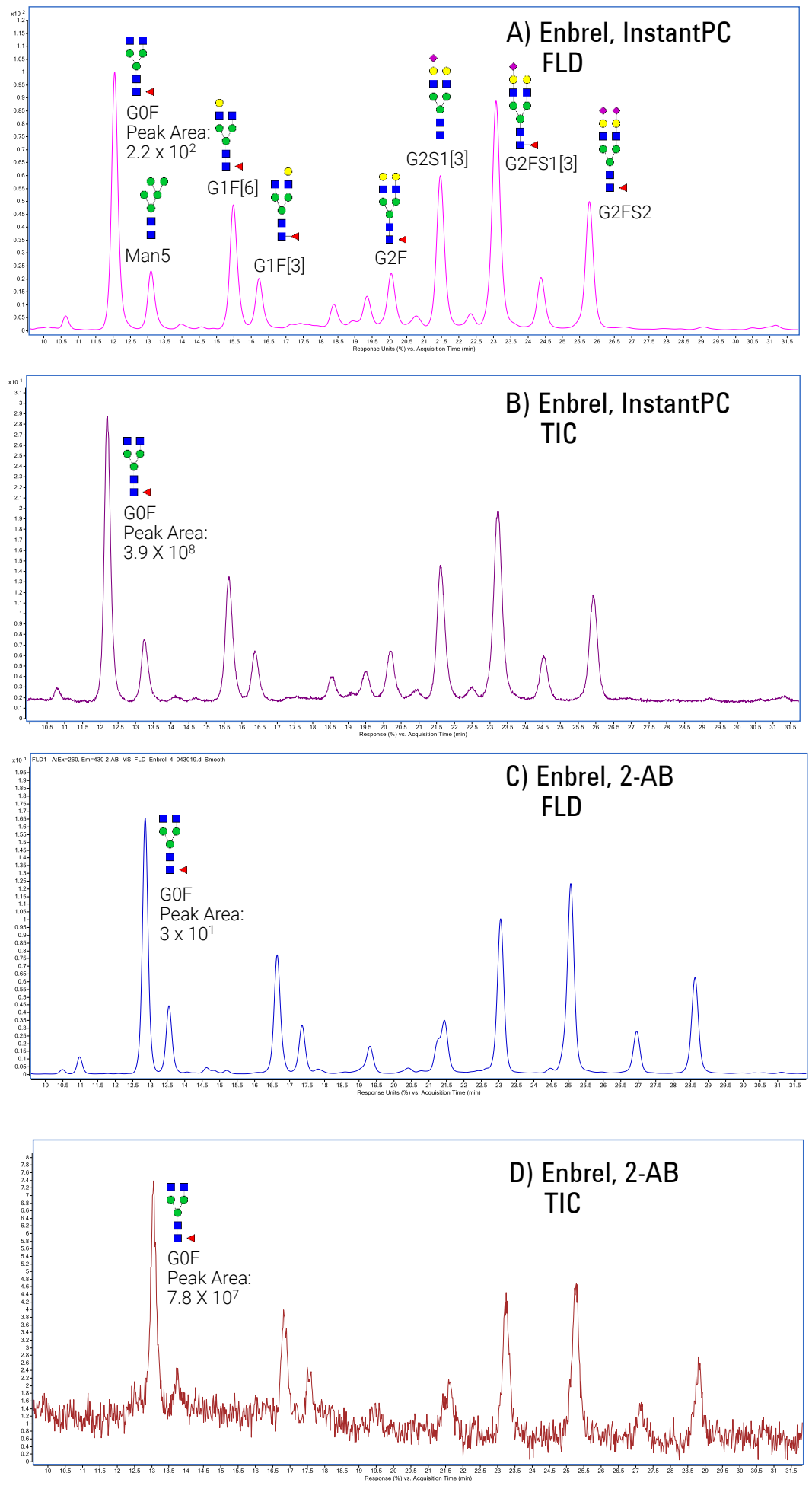


Figure 5. FLD and MS of InstantPC and 2-AB labeled N-glycans from Enbrel. A) InstantPC FLD; B) InstantPC TIC (total ion chromatogram); C) 2-AB FLD; D) 2-AB TIC.

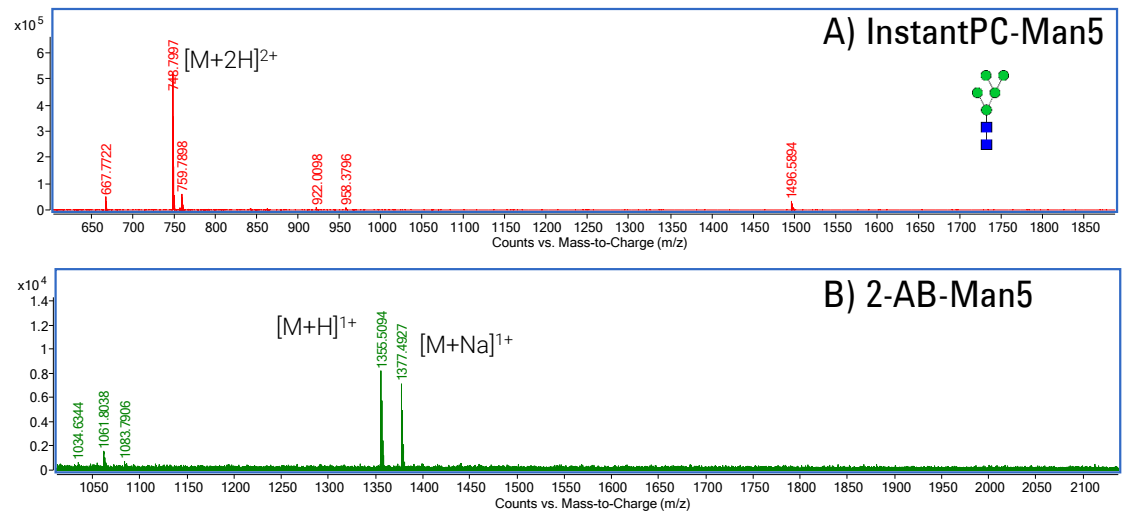


Figure 6. Mass spectrum comparison of Man5 from Enbrel, labeled with A) InstantPC and B) 2-AB.

Conclusions

- Gly-*X* sample prep enables 5 minute release of N-linked glycans suitable for labeling by both glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB.
- On-matrix 2-AB labeling eliminates dry down step.
- Glycan species were profiled by relative fluorescence peak area % and structurally assigned using high resolution tandem mass spectrometry. Reproducibility between sample preparation replicates is high.
- Compared with 2-AB, InstantPC glycans display higher FLD signal and greater MS response in positive mode, allowing for confident detection of low abundance glycan species.

References

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