Application Note

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Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

Monoclonal antibodies (mAbs) and their derivatives represent a very important class of biopharmaceutical molecules with a wide range of applications. With the dramatic increase in approved mAb products and mAb product sales over recent years, there is an increased need for comprehensive analytical characterization capabilities. mAbs are heterogeneous molecules by nature, which are composed of various types of sequences, modifications, and structural variants. Protein glycosylation is one of the major post-translational modifications (PTMs) of mAbs that plays an important role in many biological processes. The distribution and composition of the glycans bound to the mAb molecules can have an effect on therapeutic efficacy and immunogenicity; consistent glycosylation-associated quality control of therapeutic mAbs has become a high priority in pharmaceutical bioprocessing[1].

Quadrupole Time-of-Flight (Q-TOF) Liquid Chromatography/Mass Spectrometer (LC/MS) systems are widely used to analyze intact mAbs and mAb subunits, perform mAb peptide sequence mapping, and characterize PTMs due to the excellent mass accuracy and resolution in the high mass range of these instruments[2-4].

Typically, there are four levels of LC/MS workflows for glycan/glycoforms in characterization (Figure 1):

- Level 1 and level 2 focus on the analysis of glycoforms on the intact and reduced mAb molecules.
- Level 3 is the analysis of glycopeptides generated from the proteolytical digestion of mAbs, commonly part of a peptide sequence mapping workflow.
- Level 4 is the characterization of glycans that have been released by enzymatic cleavage or other mechanism.

Since we reported on glycopeptide analysis (level 3) of an IgG protein in a previous Application Note[5], three other major LC/MS-based workflows (levels 1, 2, and 4) were evaluated in this study using the NISTmAb. In this study, all three approaches aim to quantitatively understand the glycosylation present for a given protein. These workflows incorporated the:

- Agilent AssayMAP Bravo liquid handling platform
- Agilent 1290 Infinity II LC system
- Agilent PLRP-S column or AdvanceBio Glycan Mapping column
- Highly sensitive Agilent fluorescence detection (FLD)
- Agilent 6545XT AdvanceBio LC/Q-TOF system

As data were acquired on the 6545XT AdvanceBio LC/Q-TOF, they were automatically analyzed using Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves not only productivity by allowing convenient sample preparation and streamlined data acquisition, but also accuracy in data analysis.

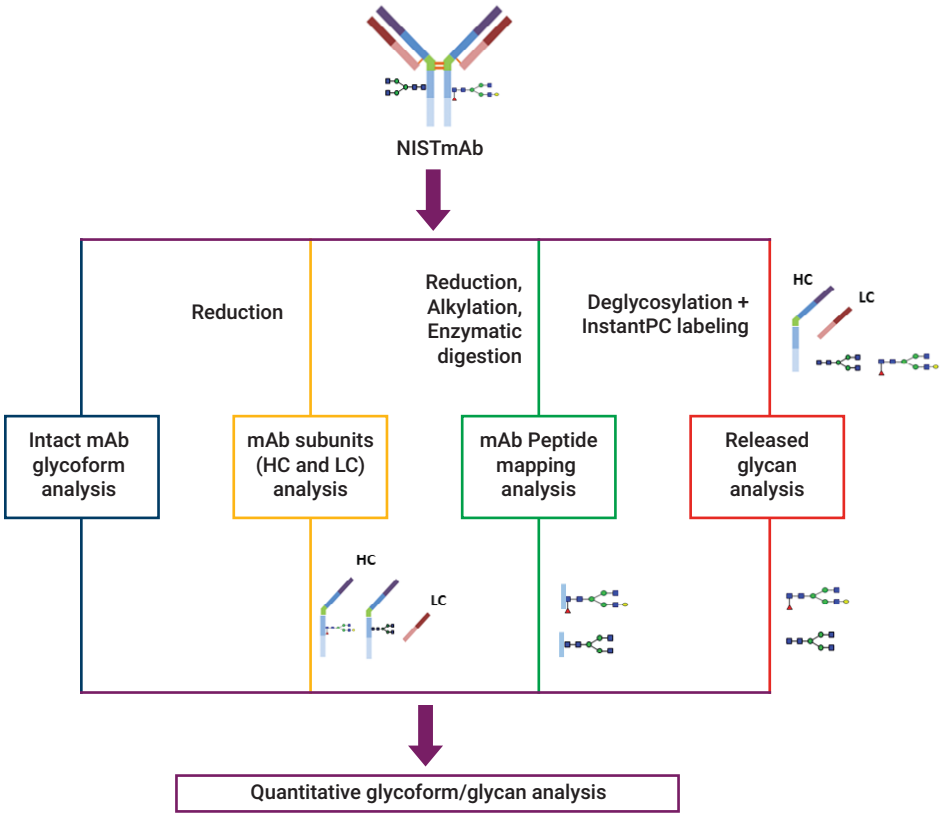


Figure 1. Various glycoforms/glycans quantitative analysis workflows.

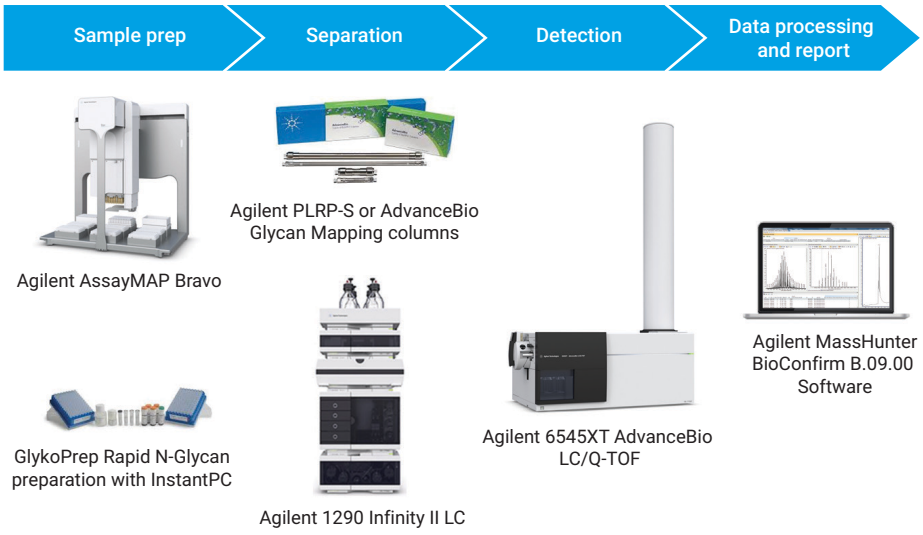


Figure 2. Analytical components of the mAb glycoform/glycan characterization workflow.

Experimental

Materials and Methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), and is often referred to as the NISTmAb. 2,2,2-Trifluoroethanol (TFE), DL-Dithiothreitol (DTT), and *tris*(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Rapid PNGase F was sourced from New England BioLabs. The GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) was purchased from ProZyme, Inc. The NISTmAb samples used in all workflows were diluted with DI water to 1.0 µg/µL.

Sample Preparation

No sample preparation was needed for the intact mAb glycoforms analysis workflow. For accurate quantitative analysis on the glycoforms of NISTmAb subunit (heavy chain), complete protein reduction was required. Therefore, a first reduction reaction with 40 mM DTT at 60 °C for 30 minutes, followed by an additional 25 mM TCEP reaction (30 minutes at room temperature) were performed. Finally, we used the Agilent AssayMAP Bravo liquid handling system (G5542A) in the released glycan quantitation workflow. The detailed procedure for the sample preparation is described in ProZyme's Application Note (product code: GPPNG-PC). After the final cleanup step, the released labeled N-glycan elution had a final concentration of 1.0 µg/µL.

LC/MS Analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B) and coupled to a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. LC separation for the intact NISTmAb and the reduced NISTmAb was obtained with an Agilent PLRP-S column (2.1 × 50 mm, 1,000 Å, 5 µm). Glycans were chromatographically separated with an Agilent AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). The fluorescence detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10. Tables 1 and 2 list the LC/MS parameters used. Approximately 0.5 µg of protein was injected for the intact and subunit analyses. The N-glycan experiments injected the free glycans released from 1–2 µg of intact protein.

Data Processing

MassHunter BioConfirm B.09.00 software featuring three major biopharma workflows (intact mAb, peptide mapping, and released glycan profiling) was used for all data processing in this study. This powerful software program simplifies downstream data analysis, enabling automatic identification and relative quantitation of targeted biomolecules. For the released glycan workflow, the Agilent Personal Compound Database and Library (PCDL) glycan database, which provides accurate glycan identification and confirmation, was used.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System			
Sample type	Intact mAb	mAb Subunits (HC and LC)	mAb Released glycans
Column	Agilent PLRP-S, 2.1 × 50 mm, 1,000 Å, 5 µm (p/n PL1912-1502)	Agilent PLRP-S, 2.1 × 50 mm, 1,000 Å, 5 µm (p/n PL1912-1502)	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm (p/n 858700-913)
Thermostat	4 °C	4 °C	4 °C
Solvent A	0.1 % Formic acid in DI water	0.1 % Formic acid in DI water	50 mM Formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	0.1 % Formic acid in 100 % acetonitrile	0.1 % Formic acid in 100 % acetonitrile	Acetonitrile
Gradient	0–1 minute, 0–20 %B 1–3 minutes, 20–50 %B 3–4 minutes, 50–70 %B	0 minutes, 25 %B 5 minutes, 45 %B 6 minutes, 60 %B 6–7 minutes, 60 %B	0–0.5 minutes, 75–71 %B 0.5–16 minutes, 71–67.5 %B 16–22 minutes, 67.5–60 %B 22–22.5 minutes, 60–40 %B 22.5–23.5 minutes, 40 %B (0.7 mL/min) 23.5–24 minutes, 40–75 %B (0.7 mL/min) 24–30 minutes, 75 %B (0.9 mL/min)
Column temperature	60 °C	60 °C	40 °C
Flow rate	0.5 mL/min	0.8 mL/min	0.4 mL/min
Injection volume	0.5 µL	1.0 µL	2.0 µL

Results and Discussion

Carbohydrate compositions, structures, and their relative quantitative levels are important for the safety and efficacy of therapeutic proteins. Detailed studies of these glycan structures will also potentially help to improve the discovery and development of novel drugs.

Characterization of mAb glycoforms at the intact protein level is the most widely used method for quick assessment and monitoring of mAb glycosylation during pharmaceutical bioprocessing.

Intact NISTmAb samples were analyzed with an Agilent PLRP-S column using the 1290 Infinity II LC system coupled to a 6545XT AdvanceBio LC/Q-TOF mass spectrometer. Raw mass spectra were deconvoluted by the Maximum Entropy algorithm in MassHunter BioConfirm B.09.00 software, as shown in Figure 3.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System			
Sample type	Intact mAb	mAb Subunits (HC and LC)	mAb Released glycans
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas temperature	350 °C	350 °C	150 °C
Gas flow	12 L/min	12 L/min	9 L/min
Nebulizer	60 psig	35 psig	35 psig
Sheath gas temperature	400 °C	350 °C	300 °C
Sheath gas flow	11 L/min	11 L/min	10 L/min
VCap	5,500 V	4,000 V	3,000 V
Nozzle voltage	2,000 V	500 V	500 V
Fragmentor	380 V	180 V	120 V
Skimmer	140 V	65 V	65 V
Quad AMU	500 *m/z*	300 *m/z*	95 *m/z*
Mass range	100–10,000 *m/z*	100–3,200 *m/z*	300–1,700 *m/z*
Acquisition rate	1.0 spectra/s	1.0 spectra/s	2.0 spectra/s
Reference mass	922.0098	922.0098	922.0098
Acquisition mode	Positive, extended (10,000 *m/z*) mass range	Positive, standard (3,200 *m/z*) mass range, HiRes (4 Gz)	Positive, low mass range, HiRes (4 Gz)

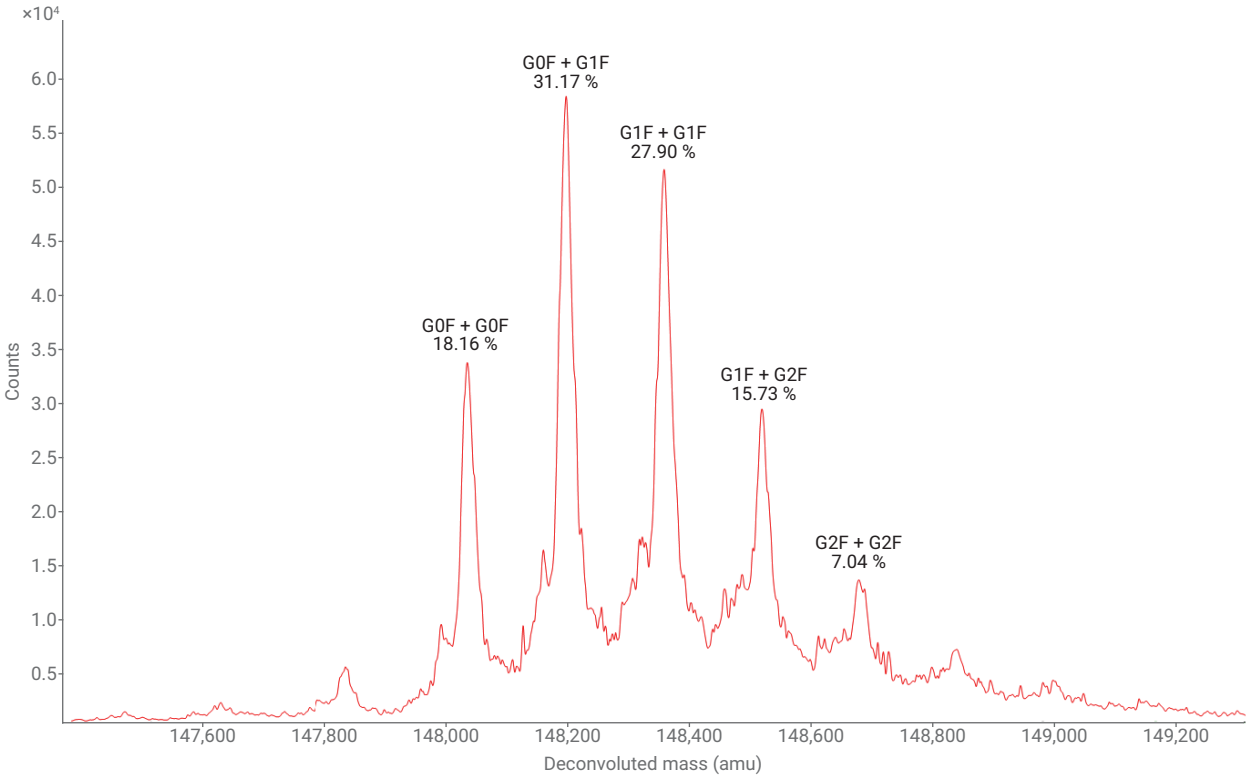


Figure 3. MS Deconvoluted spectrum (maximum entropy) of intact NISTmAb standard with relative quantitation labeled on five major glycoforms.

Typically, once the MS raw data are acquired, the BioConfirm Intact Protein Workflow can be used in an automatic mode to sum up the spectra across any chromatographic peaks, then deconvolute into the intact mass of the mAb. The biomolecule peaks were then confirmed by matching the measured masses with the theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified glycoforms was also automatically calculated using either the peak heights or peak areas of the deconvoluted mass spectra. BioConfirm can recalculate the relative quantitation percentages for any glycoform that is removed or added to the list.

Figure 4 summarizes the relative quantitation and the reproducibility results of five major glycoforms of the NISTmAb from 10 replicate sample injections of 0.5 µg on-column. The quantitative results from the peak height analysis were similar to those from the peak area calculation. However, the peak height analysis results show accuracy with the average standard deviations (SDs) of all glycoforms less than 1 %, while the average SDs of the peak area results were approximately 1.62 %.

One feature of the BioConfirm B.09.00 software allows the user to perform a relative quantitation comparison on the selected glycoforms among different samples. Figure 5 shows the mirror plot image of the deconvoluted spectra of two NISTmAb samples (1 and 2). The G1F + G1F glycoforms (shaded) were chosen for detailed analysis. The table in Figure 5 shows that both samples have very similar quantitation results using either peak height or the peak area data.

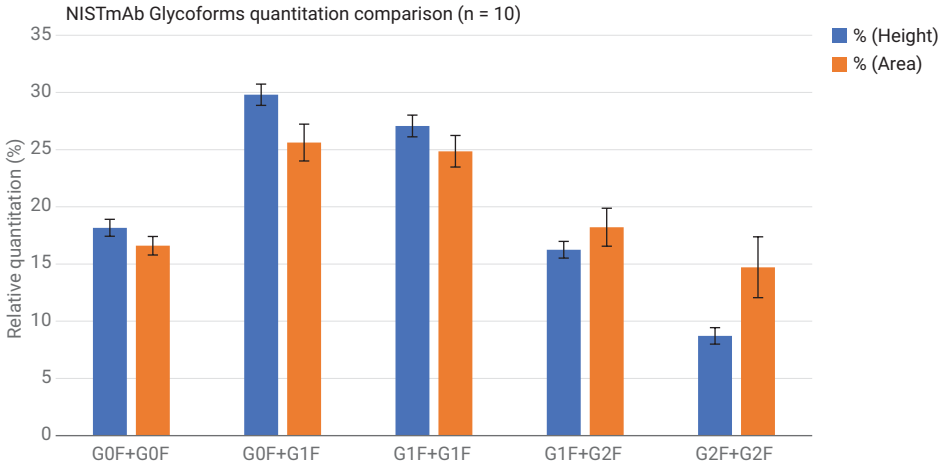
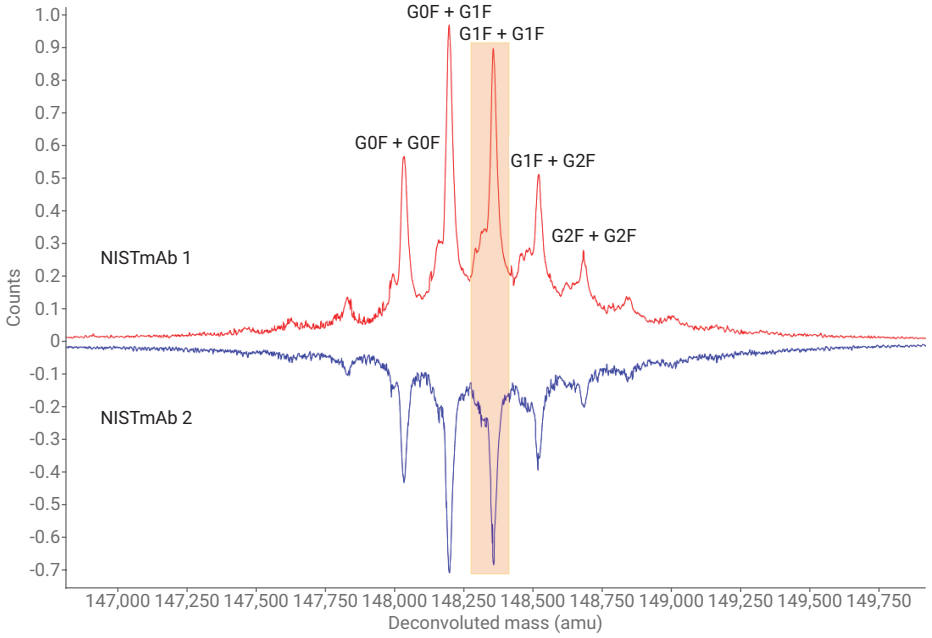


Figure 4. Quantitation results from intact NISTmAb glycoforms analysis (10 replicates).



General			% Quantitation					Sequence match
Mass	RT	File	Use for % quant	Height (MS)	% Quant (height)	Area (MS)	% Quant (area)	Pred mods
148,363.0381	2.229	NIST mab_1.d	☑	8,387	28.43	6,527,163	27.71	2*G1F(1607.5013)
148,362.5884	2.261	NIST mab_2.d	☑	6,268	29.5	4,936,695	28	2*G1F(1607.5013)

Figure 5. Quantitation results comparison of glycoforms (G1F+G1F) between two NISTmAb samples.

The NISTmAb sample was also used to perform mAb subunits analysis (level 2). To obtain accurate quantitation results on the glycoforms attached to the heavy chain of the NISTmAb, it is critical to generate the homogeneous forms of heavy (HC) and light chains (LC) of the NISTmAb. Therefore, full protein reduction with the combination of DTT and TECP reactions was performed to completely reduce all inter- and intra-disulfide bond linkages. Figure 6A shows the total ion chromatogram of the reduced NISTmAb separating the two major subunits. Excellent liquid chromatographic separation of LC and HC was achieved using a very short HPLC gradient. Figure 6B represents the deconvoluted spectrum of the NISTmAb heavy chain (shaded in light green in Figure 6A). Three major glycoforms (G0F, G1F, and G2F) were observed, and their relative abundances were calculated. Moreover, the average percent quantitation values of these three glycoforms from 10 technical replicates were also calculated to be 39.14 %, 47.68 %, and 13.18 %, respectively. The average SDs of these results were less than 0.24 % (Figure 7).

For released glycan analysis (level 4), we have developed a new workflow solution integrating UHPLC technologies, the Agilent AssayMAP Bravo liquid handling platform, the

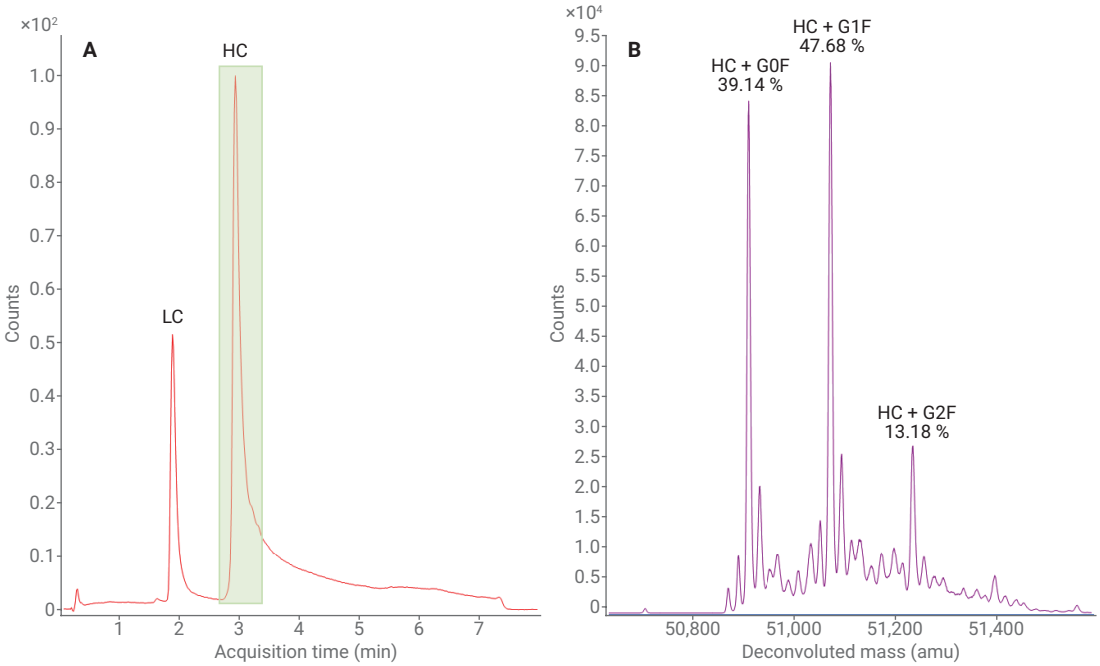


Figure 6. Total ion chromatogram (A), and MS deconvolution (B) of NISTmAb sub-units.

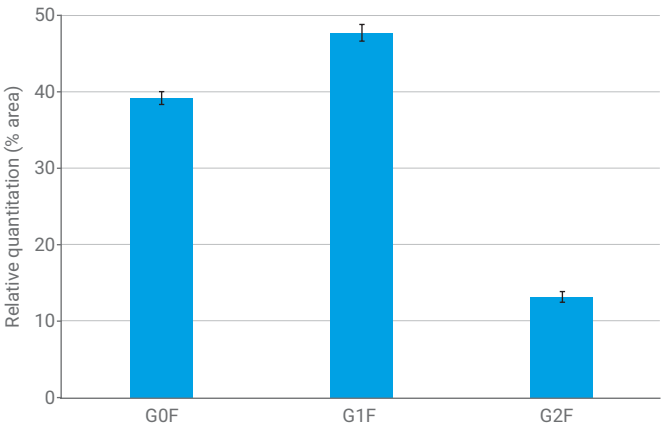


Figure 7. Quantitation results from NISTmAb sub-unit workflow.

6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software for automatic data processing[4]. Briefly, N-glycans of NISTmAb were enzymatically released by PNGaseF, followed by labeling with a fluorescent tag (InstantPC), and LC-FLD or LC/MS analysis. All sample preparations were done using the AssayMAP Bravo liquid handling system (G5542A) in a high-throughput manner. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans was used for identification using the Agilent proprietary Find-by-Formula algorithm.

Figure 8 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 8 top, zoom in) revealed that more than 15 glycan peaks were detected. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F, was comparable between the fluorescent and MS data.

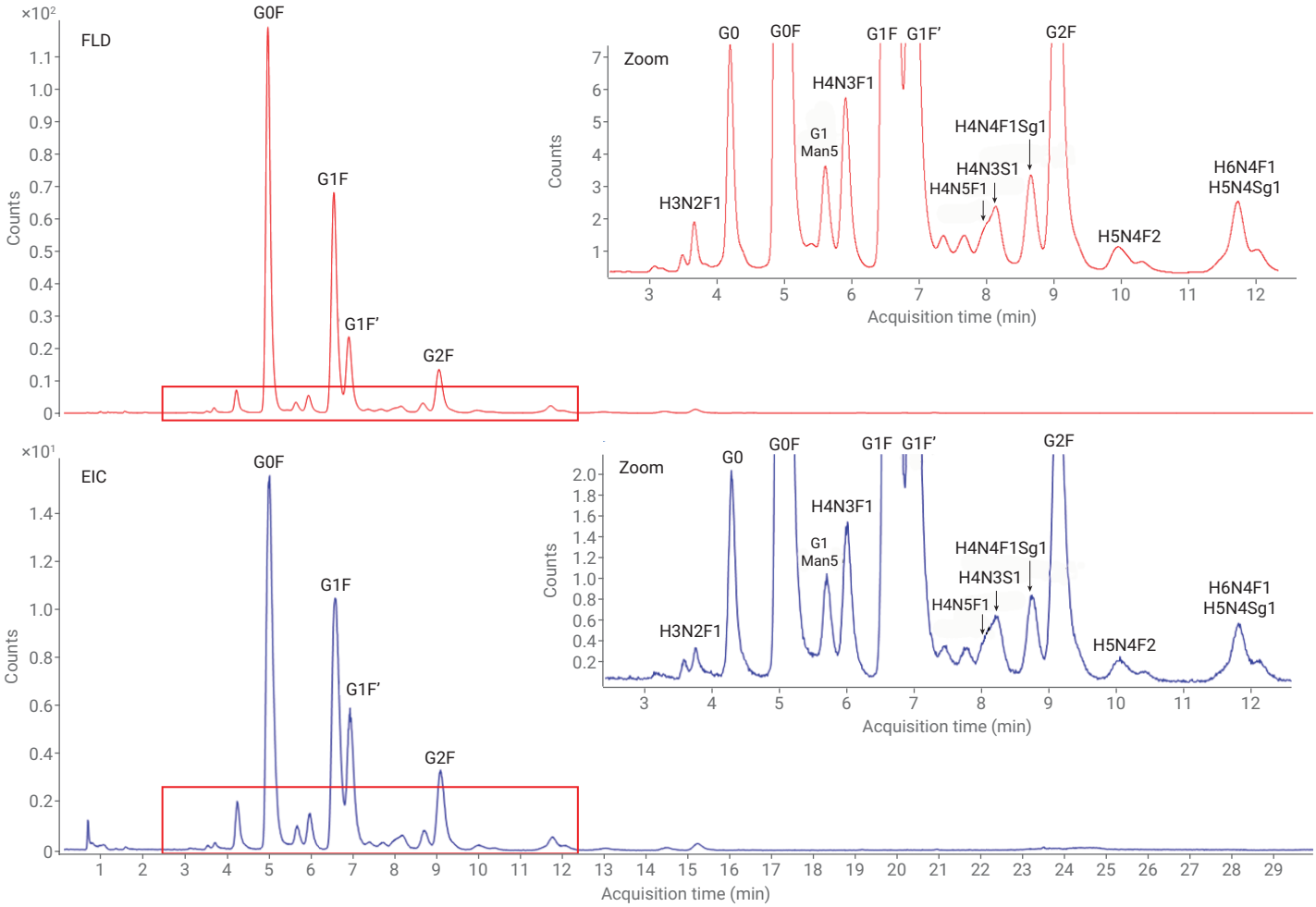


Figure 8. FLD chromatogram and mass spectra (EIC) of InstantPC labeled N-glycans from NISTmAb.

Figure 9 shows the relative sum % of the top four most abundant N-glycans of the NISTmAb sample. The relative quantitation (%) results of these glycoforms were also comparable to the results from the NISTmAb subunit workflow (level 2). The minor result discrepancy between levels 2 and 4 was likely due to the exclusion of minor glycoform peaks in the level 2 sample used for quantitative analysis. However, excellent chromatographic separation and accurate quantitation of the G1F isoforms were obtained using the AdvanceBio Glycan Mapping column. Overall, this approach can also eliminate ambiguity about glycan peak assignments and peak quantitation due to the sample heterogeneity caused by incomplete mAb reduction.

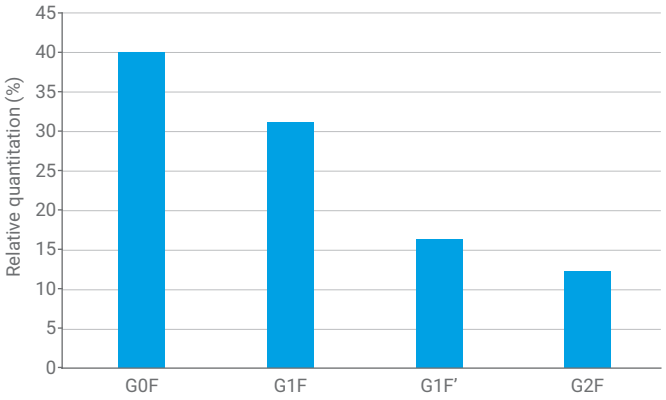


Figure 9. Quantitation results from NISTmAb released glycan workflow.

Conclusion

We have developed a complete workflow solution for antibody glycoforms characterization by integrating the Agilent AssayMAP Bravo liquid handling platform, UHPLC technologies, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software. This approach offers users flexible workflows for glycan relative quantitation at four different analytical levels:

- The intact mAb workflow provided rapid assessment of the major glycoforms of the intact mAb. The same glycoforms from various time points of the same sample or from different batch samples can easily be monitored and compared.
- The mAb subunits workflow offered detailed quantitative information about individual glycans such as G0F, G1F, and G2F. The overall high throughput of this workflow makes it an ideal method for accurate mass measurements of the majority of mAbs and their variants, including bispecific mAbs.
- The glycopeptide analysis through peptide mapping workflow resulted not only in glycan-relative quantitation but also N-glycosylation site(s) information. The Agilent AdvanceBio Glycan Mapping (HILIC) column demonstrated strong retention and increased resolution for the hydrophilic glycopeptides.
- The released glycan workflow provided high analytical sensitivity and the best quantitation for glycan analysis using both fluorescence and mass spectrometric detection. Excellence in glycans (G1F isoforms) separation, and the use of a glycan database provided in BioConfirm B.09.00 resulted in accurate glycan profiling: identification and relative quantitation.

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