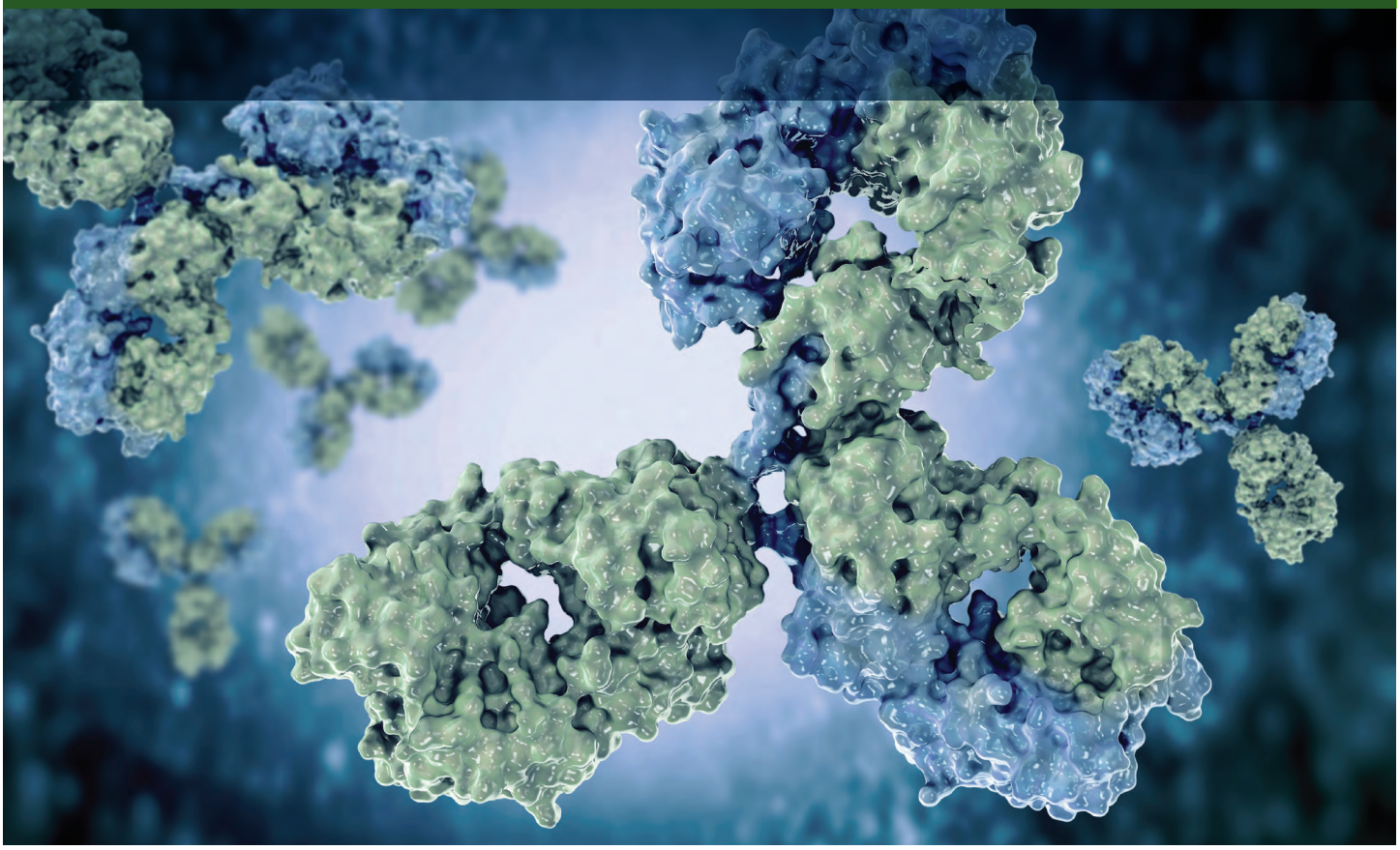
Agilent
Trusted Answers

Agilent-NISTmAb

Glycan Analysis

Agilent BioHPLC Columns
Application Compendium



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Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

Glycan Analysis

Introduction

The structure of N-linked glycans on biotherapeutic glycoproteins can play a critical role in protein recognition and cellular signaling, therefore it is not surprising that glycosylation on therapeutic proteins can significantly impact the safety, efficacy, and half-life of these drug species[1]. For example, high mannose glycans can increase clearance, impacting the pharmacokinetics of thebiotherapeutic[2]. Regulatory authorities consider glycosylation to be one of the critical quality attributes (CQA) of biomolecules. This makes N-linked glycan characterization and relative quantification within acceptable range, an essential part of the drug development process.

Protein glycosylation is a complex post-translational modification (PTM) involving attachment of glycans at specific sites on a protein, most commonly at Asn (N-linked) or Ser/Thr (O-linked) residues. The N-linked glycosylation occurs at the consensus sequence of Asn-X-Ser/Thr (where X is any amino acid except proline)[3]. Protein glycosylation is influenced by the type of host cells and fluctuations in fermentation conditions (e.g., media, pH, temperature, agitation)[4]. Depending on the sample type and detailed information necessary, glycosylation profile can be analyzed at the intact protein level, glyco-peptide, or as released glycans.

Analysis of released N-glycans is very common in characterizing therapeutic glycoproteins, and in many cases is considered a product quality attribute. In order to detect released N-glycans effectively, they are most commonly derivatized with a fluorescent dye, as they are not optically active and ionize poorly for mass spectrometric (MS) detection. Traditionally reductive amination labelling chemistry with labels such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) has been the gold standard for many years. In more recent years, glycosylamine reactive labels such as InstantPC with higher fluorescence signal and strong ionization for MS detection have entered the market. With the integration of ProZyme, Agilent can now offer convenient and easy-to-use all in one kit for N-glycan sample preparation with any of these labels, such as the AdvanceBio Gly-X N-glycan prep with InstantPC and AdvanceBio Gly-X N-glycan prep with 2-AB Express.

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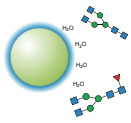

Glycan Analysis

Hydrophilic interaction chromatography

Fast, high-resolution, reproducible glycan separation

AdvanceBio Glycan Mapping

An amide HILIC column

Attribute	Advantage
2.7 µm superficially porous particle	High resolution at low back pressure
1.8 µm totally porous particles	Maximum resolution
Fluorescence and MS compatible	Easy method transfer

Column Selection

AdvanceBio Glycan Mapping	Amide bonded phase for rapid equilibration and enhanced selectivity for glycans.
1.8 µm	Based on a fully porous particle for high speed separations and high throughput applications. Stability to 1200 bar for use with the Agilent 1290 Infinity II LC.
2.7 µm	Based on Poroshell technology to give a superficially porous particle with reduced diffusion distances to give high resolution separations at lower pressures and enable the use of longer column lengths for increased separation efficiency.

Labeled N-glycan mixtures are most commonly separated via HILIC separations. HILIC, or hydrophilic interaction chromatography, uses reversed-phase type eluents with gradients starting at high organic solvent content. The mechanism of interaction of analytes with the stationary phase is a partitioning from the high organic eluent into the aqueous layer present on the surface of the stationary phase. Water is a strong eluting solvent and it is therefore important to minimize the amount of water present in the sample matrix and to allow enough time for the column to re-equilibrate and stabilize at the end of each gradient. Agilent's biocolumn portfolio offers an amide bonded phase-HILIC column for analysis of released glycans, the AdvanceBio Glycan Mapping column. This HILIC based column are available in two formats, a superficially porous columns with 2.7 µm particle size suitable for use on all HPLC instruments, and in fully porous 1.8 µm columns for maximum resolution designed to use on highly optimized UHPLC instruments. AdvanceBio Glycan Mapping column is batch tested with a glycan mixture to ensure its performance for glycan analysis. Furthermore, Agilent AdvanceBio Glycan Mapping solution provides an optimized workflow designed to deliver reproducibility in the analysis of labeled glycans, for accurate identification and quantification.

In relatively rare, or niche cases, other chromatography types may be used, such as reversed-phase for 2-AA labeled glycans, or mixed mode separations. Although it is becoming less common, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) is still used in several labs for glycan analysis. For chromatographic separations of labelled N-glycans, fluorescence detection is the dominant detection method. MS is also commonly used in development or discovery settings; however, QA/QC settings rely heavily on fluorescence detection.

Most monoclonal antibodies (mAbs) are produced in mammalian expression systems, such as Chinese hamster ovary (CHO) cells or Murine Myeloma (NS0) Cell lines[2].The NISTmAb material is a recombinant humanized IgG1κ expressed in murine suspension culture. Cell type of origin can dramatically impact the glycosylation pattern on a protein.

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As both cell lines are mammalian, the glycosylation pattern of mAbs generated from these expression systems will have similarities to human glycosylation, but as they are ultimately different species and cell types there will be appreciable differences. The NISTmAb has a more complex glycan profile than many CHO-derived biotherapeutics, both in number and diversity of glycan species[4]. For example, the NISTmAb has glycans containing Neu5Gc and α-Gal structures, which are not synthesized in humans are considered immunogenic and required to be monitored. The featured application note (5994-0372EN) highlights various glycoform/glycan quantitative analysis LC/MS workflows.

Featured Application Notes for IPC-labeled Glycan analysis:

The three application notes featured in this section each offer a different perspective on released glycan analysis as they present data on the glycans found in the NISTmAb standard as well as a mAb produced in-house at Agilent. All three use a HILIC-based separation of InstantPC-labeled glycans using the AdvanceBio Glycan Mapping column and the Agilent 1290 Infinity II LC system, selected primarily for its minimal system dead volume. The first application note (5991-8550EN) illustrates a total workflow solution for glycan analysis, including automated sample preparation on the AssayMAP Bravo, detection with the Agilent 6545XT AdvanceBio Q-TOF, and data analysis using Agilent MassHunter BioConfirm software.

The second application note (5991-6958EN) compares results obtained by fluorescence to those obtained using a QTOF mass spectrometer, and demonstrates the power of a high resolution QTOF workflow to identify unknown glycans using accurate mass and tandem MS. This application note contains data from Agilent's contribution to the glycan analysis inter-lab study hosted by NIST in 2015-20165. Comparing the results Agilent obtained to the aggregate results of the round robin study[6] illustrates the strength of Agilent's glycan analysis tools.

The third application note (5991-8071EN) shows the suitability of a more cost-effective, rugged single quadrupole MS instrument for glycan analysis for those settings where a high-power, large-footprint instrument is not ideal.

Featured Application Notes for 2-AB labeled Glycan analysis:

The application note (5994-0682EN) featured here demonstrates analysis of released N-glycans from an in-house Agilent CHO mAb and a fusion protein (Enbrel) that have been labeled with 2-AB and prepared using the Agilent AdvanceBio Gly-X 2-AB Express N-Glycan Sample Preparation kit. Samples were analyzed using a very classic approach – a HILIC separation on the AdvanceBio Glycan Mapping column with fluorescence detection using the Agilent 1290 Infinity II LC.

References

1. Liu, L. Antibody Glycosylation and its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins. *J. Pharm. Sci.* **2015**, *104*(6), 1866–1884.
2. D. Reusch and M.L. Tejada, Glycobiology 25 (12), 1325-1334 (2015).
3. Mellquist JL, Kasturi L, Spitalnik SL, Shakin-Eshleman SH. "The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency". *Biochemistry*. **1998**. *37* (19): 6833–6837.
4. 4Ghaderi, D.; Zhang, M.; Hurtado-Ziola, N.; Varki, A. Biotechnol. *Genet. Eng. Rev*. **2012**, *28*, 147–175.
5. J.M. Prien et al., "Orthogonal Technologies for NISTmAb N-Glycan Structure Elucidation and Quantitation," in State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 2. Biopharmaceutical Characterization: *The NISTmAb Case Study, J.E. Schiel et al., Eds.* (American Chemical Society, **2016**), 185-235.
6. M. L. A. De Leoz, D. L. Duewer, S. E. Stein, NIST Interlaboratory Study on the Glycosylation of NISTmAb, a *Monoclonal Antibody Reference Material* June **2015** to February **2016** https://nvlpubs.nist.gov/nistpubs/ir/2017/NIST.IR.8186.pdf

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Glycan Mapping Workflow

In this document Agilent applications chemists share their recommendations for an optimum LC system and its configuration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specific separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**

Agilent AdvanceBio Glycan Mapping 1.8 µm Columns

- AdvanceBio Glycan Mapping products include sample preparation, labelled and unlabelled standards and 1.8 µm and 2.7 µm columns.
- Both gradients provide 1.25%/mL slope.
- It may be necessary to adjust the start and end point to obtain highest resolution for samples containing different types of glycan.
- Larger glycan structures may require 75 to 55% acetonitrile gradient for optimum results for example.

AdvanceBio Glycan Mapping column, 1.8 µm, stable to 1200 bar

Description	Part No.
2.1 x 100 mm	858700-913
2.1 x 150 mm *	859700-913
Fast Guard, 2.1 mms	651750-913

* Recommended initial column size

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Agilent 1290 Infinity UHPLC System

Mobile phases

Eluent A: 100 mM ammonium formate, pH 4.5
Eluent B: acetonitrile
(mass spec compatible).

Detection (G1321B)

Agilent 1260 Infinity Fluorescence Detector,
ex 260 nm, em 430 nm, 8 µL cell.

Column compartment (G1316C)

40 °C gives longer column life; 60 °C gives sharper peaks but significantly reduces lifetime. Selectivity and resolution may change with temperature.

Sample injection (G4226A)

1 to 2 µL injection for maximum resolution. Samples should first be dissolved in H2O then made up to 70:30 ACN:Water. Chiller should be used.

Pump (G4220A)

0.5 mL/min for high resolution separations; up to 1.0 mL/min for high speed. High aqueous clean up should ALWAYS be run at reduced flow rate.

Suggested gradient for resolution

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

Suggested gradient for speed

Time	Eluent A	Eluent B	Flow
0	25%	75%	1.0 mL/min
12	40%	60%	1.0 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
14	25%	75%	0.5 mL/min
15	25%	75%	1.0 mL/min
20	25%	75%	1.0 mL/min

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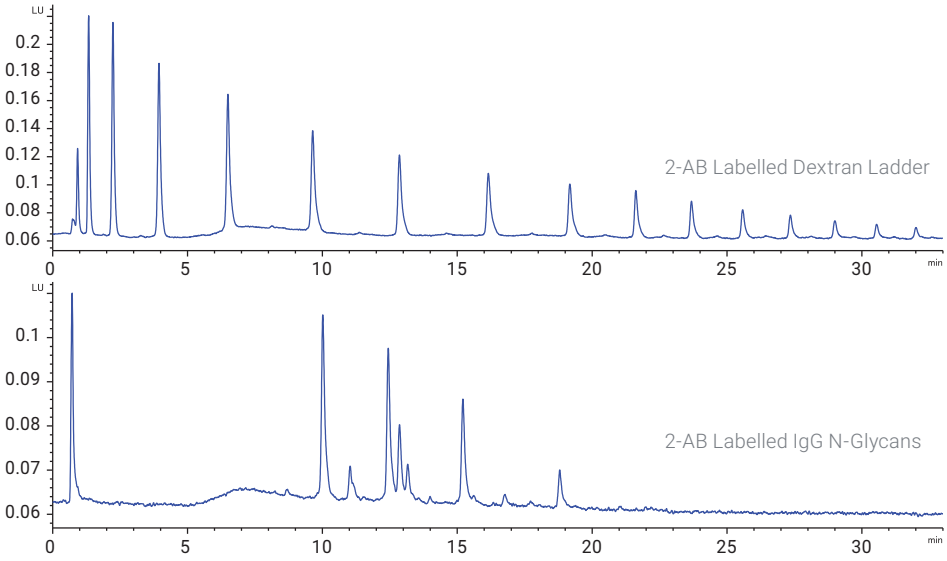
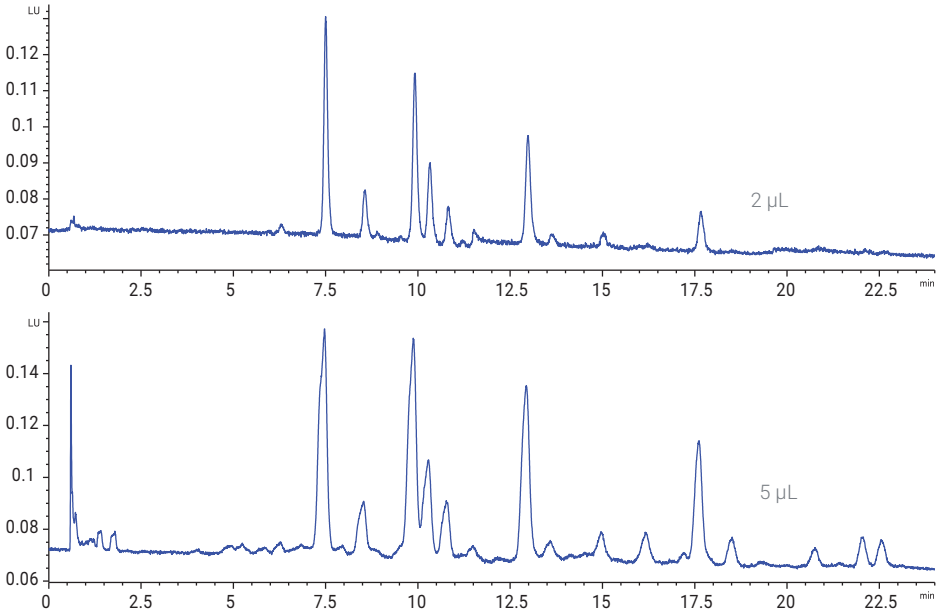
Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

Samples should be prepared by dissolving in water and then adding acetonitrile to give a final composition 30:70 water:acetonitrile. The small column dimension, 2.1 x 150 mm, still requires small injection volumes. The figure below on the left demonstrates the outcome from injecting 5 µL – peaks become broader and resolution is lost – compared to 2 µL injection.



High-resolution separation of 2-AB Labeled Dextran Ladder (p/n 5190-6998) and 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).



Over-injection of 2-AB Labeled Human IgG N-Glycan Library (2 µL vs. 5 µL).

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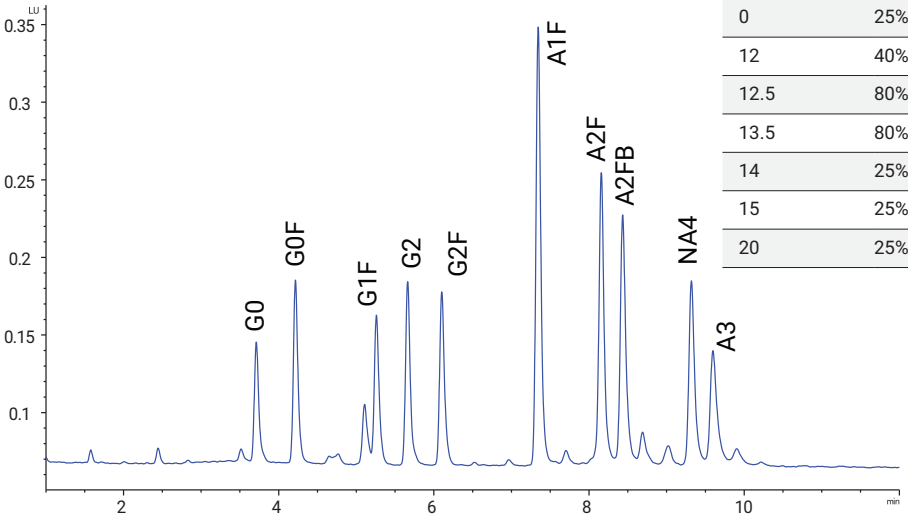
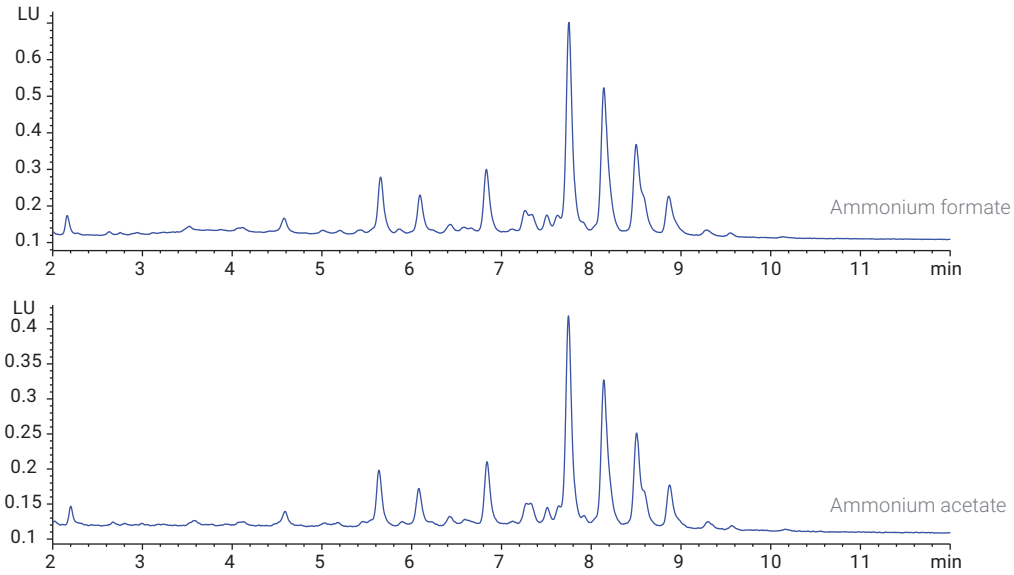


Time	Eluent A	Eluent B	Flow
0	25%	75%	1.0 mL/min
12	40%	60%	1.0 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
14	25%	75%	0.5 mL/min
15	25%	75%	1.0 mL/min
20	25%	75%	1.0 mL/min

High speed separation of 2-AB Labeled N-Glycans (tentative peak assignment).



Glycans, such as those found in bovine fetuin, can be eluted with ammonium formate or ammonium acetate mobile phases.

Agilent AdvanceBio Glycan Mapping 2.7 µm Columns

AdvanceBio Glycan Mapping, 2.7 µm, stable to 600 bar

Description	Part No.
2.1 x 100 mm	685775-913
2.1 x 150 mm *	683775-913
2.1 x 250 mm	651750-913
Fast Guard, 2.1 mm, 2.7 µm	821725-906

* Recommended initial column size

Description	Part No.
4.6 x 100 mm	685975-913
4.6 x 150 mm	683975-913
4.6 x 250 mm	680975-913

AdvanceBio Glycan Mapping products include sample preparation, labeled and unlabelled standards, and 1.8 µm and 2.7 µm columns.

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Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Eluent A: 100 mM ammonium formate, pH 4.5
Eluent B: acetonitrile
(mass spec compatible)

Pump (G5611A)

0.5 mL/min for high resolution separations; up to 1.0 mL/min for high speed. High aqueous clean up should ALWAYS be run at reduced flow rate.

Sample injection (G5667A)

1 to 2 µL injection for maximum resolution. Samples should first be dissolved in H_2O then made up to 70:30 ACN:Water. Chiller should be used.

Column compartment (G1316C)

40 °C gives longer column life; 60 °C gives sharper peaks but significantly reduces lifetime. Selectivity and resolution may change with temperature.

Detection (G1316C)

Agilent 1260 Infinity Fluorescence Detector, ex 260 nm, em 430 nm, 8 µL cell

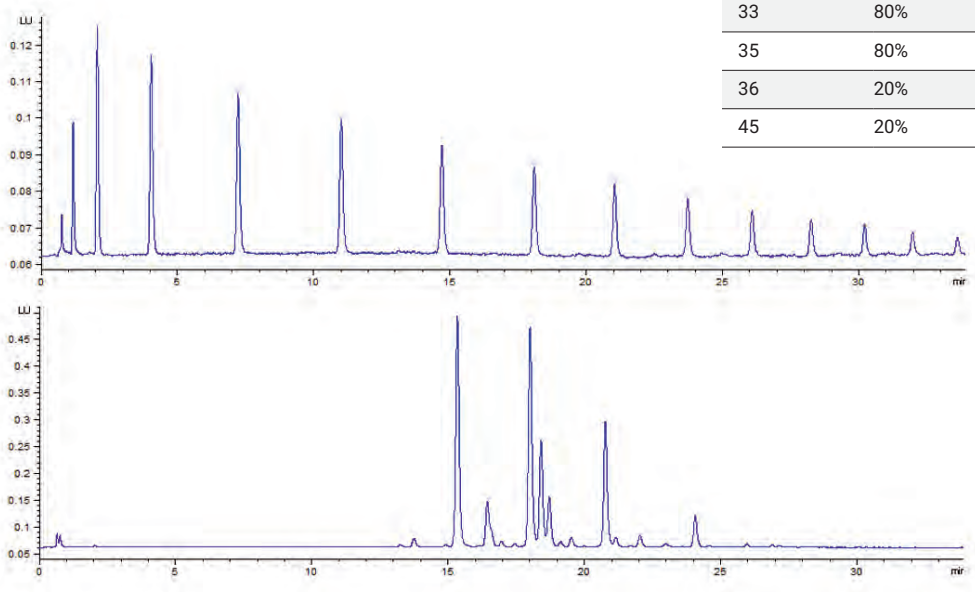
Suggested gradient for resolution

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

Suggested gradient for speed

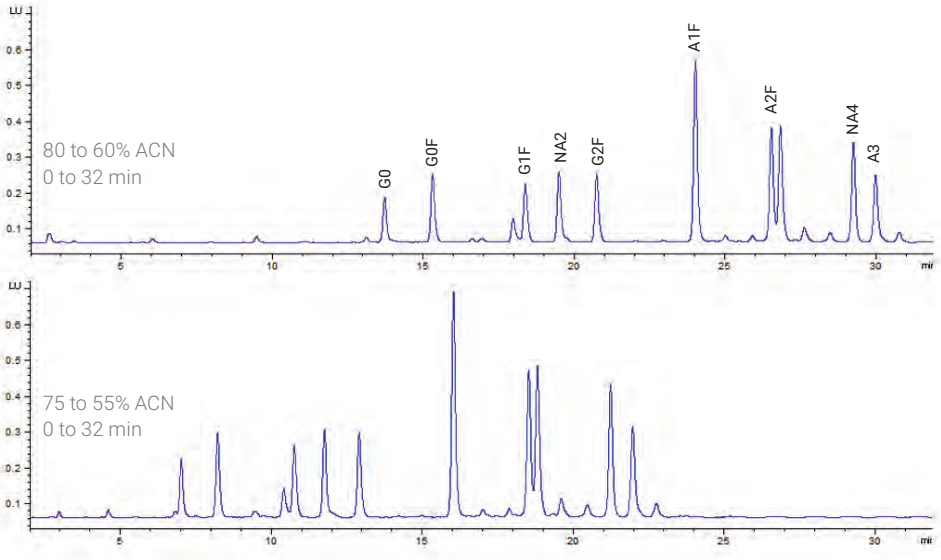
Time	Eluent A	Eluent B	Flow
0	20%	80%	0.7 mL/min
12	40%	60%	0.7 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
16	20%	80%	0.5 mL/min
17	20%	80%	0.7 mL/min
20	20%	80%	0.7 mL/min

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min



High-resolution separation of 2-AB Labeled Dextran Ladder (p/n 5190-6998) and 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).

Time	Eluent A	Eluent B	Flow
0	25%	75%	0.5 mL/min
32	45%	55%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	25%	75%	0.5 mL/min
45	25%	75%	0.5 mL/min



Effect of gradient adjustment on larger (later eluting) 2-AB Labeled N-Glycans

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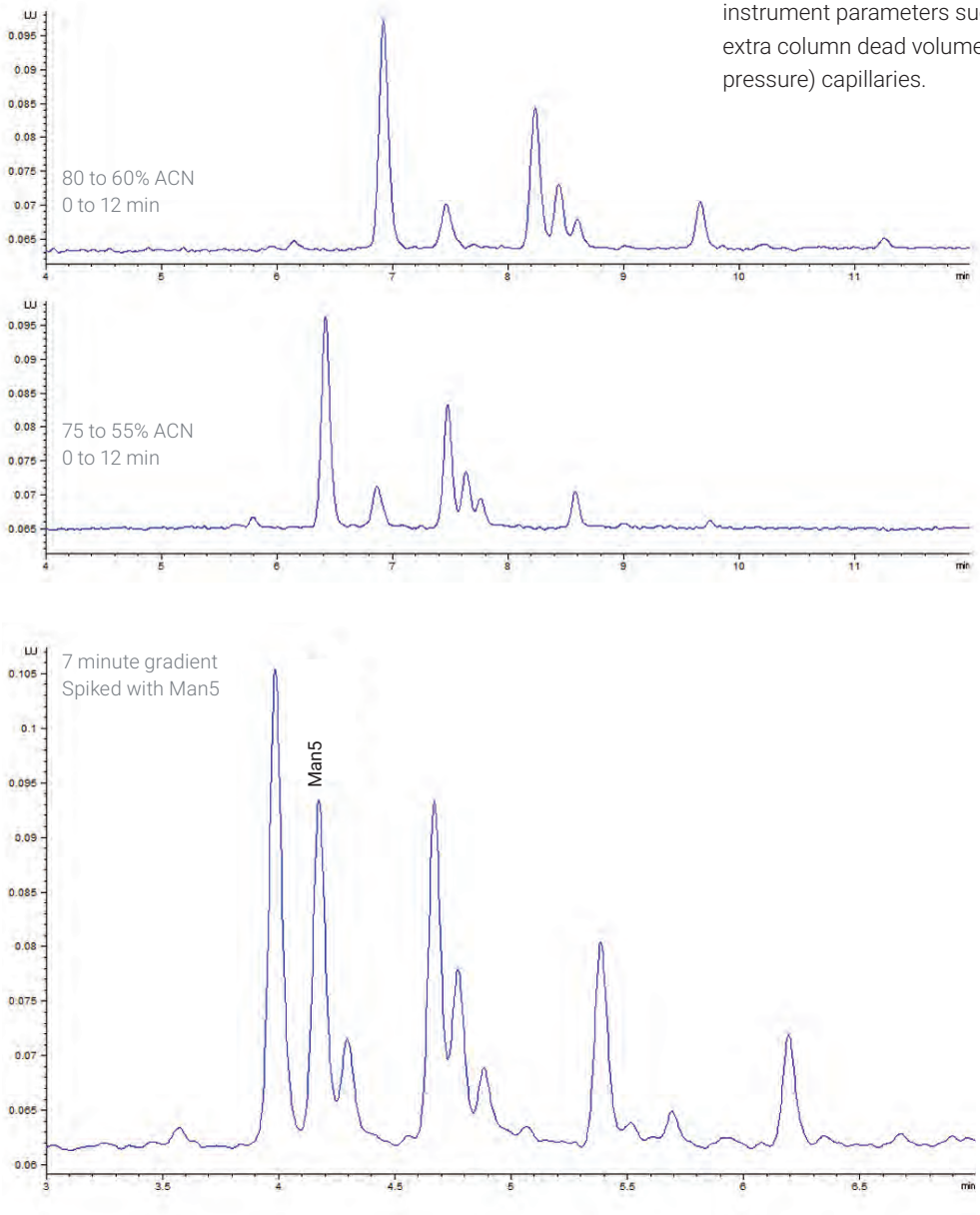
High speed separations can be more difficult to achieve due to instrument parameters such as pump gradient dwell time, and extra column dead volume through use of wider bore (lower pressure) capillaries.



High speed and very high speed separations of 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).

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Application Note

Biotherapeutics and Biosimilars

Agilent

Trusted Answers

Glycopeptide Characterization for Various Monoclonal Antibodies Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Authors

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Agilent Technologies, Inc.
Santa Clara, CA, USA

Introduction

Monoclonal antibodies (mAbs) and their derivatives represent a very complex but important class of biopharmaceutical molecules with a wide range of applications. As mAbs are heterogeneous molecules by nature, comprehensive analytical characterization is required. The full range of biotherapeutics characterization usually includes confirmation of the protein sequences, protein post-translational modification (PTM) locations, and their relative quantitative information. Protein glycosylation is one of the major PTMs of an mAb, and is involved in many biological regulatory processes as well as therapeutic efficacy and immunogenicity. Therefore, it is important to understand the various glycans' distribution and composition for pharmaceutical bioprocessing.

Quadrupole time-of-flight (Q-TOF) liquid chromatography/mass spectrometer (LC/MS) systems are often used to analyze intact mAbs and mAb subunits, perform mAb peptide sequence mapping, and characterize PTMs due to their excellent mass accuracy and resolution in the high-mass range[2,3].

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Typically, four levels of LC/MS workflows for glycoform/glycan characterization are used:

- Levels 1 and 2 focus on the analysis of glycoforms on intact and reduced mAb molecules. The intact mAb workflow provides rapid assessment of the major glycoforms of the intact mAb, while the mAb subunit workflow offers detailed quantitative information about individual glycans such as G0F, G1F, and G2F.
- Level 3 is the analysis of glycopeptides generated from the proteolytical digestion of mAbs, commonly part of the peptide sequence mapping workflow[4]. This workflow shows results not only in glycan-relative quantitation, but also N-glycosylation site(s) information.
- Level 4 is the characterization of glycans that have been released by enzymatic cleavage or other mechanisms. It provides high analytical sensitivity and the best quantitation for glycan analysis (Figure 1)[5].

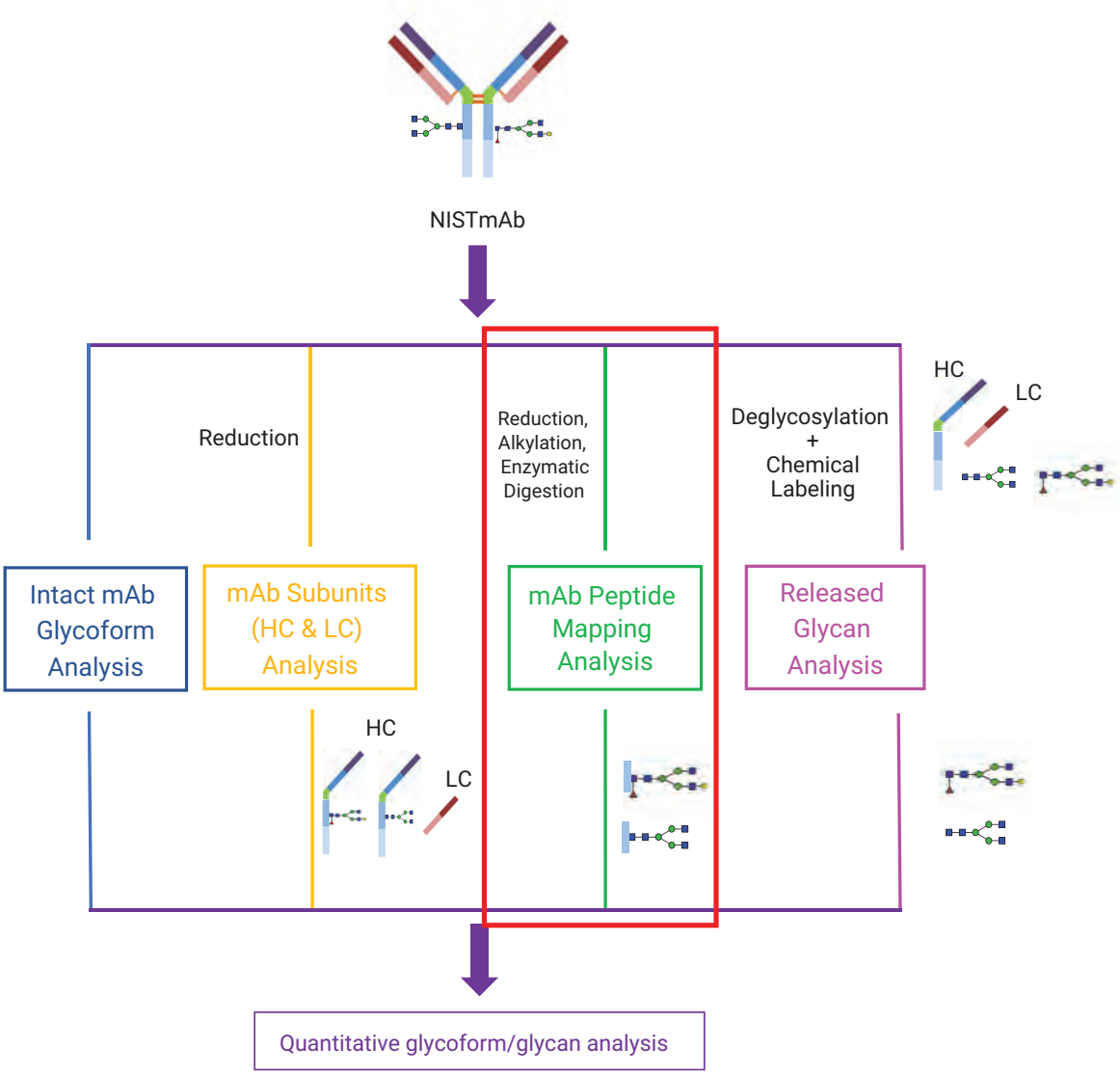

Figure 1. Various glycoform/glycan quantitative analysis workflows. The glycopeptide workflow is highlighted in the red box.

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Peptide mapping of mAbs has widely been used as an analytical technique for the comprehensive characterization of protein biotherapeutics. This technique provides not only the complete amino acid sequences of mAbs and their variants, but also information on PTMs and locations. In routine analysis, peptides resulting from proteolytic digestion are typically separated by reversed-phase (RP) chromatography. RP-C18 or C8 columns are the most commonly used due to their excellent chromatographic separation power for regular peptides as well as peptides with PTMs such as oxidation and deamidation.

However, some protein modifications are not so easy to resolve through RP-type separation. Glycopeptides, which post relatively higher hydrophilicity, demonstrate very low retention and poor resolution on RP columns. In this case, hydrophilic interaction liquid chromatography (HILIC) with an amide-bonded stationary phase is often used as it can provide significantly more retention for glycosylated peptides.

This work demonstrates an optimized LC/MS workflow for mAb glycopeptide characterization (level 3) using the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent MassHunter BioConfirm software for various glycopeptide identification and their relative quantitation (Figure 2). HPLC separation of glycopeptides from three different mAbs (NISTmAb, Trastuzumab, and A CHO cell cultured human IgG1 mAb) were compared on both the Agilent AdvanceBio Peptide Mapping (RP-C18) column and the Agilent AdvanceBio Glycan Mapping (HILIC) column.

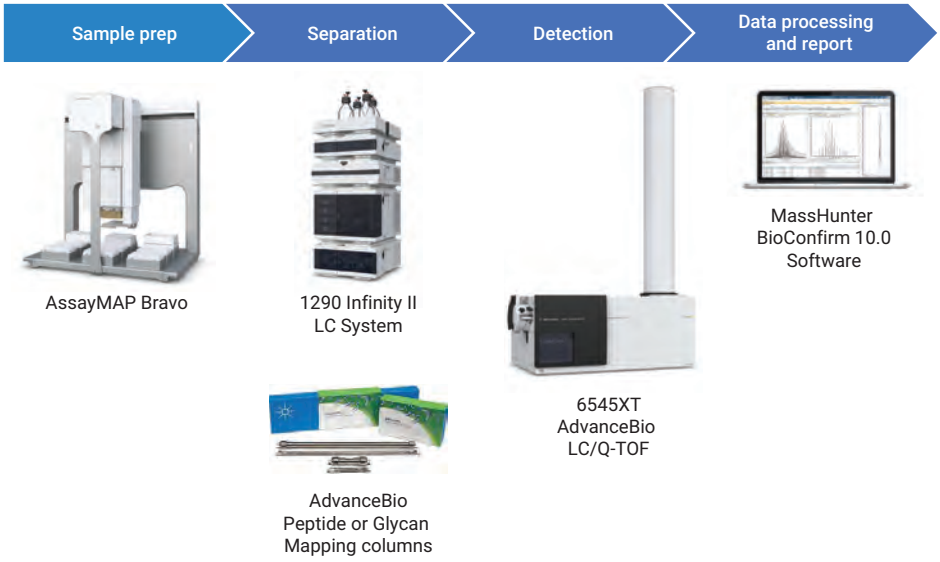

Figure 2. Analytical components of the mAb glycopeptide characterization workflow.

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Experimental

Materials and methods

Three mAb samples were used in this study:

- The mAb standard, RM 8671, was from National Institute of Standards & Technology (NIST), aka NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA). CHO mAb1 (A-mAb) was obtained from a collaborator.

2,2,2-Trifluoroethanol (TFE), DL-dithiothreitol (DTT), iodoacetamide (IAA), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. High-quality mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. AssayMAP C18 cartridges were from Agilent Technologies.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system.

Instrumentation

Three mAb samples were used in this study:

- Agilent AssayMAP Bravo system (G5542A)
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Sample preparation

The AssayMAP Bravo liquid handling system was used to dilute, digest, and desalt the mAb samples. Samples were then dried down and resuspended with 0.1 % formic acid (FA) in DI water for analysis on the Peptide Mapping column. The digested samples that needed to be analyzed by the AdvanceBio Glycan Mapping (HILIC) column were resuspended with 80 % acetonitrile solution, which allowed effective sample loading and better chromatographic separation.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. LC separation was obtained with either an AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) or an AdvanceBio Glycan Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1–3 list the LC/MS parameters used. Approximately 2 µg of protein digest was injected onto the AdvanceBio Peptide Mapping column, and 5 µg of protein digest was used on the AdvanceBio Glycan Mapping column for the glycopeptide analyses.

Two separate sample data acquisitions were run for glycopeptide quantitative analysis: one with MS/MS data acquisition mode (using the shaded parameters in Table 3) for peptide identification; the other, with MS-only acquisition mode, was for glycopeptide quantitation.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System		
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 653750902)	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 683775913)
Thermostat	4 °C	4 °C
Solvent A	0.1 % Formic acid in water	0.1 % Formic acid in acetonitrile
Solvent B	0.1 % Formic acid in acetonitrile	0.1 % Formic acid in water
Gradient	0–15 minutes, 0–10 %B 15–45 minutes, 10–40 %B 45–55 minutes, 40–90 %B	0–30 minutes, 5–40 %B 30–40 minutes, 40–60 %B 40–55 minutes, 60–90 %B
Column temperature	60 °C	50 °C
Flow rate	0.4 mL/min	0.4 mL/min
Injection volume	8.0 µL	2.0 µL
Amount on column	2 µg	5 µg

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Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	250 °C
Drying gas	10 L/min
Nebulizer (psig)	25
Sheath gas temperature	250 °C
Sheath gas flow	12 L/min
VCap	3,500 V
Nozzle voltage	0 V
Fragmentor	170 V
Skimmer	65 V
Quad AMU	95
Reference mass	121.0509 922.0098

Data processing

Raw data acquired from LC/MS/MS were processed using MassHunter BioConfirm 10.0 software. This software simplifies data analysis, enabling automatic identification and relative quantitation of targeted biomolecules for all major biopharma workflows.

Table 3. MS/MS Acquisition parameters.

Parameter	Value
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	m/z 150–1,700
Acquisition rate	8 spectra/sec
Auto MS/MS range	m/z 50–1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 m/z)
Precursors/cycle	Top 10
Collision energy	3.6*(m/z)/100–4.8
Threshold for MS/MS	2,000 counts and 0.001%
Dynamic exclusion	On; 3 repeat then exclude for 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By abundance only; +2, +3, >+3

Data acquired for glycopeptide quantitation analysis used the MS-only acquisition mode. Highlighted parameters were used for peptide identification.

Results and discussion

mAb glycoform profiling through the routine peptide mapping approach has been a widely used method. To demonstrate the effectiveness of glycopeptide separation by the HILIC column compared to the conventional RP-C18 column, three humanized IgG-1 type of mAbs were selected in this study. All mAbs were reduced, alkylated, and digested with a Trypsin + Lys-C enzymes mix using the same protocol in the AssayMAP Bravo liquid handling system. The digested mAb samples were then injected and separated by both the RP-C18 and the HILIC columns with the same HPLC run time (60 minutes). Figures 3 and 4 illustrate the chromatographic retention differences between the regular peptides and glycopeptides in the RP-C18 and the HILIC conditions.

Under routine RP HPLC conditions, peptides are separated by their hydrophobicity. The less hydrophobic peptides elute earlier than the more hydrophobic peptides. Since our HPLC gradient was optimized for the mAb tryptic digested samples, most of the peptides were separated nicely in the HPLC run. The glycopeptides are more hydrophilic and, thus, had shorter retention on the RP column. Figure 3 shows that all glycopeptides were eluted in the early gradient, with an approximately one-minute retention time window.

The HILIC separation is an orthogonal method to the RP, where the HPLC gradient is reversed. The lyophilized mAb digests should be dissolved in a high organic content solution to have better sample loading retention. High resolution in separation was achieved, and all major glycopeptide peaks were eluted between 28–34 minutes, as shown in Figure 4.

For LC/MS data analysis, the Peptide Digest Workflow in MassHunter BioConfirm 10.0 software was used. This software program enables the quick setup for batch sample analysis. A modification file of most major PTMs, including oxidation, deamidation, and many glycans imported from a personal compound database (PCD), can be generated easily. The Agilent proprietary Peptide Feature Extraction (PFE) algorithm was used for the identification of biomolecules, which were then confirmed by matching the measured masses with theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified peptides (including the glycopeptides) was also automatically calculated using either peak heights or peak areas of the mass spectra. Figure 5 is a screen capture of the BioConfirm 10.0 software layout showing the compound list of matched glycopeptides of NISTmAb. This program allows quick review of detailed peptide information including mass, retention times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram format.

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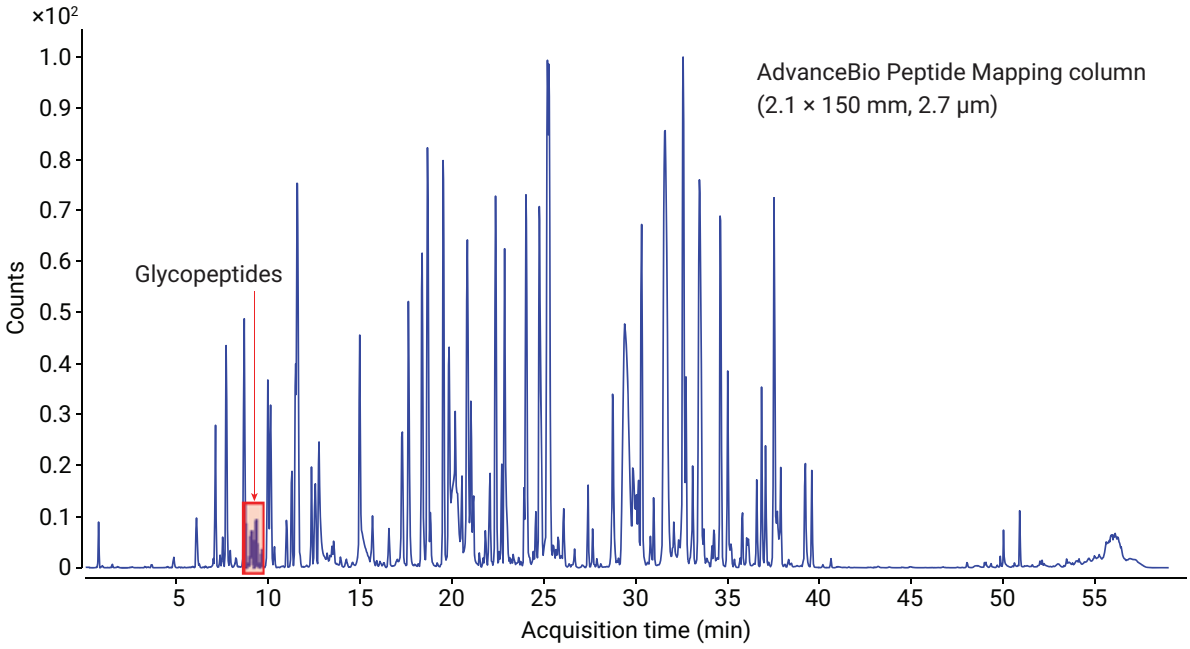

Figure 3. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Peptide Mapping (RP-C18, 2.1 × 150 mm, 2.7 µm) column.

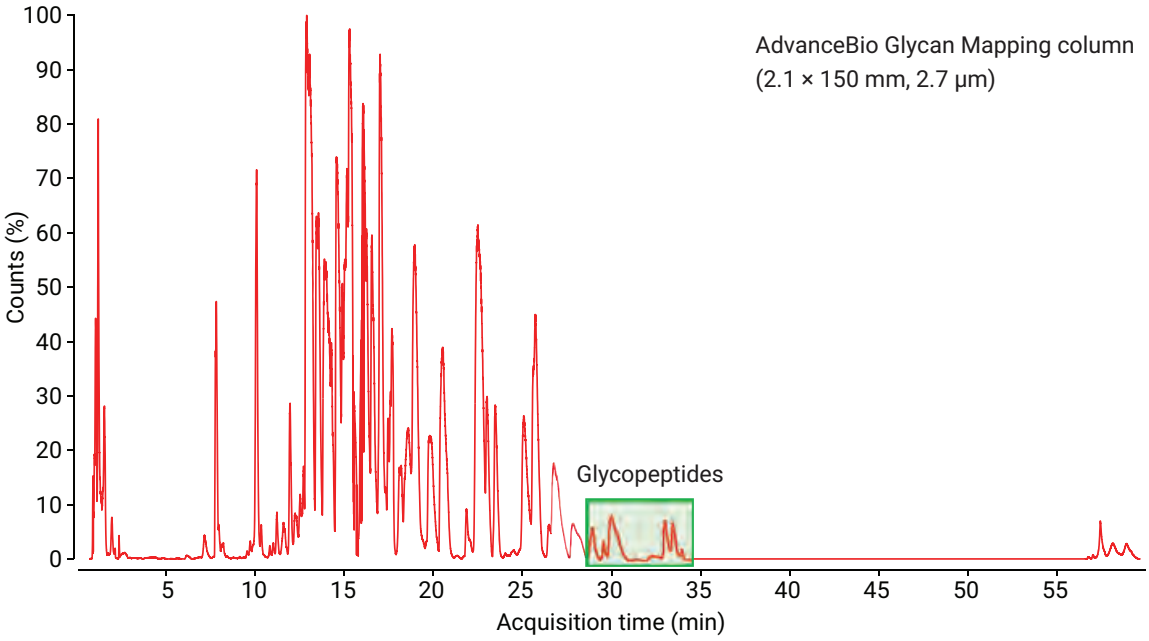

Figure 4. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Glycan Mapping (HILIC, 2.1 × 150 mm, 2.7 µm) column.

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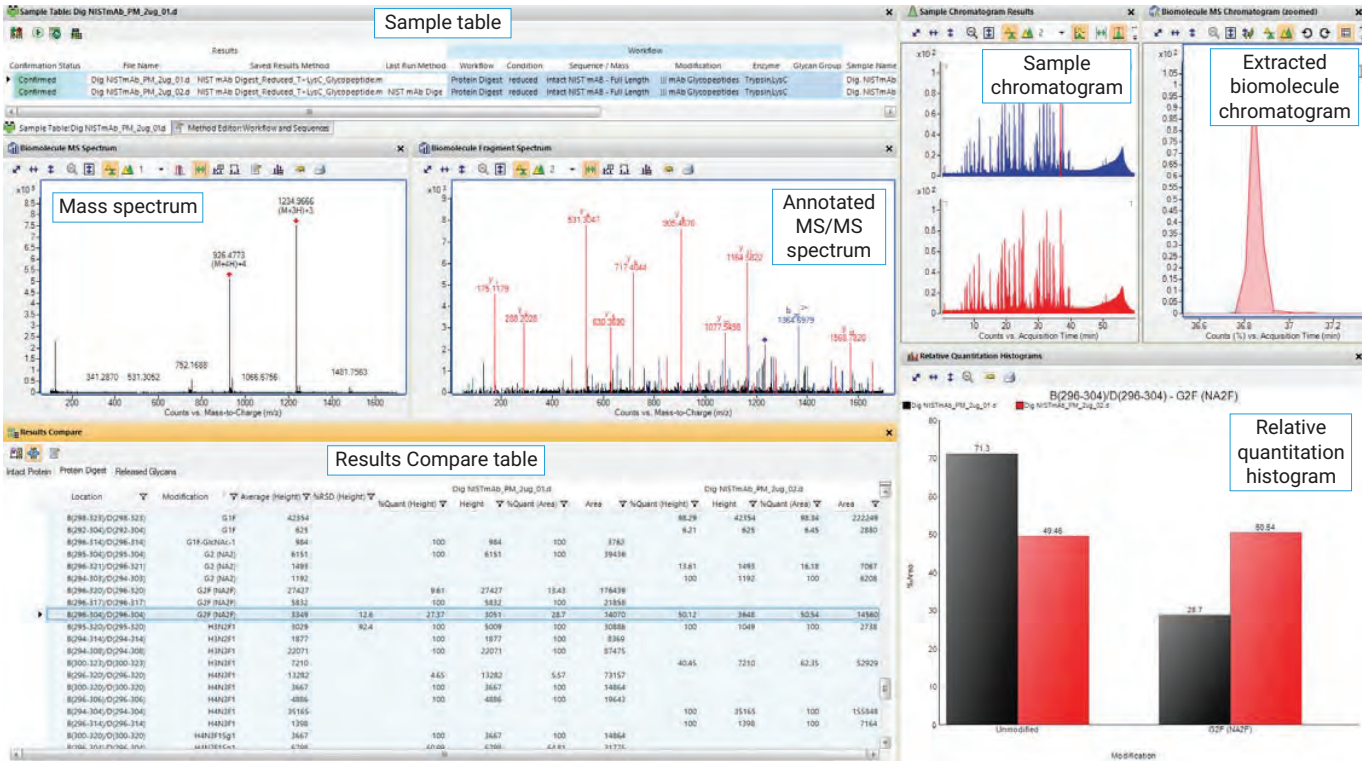

Figure 5. Screen capture of MassHunter BioConfirm 10.0 software with representative glycopeptide profiling results and histogram of relative quantitation on glycopeptides.

For LC/MS data analysis, the Peptide Digest Workflow in MassHunter BioConfirm 10.0 software was used. This software program enables the quick setup for batch sample analysis. A modification file of most major PTMs, including oxidation, deamidation, and many glycans imported from a personal compound database (PCD), can be generated easily. The Agilent proprietary Peptide Feature Extraction (PFE) algorithm was used for the identification of biomolecules, which were then confirmed by matching the measured masses with theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified peptides (including the glycopeptides) was also automatically calculated using either peak heights or peak areas of the mass spectra. Figure 5 is a screen capture of the BioConfirm 10.0 software layout showing the compound list of matched glycopeptides of NISTmAb. This program allows quick review of detailed peptide information including mass, retention times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram format.

Detailed inspection of raw MS data from Figures 3 and 4 reveals that there were two major group of glycopeptides (EEQYNSTYR and TKPREEQYNSTYR) with various glycans attached at the asparagine (N300 of heavy chain) position. In the RP separation, three glycopeptides with sequence of EEQYNSTYR, and six glycopeptides in TKPREEQYNSTYR were identified (Figure 6). However, the same group of glycopeptides were coeluted, and poor chromatographic resolution was observed. Conversely, the HILIC column demonstrated great resolution for the separation of the same sets of glycopeptides (Figure 7).

Although more than nine glycopeptides (Figures 6 and 7) were detected and identified in different LC conditions, a set of six major abundant glycopeptides with the sequence of TKPREEQYNSTYR were selected for relative quantitation analysis (tables in Figures 6 and 7) to have fair comparison results.

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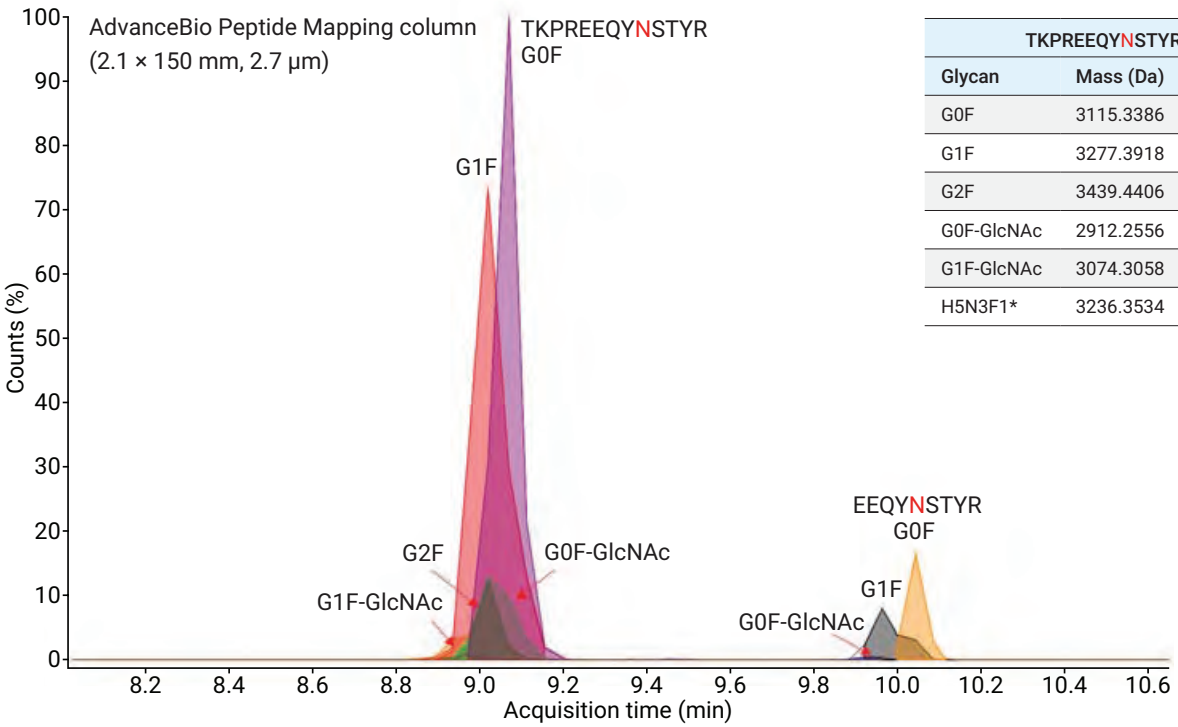
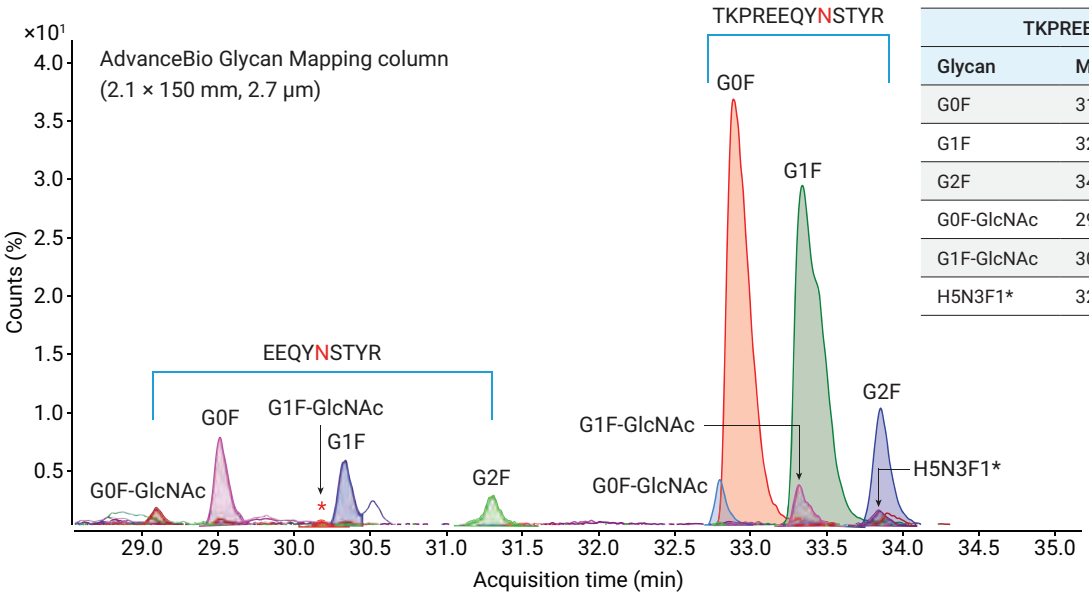


TKPREEQYNSTYR (292-304)			
Glycan	Mass (Da)	RT (min)	Quant (%)
G0F	3115.3386	9.066	41.82
G1F	3277.3918	9.016	41.70
G2F	3439.4406	9.016	9.08
G0F-GlcNAc	2912.2556	9.016	2.87
G1F-GlcNAc	3074.3058	9.016	3.35
H5N3F1*	3236.3534	8.930	1.18

Figure 6. MS extracted compound chromatograms (ECCs) and relative % quantitation of the identified glycopeptides from RP LC separation. H5N3F1* may be denoted as FM4A1G1 or FA1G1Ga1 in other publications.



TKPREEQYNSTYR (292-304)			
Glycan	Mass (Da)	RT (min)	Quant (%)
G0F	3115.3386	32.923	43.07
G1F	3277.3918	33.334	39.89
G2F	3439.4406	33.877	9.31
G0F-GlcNAc	2912.2556	32.797	3.10
G1F-GlcNAc	3074.3058	33.321	3.37
H5N3F1*	3236.3534	33.864	1.25

Figure 7. MS ECCs and relative % quantitation of the identified glycopeptides from HILIC separation.

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Figure 8 summarizes the relative quantitation and reproducibility results of the six major glycopeptides of the NISTmAb from three replicate sample injections of 2 µg (RP-C18) and 5 µg (HILIC) on-column, respectively. The quantitative results from the peak area of the RP method were similar to those from the HILIC method. However, due to the better glycopeptide separation, the HILIC results represented higher quantitation accuracy and smaller average standard deviations (SDs) for all glycopeptides (<0.2 %); the average SDs of the RP method results were approximately 0.56 %.

We used the same HILIC method for glycopeptide relative quantitative comparison among three mAbs (NISTmAb, Herceptin, and A-mAb). Figure 9 shows the relative % quant of the top six most abundant glycopeptides. Unlike the NISTmAb that posted similar abundances of G0F and G1F (43 % and 40 %), the Herceptin sample contained a very high level of G0F (>65 %) and low level of G2F (~2 %). In addition, no H5N3F1 could be detected in either Herceptin or A-mAb samples. Two degraded glycan molecules (G0F-GlcNAc and G1F-GlcNAc) were found at trace levels (<0.5 %) as well in the A-mAb sample.

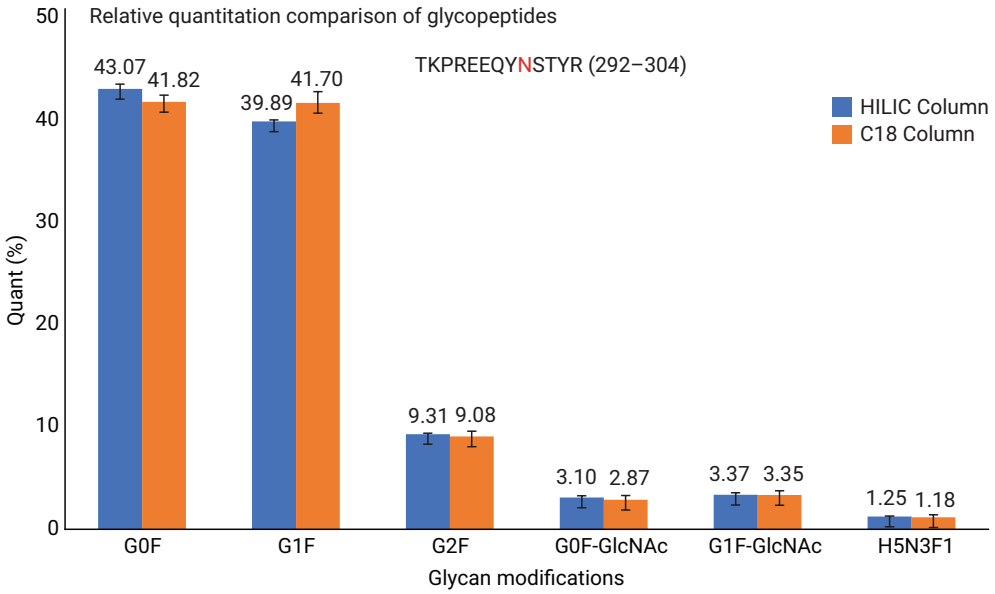

Figure 8. Relative quantitative comparison of NISTmAb glycopeptides analysis (RP-C18 versus HILIC, three replicates).

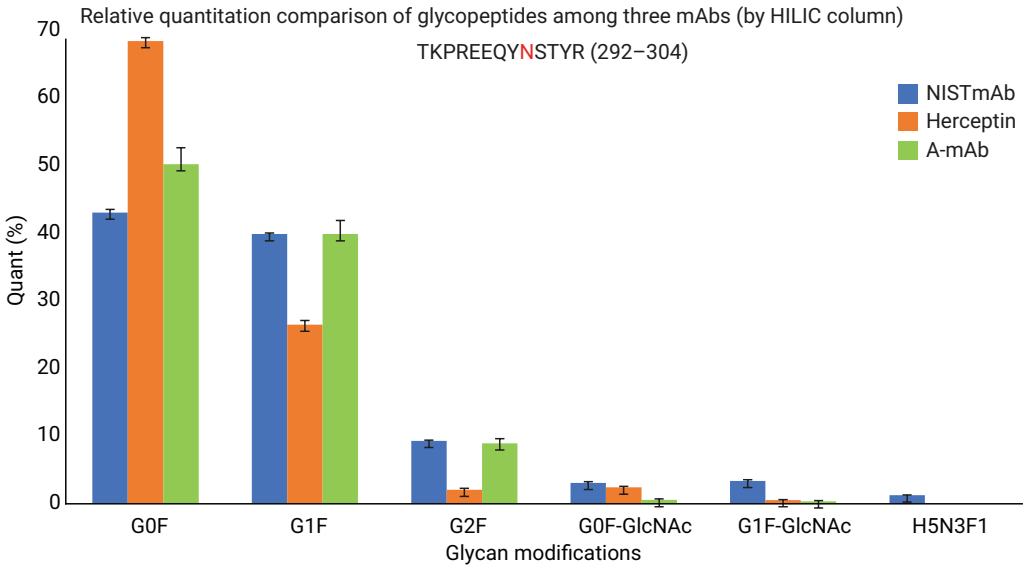

Figure 9. Relative % quantitation of the top six glycopeptides in each of the three mAb samples. All digested mAb samples were separated by the HILIC column (three replicates).

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Conclusion

A complete workflow solution for mAb glycopeptide characterization by integrating the AssayMAP Bravo liquid handling platform, 1290 Infinity II LC, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software has been developed. The major benefits of this new workflow include:

- The AdvanceBio Glycan Mapping (HILIC) column demonstrated strong retention and increased resolution for hydrophilic glycopeptides. Various glycoforms of the same peptide were well resolved.
- The glycopeptide analysis through peptide mapping workflow resulted in not only glycan relative quantitation, but also N-glycosylation site(s) information.
- The automated data processing capability of BioConfirm 10.0 resulted in accurate glycopeptide profiling—identification and relative quantitation. A batch of samples or different mAb digests can easily be analyzed and compared.

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2. Precise Characterization of Intact Monoclonal Antibodies by the Agilent 6545XT AdvanceBio LC/Q-TOF, *Agilent Technologies*, publication number 5991-7813EN.
3. Making Peptide Mapping Routine with the Agilent 6545XT AdvanceBio LC/Q-TOF, *Agilent Technologies*, publication number 5991-7815EN.
4. Dong, Q; et al. In-Depth Characterization and Spectral Library Building of Glycopeptides in the Tryptic Digest of a monoclonal Antibody Using 1D and 2D LC-MS/MS. *J. Proteome Res.* **2016**, *15*, 1472-1486.
5. A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis, *Agilent Technologies*, publication number 5991-8550EN.
6. Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, *Agilent Technologies*, publication number 5991-2957EN.
7. Highly Confident Peptide Mapping of Protein Digests Using Agilent LC/QTOFs, *Agilent Technologies*, publication number 5991-8552EN.

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A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis

Authors

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Introduction

Monoclonal antibodies (mAbs) and their derivatives comprise a very important class of biopharmaceutical molecules with a wide range of applications. Due to the heterogeneous nature of these mAbs, comprehensive analytical characterization is required. These analyses include determining the complete amino acid sequences of the mAbs and their variants, as well as characterization of post-translational modifications (PTMs) including glycosylation, oxidation, and deamidation. Glycosylation plays an important role in many biological processes. It also affects the therapeutics' efficacy, stability, pharmacokinetics, and immunogenicity[1]. Glycan characterization usually involves techniques such as NMR, HPLC, or mass spectrometry (MS). Since glycans are very diverse in composition/structures and are poorly ionized by electrospray, the MS-based approach for glycan characterization has been challenging. InstantPC is a novel fluorescence tag from ProZyme Inc. (Figure 1) that has been developed to improve MS ionization efficiency, and sensitivity for N-glycan molecules.

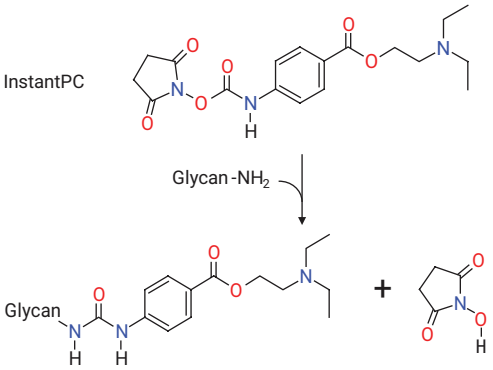

Figure 1. Diagram of InstantPC-labeled N-glycans released from an mAb.

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The traditional method of glycan analysis is laborious, and involves many steps, starting with enzymatic glycan release by PNGaseF (overnight), followed by sample cleanup, labeling with a fluorescence tag by reductive amination (2-AB or InstantPC), and finally cleanup of the released labeled N-glycans prior to LC-FLD or LC/MS analysis[2,3]. Despite the significant improvement of MS sensitivity using fluorescent tags, the labor intensiveness of manual sample preparation, low reproducibility, and limitation to scale-up on sample processing have been major issues for the biopharmaceutical industry.

This study demonstrates how to increase sample throughput for glycan characterization workflows using the Agilent AssayMAP Bravo liquid handling platform. The solution incorporates the Agilent 1290 Infinity II LC system, Agilent AdvanceBio Glycan Mapping column, Agilent highly sensitive fluorescence detection (FLD), and the Agilent 6545XT AdvanceBio LC/Q-TOF. The Q-TOF data are analyzed automatically with Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves productivity by allowing convenient sample preparation, streamlined data acquisition, and data analysis. This solution provides the flexibility to perform quantitation based on FLD or MS signals with accurate mass peak assignment from an N-glycan mass database.

Experimental

Sample preparation

Four monoclonal antibody (mAb) samples were used in this study:

- The monoclonal antibody standard, RM 8671, was from National Institute of Standards & Technology (NIST) A.K.A. NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA).
- Sigma SiLu mAb was purchased from Sigma-Aldrich (SiLu Lite, P/N: MSQC4).
- CHO mAb1 was expressed and purified from the Agilent R&D lab.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system (G5542A) with the GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from ProZyme Inc. A detailed procedure for the sample preparation is described in ProZyme's application note (product code: GPPNG-PC). After the final cleanup step, the eluted, released, labeled N-glycans had a final volume of 50 µL, so that each 1 µL of the prepared sample contained N-glycans from 1 µg of mAb.

Figure 2. mAb Glycan characterization workflow.

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LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B), coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10. Glycans were chromatographically separated with an AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). Tables 1 and 2 list the LC/MS parameters used. Approximately 1–2 µL of each N-glycan sample were injected for LC/MS analysis.

Data processing

The InstantPC-labeled released N-glycans were analyzed using the Released Glycans Workflow of MassHunter BioConfirm B.09.00 software. This analytical workflow uses the Agilent Personal Compound Database (PCD) glycan database. The PCD glycan database provides accurate glycan identification and confirmation. Finally, a summarized report of the analyses was generated in PDF format using the Report Builder program in BioConfirm B.09.00.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm
Thermostat	4 °C
Solvent A	50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	Acetonitrile
Gradient	0–0.5 minutes, 75–71 %B 0.5–16 minutes, 71–67.5 %B 1–22 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	40 °C
Flow rate	0.4 mL/min
Injection volume	2.0 µL

Agilent 1260 Infinity Fluorescence Detector (G1321B) was used. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	150 °C
Drying gas	9 L/min
Nebulizer (psig)	35
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
VCap	3,000 V
Nozzle voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Quad AMU	95
Acquisition mode	Low mass range, HiRes (4 GHz)
Mass range	m/z 300–1,700
Acquisition rate	2 spectra/sec

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Results and Discussion

LC-FLD analysis of released labeled glycans is one of the most widely used approaches to determining therapeutic protein glycosylation. We have previously published application notes showing optimized separation of several mAb glycan profiles using various column dimensions and run conditions[4,5]. The separation method in this report represents the best overall performance with maximum peak resolution and excellent robustness for the different mAb N-glycan samples in this study.

Figure 3 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 3 top, zoom in) reveals 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 7).

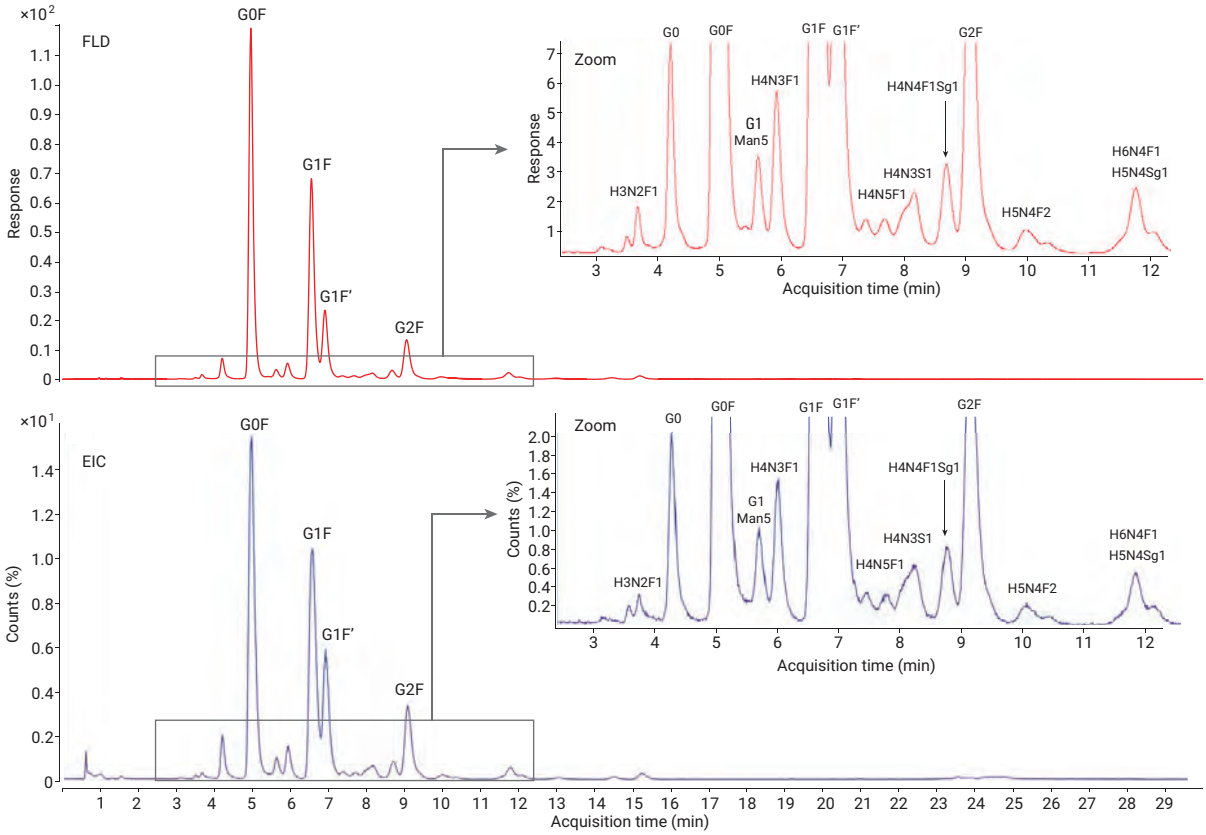

Figure 3. FLD chromatogram and mass spectra (EIC) of InstantPC-labeled N-glycans from NISTmAb.

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While fluorescence detection does not allow for direct structure elucidation, MS analysis of mAb glycans can be used to determine glycan monosaccharide composition. In the case of many mAb N-glycans, this composition is sufficient to achieve a high-confidence structural assignment. The combination of the positively charged InstantPC tag and sensitive Agilent Jet Stream (AJS) electrospray ionization (ESI) source technology dramatically increases MS detection sensitivity for N-glycans. In addition, we have optimized the MS parameters to maximize the sensitivity of the InstantPC-labeled N-glycans while minimizing in-source fragmentation of these fragile molecules.

The optimized conditions have significantly improved the MS spectrum quality, leading to accurate N-glycans identification and relative quantification results. Figure 4 shows the MS spectrum of an InstantPC-labeled N-glycan (G2F) where only the doubly charged ions of its protonated form, $[M+2H]^{2+}$, as well as its adducts $[M+H+Na]^{2+}$ and $[M+H+K]^{2+}$ were observed (Note: InstantPC tag causes a mass increment of 261.1477 Da compared to the free reducing end form of the glycan).

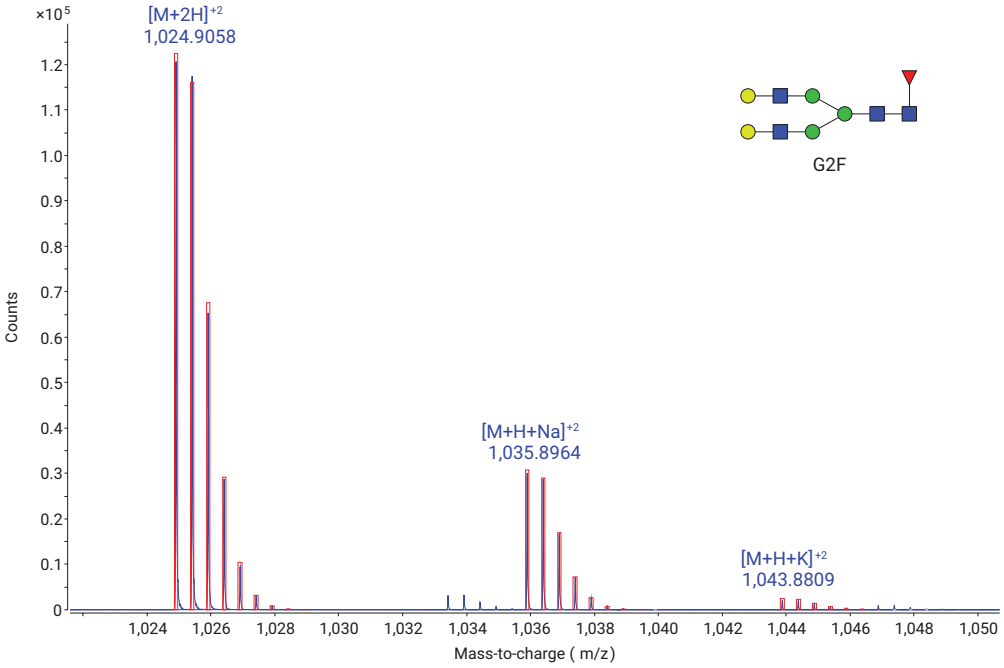

Figure 4. Representative spectrum of an InstantPC-labeled N-glycan (G2F). Excellent isotopic fidelity of the charge states of the InstantPC-labeled G2F glycan and its adducts. The red boxes represent the theoretical isotopic pattern, and the blue lines represent the actual raw MS spectrum.

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We have introduced a workflow in MassHunter BioConfirm software for released glycan profiling. This workflow enables the easy setup of sample batch analysis. The software can accommodate many commercial or customized fluorescent tags. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans is used for identification using the Agilent proprietary Find by Formula algorithm. Subsequently, a summary analytical report can be created in a customer-defined report format. Figure 5 shows the extracted ion chromatograms (EICs) of the identified glycans.

The Biomolecules results table (Figure 6) in BioConfirm allows quick review of detailed glycans information including names, mass, retention time, peak area, composition, and database matching score. Multiple IDs are displayed for glycans with possible isoform structures. It also allows users to review the TIC of the sample as well as the individual glycan MS spectra.

In addition, multiple data files can be processed and analyzed in batch mode. The user may use peak areas of the selected glycans in the results table for relative quantitative analysis.

InstantPC-labeled glycans were previously shown to give similar relative quantitation results for MS and FLD analysis[4]. The FLD chromatogram for the CHO mAb1 sample was integrated using the Agilent MassHunter Qualitative Analysis software. The relative sum abundance of the top seven most abundant N-glycans was calculated and compared against the same data from the MS analysis (Figure 7). To get equivalent results, do not saturate the MS detector. An ideal quantity for this workflow would be to inject N-glycans released from approximately 0.5 µg of mAb.

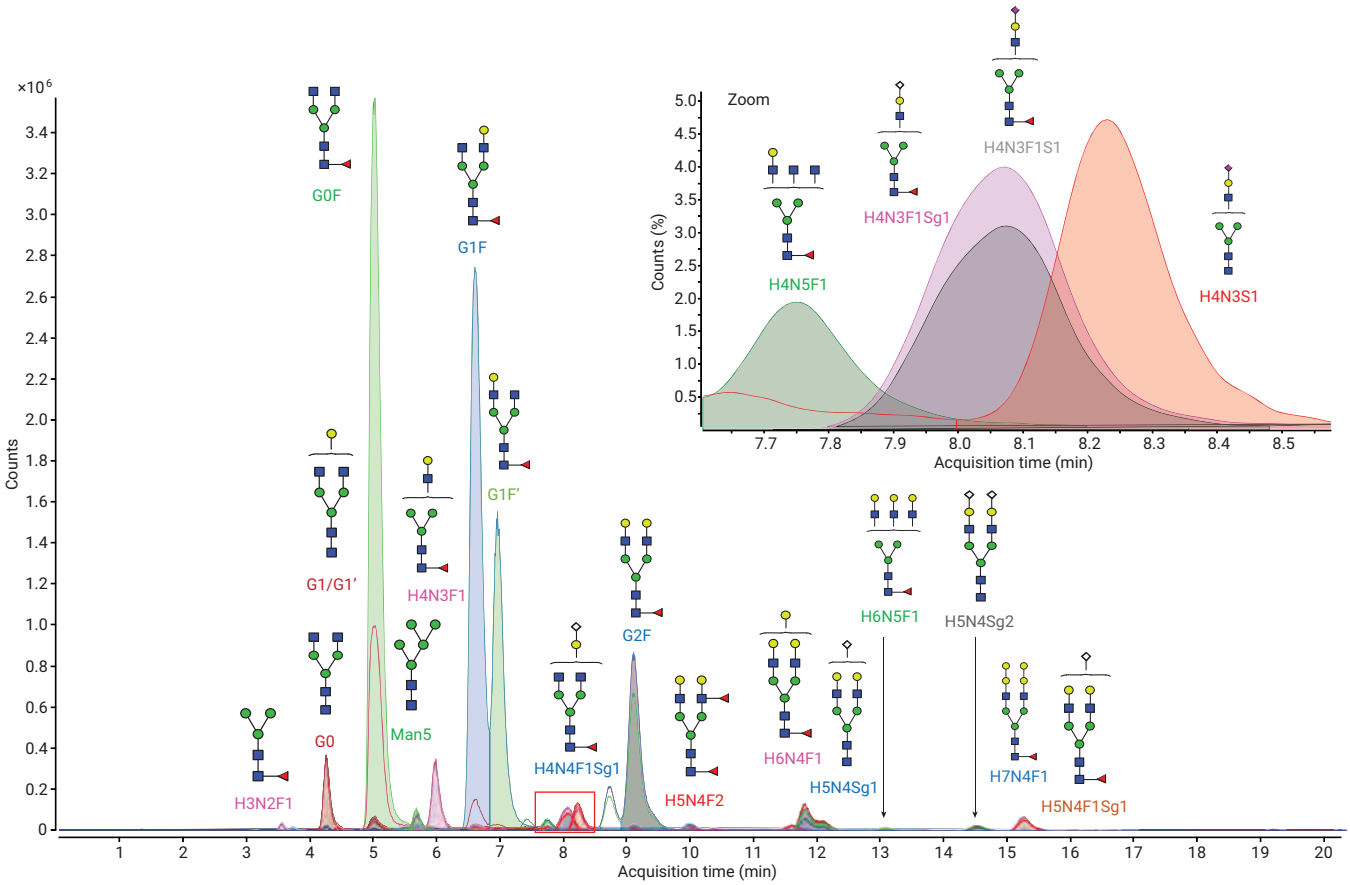

Figure 5. Extracted ion chromatograms of the identified glycans from NISTmAb. Inset: zoom of EICs of identified glycans eluted in the retention time range of 7.6–8.6 minutes.

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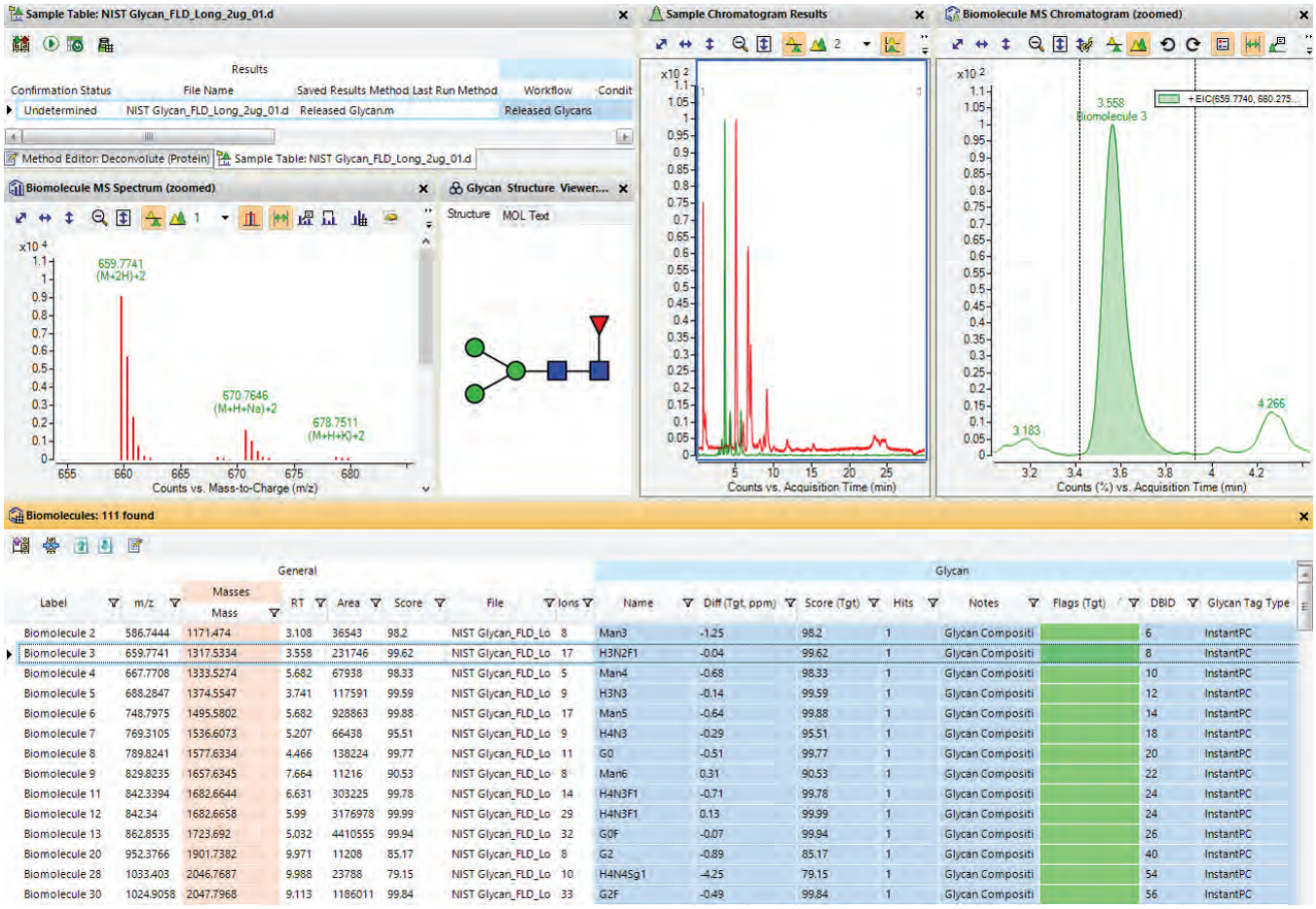

Label	m/z	Mass	RT	Area	Score	File	Ions	Name	Diff (Tgt, ppm)	Score (Tgt)	Hits	Notes	Flags (Tgt)	DBID	Glycan Tag Type
Biomolecule 2	586.7444	1171.474	3.108	36543	98.2	NIST Glycan_FLD_Lo	8	Man3	-1.25	98.2	1	Glycan Compositi		6	InstantPC
Biomolecule 3	659.7741	1317.5334	3.558	231746	99.62	NIST Glycan_FLD_Lo	17	H3N2F1	-0.04	99.62	1	Glycan Compositi		8	InstantPC
Biomolecule 4	667.7708	1333.5274	5.682	67938	98.33	NIST Glycan_FLD_Lo	5	Man4	-0.68	98.33	1	Glycan Compositi		10	InstantPC
Biomolecule 5	688.2847	1374.5547	3.741	117591	99.59	NIST Glycan_FLD_Lo	9	H3N3	-0.14	99.59	1	Glycan Compositi		12	InstantPC
Biomolecule 6	748.7975	1495.5802	5.682	928863	99.88	NIST Glycan_FLD_Lo	17	Man5	-0.64	99.88	1	Glycan Compositi		14	InstantPC
Biomolecule 7	769.3105	1536.6073	5.207	66438	95.51	NIST Glycan_FLD_Lo	9	H4N3	-0.29	95.51	1	Glycan Compositi		18	InstantPC
Biomolecule 8	789.8241	1577.6334	4.466	138224	99.77	NIST Glycan_FLD_Lo	11	G0	-0.51	99.77	1	Glycan Compositi		20	InstantPC
Biomolecule 9	829.8235	1657.6345	7.664	11216	90.53	NIST Glycan_FLD_Lo	8	Man6	0.31	90.53	1	Glycan Compositi		22	InstantPC
Biomolecule 11	842.3394	1682.6644	6.631	303225	99.78	NIST Glycan_FLD_Lo	14	H4N3F1	-0.71	99.78	1	Glycan Compositi		24	InstantPC
Biomolecule 12	842.34	1682.6658	5.99	3176978	99.99	NIST Glycan_FLD_Lo	29	H4N3F1	0.13	99.99	1	Glycan Compositi		24	InstantPC
Biomolecule 13	862.8535	1723.692	5.032	4410555	99.94	NIST Glycan_FLD_Lo	32	G0F	-0.07	99.94	1	Glycan Compositi		26	InstantPC
Biomolecule 20	952.3766	1901.7382	9.971	11208	85.17	NIST Glycan_FLD_Lo	8	G2	-0.89	85.17	1	Glycan Compositi		40	InstantPC
Biomolecule 28	1033.403	2046.7687	9.988	23788	79.15	NIST Glycan_FLD_Lo	10	H4N4Sg1	-4.25	79.15	1	Glycan Compositi		54	InstantPC
Biomolecule 30	1024.9058	2047.7968	9.113	1186011	99.84	NIST Glycan_FLD_Lo	33	G2F	-0.49	99.84	1	Glycan Compositi		56	InstantPC

Figure 6. Screenshot of Agilent MassHunter BioConfirm B.09.00 software with representative glycan profiling results.

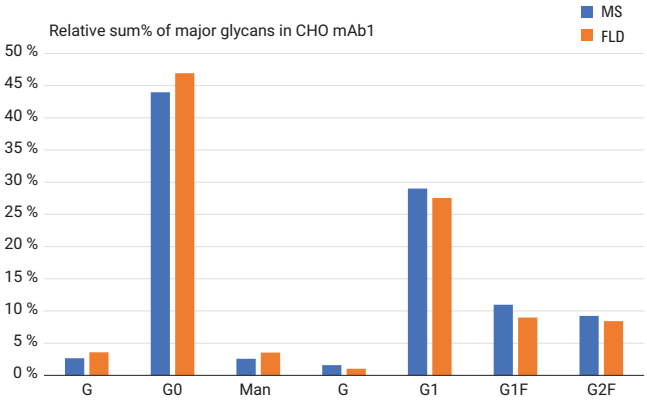

Figure 7. Relative sum % of the major N-glycans in the CHO mAb1 (0.5 µg), comparing results from MS-based quantitation (blue) with FLD-based quantitation (orange).

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To summarize and compare the MS results, the top five most abundant N-glycans for each mAb sample were used to calculate relative sum %. Figure 8 presents the data.

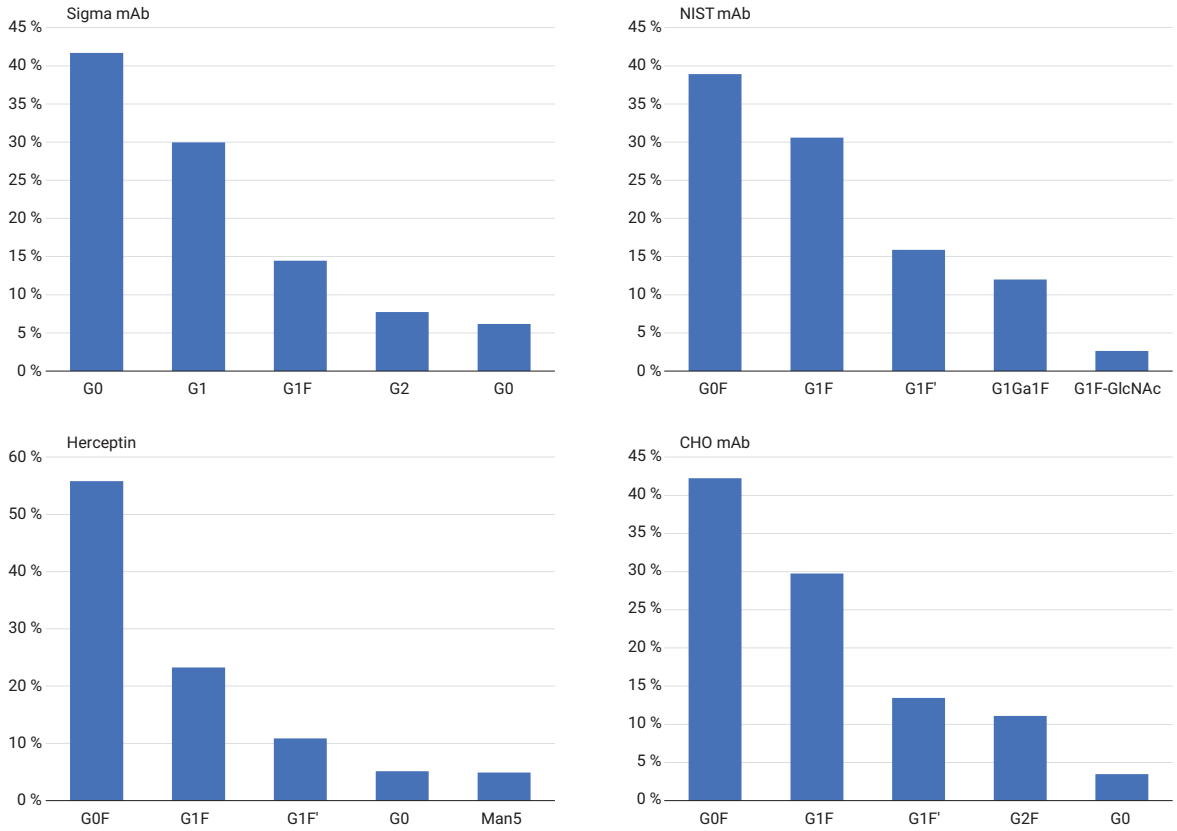

Figure 8. Relative sum % of the top five N-glycans in each of the four mAb samples. Note: The NISTmAb contained a structure suspected to be G1F with an additional alpha-1,3-galactose, and this was labeled as G1Ga1F.

The BioConfirm B.09.00 software allows users to generate their own glycan profile reports using the Report Builder program. Figure 9 shows an example of a released glycan report. In the Report Builder, users can customize the report sections with information such as Sample Information, Sample Chromatogram, Biomolecule Summary, and Biomolecule Details.
The corresponding glycan structures are displayed along with the identified glycans.

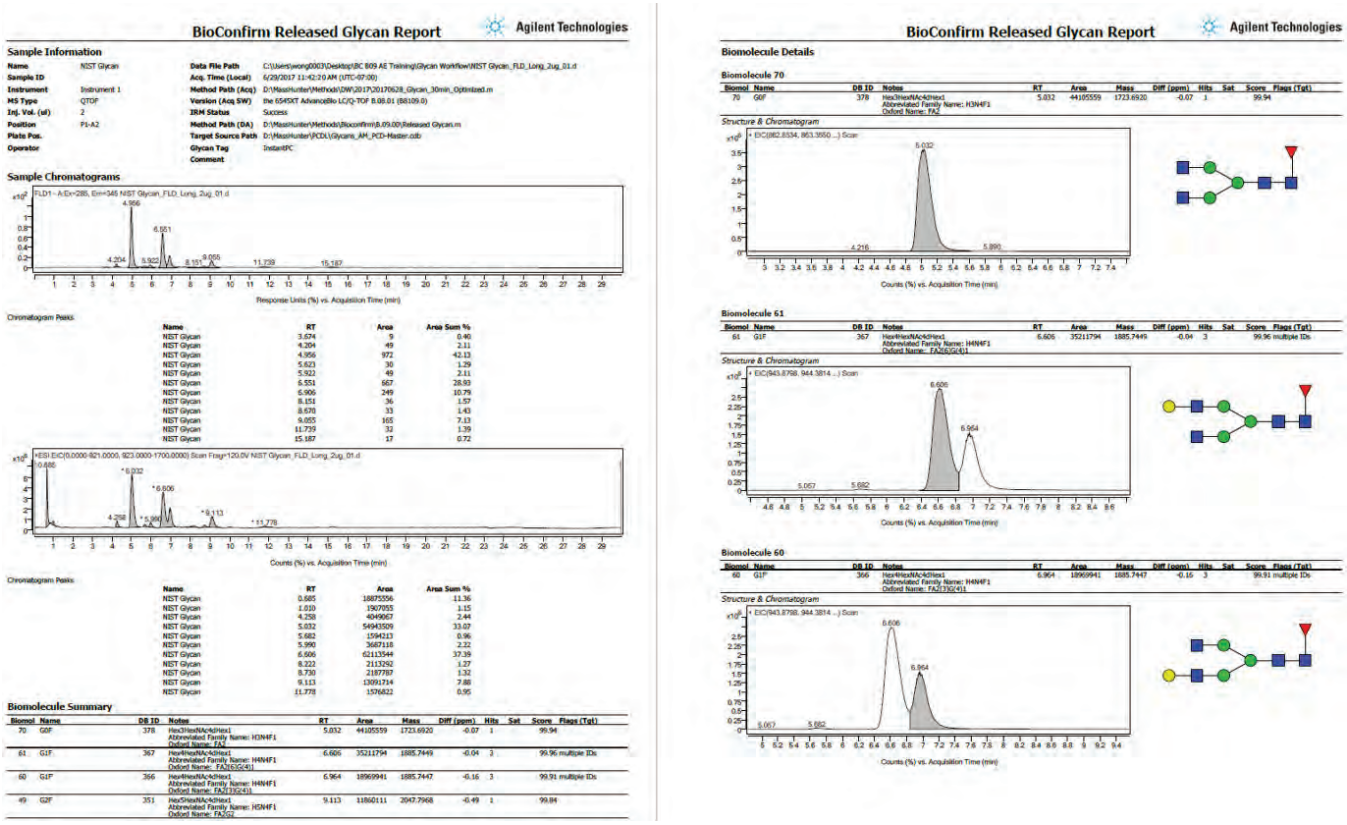

Figure 9. Agilent MassHunter BioConfirm B.09.00 Software – Released Glycan Report.

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Conclusion

This study demonstrated the performance of the Agilent AssayMap Bravo, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software, when used as an integrated solution for released glycan analysis.

- This workflow combines high-throughput sample preparation with excellent chromatographic separation using the Agilent AdvanceBio Glycan Mapping column.
- The easy setup and use of the glycan database included with BioConfirm B.09.00 provided the ability to accurately profile, identify, and perform relative quantification.
- The 6545XT-based glycan analysis generated similar quantitative results to that of fluorescence analysis, making it possible to compare different N-glycans across different mAb samples.
- The Report Builder function in BioConfirm B.09.00 provides the ability to create custom reports.

In conclusion, the Agilent solution automated the entire process of N-linked glycan analysis from sample preparation to data analysis with high precision. This approach provided high sensitivity and best quantitation for glycan analysis using fluorescence and additional identification by mass spectrometric detection.

References

1. Rademacher, T. W; Williams, P; DwekMark, R. A. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *P. Natl. Acad. Sci.* **1994**, 91, pp 6123-6127.
2. Anumula, K. R. "Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates" *Anal. Biochem.* **2006**, *350*, 1-23.
3. N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection, *Agilent Technologies*, publication number 5991-5253EN.
4. Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection, *Agilent Technologies*, publication number 5991-6958EN.
5. Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT, *Agilent Technologies*, publication number 5991-8071EN.

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Application Note

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Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XTs

Authors

Oscar Potter, Gregory Staples, Jordy Hsaio, and Te-Wei Chu

Introduction

N-glycosylation of therapeutic proteins is monitored during product development, process development, and QC. Many analysts perform enzymatic N-glycan release followed by labeling with a fluorescent tag to run HILIC-FLD analysis. The InstantPC tag from Agilent (formerly ProZyme) allows manual sample prep within 1 hour, or alternatively, allows high-throughput parallel processing with the Agilent AssayMap Bravo liquid handling platform. High-resolution separation can be completed in short cycle times using the Agilent AdvanceBio Glycan Mapping column (see 5991-4886EN). While fluorescence detection is a popular detection technique, analysts running HILIC-FLD workflows may face difficult challenges in the form of ambiguous peak assignment, particularly in the case of new sample types or unexpected peaks. In such cases, analysts might resort to sending the samples through complementary analyses such as LC-Q-TOF or MALDI-TOF, but this loses time, and incurs additional expenses.

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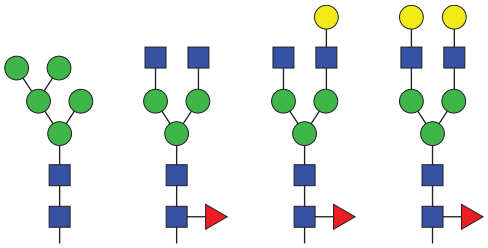

We previously demonstrated an enhanced workflow where a HILIC-FLD system was hyphenated online to a high-resolution Q-TOF mass spectrometer (see 5991-6958EN). This approach couples the benefits of robust fluorescence based quantitation with the powerful structural elucidation capabilities of mass spectrometry. However, analysts who seek a routine cost-effective solution may prefer to work with a rugged, small-footprint mass selective detector (MSD) based on single quadrupole technology. Therefore, we present a method where a HILIC-FLD system is coupled to a rugged, sensitive, and stackable Agilent LC/MSD XT single quadrupole. The MSD data provide mass information to solve ambiguous peak assignments and detect coeluting structures. The method operates at less than 600 bar to maintain compatibility with a range of existing LC systems.

Agilent LC/MSD XT

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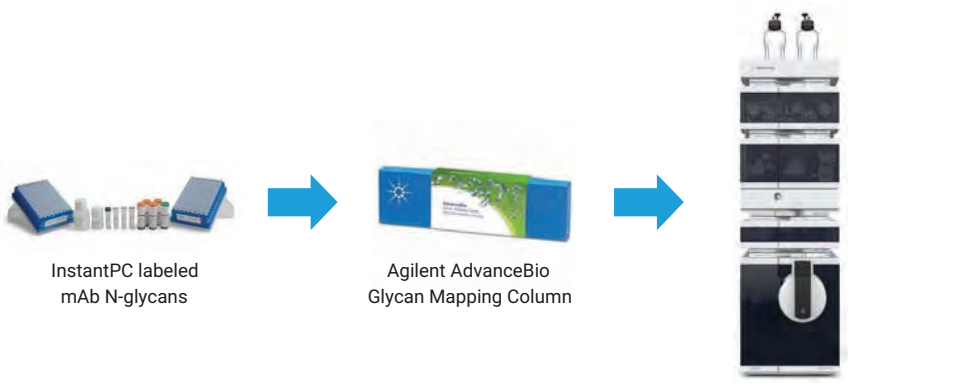

Figure 1. Flow chart of the sample preparation, LC column, and instruments used for identification and quantification of InstantPC labeled N-glycans.

Experimental

Sample preparation

Samples of monoclonal antibody (mAb) were expressed in our own lab (CHO mAb 1) or purchased from Sigma-Aldrich (SiLu Lite P/N MSQC4) and from NIST (NISTmAb, Reference Material 8671). All samples were adjusted to 1 µg/µL prior to processing by GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from Agilent (formerly ProZyme) (GPPNG-PC). The sample handling was automated using the AssayMap Bravo Liquid Handling Platform (G5542A). The final step of this protocol elutes the labeled glycans in 50 µL, so that each µL of this final sample solution represents N-glycans released from 1 µg of mAb. Conveniently, this workflow has the advantage of not requiring any centrifugation or dry down steps.

Chromatography conditions

Pump

Agilent 1260 Infinity II Binary Pump G7112B

Mobile phase A

50 mM Formic acid adjusted to pH 4.5 with ammonium hydroxide

Mobile phase B

Acetonitrile

Sampler

Agilent 1260 Infinity II Multisampler (G7167A) with thermostat set at 11 °C

Columns

- AdvanceBio Glycan Mapping Column
  1.8 µm, 2.1 × 100 mm used with method A
- AdvanceBio Glycan Mapping Column
  2.7 µm, 2.1 × 150 mm used with method B
- AdvanceBio Glycan Mapping Column
  1.8 µm, 2.1 × 150 mm used with method C

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Column heater

Agilent 1260 Infinity II G7116 thermostatic column compartment with G7116-60015 solvent preheater set to 40 °C

Columns were plumbed using 100 µm id SSTL tubing to an Agilent 1260 Infinity Fluorescence Detector (G1321B) with a 8 µL flow cell (G1321-60005). The detector was set to λEx at 285 nm, λEm at 345 nm with PMT gain = 10.

LC/MSD XT Parameters

Parameter	Value
Source	Agilent Jet Stream in positive mode
Sheath gas	300 °C at 10.0 L/min
Dry gas temperature	150 °C at 9.0 L/min
Nebulizer pressure	35 psig
VCap	2,500 V Nozzle: 500 V
Mass range	500–1,400 m/z
Fragmentor	100 V
Gain EMV	1.0
Step size	0.10
Peak width	0.2

Software

OpenLab CDS Chemstation Edition Rev C.01.07 SR3

Reagents

All reagents and solvents used were of the highest purity available.

Gradient tables

Method A (for 2.1 × 100 mm 1.8 µm column)

Time	Eluent A	Eluent B	Flow
0.00	25.0%	25.0%	0.70 mL/min
0.50	27.0%	27.0%	0.70 mL/min
4.00	28.0%	28.0%	0.70 mL/min
9.00	33.0%	33.0%	0.70 mL/min
9.20	50.0%	50.0%	0.70 mL/min
9.70	50.0%	50.0%	0.70 mL/min
10.00	25.0 %	75.0%	0.70 mL/min
11.50	25.0%	75.0%	0.70 mL/min
11.80	25.0%	75.0%	0.80 mL/min
14.50	25.0%	75.0%	0.90 mL/min
15.30	25.0%	75.0%	0.70 mL/min

Stop time = 18 minutes

Method B (for 2.1 × 150 mm 2.7 µm column)

Time	Eluent A	Eluent B	Flow
0.00	27.0%	73.0%	0.50 mL/min
1.00	28.5%	71.5%	0.50 mL/min
9.00	29.5%	70.5%	0.50 mL/min
22.00	41.0%	59.0%	0.50 mL/min
22.50	50.0%	50.0%	0.80 mL/min
23.50	50.0%	50.0%	0.70 mL/min
23.70	27.0%	73.0%	0.70 mL/min
25.00	27.0%	73.0%	0.70 mL/min
25.50	27.0%	73.0%	0.80 mL/min
27.50	27.0%	73.0%	0.90 mL/min
28.00	27.0%	73.0%	0.50 mL/min

Stop time = 30 minutes

Method C (for 2.1 × 150 mm 1.8 µm column)

Time	Eluent A	Eluent B	Flow
0.00	22.0%	78.0%	0.60 mL/min
0.50	26.0%	74.0%	0.60 mL/min
13.00	27.5%	72.5%	0.60 mL/min
28.00	39.0%	61.0%	0.60 mL/min
28.50	50.0%	50.0%	0.50 mL/min
28.60	50.0%	50.0%	0.40 mL/min
28.80	22.0%	78.0%	0.40 mL/min
31.00	22.0%	78.0%	0.50 mL/min
31.50	22.0%	78.0%	0.55 mL/min
33.50	22.0%	78.0%	0.60 mL/min

Stop time = 37 minutes

Results and Discussion

Separation

We optimized the separation of typical therapeutic mAb glycan profiles on three different columns, targeting various cycle times. The methods begin with a shallow gradient that maximizes resolution of the common neutral glycans that appear in most therapeutic mAb samples. The gradient slope was increased midway through the run to elute any larger, acidic glycan structures more efficiently. These methods were all designed to stay well under 600 bar to maximize robustness, as well as allowing for compatibility with a wide range of LC instruments. While all three separation methods are shown in Figure 2, we chose to use Method C for the remainder of the experiments because it was found to give the best overall performance for our three mAb N-glycan samples.

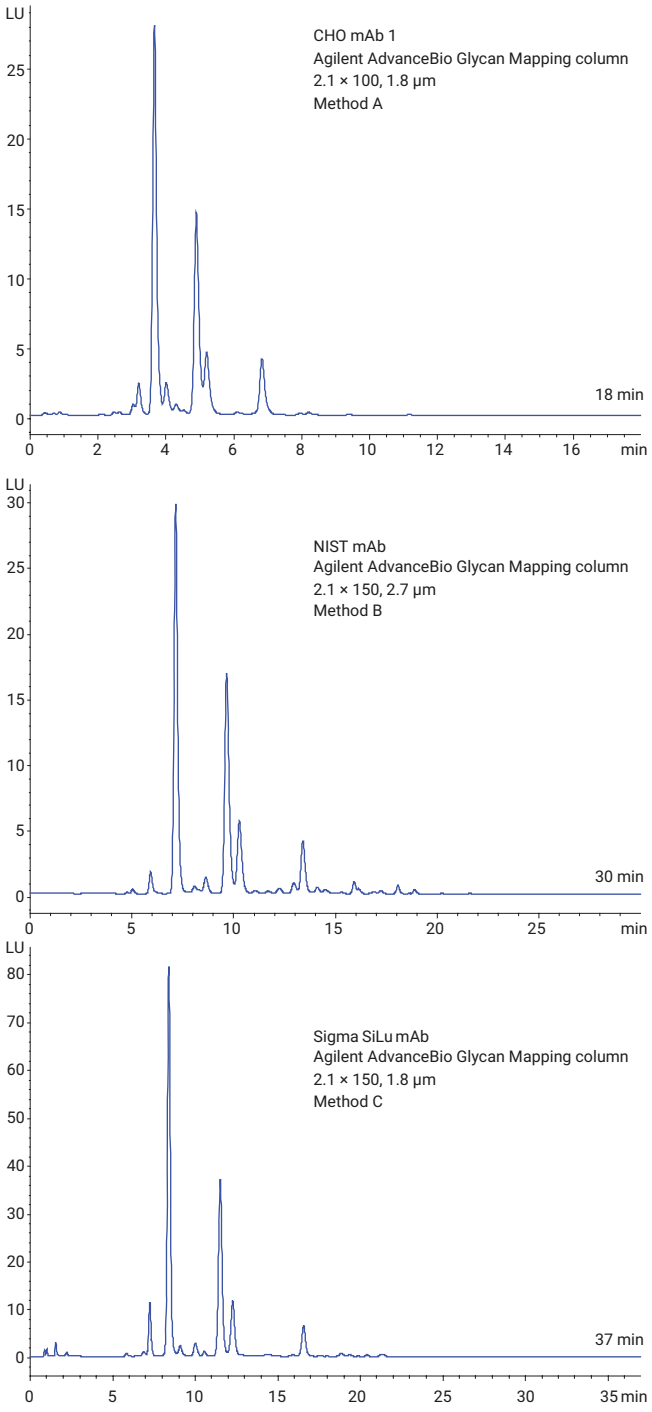

Figure 2. Three separation methods using different column formats with three mAb samples.

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Peak assignment

The data from all three samples (using method C) were integrated, and the peaks were assigned to glycan compositions using an approach similar to that shown in application note 5991-5253EN.

Note: InstantPC labeling causes a mass increment of 261.1477 m/z versus the free reducing-end form of the glycans.

Table 1 was constructed showing the composition of the glycans that were detected across the three samples. Glycan compositions are shown in the form: HxNxFxSgx + Core (where H = Galactose or Mannose, N = N-acetylglucosamine, F = Fucose, Sg = N-glycolylneuraminic acid, and Core = trimannosyl, a core common to all N-glycans). In many cases, these compositions allowed us to propose glycan structures by supplementing basic knowledge of therapeutic mAb glycosylation patterns and HILIC retention order. Since most therapeutic mAbs contain a similar, limited set of common N-glycan structures, Table 1 can serve as a useful reference for assigning N-glycans in this workflow.

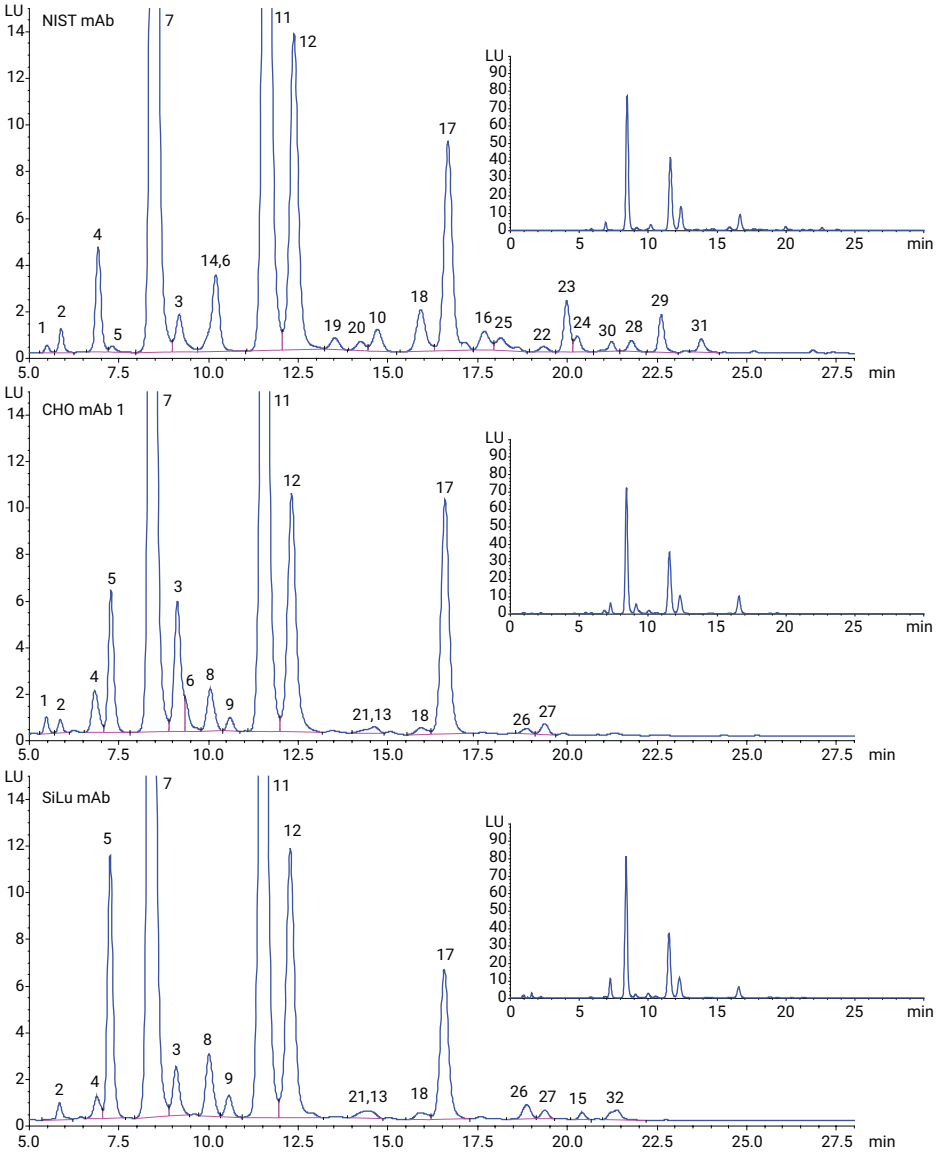

Figure 3. Zoomed FLD chromatograms of the three mAb N-glycan samples showing detection of major and minor glycans for method C. Insets show the zoomed-out data. Annotations refer to Table 1.

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LC/MSD XT Sensitivity

Traditionally, analysts have avoided using single quadrupole mass spectrometers for LC/MS analysis of N-glycans due to concerns about sensitivity. However, two technical innovations have led to dramatic improvements in the limit of detection for N-glycans. The first is the availability of a highly sensitive ion source, Agilent Jet Stream, which uses a super-heated sheath gas flow around the electrospray plume to dramatically improve ionization. The second innovation is the availability of a InstantPC tag from Prozyme Inc. that radically increases ionization efficiency versus traditional fluorescent labels.

We optimized the MSD parameters to maximize sensitivity. InstantPC-labeled N-glycans were observed almost exclusively as doubly charged ions of the forms $[M+2H]^{2+}$, with some $[M+NH_4+H]^{2+}$, and $[M+H+Na]^{2+}$. Increasing the dry gas temperature or fragmentor voltage can cause collision-induced dissociation of the N-glycans, offering powerful structural elucidation strategies similar to those achievable with a Q-TOF (see 5991-5253EN).

Diagnostic signals were obtained for glycans making up as little as 0.1 % of the profile. When comparing this sensitivity with alternative workflows, remember that these results were achieved without adding a preconcentrating step to the end of the sample preparation workflow, therefore saving considerable time. Sensitivity could be boosted even further by drying the samples and reconstituting them in a lower volume of sample matrix.

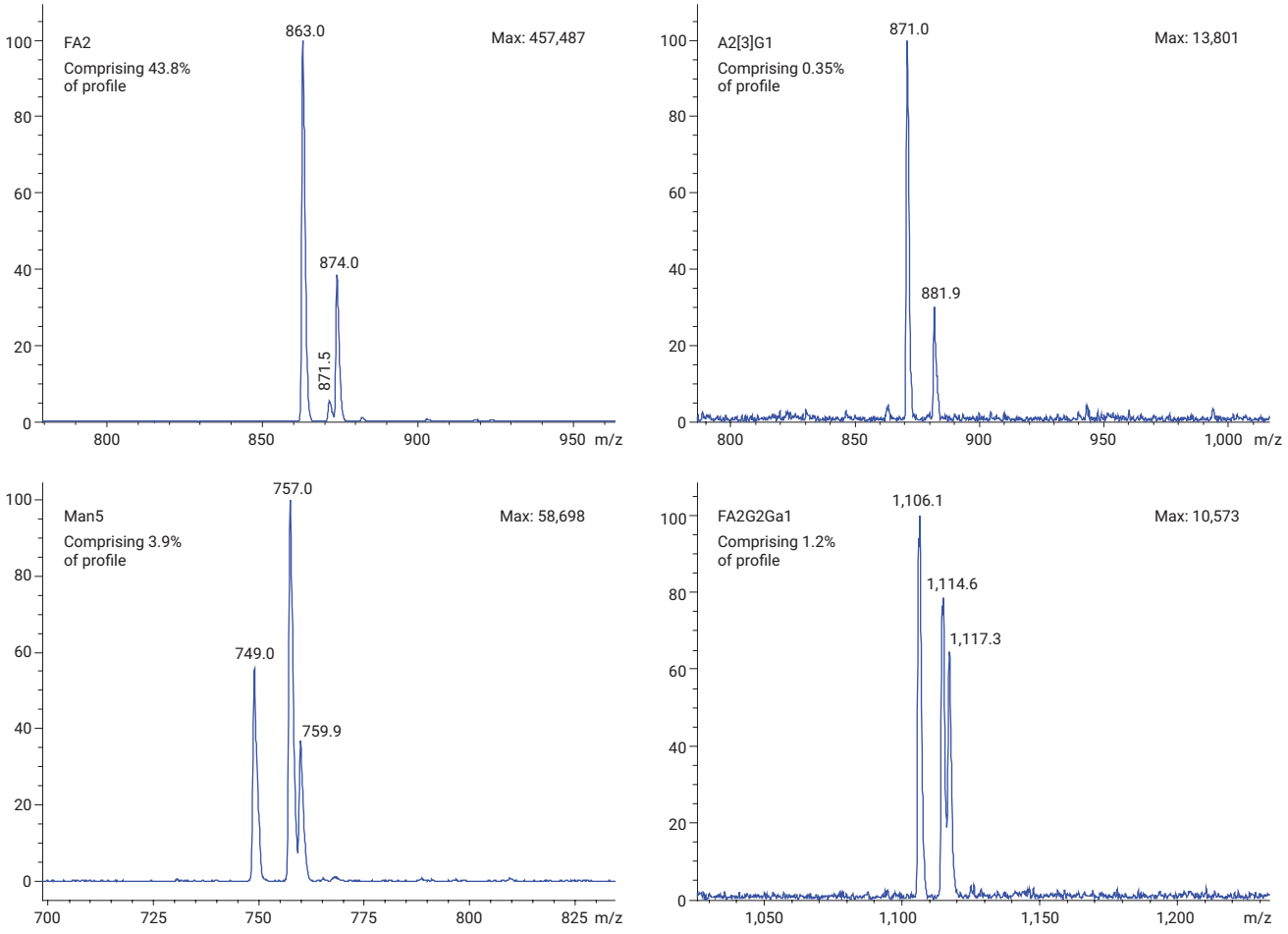

Figure 4. Mass spectra of four representative glycans from 3 µg of mAb at various levels of relative abundance. The signal for A2[3]G1, which makes up just 0.35 % of the glycan profile, is clearly observable with a high signal-to-noise ratio.

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Table 1. Glycan Compositions

ID	Observed $[M+2H]^{2+}$	Proposed composition	Theoretical $[M+2H]^{2+}$	Proposed structure	
				Oxford	Alternative
1	659.9	F1+Core	659.774	F1M3	G0F-2GlcNAc
2	688.4	N1+Core	688.284	A1	G1-GlcNAc
3	748.9	H2+Core	748.798	M5	Man5
4	761.5	N1F1+Core	761.313	FA1	G0F-GlcNAc
5	790.0	N2+Core	789.824	A2	G0
6	842.4	H1N1F1+Core	842.340	FA1G1	G1F-GlcNAc
7	863.0	N2F1+Core	862.853	FA2	G0F
8	871.0	H1N2+Core	870.851	A2[6]G1	G1
9	871.0	H1N2+Core	870.851	A2[3]G1	G1'
10	923.6	H2N1F1+Core	923.366	-	-
11	944.0	H1N2F1+Core	943.879	FA2[6]G1	G1F
12	944.0	H1N2F1+Core	943.879	FA2[3]G1	G1F'
13	952.0	H2N2+Core	951.877	A2G2	G2
14	964.7	N3F1+Core	964.393	FA2B	G0FB
15	991.9	H5+Core	991.877	M8	Man8
16	996.1	H1N1Sg1F1+Core	995.885	FA1G1Sg1	G1Sg1F-GlcNAc
17	1025.1	H2N2F1+Core	1024.906	FA2G2	G2F
18	1025.1	H2N2F1+Core	1024.906	FA2G1Ga1	G1F+αGal
19	1045.6	H1N3F1+Core	1045.419	FA2[6]B1G1	G1FB
20	1045.7	H1N3F1+Core	1045.419	FA2[3]B1G1	G1FB'
21	1089.5	H1N1F1S1+Core	1089.427	FA1G1S1	G1S1F-GlcNAc
22	1097.7	H1N2F1Sg1+Core	1097.425	FA2G1Sg1	G1Sg1F
23	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1	G2F+αGal
24	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1 iso	G2F+αGal'
25	1126.5	H2N3F1+Core	1126.446	FA2BG2	G2FB
26	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1	A1F
27	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1 iso	A1F iso
28	1178.7	H2N2F1Sg1 + Core	1178.451	FA2G2Sg1	Ag1F
29	1187.1	H4N2F1+Core	1186.959	FA2G2Ga2	G2F+(αGal)2
30	1207.7	H3N3F1+Core	1207.472	FA2BG2Ga1	G2FB+αGal
31	1259.4	H3N2F1Sg1+Core	1259.477	FA2G2Sg1Gal1	Ag1F+αGal
32	1316.2	H2N2F1S2+Core	1316.001	FA2G2S2	A2F

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Result tables

Peaks in the FLD chromatograms were reported as relative sum % of the total glycan profiles. Figure 5 shows the results.

Some of the profiles contained peaks representing coeluting glycan structures. These coelutions involved minor glycans, and quantitation based on FLD alone would report the peak area as the combined contribution of two structures.

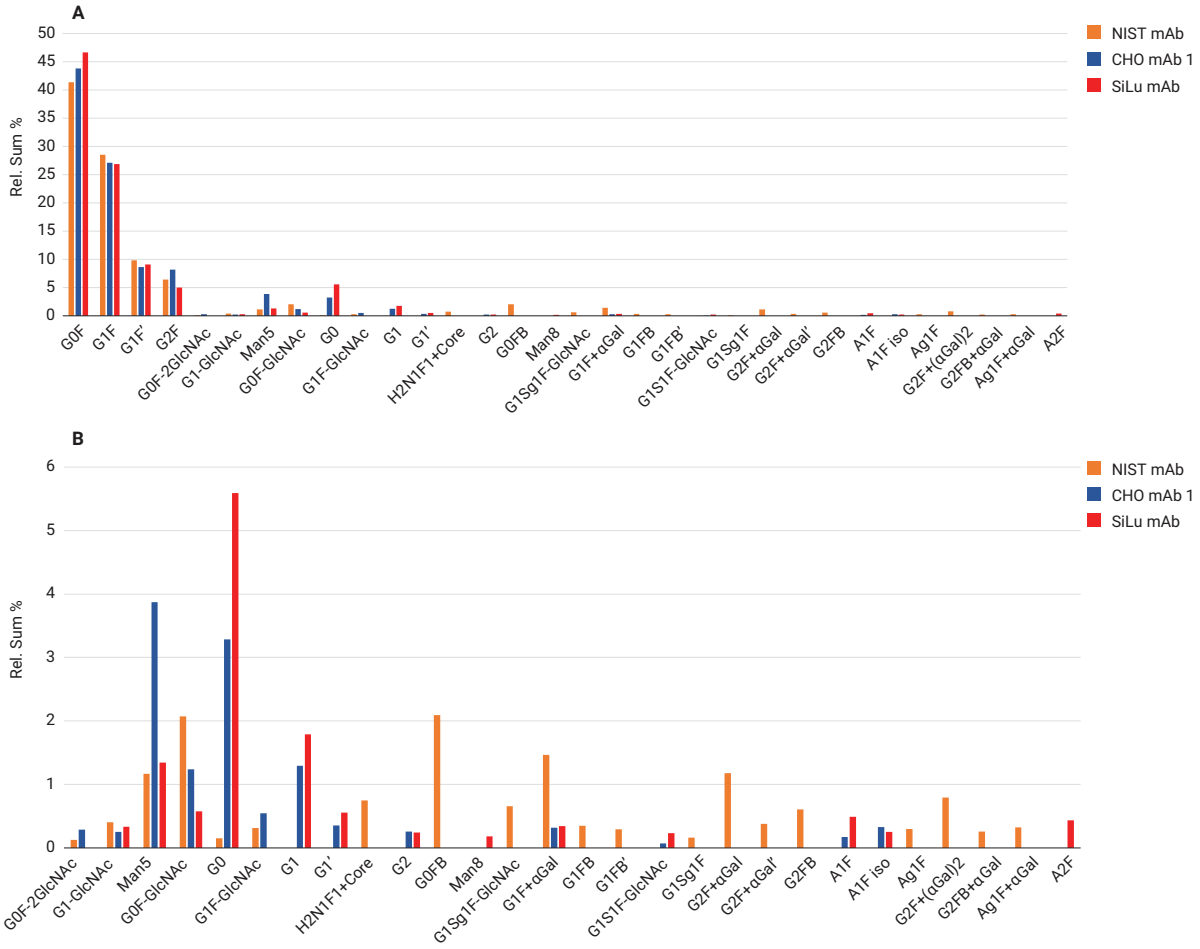

Figure 5. A) Relative abundance of N-glycans in the three mAb samples.
B) Zoomed-in chart showing only the minor components.
Minor glycans were detected easily at less than 1 %.

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For analysts who need accurate quantitation of these low abundance coeluting structures, we propose using the LC/MSD XT data to supplement the FLD. The mass spectrum can be integrated across the time period corresponding to the coeluting FLD peaks. The FLD area can then be apportioned to the different structures based on the combined relative intensity of the doubly charged N-glycan ions in this spectrum. We have previously shown that the MS signals for InstantPC labels closely correspond to their true abundance as defined by fluorescence intensity (see 5991-6958EN).

Conclusion

The Agilent LC/MSD XT based on single quad technology can be hyphenated online to a typical UHPLC HILIC-FLD system to provide the option of mass spec-based identification for every peak in every sample. Using an Agilent Jet Stream ion source in combination with the InstantPC glycan tag provides ample sensitivity to detect MS signals for both major and minor peaks in typical antibody samples without needing to concentrate the sample. For typical monoclonal antibody samples, this allows confident assignment of glycan structures. While this application note used the traditional approach of basing relative quantitation on robust fluorescence detection, the MS data can be used to assist quantitation in the case of coeluting peaks.

Application Note

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Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection

Authors

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Gregory Staples
Agilent Technologies, Inc.
Santa Clara, California, USA

Introduction

This application note describes the analysis of monoclonal antibody N-glycosylation using a novel instant mass tag (InstantPC) that permits detection using both fluorescence and mass spectrometry (MS). We have optimized a HILIC separation method for the purpose of comparing the two detection methods. Our results indicate that fluorescence and MS relative quantification of InstantPC-labeled glycans is highly similar. The sample prep procedure was conducted in quadruplicate for two different samples, and the results showed outstanding reproducibility with low RSDs even for minor components. The high MS sensitivity afforded by the InstantPC label facilitates identification of unknown glycans using accurate mass and tandem MS.

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Introduction

Monoclonal antibodies (mAbs) are modified by N-glycans during biosynthesis in cell culture. Typical mAbs contain two N-glycosylation sites, one in each of the Fc regions of the molecule. Some mAbs contain additional glycosylation sites, including N- or O-glycosylation in the Fab region. Glycans can affect the function of the mAb, so it is important to monitor the glycosylation profile using appropriate analytical methods.

Popular methods for glycan analysis involve NMR, CE-LIF, HPLC with fluorescence detection (FLD), and more recently, LC/MS. Both CE-LIF and HPLC-FLD require that the glycans are labeled with a dye to permit optical detection. Conventionally, the dyes that have been used also increase the ionization efficiency of glycans in comparison to the unlabeled species, but only to the point where the most abundant compositions can be detected using MS. More recently, a novel dye (InstantPC from Prozyme Inc., depicted in Figure 1) has been developed, which moderately improves fluorescence activity and greatly improves ionization efficiency for MS analysis. Using such a tag, researchers can now use MS (in the form of accurate mass or tandem MS) for identification of glycans from LC separations. Furthermore, they have the option of relative quantification using MS rather than fluorescence detection.

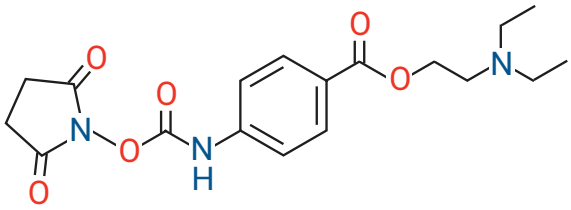

Figure 1. Diagram of InstantPC (ProZyme, Inc), an amine reactive instant label for fluorescence and MS detection of glycans.

This application note investigates the performance of InstantPC in the context of relative quantification of N-glycans released from two mAb preparations. Quadruplicate samples of the mAb samples were processed using the InstantPC kit from ProZyme, Inc. The samples were then separated by HILIC on a UHPLC system using FLD and MS detection. The LC separation conditions were optimized for maximum chromatographic separation. In doing so, the goal was to decrease the number of overlapping peaks that would otherwise not be discernable using FLD detection alone. As a result, we were able to compare the relative quantification results from the two detection methods for nearly all significant glycan structures. Accurate mass and tandem MS spectra were acquired for all glycan compositions, and were used for identification of the glycans present in the mAb preparations. Figure 2 shows the entire workflow.

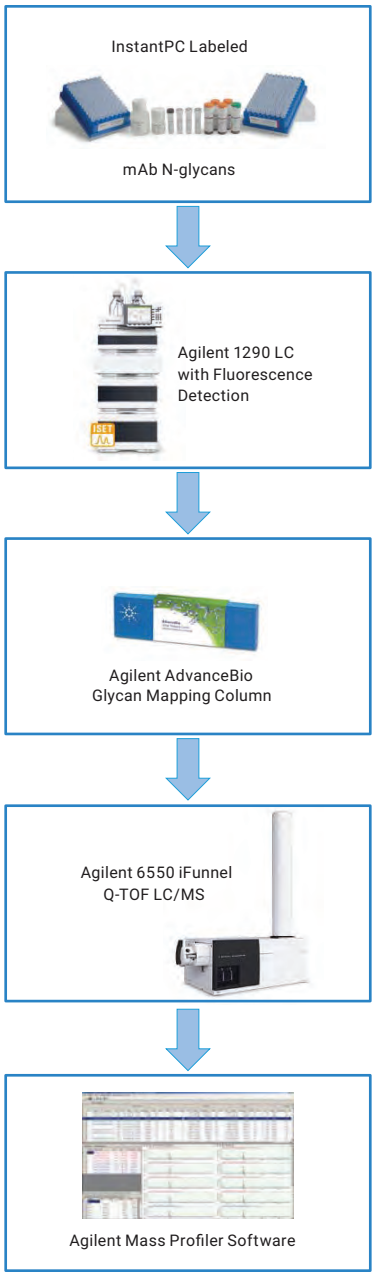

Figure 2. Workflow used for identification and quantification of InstantPC-labeled N-glycans from mAbs.

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Experimental

The Agilent LC/MS System used in this work comprised the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

MS system

Agilent 6550 iFunnel Q-TOF LC/MS system with dual-nebulizer AJS source

Columns

Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm connected to a second AdvanceBio Glycan Mapping column, 2.1 × 100 mm

Software

- Agilent PCDL Manager (Version B.07.00 Build 7024.0) and Agilent Mass Profiler (Version B.07.01 Build 99.0)
- Agilent MassHunter Workstation Software, Version B.05.01, Build 5.01.5125.1

Solvents and samples

All reagents and solvents used were of the highest purity available.

Chromatographic conditions

Parameter	Value
Mobile phase A	50 mM ammonium formate pH 4.4
Mobile phase B	Acetonitrile
Gradient	Time (min) %B 0 75 32 69 48 60 48.5 25 50.5 25 52 75
Autosampler temperature	4 °C
FLD	Ex. 285 Em. 345
Injection	2 µL in 20 % DMSO (1 µg of IgG equivalent)
Column temperature	40 °C
Flow rate	0.4 mL/min

Results and Discussion

FLD chromatograms from both mAb 1 and mAb 2 revealed that each molecule was modified by a very similar set of glycoforms, as shown in Figure 3. Some structures have been annotated in the figures, and represented by symbols according to the guidelines of the Consortium for Functional Glycomics (CFG) [1].

Using accurate mass and tandem mass spectrometry information, FLD peaks were assigned to glycan compositions in the form:

HxNxFxSgx + Core

H = galactose or mannose, N = N-acetylglucosamine, F = fucose, Sg = N-glycolylneuraminic acid, and
Core = trimannosyl core common to all N-glycans.

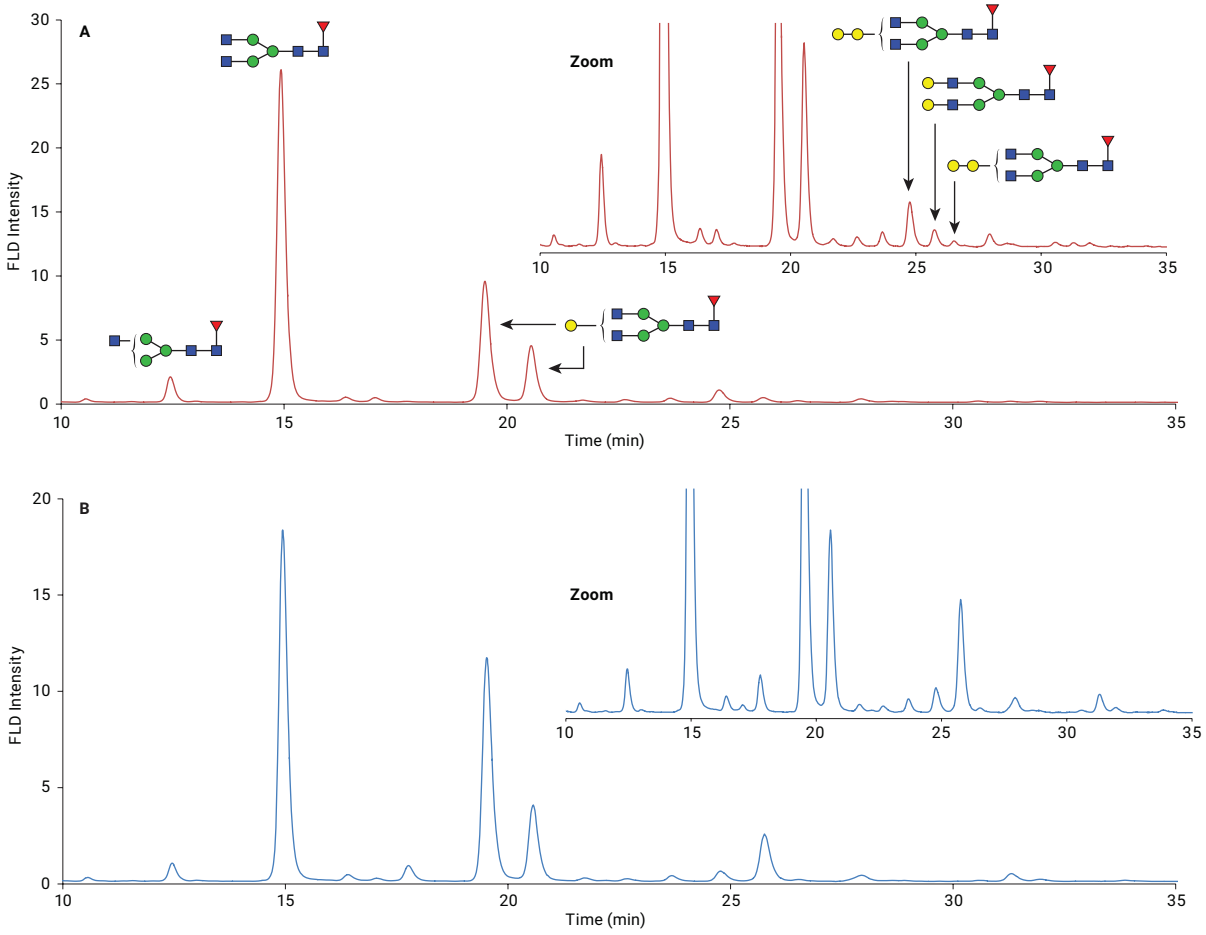

Figure 3. FLD chromatograms of InstantPC-labeled N-glycans released from mAb 1 and mAb 2.
A) FLD chromatogram for mAb 1.
B) FLD chromatogram for mAb 2.

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Based on peak area from the FLD chromatograms, each composition was quantified as a relative sum percentage based on the total FLD area for all compositions. The results are shown as a histogram in Figure 4.

Overall, 21 glycan compositions were quantified based on the FLD data. The criteria used for inclusion in the FLD quantification were a relative abundance of 0.1 % or greater, and reasonable resolution from neighboring peaks.

One exception was the pair H2N1F1Sg1 + Core/H2N3F1 + Core. These coeluting compositions were abundant enough to merit inclusion in the FLD quantification. Therefore, the FLD signal area from this peak was divided into two portions according to the relative abundance of each as determined by MS.

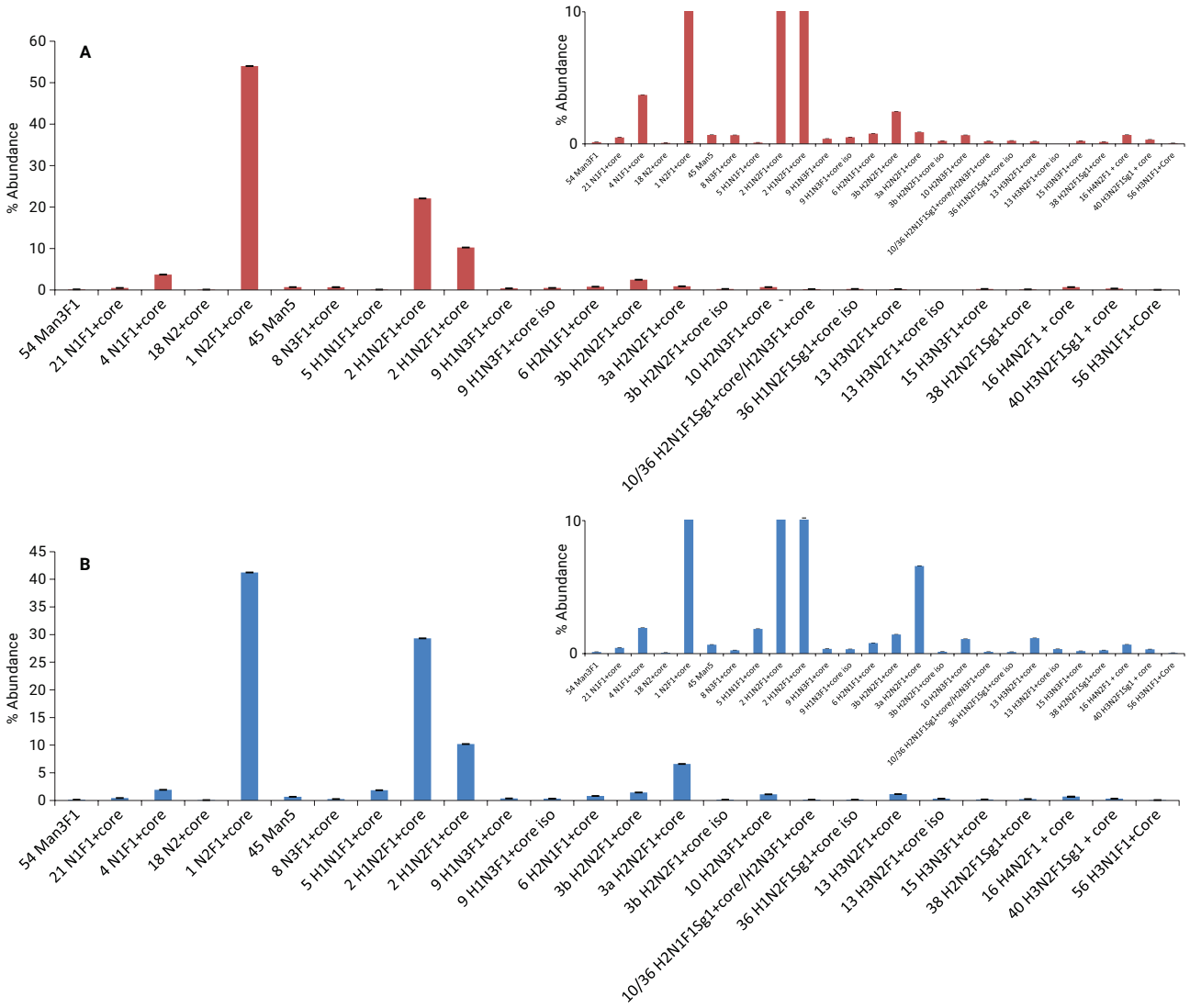

Figure 4. A) Relative FLD quantification of mAb 1 glycans. B) Relative FLD quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Integration of FLD signals was performed using Agilent MassHunter Qualitative Analysis Software. Insets show the same data zoomed to better display components with <10 % relative abundance.

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InstantPC imparts high ionization efficiency to N-glycans. Thus, it is possible to perform relative quantification using the peak area from extracted ion chromatograms from MS detection. To assess this possibility, we compared FLD chromatograms with ion chromatograms. Figure 5 shows that the FLD and MS chromatograms were highly similar. There is a corresponding MS peak for every FLD peak that was detected.

Encouraged by the high similarity seen in Figure 5, we performed relative quantification of glycans from mAb 1 and mAb 2 based on the MS data. Ion chromatograms for each feature (defined as a mass-retention time pair, which includes signals from all charge states and adducts) were created using Agilent Mass Profiler software. In this case, no lower threshold for detection was imposed. The features determined using Mass Profiler were identified using a Personal Compound Database (PCD) constructed for these experiments.

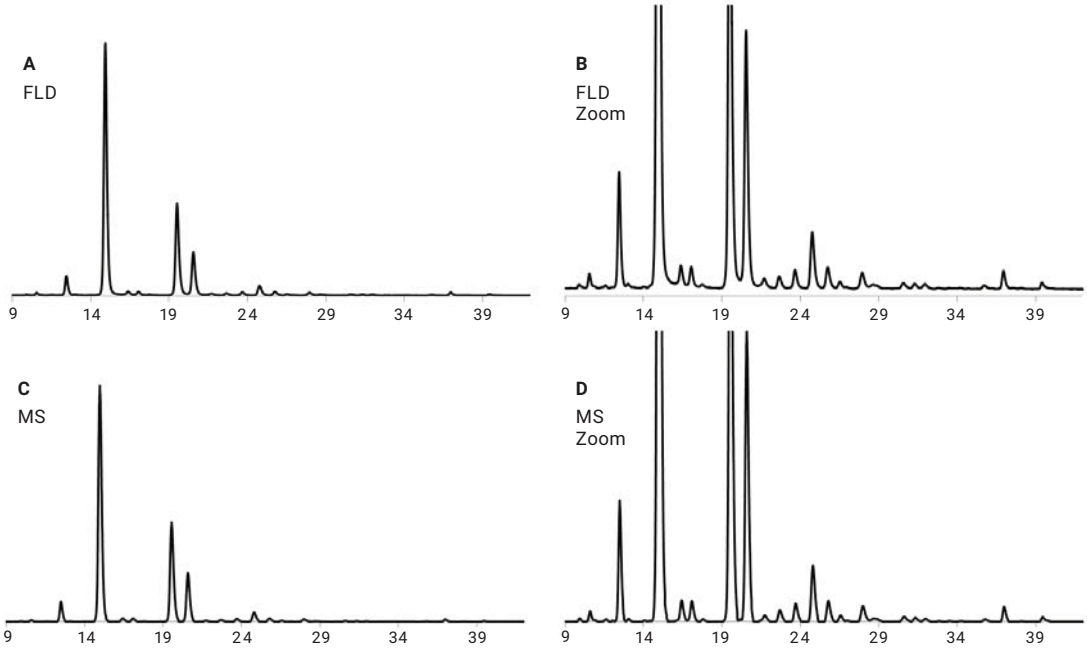

Figure 5. Comparison of FLD and MS chromatograms for mAb 1.
A) FLD chromatogram of mAb 1 glycans.
B) Zoom of FLD chromatogram of mAb 1 glycans.
C) MS chromatogram of mAb 1 glycans.
D) Zoom of MS chromatogram of mAb 1 glycans.

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The PCD contains accurate mass and retention time information for mAb glycans. The database was constructed based on a combination of tandem MS information from the current work in addition to knowledge of glycan biosynthetic rules. Figure 6 shows an example of the utility of tandem MS for assigning glycan compositions. In particular, the example shown in Figure 6B illustrates a common case where mass alone may be insufficient for assignment of composition, due to the fact that the mass of NeuGc + fucose is isobaric with that of NeuAc + galactose. Tandem MS resolves the ambiguity, because the presence of the fragment ion at ***m/z*** 673 provides strong evidence that the structure contains an antenna with NeuGc.

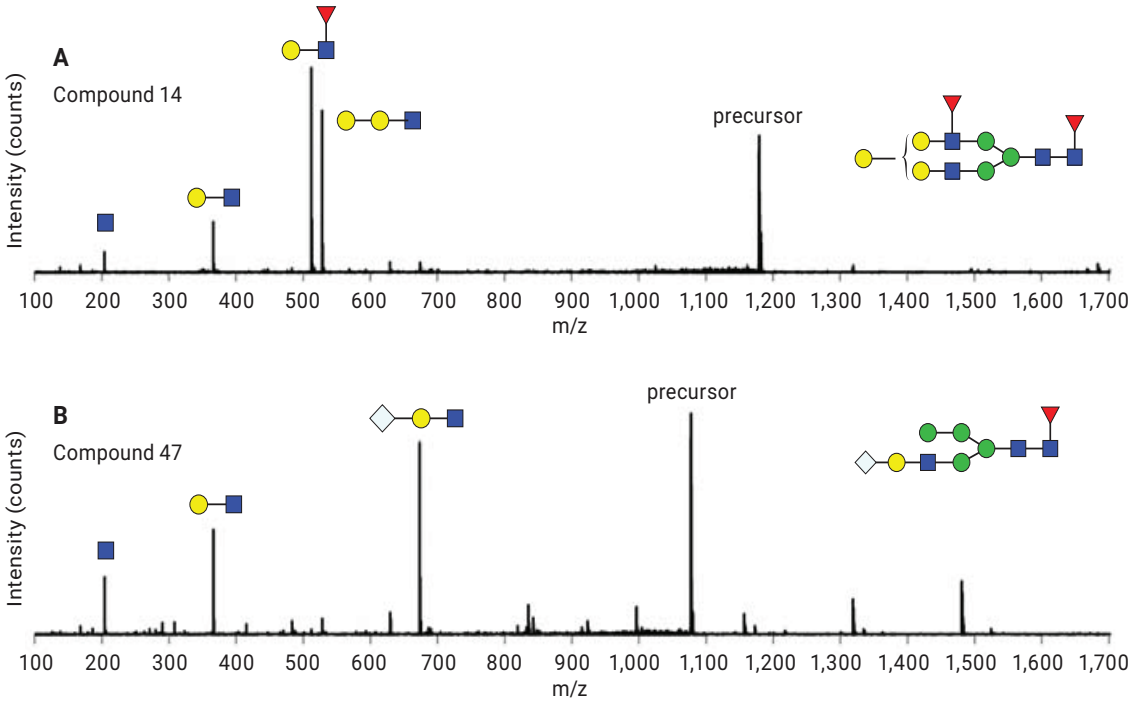

Figure 6. Tandem MS data were acquired for all glycans. MS/MS aided in compound identification when accurate mass was insufficient. The two examples above are consistent with gal-gal and outer arm fucose (A) and NeuGC (B) modifications.

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Figure 7 shows the results of the MS-based quantification of mAb 1 and mAb 2 glycans.

As a result of the mass selectivity provided by Q-TOF detection, it was possible to quantify more compositions than from the FLD detection. In this case, a total of 35 compositions were quantified. The average RSD was 3.2 % for mAb 1 and 3.9 % for mAb 2 for all features independent of abundance. For those features equal to or greater than 0.1 % relative abundance, RSDs were 2.7 % and 3.4 % respectively.

Finally, we directly compared the relative quantification of glycans from mAb 1 and mAb 2 using FLD and MS. Figure 8 shows the results from each method plotted on a single histogram.

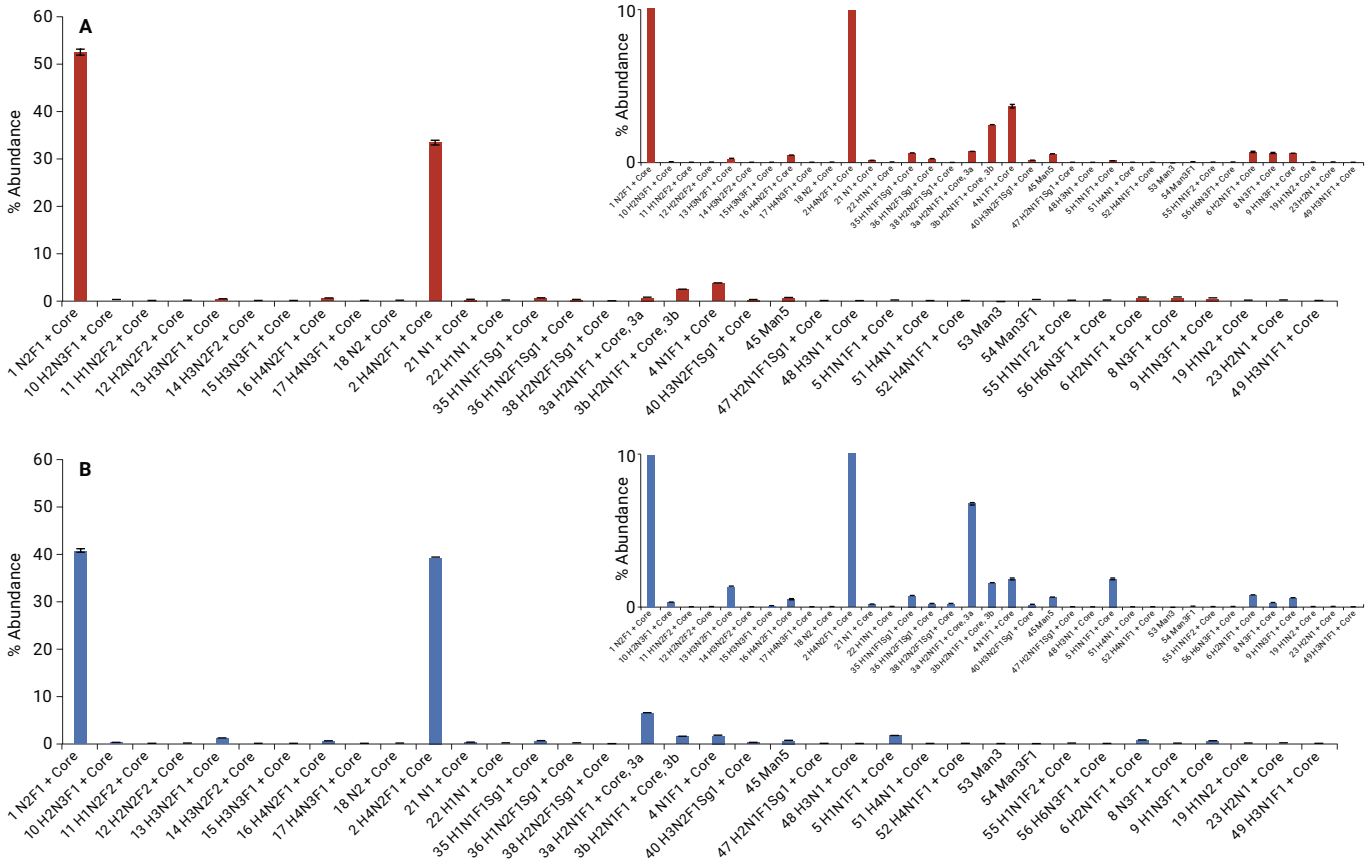

Figure 7. Relative MS quantification of InstantPC labeled N-glycans released from mAb 1 and mAb 2.
A) Relative MS quantification of mAb 1 glycans.
B) Relative MS quantification of mAb 2 glycans.
Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow.
Insets show the same data zoomed to better display components with <10 % relative abundance.

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Conclusion

As shown in Figure 8, the relative quantification results from FLD and MS were highly similar. Some small differences in the results from the two methods can be explained by the different numbers of compositions quantified in the two methods (21 from FLD, 35 from MS). Based on the results of this study, the combination of Prozyme's InstantPC label and an Agilent LC/MS system provides the researcher with the capability to perform MS-based quantification of glycans from mAbs. Still, FLD will likely remain a gold standard detection method for this compound class. In that case, high quality Q-TOF MS data greatly facilitate peak assignment by offering accurate mass and tandem mass information for each of the InstantPC-labeled glycans detected using FLD.

Acknowledgements

We would like to thank NIST for providing the two mAb samples used in this work.

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1. http://glycomics.scripps.edu/CFGnomenclature.pdf

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

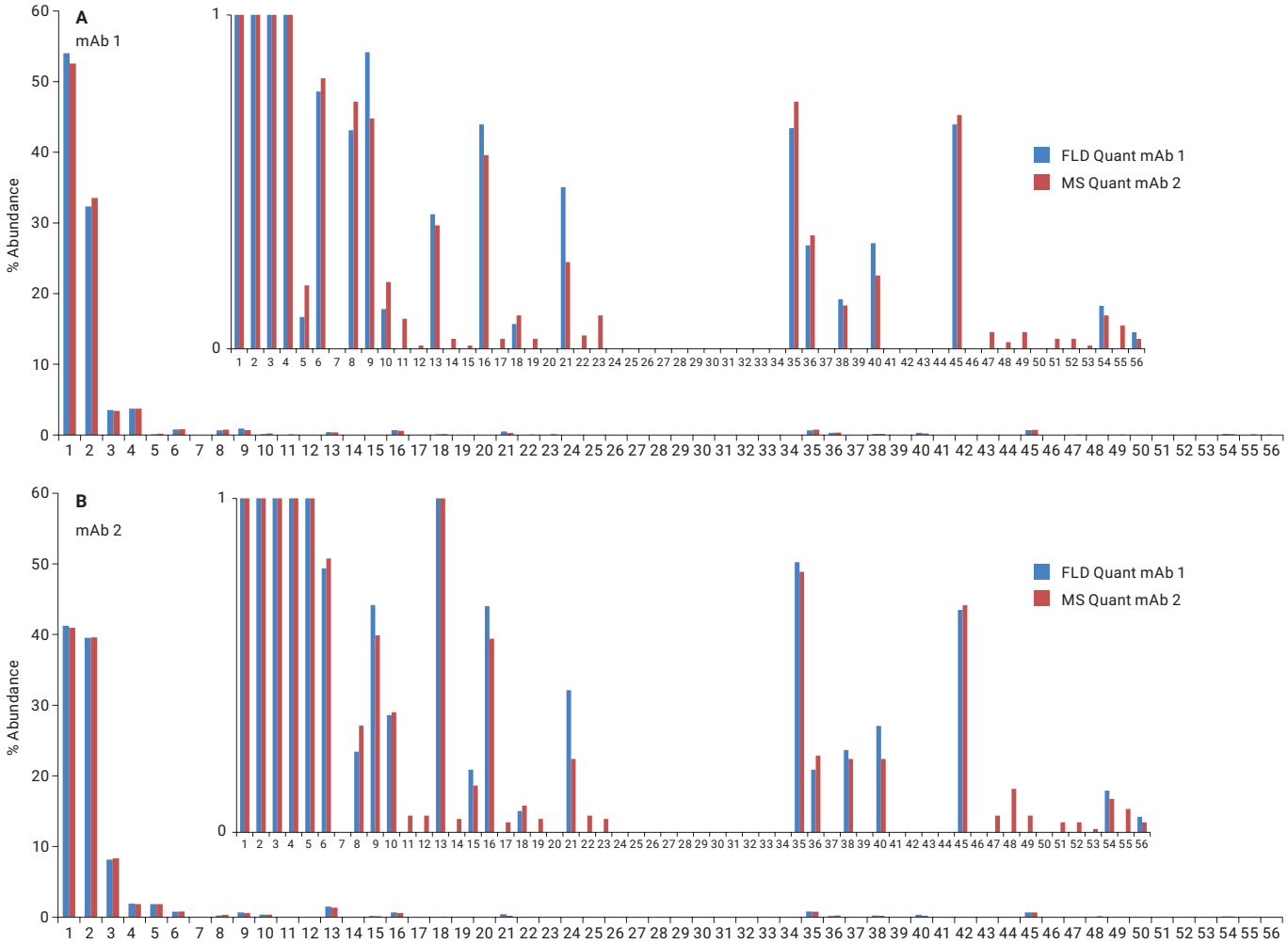

Figure 8. Comparison of fluorescence and relative MS abundance (area sum percentage) of InstantPC-labeled N-glycans from mAb 1 and mAb 2. The X-axis represents individual glycan compositions quantified in the study.

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Application Note

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Preparation of Released N-Glycan Samples from Monoclonal Antibodies Using Agilent AdvanceBio Gly-*X* 2-AB Express for LC-Fluorescence Analysis

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Anne Blackwell
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Abstract

This Application Note describes the preparation of N-glycans from biotherapeutic glycoproteins for released glycan analysis. N-Glycan analysis is critical to the development and production of therapeutic proteins, as glycan composition may directly impact the safety and efficacy of the product. This protocol describes the use of the Agilent AdvanceBio Gly-*X* 2-AB Express kit for release of glycans using PNGase F, labeling through reductive amination, and cleanup of free dye within two hours rather than a full day or longer. The label used is 2-aminobenzamide (2-AB), valued for its well established use and consistency with large bodies of historical glycan analysis data.

Introduction

Glycosylation is a common feature of many biotherapeutic proteins that can affect pharmacokinetics, pharmacodynamics, and immunogenicity[1], and is frequently a critical quality attribute[2]. As such, biotherapeutic glycosylation must be carefully characterized and monitored throughout the development and production process.

Glycans are commonly derivatized prior to analysis, as they are not inherently UV-absorbing or fluorescent, and ionize poorly for MS detection. A handful of fluorescent labels have become widely used, including 2-AB and 2-AA, which modify released N-glycans through reductive amination[3]. Labels with both higher fluorescence and MS sensitivity have been introduced recently[4].

Earlier protocols for 2-AB labeling are often long, with multiple prolonged incubation periods. This not only consumes a large amount of the user's time, but also makes it impossible to obtain results and make decisions based on those results quickly. Deglycosylation was often incomplete without a long incubation period, therefore it became common to allow digestion to take place overnight. Reductive amination to affix a 2-AB label included a preceding step to dry the glycans prior to labeling, and labeling reactions were often allowed to incubate for hours. Additionally, older cleanup cartridges for removal of excess 2-AB reagent prior to LC analysis were cumbersome, and not suited for high-throughput or automated workflows.

The protocol for the Agilent AdvanceBio Gly-*X* 2-AB Express kit includes all the high-level steps for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

Materials

HPLC grade acetonitrile was purchased from Sigma-Aldrich. Water was purified using a Milli-Q A10 water purification system (Millipore).

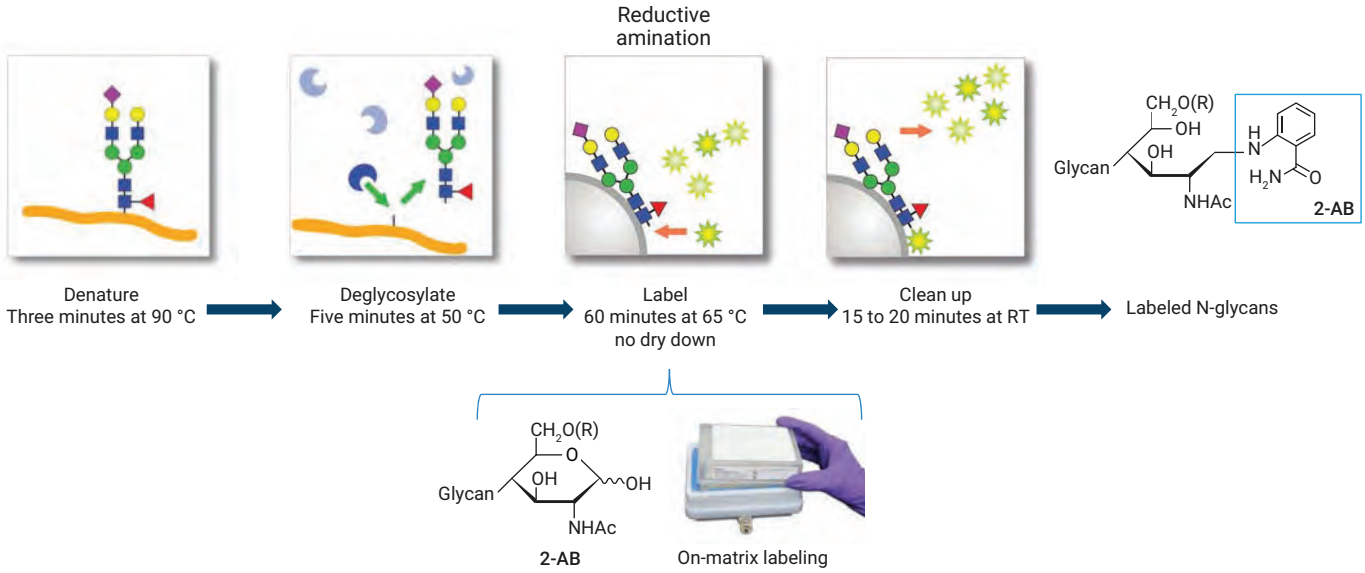

Figure 1. AdvanceBio Gly-*X* 2-AB Express workflow for release and labeling of N-glycans.

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N-Glycan sample preparation

Labeled N-glycan samples were prepared using the AdvanceBio Gly-*X* 2-AB Express kit (p/n GX96-2AB). Figure 2 shows the kit components.

AdvanceBio Gly-*X* N-glycan sample preparation involves a series of enzymatic and chemical steps, beginning with denaturation of the target protein (Figure 3). A denaturing reagent is added, and the sample is incubated at 90 °C for three minutes. Effective unfolding of the protein allows for highly efficient, in-solution cleavage of N-glycans using the enzyme PNGase F in only five minutes[4]. PNGase F is specific to N-linked glycans, so only N-glycans are removed from the protein (Figure 4), while any O-linked glycans and nonenzymatic glycosylation remain attached to the protein.

The labeling and cleanup steps take place on a HILIC-based solid phase stationary support. Released N-glycans are converted to -OH form in solution prior to loading onto the stationary phase, followed by the 2-AB labeling reagents, and the phase is incubated for one hour at 65 °C (Figure 5). After labeling is complete, the excess reagents are rinsed away through a series of acetonitrile washes. The labeled N-glycans are then eluted with water (Figure 6). On-matrix labeling eliminates the need to dry the released glycans prior to 2-AB labeling.

Instrumentation

Samples were analyzed using an Agilent AdvanceBio Glycan Mapping column on an Agilent LC composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent Infinity multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)

Figure 2. Components of the AdvanceBio Gly-*X* 2-AB Express N-glycan sample preparation kit.

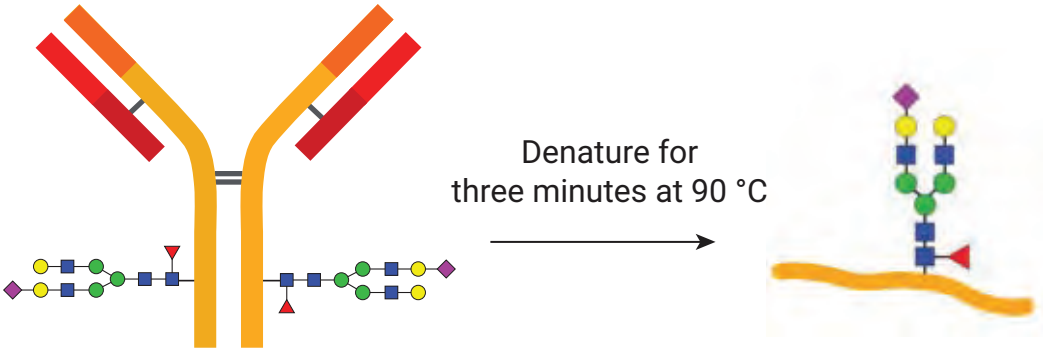

Figure 3. The sample protein is first denatured to effectively deglycosylate in the subsequent step.

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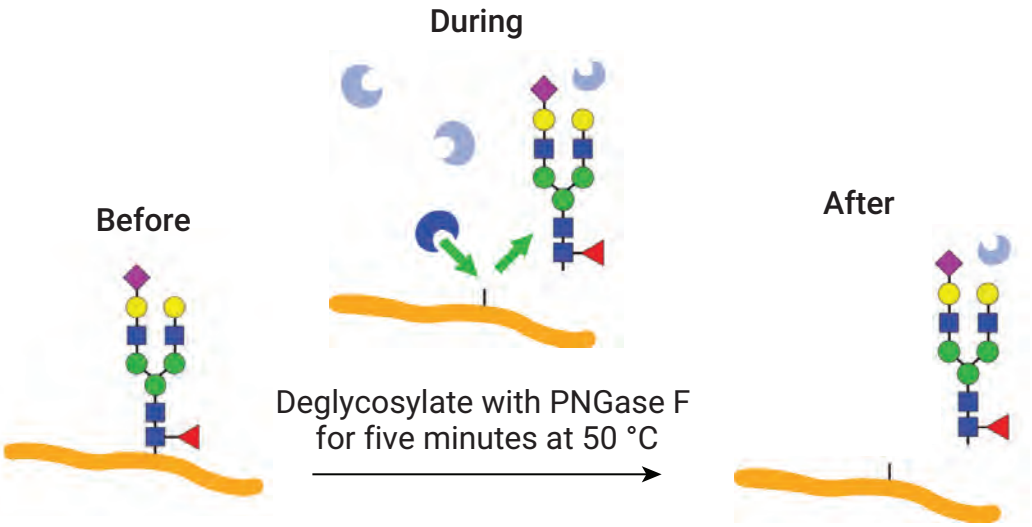

Figure 4. N-glycans are rapidly cleaved from the protein using PNGase F.

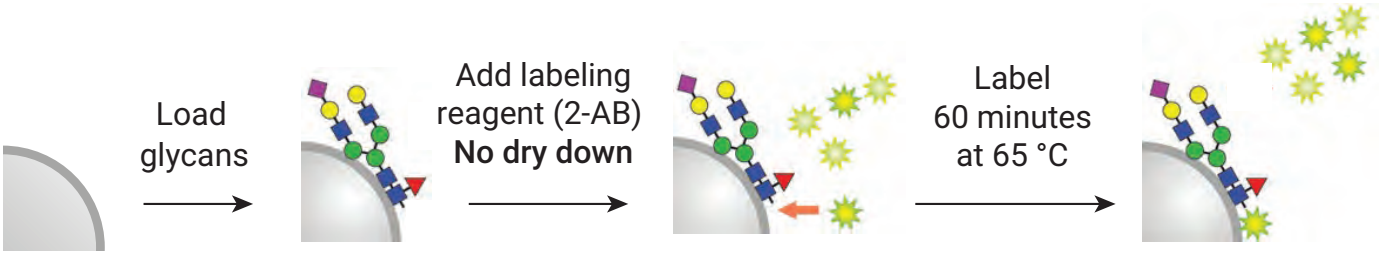

Figure 5. The sample is loaded onto a HILIC-based solid phase support on a vacuum-based cleanup plate.
The 2-AB labeling reagents are then added to the solid phase support and incubated for one hour. No dry-down step is necessary.

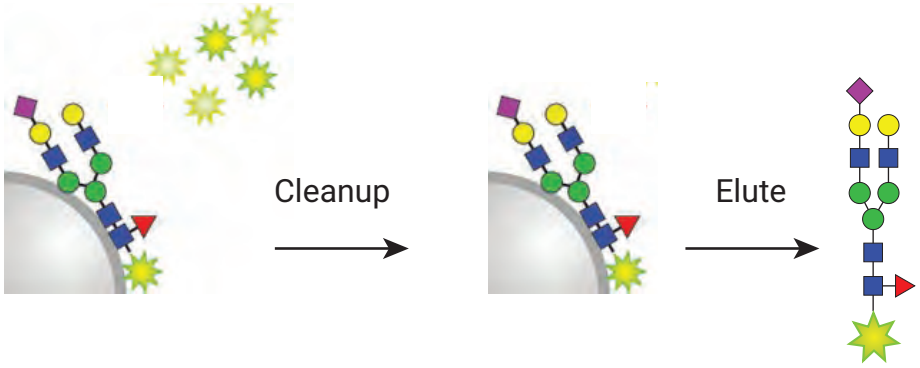

Figure 6. Excess labeling reagents are rinsed away, and labeled glycans are eluted from the solid stationary phase.

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Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Results and discussion

MabThera and Enbrel 2-AB N-glycan samples were analyzed by LC/FLD. Figure 7 shows representative chromatograms. MabThera has a simpler glycosylation pattern, with Enbrel showing higher relative levels of sialylated glycans. Reproducibility of the sample preparation is of utmost importance, so samples may be compared across production lots. Variability measured needs to truly originate from changes in the sample, rather than as an artifact of sample handling or analysis.

Table 1. LC method.

Parameter	Value
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
Column Temperature	40 °C
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) acetonitrile
Flow Rate	0.5 mL/min
Gradient Program	Time (min) %B Flow rate (mL/min) 0.0 82 0.4 2.0 82 0.4 2.5 77 0.4 48.0 62 0.4 49.0 40 0.4 51.5 40 0.4 52.0 82 0.4 54.0 82 0.6 58.0 82 0.6 58.5 82 0.4
Injection Volume	1 µL (equivalent to glycans from 0.4 µg of protein)
Detection	Agilent 1290 Infinity II FLD Excitation 260 nm Emission 430 nm

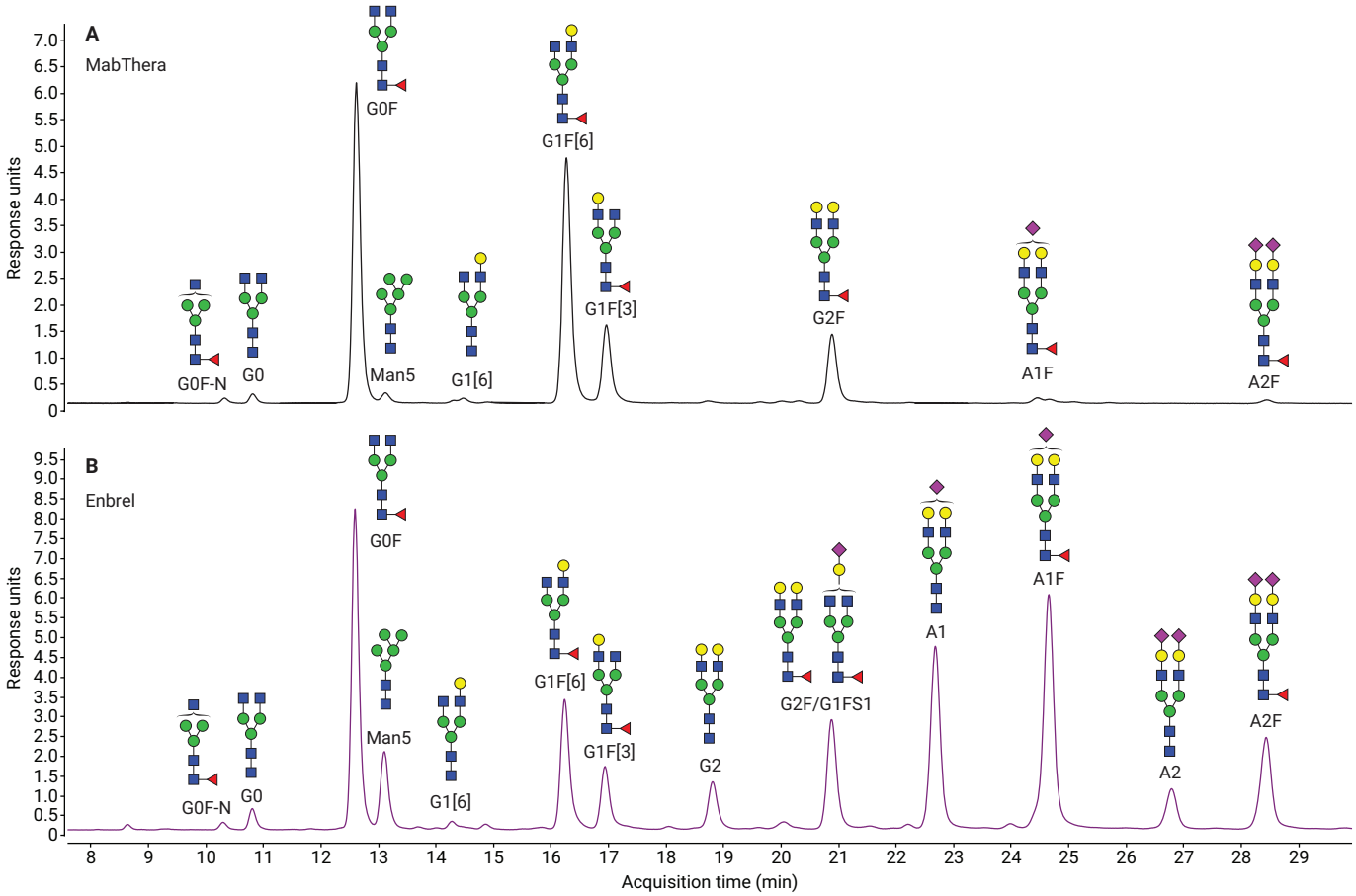

Figure 7. Representative chromatograms showing separation of 2-AB labeled N-glycans from A) MabThera and B) Enbrel.

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Table 2 shows the relative percent area of the major N-glycan species detected in three preparations of a MabThera sample. The average percent area, along with standard deviation and relative standard deviation (%CV), are reported. The variations between sample preparations are all low, except for the lowest abundance glycans. Precision is more challenging near the limit of detection, so higher variation is to be expected for these peaks.

For any researcher to change sample preparation methods, the data obtained must be either equivalent, or in some way superior to the results of the previous method. With other labeling chemistries available, a major driver behind continuing to use 2-AB is the ability to compare results to older data obtained with other 2-AB labeling protocols. Figure 8 shows the relative percent area of the N-glycans detected in triplicate preparations of Enbrel. The relative abundances are very similar between the samples prepared using AdvanceBio Gly-*X* 2-AB Express and ProZyme GlykoPrep 2-AB, an earlier generation of 2-AB sample preparation available from ProZyme.

Table 2. Relative % area of major N-glycan species from three preparations of a MabThera sample.

		Relative % Area					
Glycan	RT	1	2	3	Average	Standard Deviation	%CV
G0F-N	10.32	0.56	0.57	0.57	0.57	0.01	1.02
G0	10.81	1.09	1.01	0.98	1.03	0.06	5.54
G0F	12.62	39.85	39.31	39.33	39.50	0.31	0.78
Man5	13.12	0.74	0.62	0.69	0.68	0.06	8.82
G1[6]	14.47	0.66	0.6	0.62	0.63	0.03	4.88
G1F[6]	16.27	34.65	34.81	34.67	34.71	0.09	0.25
G1F[3]	16.96	10.65	10.47	10.6	10.57	0.09	0.88
G2F	20.89	10.15	10.78	10.83	10.59	0.38	3.58
A1F	24.45	1.13	1.26	1.17	1.19	0.07	5.61
A2F	28.44	0.52	0.57	0.54	0.54	0.03	4.63

Conclusion

N-Glycan samples can be fully prepared for analysis within two hours with Agilent AdvanceBio Gly-*X* 2-AB Express, versus older methods that take a full day, including an overnight incubation and dry down prior to labeling. Data produced for a variety of glycoproteins are highly reproducible, and consistent with data obtained from older 2-AB sample preparation methods.

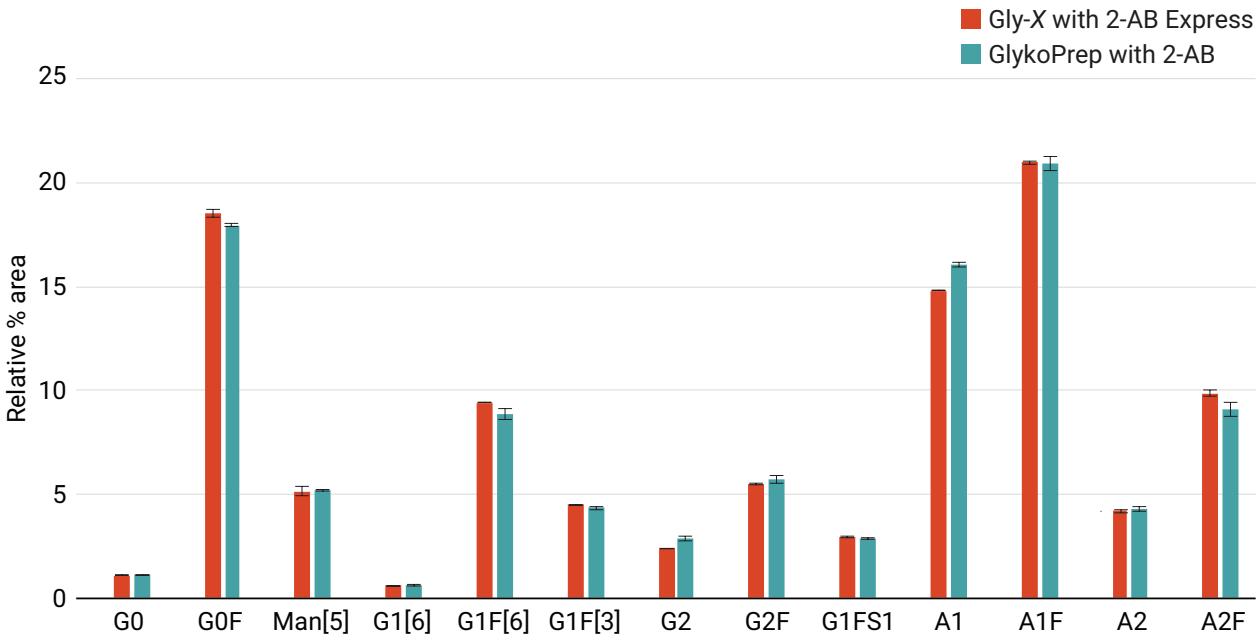

Figure 8. Samples produced using the AdvanceBio Gly-*X* 2-AB Express kit produce data equivalent to samples prepared using older methods, such as ProZyme GlykoPrep 2-AB, shown here for N-glycans from Enbrel. n = 3 for all data.

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