Agilent

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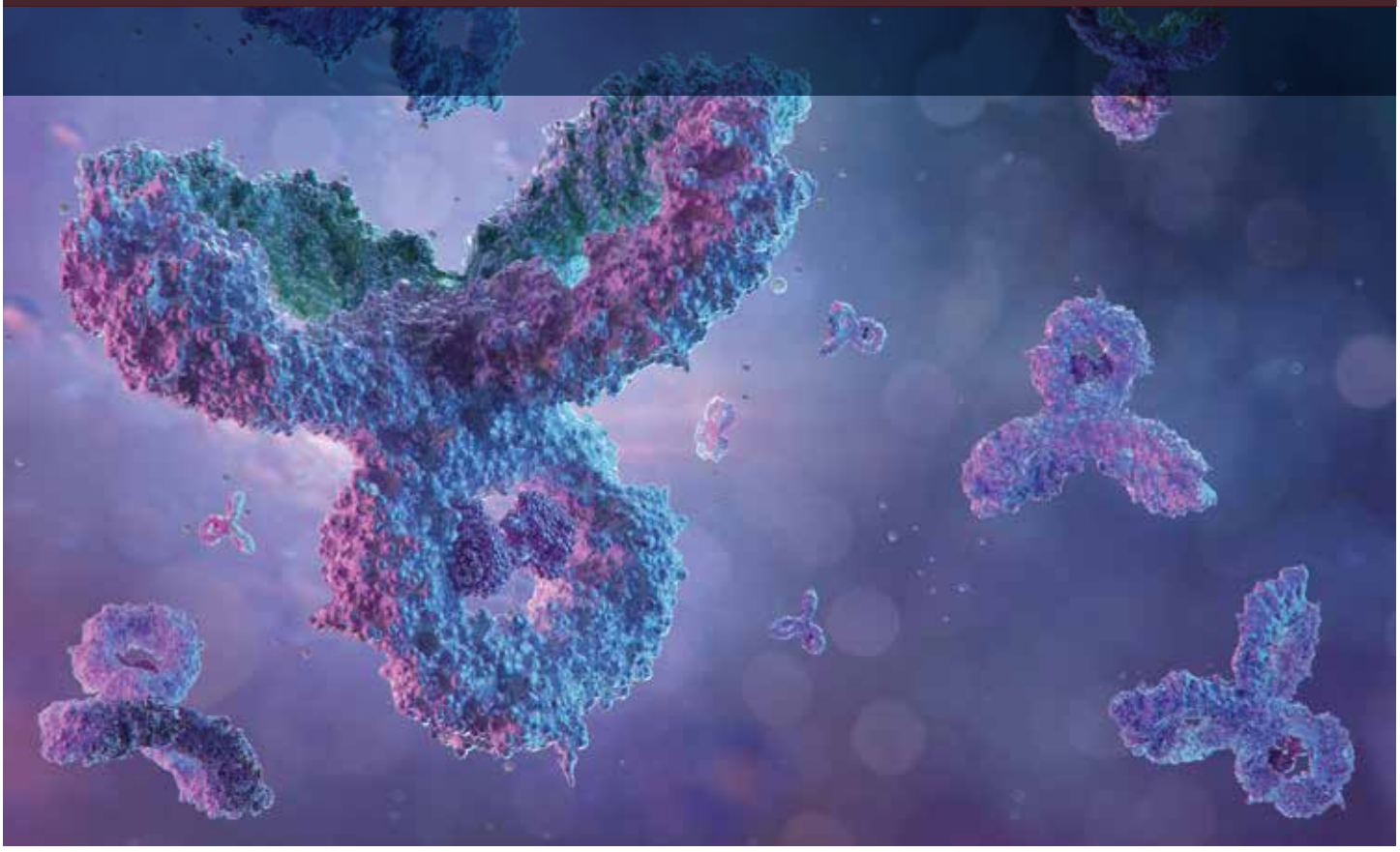
Agilent Biocolumns

Glycan Analysis

Application Compendium



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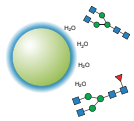
Glycan Analysis

Background

Glycosylation is an important post-translational modification as glycans play a key role in protein recognition and biotherapeutic efficacy. Glycosylation patterns in human cells generally follow a typical antenna-like pattern based on a common core structure, and it is the goal of recombinant protein manufacturers to try to replicate that profile using cell culture. Mammalian cell lines are required but the glycosylation pathway is complex, and not all clones will generate the desired glycan profile. Regulatory authorities recognize this as a major challenge and provide instructions in how to determine the glycan fingerprint. This involves the use of a specific enzyme, PNGase F, to cleave N-linked glycans, labeling them with a fluorophore to increase detection sensitivity, and then separating them using hydrophilic interaction chromatography (HILIC) columns (often in combination with a fluorescence detector, although mass spectrometry may also be used).

Agilent provides several kits containing all the components needed to manually perform the deglycosylation and labeling reaction with a variety of labels including 2-aminobenazamide (2-AB), InstantPC, and APTS. Alternatively, much higher throughput can be obtained using a fully automated AssayMAP platform capable of handling a greater number of samples in a fraction of the time. This approach is illustrated in the application note featured on page 66.



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

Getting Started

HILIC, or hydrophilic interaction chromatography, is a normal phase chromatography. In some ways the opposite of the more familiar reversed phase chromatography, with gradients starting at high organic solvent content rather than high aqueous 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 also the strong eluting solvent. Ample re-equilibration time at the end of the method and minimized sample injection volumes are critical for reproducible chromatography and good peak shape. AdvanceBio Glycan Mapping columns are available in superficially porous 2.7 µm columns suitable for use on all HPLC instruments, or in fully porous 1.8 µm columns designed for use on UHPLC instruments. Appropriate method conditions to achieve the optimum resolution for closely related glycan structures can be found in the Workflow "How-To" guides detailed in this section.

Glycan Mapping: A "How-To" Guide

Introduction

Post-translational modifications to the primary amino acid sequence, including glycosylation, have functional consequences and can impact efficacy and immunogenicity of a biopharmaceutical. The structure of the glycan also contributes to the clearance rates of the protein in plasma and the ability of the monoclonal antibody to trigger the immune response required for efficacy. Many regulatory authorities consider glycosylation to be one of the critical quality attributes of biomolecules. Therefore, it must be characterized and quantified, with acceptable ranges determined, as part of the development process for a glycoprotein innovator, biosimilar, or biobetter pharmaceutical.

Agilent's AdvanceBio Glycan Mapping solutions provide optimized workflows designed to deliver reproducibility in the analysis of fluorescently-labeled glycans for accurate identification and quantification.

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Sample Preparation - AdvanceBio N-Glycan Sample Preparation Kits

The mapping of the N-linked glycan component of a glycoprotein, including monoclonal antibodies, requires the N-glycans to be enzymatically cleaved from the protein amino acid backbone using PNGase F. The cleaved N-glycans can be analyzed by hydrophilic interaction chromatography (HILIC) with MS detection. More commonly, N-glycans can labeled with one of a variety of fluorophores and analyzed using HILIC chromatography followed by either fluorescence or MS detection.

AdvanceBio glycan sample preparation kits provide all the reagents needed to prepare samples. Agilent's sample preparation kits are modular, generally separated into sub-kits for deglycosylation, labeling and cleanup. In addition, modules for each of the separate steps of the workflow have separate part numbers for flexibility. The workflow yields samples that are suitable for analysis by liquid chromatography, typically by HILIC.

Column Selection

Agilent AdvanceBio Glycan Mapping columns are designed and manufactured to deliver fast, high resolution, reproducible glycan identification using HILIC chromatography. AdvanceBio Glycan Mapping columns apply technology that optimizes results for MS and fluorescence detection. Choose from two UHPLC configurations: 2.7 µm superficially porous, for high resolution and lower backpressure, or 1.8 µm for highest resolution.

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.

Speed of Analysis

The AdvanceBio Glycan Mapping 1.8 µm columns provide high throughput N-glycan analysis where speed is the primary concern either due to the number of samples or to the immediate requirement for data. These columns deliver superior results in 40 % less time than the competition.

Conditions

Parameter	Value
Column A	AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm
Column B	Competitor sub-2 µm glycan column
Instrument	Agilent 1290 Infinity LC with 1260 Infinity Flourescence Detection
Column Temperature	55 °C
Sample thermostat	105 °C
Mobile Phase	A: 100 mM NH_4Formate. pH 4.5 B: ACN
FLD	Excitation = 260 Emission = 430
Injection Volume	2 µl in 70:30 ACN: 100mM NH_4Formate
Sample	Agilent 2-AB labeled N-linked Human IgG glycan library (p/n5190-6996)

Resolution

High resolution separations are achieved using the AdvanceBio glycan mapping 2.7 µm media, in longer 250 mm columns. This increased resolution enables accurate quantitation of target glycans and changes to the protein glycosylation profile, which may have occurred during expression.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	40	80	0.5

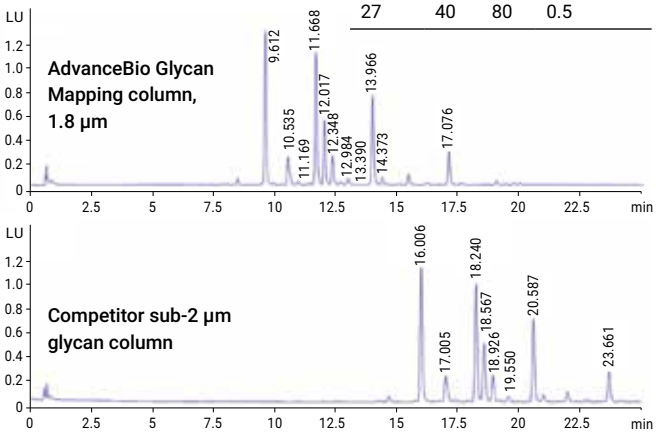

Figure 1. The Agilent AdvanceBio Glycan Mapping column delivers better resolution, narrower bands, and higher peak capacity than the non-Agilent sub-2 µm column in a 2.1 x 150 mm configuration.

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Ensure consistent results with glycan standards for performance testing and retention mapping

Missed information in the early stages of development can cause major setbacks downstream. What's more, production process inconsistency may lead to changes in glycosylation, which can negatively impact immunogenicity and efficacy. Agilent reference standards help you make sure that critical data have been captured, and that every workflow component is working optimally. Agilent offers a full line of pre-labeled glycan standards and libraries as well as unlabeled glycan standards. A full list including structure diagrams and part numbers is available on pages 12-15.

Separation of a 2-AB labeled dextran ladder

Conditions

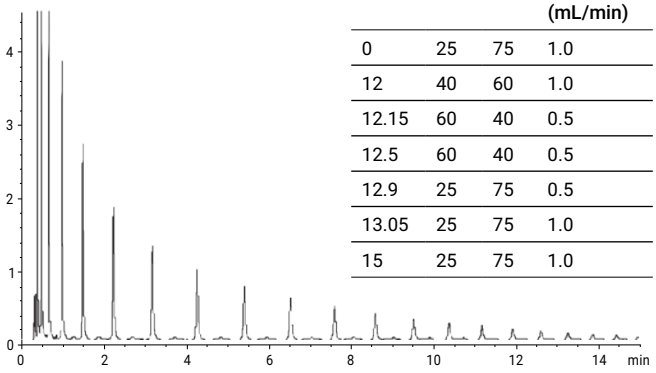
Parameter	Value
Column	AdvanceBio Glycan Mapping, 859700-913 2.1 × 150 mm, 1.8 µm
Mobile Phase	A: 100 mM NH_4Formate. pH 4.5 B: ACN
FLD	Excitation = 260 Emission = 430
Injection Volume	2 µl (10pmol total glycan/1 µl 75:25 ACN:water)
Sample	Agilent 2-AB labeled dextran ladder (p/n GKSB-503)

Flexible, high-performance LC instruments

Robust and easy to use:
The 100 % bio-inert Agilent 1260 Infinity II Bio-inert LC delivers outstanding results with its low surface activity, corrosion resistance, active seal wash, and quaternary buffer mixing.

New Benchmarks in Efficiency:
The Agilent 1290 Infinity II LC is the next generation in UHPLC, providing maximum analytical, instrument, and laboratory efficiency, with pressures up to 1300 bar and flows up to 5 mL/min.

Better efficiency and interaction-free results: Agilent bio-inert LC supplies improve chromatographic reliability with sharper peaks and high reproducibility.



Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

Figure 2. This analysis uses the agilent dextran ladder standard, together with an AdvanceBio Glycan Mapping column to correlate retention times of unkown glycans. From 5990-9384EN.

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Conditions

Mobile phase

AdvanceBio Glycan Mapping columns are shipped with storage buffer acetonitrile: water and are ready to use for HILIC separations. HILIC columns are compatible with aqueous buffers and acetonitrile, which are most commonly used for glycan analysis. A typical mobile phase for glycan analysis is:

Buffer A: 100 mM ammonium formate in water, pH 4.5

Buffer B: Acetonitrile (mass spectrometry compatible)

The operating pH range of AdvanceBio Glycan Mapping columns is pH 2 to 7. AdvanceBio Glycan Mapping columns are silica-based columns with an amide HILIC phase. All silica has some solubility in pH>6 aqueous mobile phases, therefore using the column above pH 7 reduces the column lifetime.

Sample injection

For maximum resolution, inject 1 to 2 µL of your samples. Samples should first be dissolved in H_2O then made up to 70:30 acetonitrile: water. The autosampler should be cooled to preserve sample integrity. In addition, samples should be filtered before injection into the column. For UHPLC analysis, we recommend injecting 1 µL aqueous. The column inlet frit is nominally 0.5 µm for the AdvanceBio Glycan Mapping 1.8 µm columns, and 2 µm for the AdvanceBio Glycan Mapping 2.7 µm columns. Samples should therefore be filtered through a 0.2 µm sample filter.

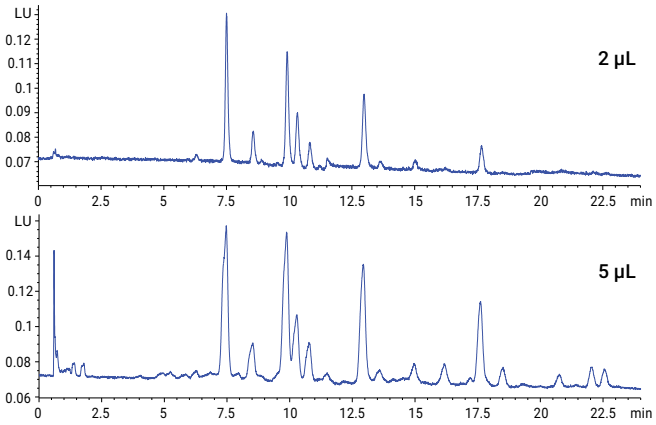

Figure 3. Demonstrates the outcome from injecting 5 µL (bottom chromatogram) on a AdvanceBio Glycan Mapping 1.8 µm, 2.1 x 150 mm column, peaks become broader and resolution is lost compared to 2 µL injection (top chromatogram). From 5991-6183EN.

Flow rates

For high resolution separations, a flow rate of 0.5 mL/min should be used. Whereas for high speed separations up to 1.0 mL/min can be used. Always run high aqueous cleanup at reduced flow rates.

Maximum operating pressure is 1200 bar for the 1.8 µm column, and 600 bar for the 2.7 µm column. Optimal column lifetime is achieved when operating up to 80 % of the maximum pressure.

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AdvanceBio Glycan Mapping 2.7 µm column suggested gradients

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
18	20 %	80 %	0.7 mL/min

AdvanceBio Glycan Mapping 1.8 µm column suggested gradients

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

It may be necessary to adjust the start and end point to obtain highest resolution for samples containing different types of glycans. Larger glycan structures may require 75 to 55 % acetonitrile gradient for optimum results for example.

Temperature

The typical operating temperature is 60 °C to achieve sharp peaks. Higher temperatures can be used but may shorten column lifetime. For longer column lifetimes, 40 °C is recommended. Selectivity and resolution may change with temperature.

Detection

Mass spectrometry can be used to detect N-glycans. However, fluorescence detection is the most commonly used analytical method employed for labeled N-glycans. Excitation and emission wavelengths are dye-specific, and values for the Agilent 1260 Infinity fluorescence detector can be found in the instruction manual pertaining to the sample preparation kit.

Maximizing column lifetime and performance

Column Conditioning

The AdvanceBio Glycan Mapping columns are designed for the separation of N-linked glycans cleaved from glycoproteins and glycopeptides. For the HILIC mechanism to work effectively, the column must be fully equilibrated before use.

- Flush the column with 100 % acetonitrile for a minimum of 10 column volumes
- Flush the column aqueous phase containing 15 % acetonitrile for a further 10 column volumes
- Finally, flush the column with the mobile phase to be used at the start of the analysis for 20 column volumes.

To check that the column is fully equilibrated, two to three analysis runs may be made done to check for reproducibility.

Troubleshooting high backpressure

If the solvent flow appears to be restricted (unusually high column backpressure), first check to see that solvent flow is unobstructed up to the column inlet. If the restriction is before the column, replace the appropriate piece of tubing or filter that is plugged. If the column is plugged, do not backflush the column, instead replace the column.

Storing your column

Acetonitrile: water (95:5) is recommended as the long-term storage solvent. It may be necessary to flush the column with 60 % acetonitrile: 40 % water to remove buffer before switching to the storage solvent. Before storing the column, tightly cap the end fittings with the end plugs to prevent the packing from drying out.

Columns can be safely stored for short periods in the mobile phases. However, to protect equipment, it is best to remove salts from the instrument and column by purging the column with the same mobile phase without the buffer. For example, using 90:10 ACN:H_2O to remove a 90:10 acetonitrile:0.01 M formate buffered mobile phase.

For short term storage, re-equilibration is faster when the column is stored in 80 % acetonitrile:20 % 5 mM ammonium formate. Several (3 to 6) injections should be made to verify column equilibration.

Ordering information

AdvanceBio Glycan Mapping Columns

1.8 µm, stable to 1200 bar

Description	Part number
2.1 x 100 mm	858700-913
2.1 x 150 mm*	859700-913
Fast Guards, 2.1 mm, 1.8 µm	651750-913

* Recommended initial column size

2.7 µm superficially porous, stable to 600 bar

Description	Part number
2.1 x 100 mm	685775-913
2.1 x 150 mm*	683775-913
2.1 x 250 mm	651750-913
Fast Guards, 2.1 mm, 2.7 µm	821725-906
4.6 x 100 mm	685975-913
4.6 x 150 mm	683975-913
4.6 x 250 mm	680975-913

* Recommended initial column size

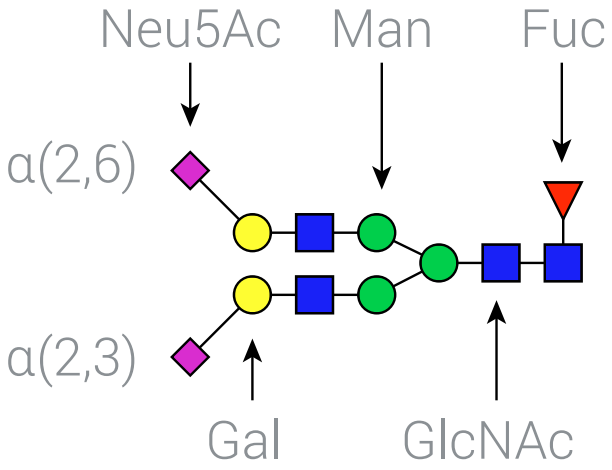
For glycan standards, see 5994-2202EN on pages 12-15

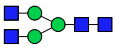
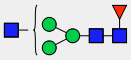
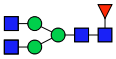
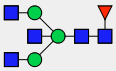
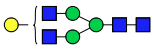
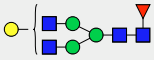
AdvanceBio Glycan Standards InstantPC, 2-AB, 2-AA, APTS, InstantAB, Unlabeled

Glycan standard structures

Glycan graphical representations follow the recommendations of the Consortium for Functional Glycomics[3] (CFG) and were drawn using GlycoWorkbench 2.1[4]. Neu5Ac = N-acetylneuraminic acid;
Gal = galactose;
Man = mannose;
GlcNAc = N-acetylglucosamine;
Fuc = fucose.

The α(2,3) sialic acid linkage is found on glycoproteins produced in Chinese hamster ovary (CHO) cells[5]. In contrast, human intravenous immunoglobulin (IVIG) IgG Fc N-glycans are predominantly α(2,6)-sialylated[6].



Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
Complex-type Native N-Glycans									
G0-N	NGA2-N	A1			GKPC-401		GKSB-401		GKSP-401
G0	NGA2	A2		GKC-004300	GKPC-301	GKIB-301	GKSB-301	GKSA-301	GKSP-301
G0F-N	NGA2F-N	F(6)AI			GKPC-402		GKSB-402		GKSP-402
G0F	NGA2F	F(6)A2		GKC-004301	GKPC-302	GKIB-302	GKSB-302	GKSA-302	GKSP-302
G0FB	NGA2FB	F(6)A2B		GKC-004311			GKSB-303		
G1	NA2G1	A2G1		GKC-014300	GKPC-317	GKIB-317	GKSB-317		GKSP-317
G1F	NA2G1F	F(6)A2G1		GKC-014301	GKPC-316	GKIB-316	GKSB-316	GKSA-316	GKSP-316

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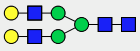
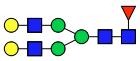
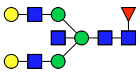
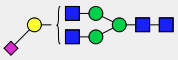
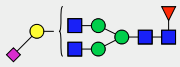
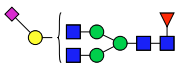
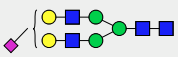
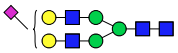
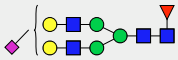
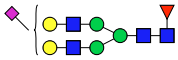
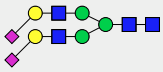
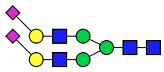
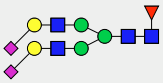
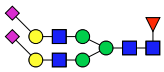
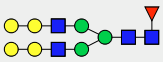
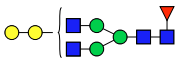
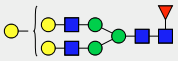
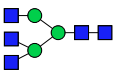
Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
G2	NA2	A2G(4)2		GKC-024300	GKPC-304	GKIB-304	GKSB-304	GKSA--304	GKSP-304
G2F	NA2F	F(6)A2G(4)2		GKC-024301	GKPC-305	GKIB-305	GKSB-305	GKSA-305	GKSP-305
G2FB	NA2FB	F(6)A2BG(4)2		GKC-024311			GKSB-306		
G1S1 α(2,3)		A2G(4)1S(3)1			GKPC-329				
G1S1 α(2,6)		A2G(4)1S(6)1			GKPC-319				
G1FS1 α(2,3)		FA2G(4)1S(3)1			GKPC-330				
G1FS1 α(2,6)		FA2G(4)1S(6)1			GKPC-320				
G2S1 α(2,3)	A1(α2,3)	A2G(4)2S(3)1			GKPC-321				
G2S1 α(2,6)	A1(α2,6)	A2G(4)2S(6)1		GKC-124300	GKPC-311	GKIB-311	GKSB-311	GKSA-311	GKSP-311
G2FS1 α(2,3)	A1F(α2,3)	F(6)A2G(4)2S(3)1			GKPC-325				
G2FS1 α(2,6)	A1F(α2,6)	F(6)A2G(4)2S(6)1		GKC-124301	GKPC-315	GKIB-315	GKSB-315	GKSA-315	GKSP-315
G2S2 α(2,3)	A2(α2,3)	A2G(4)2S(3)2			GKPC-322				
G2S2 α(2,6)	A2(α2,6)	A2G(4)2S(6)2		GKC-224300	GKPC-312	GKIB-312	GKSB-312	GKSA-312	GKSP-312
G2FS2 α(2,3)	A2F(α2,3)	F(6)A2G(4)2S(3)2			GKPC-323				
G2FS2 α(2,6)	A2F(α2,6)	F(6)A2G(4)2S(6)2		GKC-224301	GKPC-313	GKIB-313	GKSB-313	GKSA-313	GKSP-313
G2F w/2 α-gal	NA2Ga2F	F(6)A2G(4)2Ga(3)2			GKPC-318		GKSB-318		GKSP-318
G1F w/1 α-gal	NA2G 1FGa1	F(6) A2G(4)1Ga(3)1			GKPC-403				
G2F w/1 α-gal	NA2FGa1	F(6)A2G(4)2Ga(3)1			GKPC-404				
A3	NGA3	A3		GKC-005300		GKIB-307	GKSB-307	GKSA-307	

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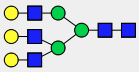
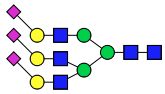
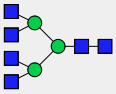
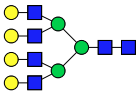
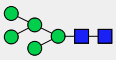
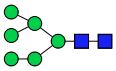
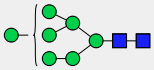
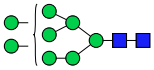
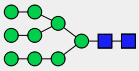
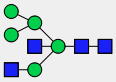
Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
G3	NA3	A3G(4)3		GKC-035300			GKSB-308	GKSA-308	
G3S3 α(2,6)	A3(a2,6)	A3G(4)3S(6)3		GKC-335300			GKSB-314		
A4	NGA4	A4		GKC-006300			GKSB-309	GKSA-309	
G4	NA4	A4G(4)4		GKC-046300			GKSB-310		
High Mannose-type Native N-Glycans									
Man5	MAN-5	M5		GKM-002500	GKPC-103	GKIB-103	GKSB-103	GKSA-103	GKSP-103
Man6	MAN-6	M6		GKM-002600	GKPC-104	GKIB-104	GKSB-104	GKSA-104	GKSP-104
Man7	MAN-7	M7		GKM-002700	GKPC-105	GKIB-105	GKSB-105	GKSA-105	GKSP-105
Man8	MAN-8	M8		GKM-002800	GKPC-106	GKIB-106	GKSB-106	GKSA-106	GKSP-106
Man9	MAN-9	M9		GKM-002900	GKPC-107	GKIB-107	GKSB-107	GKSA-107	GKSP-107
Hybrid-type Native N-Glycan									
Hybrid	HYBR	M5A1B					GKSB-111		
Native N-Glycan Cores									
NF	NF			GKR-001001					
NN	NN			GKR-002000			GKSB-100		
NNF	NNF			GKR-002001					
Man1	MNN	M1		GKR-002100					
Man1F	MNNF	F(6)M1		GKR-002101					
Man3				GKR-002300			GKSB-101		
Man3F				GKR-002301			GKSB-102		

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Glycans	Unlabeled	InstantPC	InstantAB	2-AB	2-AA	APTS
N-Glycan Libraries						
Human IgG N-Glycan Library	GKLB-005	GKPC-005	GKIB-005	GKSB-005	GKSA-005	GKSP-005
CHO mAb N-Glycan Library		GKPC-020				
CHO mAb N-Glycan Library plus CHO mAb Glycoprotein		GKPC-020-P				
Human α1-acid glycop rotein N-Glycan Library	GKLB-001		GKIB-001	GKSB-001	GKSA-001	
Bovine Fetuin N-Glycan Library	GKLB-002		GKIB-002	GKSB-002	GKSA-002	
RNase B N-Glycan Library (High Mannose)			GKIB-009			
Biantennary and High Mannose Partitioned Library			GKIB-520	GKSB-520		GKSP-520
Sialylated Biantennary N-Glycan Library			GKIB-232	GKSB-232		GKSP-232
α(2,6) Sialylated Biantennary N-Glycan Library				GKSB-262		GKSP-262
α(2,3) Sialylated Triantennary N-Glycan Library		GKPC-233	GKIB-233	GKSB-233		GKSP-233
α(2,6) Sialylated Triantennary N-Glycan Library		GKPC-263		GKSB-263		GKSP-263
α(2,3) Sialylated Tetraantennary N-Glycan Library		GKPC-234	GKIB-234	GKSB-234		GKSP-234
α(2,6) Sialylated Tetraantennary N-Glycan Library		GKPC-264		GKSB-264		GKSP-264
Alignment Standards						
Glucose Unit (GU) Ladder		GKPC-503	GKIB-503	GKSB-503	GKSA-503	GKSP-503
Internal Migration Standards for Capillary Electrophoresis (CE)						GKSP-500

References

1. Harvey DJ, *et al*. Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. *Proteomics*. **2009**, *9*(15):3796–801.
2. Not all unlabeled glycans are shown.
3. Varki A, et al. Symbol Nomenclature for Graphical Representations of Glycans. *Glycobiology*. **2015** *Dec; 25*(12): 1323–1324.
4. Ceroni A, *et al*. GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. *J Proteome Res*. **2008** *Apr;7*(4):1650-9.
5. Lee EU, *et al*. Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of beta-galactoside alpha 2,6-sialyltransferase. *J Biol Chem*. **1989**, *264*(23), 13848-55.
6. Anthony RM, *et al*. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science*. **2008**, *320*(5874), 373-6.

Application Note

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Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS

Authors

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Agilent Technologies, Inc

Introduction

This Application Note describes the preparation and analysis of released N-glycans from biotherapeutic glycoproteins using two labels, InstantPC and 2-aminobenzamide (2-AB). N-Glycan analysis is vital to the development and production of biotherapeutics, as glycosylation can influence the therapeutic function of the final drug product. The workflows described here use the Agilent AdvanceBio Gly-X with InstantPC and Gly-X 2-AB Express kits (formerly ProZyme) for the release of N-glycans using PNGase F followed by instant glycosylamine labeling with InstantPC or reductive amination labeling with 2-AB Express, respectively. Labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC), with detection using both fluorescence and mass spectrometry (MS). Gly-X sample preparation offers a high level of reproducibility and throughput, with a one hour preparation time for InstantPC and two hours for 2-AB Express. In addition, the InstantPC label offers improved fluorescence response and MS ionization efficiency.

Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA).[1] N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD), and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation. Many of the frequently used fluorescent tags such as 2-AB[2] are limited concerning MS sensitivity compared with recently introduced dyes such as InstantPC, and pre-existing N-glycan sample preparation workflows can be time-consuming.[3] However, 2-AB has been used for over 20 years and so is well-established in the literature and in many laboratories.

This Application Note presents streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans coupled with analysis using Agilent LC/FLD/MS instrumentation. Gly-X N-glycan sample preparation kits for InstantPC or 2-AB Express labeling (formerly ProZyme) include all reagents for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

N-Glycan sample preparation

Agilent AdvanceBio Gly-X N-glycan prep with InstantPC (p/n GX96-IPC) and Gly-X 2-AB Express (p/n GX96-2AB) Kits were used to prepare labeled N-glycans from monoclonal antibody rituximab (Rituxan, lot number M190170) and Fc fusion protein etanercept (Enbrel, lot number 1092537), 40 µg protein per preparation. Four replicates of each sample were analyzed with fluorescence/MS detection and relative percent glycan peak areas calculated.

InstantPC and 2-AB labeled samples were prepared by standard manual protocols. The Gly-X in-solution deglycosylation protocol uses a three-minute denaturation at 90 °C, opening up the glycoprotein target to enable a five-minute deglycosylation reaction at 50 °C with PNGase F. Following in-solution deglycosylation, InstantPC labeled samples are prepared by one-minute glycosylamine labeling of released N-glycans (Figure 2), followed by vacuum-driven cleanup of free dye using HILIC solid-phase extraction (SPE).

N-Glycan samples labeled with 2-AB were prepared using the standard Gly-X 2-AB Express protocol with reductive amination chemistry. Following the Gly-X five-minute deglycosylation with PNGase F, released N-glycans are converted from the glycosylamine form ($-NH_2$) to free reducing end form (–OH) to allow for 2-AB labeling with reductive amination. N-Glycans are then desolvated by vacuum filtration onto a solid-state matrix followed by an on-matrix 2-AB labeling step. This process eliminates the need for glycan drying prior to the 2-AB labeling step, thereby reducing total sample preparation time.

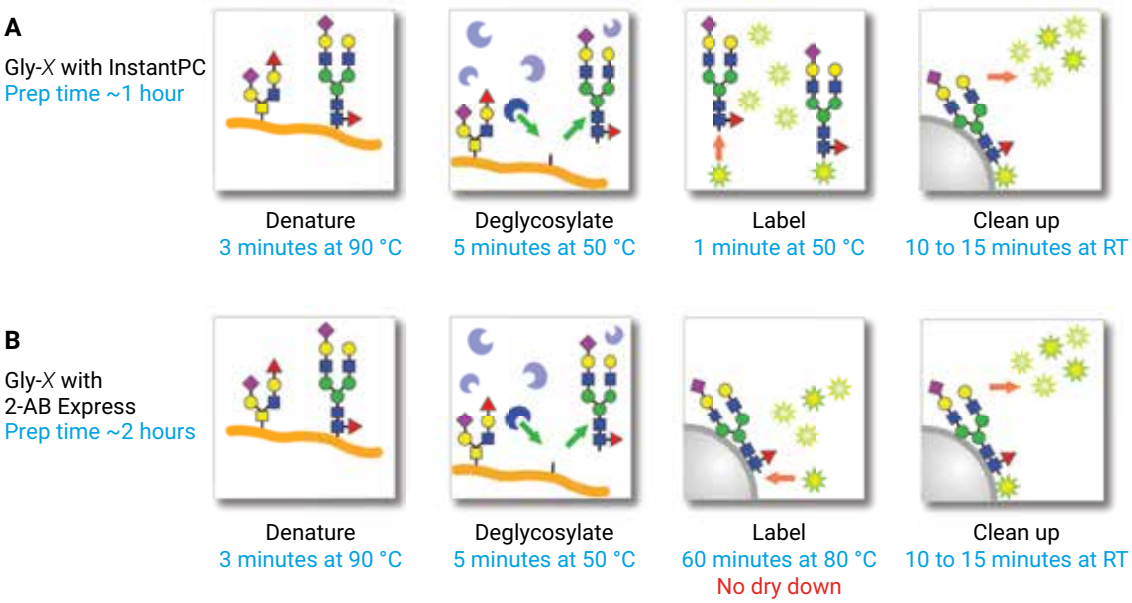

Figure 1. Gly-X N-glycan sample prep.
A) InstantPC workflow with in-solution deglycosylation and labeling followed by on-matrix cleanup;
B) 2-AB workflow with deglycosylation in-solution, followed by on-matrix labeling and cleanup.

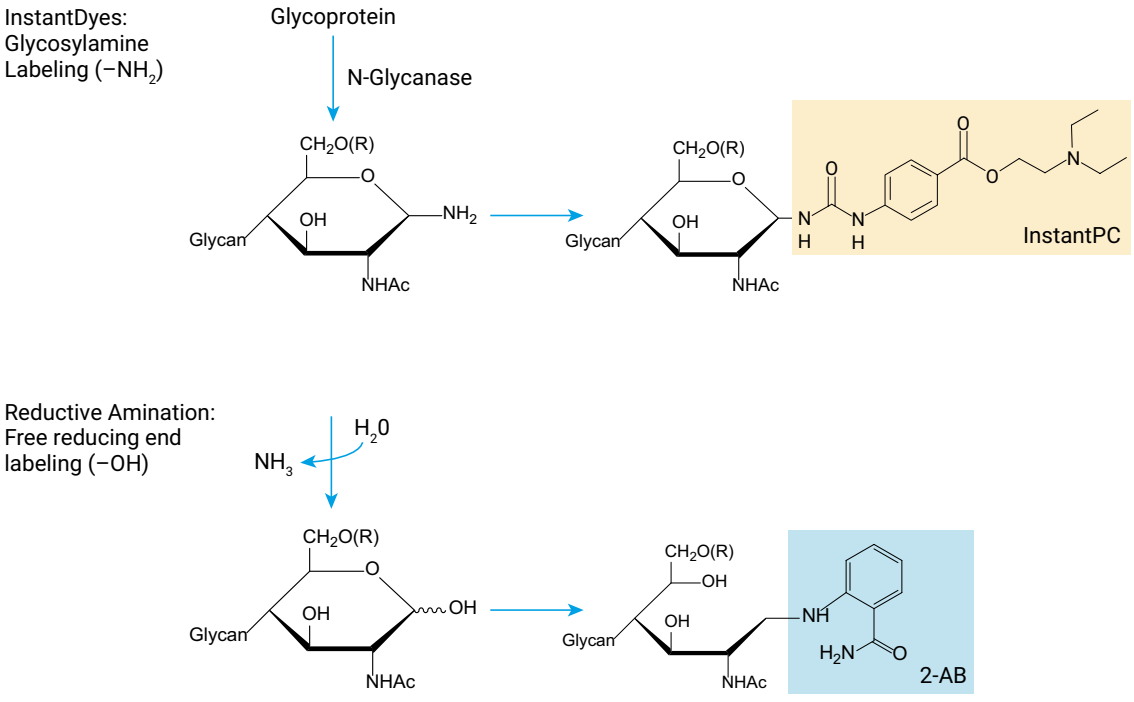

Figure 2. Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

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N-Glycan analysis

InstantPC and 2-AB labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913) with an Agilent 1290 Infinity II LC system with in-line fluorescence detection (Table 1) coupled to an Agilent AdvanceBio 6545XT LC/Q-TOF (Table 2).

All HILIC separations were conducted under the conditions described in Table 1. A fixed flow splitter (IDEX Health & Science p/n UH-427) was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. Agilent MassHunter BioConfirm software was used for data processing, with a personal compound database (PCD).

Materials

LC/MS grade acetonitrile and water were purchased from Honeywell Research Chemicals.

Instrumentation

Labeled N-glycan samples were separated using an Agilent AdvanceBio Glycan Mapping column (Table 1 shows the method details) on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 2)

Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Table 1. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions.

Conditions, Agilent 1290 Infinity II UHPLC HILIC/FLD

Parameter	Value	
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)	
Column Temp:	40 °C	
Mobile phase:	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile	
Gradient:	Time (min) 0 2 48 49 51.5 52 60	% B 80 75 62 40 80 80 80
Flow rate:	0.5 mL/min	
Injection volume:	1 µL (equivalent to glycans from 0.4 µg protein)	
Detection:	Agilent 1260 Infinity II FLD InstantPC: λEx 285 nm, λEm 345 nm 2-AB: λEx 260 nm, λEm 430 nm	

Table 2. Agilent 6545XT Q-TOF parameters.

Agilent 6545XT Q-TOF parameters	
Source	Dual AJS ESI
Gas temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	m/z 600 to 3,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

Results and Discussion

HILIC Separation of InstantPC and 2-AB N-Glycans

HILIC separation of labeled N-glycans from Rituxan and Enbrel labeled with InstantPC or 2-AB results in well resolved peaks for major glycan species with the 60-minute method used (Figures 3 and 4). Rituxan (Figure 3A, InstantPC; Figure 4A, 2-AB), an IgG, has an N-glycan profile typical of monoclonal antibodies with one N-glycosylation site in the Fc region produced in Chinese hamster ovary (CHO) cells: predominantly neutral biantennary complex N-glycans with core fucose, some Man5, and a relatively low proportion of sialylated glycans. The N-glycan profile of Enbrel (Figure 3B, InstantPC; Figure 4B, 2-AB), an Fc fusion protein, contains a higher level of sialylated glycans owing to two additional N-glycosylation sites in the fusion partner, TNF-α receptor (TNFR) extracellular domain, in addition to the single N-glycan site in the Fc portion.5

The HILIC retention time of 2-AB N-glycans is shorter than for InstantPC N-glycans, although the elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and Man5/G1, which are often monitored during the development process of biotherapeutics, are well separated with both InstantPC and 2-AB labels, leading to confident determination of relative percentage composition. G1F isomers G1F[6] and G1F[3] are also separated. Relative percent areas, standard deviation, and relative standard deviation are reported in Tables 3 through 6, and show a low degree of variability between the four sample preparation replicates. This variability rises for lower abundance glycans.

An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from Enbrel (Figure 3B) compared to 2-AB (Figure 4B) using the previously described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics Rituxan and Enbrel results in comparable relative percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2, and G2FS2.

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

InstantPC displays higher fluorescence and MS signal compared to 2-AB (Figure 5), when using the same amount of glycoprotein starting material (40 µg), and injecting the same relative volume for HILIC separations (1 µL of 100 µL kit eluent). Individual spectra for InstantPC and 2-AB labeled Man5 illustrates the higher MS signal of InstantPC (Figure 6).

Table 3. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

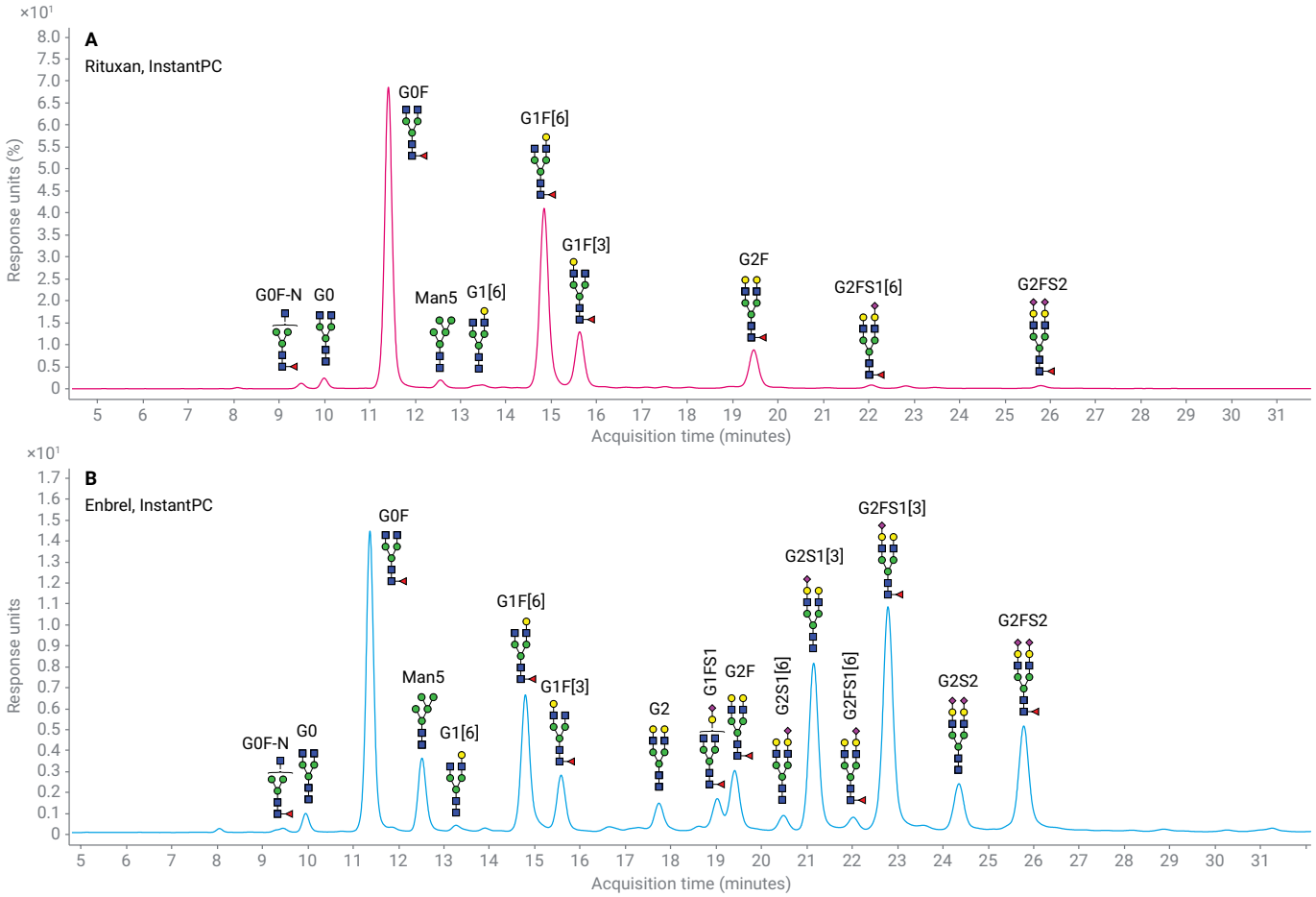

Figure 3. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in Table 3 and Table 4, n = 4.

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Table 3. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

Table 5. Figure 4A relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

Table 6. Figure 4B relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

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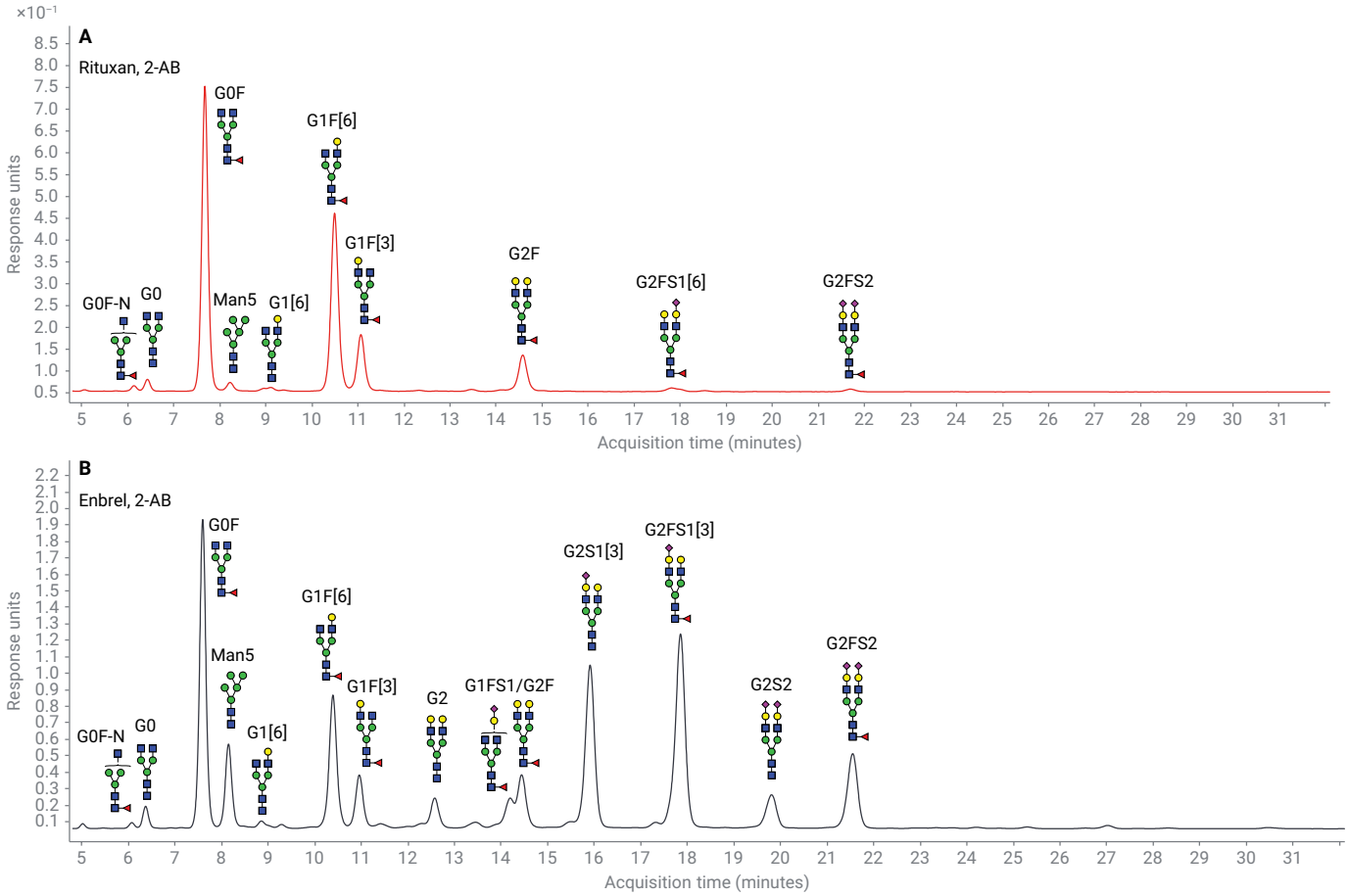

Figure 4. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in Table 5 and Table 6, n = 4.

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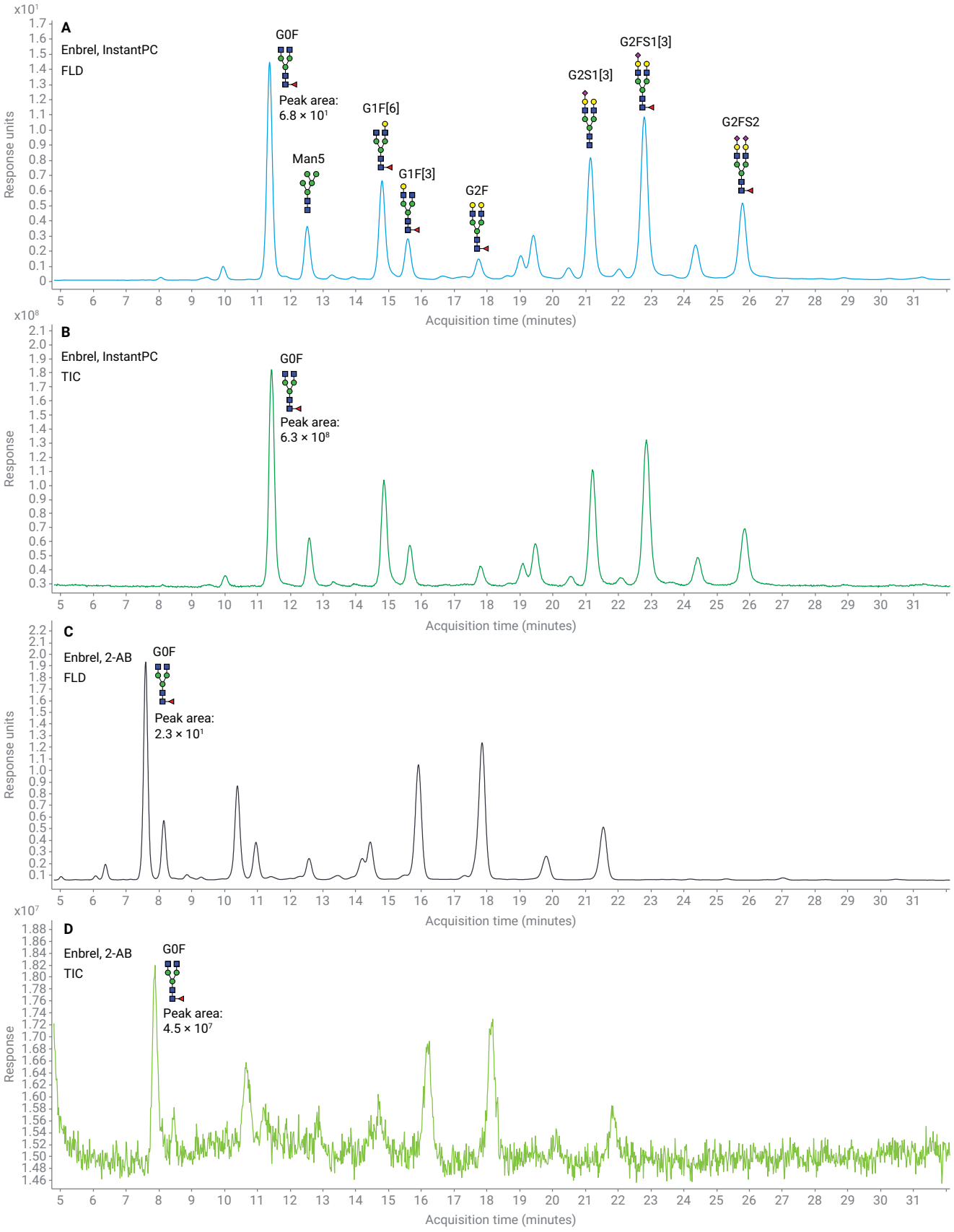

Figure 5. FLD and MS of InstantPC and 2-AB labeled N-glycans from Enbrel. A) InstantPC FLD; B) InstantPC TIC (total ion chromatogram); C) 2-AB FLD; D) 2-AB TIC.

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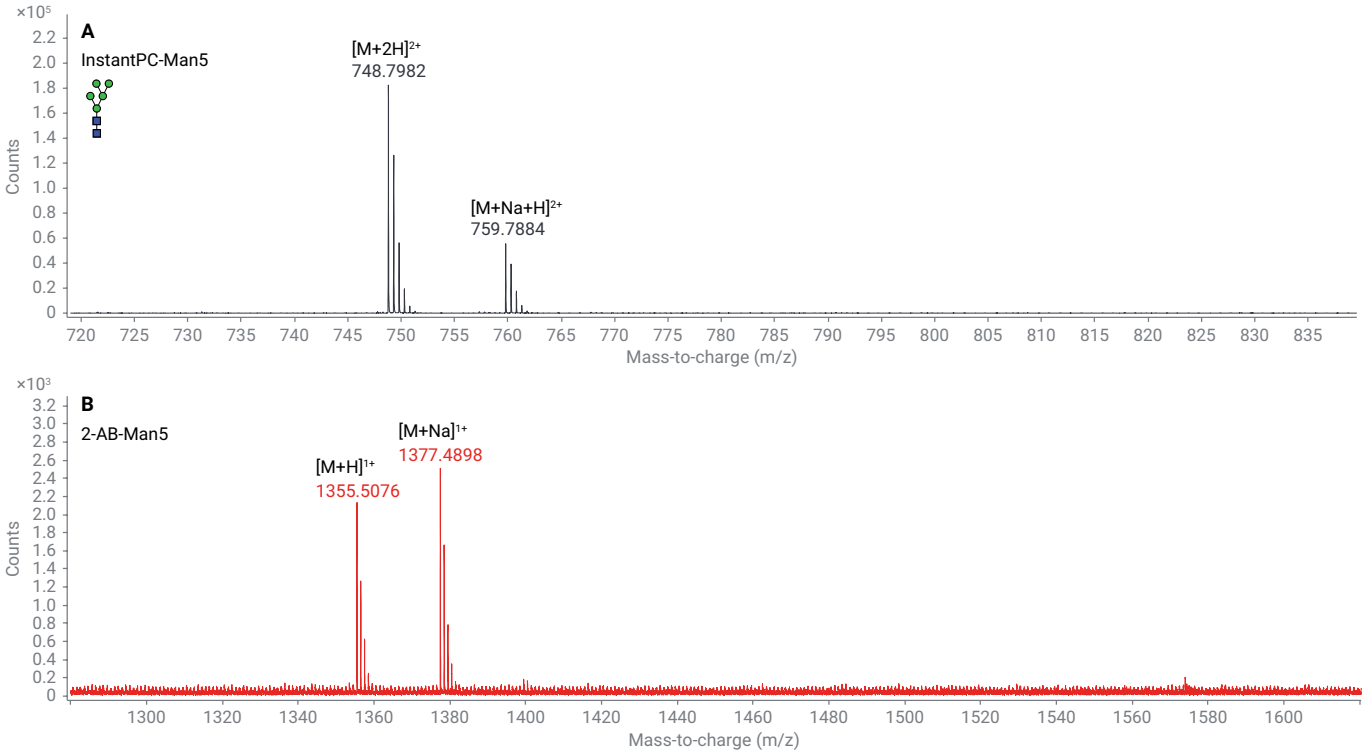

Figure 6. Mass spectrum comparison of Man5 from Enbrel, labeled with A) InstantPC and B) 2-AB.

Conclusions

Glycosylation is a feature of many biotherapeutic proteins and is often a CQA that must be monitored. N-Glycan analysis is important in the development and production of therapeutic proteins. Gly-X N-glycan sample preparation workflows enable a five minute release of N-linked glycans suitable for labeling both by glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB. These workflows allow for instant glycosylamine labeling with InstantPC or no dry down on-matrix reductive amination labeling with 2-AB. Glycan species were profiled by relative fluorescence peak area % and peak assignments confirmed by high resolution mass spectrometry. Compared to 2-AB, InstantPC labeled glycans display higher FLD signal and greater MS ionization efficiency in positive mode, allowing for confident detection of low abundance glycan species. Although the performance benefits of InstantPC are clear, 2-AB is an N-glycan label that has been used for many years. Therefore, a rapid 2-AB workflow enables continuity with historical 2-AB N-glycan data sets.

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Application Note

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An Improved Workflow for Profiling and Quantitation of Sialic Acids in Biotherapeutics

Authors

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Agilent Technologies, Inc.

Introduction

This application note describes use of the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) to profile and perform absolute quantitation of sialic acids present in biotherapeutic glycoproteins as well as the NISTmAb. This kit uses a new and improved high-throughput workflow for the preparation, separation, and detection of sialic acids labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of biotherapeutic glycoproteins.[1]

The workflow described here demonstrates the AdvanceBio Sialic Acid profiling and quantitation kit for release of terminal sialic acid by acid hydrolysis, followed by DMB labeling and both qualitative and quantitative analysis. DMB-labeled sialic acids from samples and standards are separated by reversed-phase liquid chromatography (LC) and quantitated using fluorescence detection (FLD) and structurally confirmed by mass spectrometry (MS).

Introduction

The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics.[2] Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory.[3–5] Two forms commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species, while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to monitor the absolute quantity of sialic acid, as well as the levels of different sialic acid species present in therapeutic glycoproteins.

Presented here is a new high-throughput workflow based on a 96-well plate format for the release, labeling, and analysis of sialic acids from therapeutic glycoproteins using rituximab, etanercept, NISTmAb, and cetuximab as examples. Sialic acid residues are released then labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in a two-step procedure. DMB-labeled sialic acids are then separated and analyzed using a rapid 10-minute method based on reversed-phase ultrahigh-performance liquid chromatography (UHPLC) with FLD detection for quantitation and optional MS detection for mass analysis. The workflow offers both qualitative characterization of Neu5Ac, Neu5Gc, and other sialic acid species using a sialic acid reference panel (SARP), as well as absolute quantitation with picomole level sensitivity using included Neu5Ac and Neu5Gc quantitative standards. The workflow enables reliable and reproducible high-throughput sample preparation for the profiling and quantitation of sialic acids. This kit provides a broad detection range and improved sensitivity for molecules with low levels of sialylation.

Experimental

Sample preparation

Samples were prepared using a using a 96-well plate format. Sialic acids were released from rituximab (Rituxan, lot number M190170), etanercept (Enbrel, lot number M190088), NISTmAb (lot number 14HB-D-002), and Erbitux (cetuximab, lot number MI60886) through an acid hydrolysis reaction. The method eliminates the need for a dry-down step, thereby decreasing overall sample preparation time by 1 to 2 hours. The sample amount is typically 200 µg of glycoprotein with low-level sialylation and 5 µg of highly sialylated glycoprotein. Serial dilutions of sialic acid reference standards Neu5Ac and Neu5Gc were used to prepare a standard curve and to determine the limit of quantitation (LOQ) and limit of detection (LOD) for the assay. Released sialic acids, SARP, and standards were then derivatized with DMB following the workflow illustrated in Figure 1, release and labeling steps were carried out in a thermocycler.

LC/FLD/MS analysis of DMB-labeled sialic acids

DMB-labeled sialic acids from Rituxan, Enbrel, NISTmAb, and Erbitux were analyzed using reversed-phase (RP) separation with an Agilent 1290 Infinity II LC system with fluorescence detection (FLD) for quantitation. All RP-UHPLC separations were conducted under the conditions described in Table 1. Additional inline analysis using an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer (Table 2) was also performed to confirm elution order of the DMB-labeled sialic acid species present in the SARP. A fixed flow splitter was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. The data was analyzed with Agilent OpenLab CDS 2.3 and MassHunter Qualitative Analysis 10.0 software. Neu5Gc and Neu5Ac were quantified using the calibration curves.

Materials

Acetonitrile (LC/MS grade, Honeywell Burdick & Jackson) was purchased from VWR. Methanol (Optima LC/MS grade) was purchased from Fisher Scientific. Nanopure water generated in-house was used for all experiments.

Instrumentation

DMB-labeled sialic acid samples were separated using an Agilent InfinityLab Poroshell 120 EC-C18 column (2.1 × 75 mm, 2.7 µm; p/n 697775-902) using the method details in Table 1, on an Agilent LC/MS setup 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)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 2)

Software

- Agilent OpenLab CDS 2.3
- Agilent MassHunter Qualitative Analysis 10.0

Table 1. Reversed-phase UHPLC conditions.

Conditions, Agilent 1290 Infinity II LC System

Parameter	Value			
Column:	Agilent InfinityLab PoroShell 120EC-C18, 2.1 × 75 mm, 2.7 µm (p/n 697775-902)			
Column Temp:	30 °C			
Mobile phase:	A) Methanol:acetonitrile:water (4:8:88) B) Acetonitrile			
Gradient:	Time (min) 0 6 6.25 7.3 7.5 10	% A 100 100 20 20 100 100	% B 0 0 80 80 0 0	 Isocratic elution Wash Re-equilibration
Flow rate:	0.4 mL/min			
Injection volume:	10 µL			
Detection:	Agilent 1260 Infinity II FLD λEx 373 nm, λEm 448 nm			

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

6545XT AdvanceBio LC/Q-TOF parameters	
Source	Dual AJS ESI
Gas temperature	350 °C
Drying Gas Flow	11 L/min
Nebulizer	15 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Vcap	1,400 V
Nozzle Voltage	1,800 V
Fragmentor	120 V
Skimmer	65 V
Mass Range (MS)	m/z 400 to 1,000
Mass Range (MS/MS)	m/z 100 to 550
Acquisition Mode	High resolution (4 GHz)

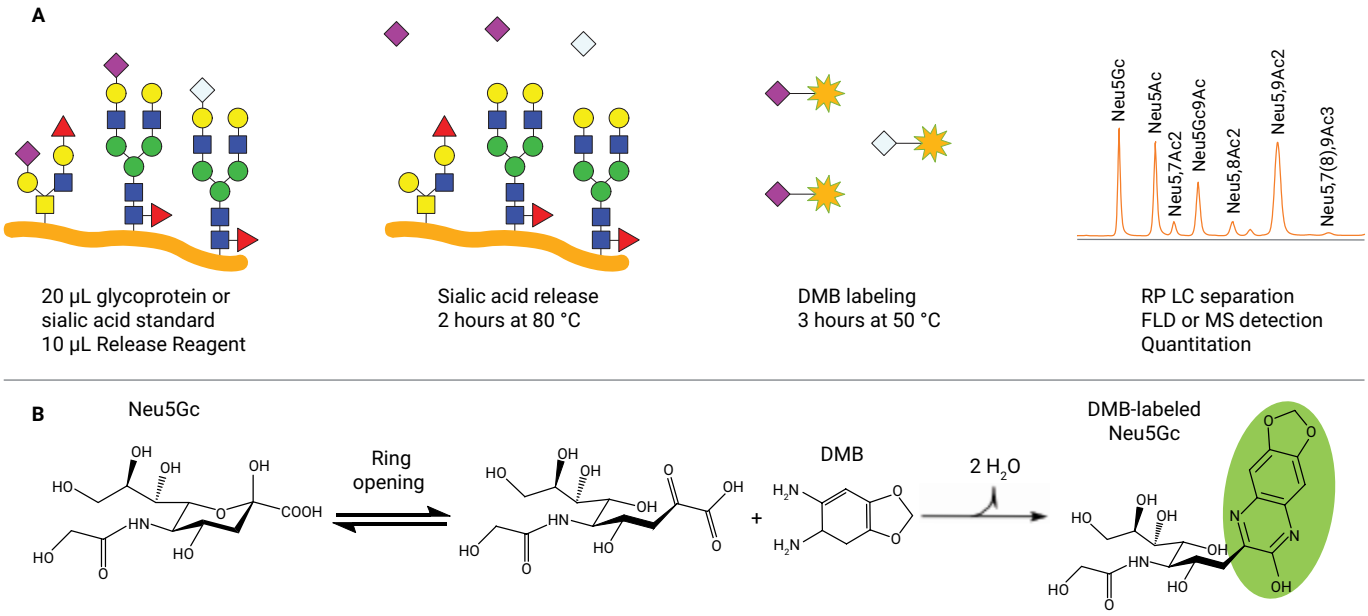

Figure 1. Sialic acid release and DMB labeling workflow (A) overview (B) DMB labeling mechanism of sialic acid Neu5Gc.

Results and discussion

LC/FLD/MS analysis of DMB-labeled sialic acids

RP-UHPLC analysis of DMB-labeled SARP results in the separation and detection of seven sialic acid derivatives: Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc,9Ac, Neu5,8Ac2, Neu5,9Ac2, and Neu5,7(8),9Ac3. While differences in retention times may be observed with different columns, flow rate, solvents, or laboratory conditions, the elution order of DMB-derivatized sialic acids remains consistent. The reference panel is used to evaluate the resolution and accuracy of the chromatographic system at the beginning of the sample sequence. A typical FLD chromatogram of DMB-labeled SARP is shown in Figure 2A. Identification of the DMB-sialic acid derivatives was confirmed by mass spectrometry (Figure 2B).

Analysis of sialic acid content of biotherapeutics and NISTmAb

DMB-labeled sialic acids identified by applying the workflow to Rituxan, Enbrel, NISTmAb, and Erbitux are shown in Figure 3. Both Rituxan (Figure 3A) and Enbrel (Figure 3B) contain primarily Neu5Ac while NISTmAb (Figure 3C) and Erbitux (Figure 3D) contain primarily Neu5Gc. Mass spectra of major peaks in DMB-labeled samples from Enbrel and Erbitux confirm their identities as Neu5Ac and Neu5Gc, respectively (Figure 4).

Quantitative analysis of sialic acid content

Based on the chromatographic separation and fluorescence response of DMB-labeled Neu5Ac and Neu5Gc standards, a quantitative calibration curve was generated (Figure 5). The LOD and LOQ were calculated using the noise determined by OpenLab CDS 2.3 using P2P noise calculation. The detectable mole quantities of Neu5Gc and Neu5Ac from Rituxan, Enbrel, NISTmAb, and Erbitux was determined based on integrated peak areas and listed in Table 3. Total sialic acid quantitation results are consistent with those obtained from the AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ) (Table 4). The kit also shows improved performance compared to an older DMB labeling workflow (p/n GKK-407) (Table 5) by allowing an increased concentration of glycoprotein per sample well as a decrease in sample dilution prior to analysis, resulting in an increase in fluorescence signal for DMB labeled sialic acids.

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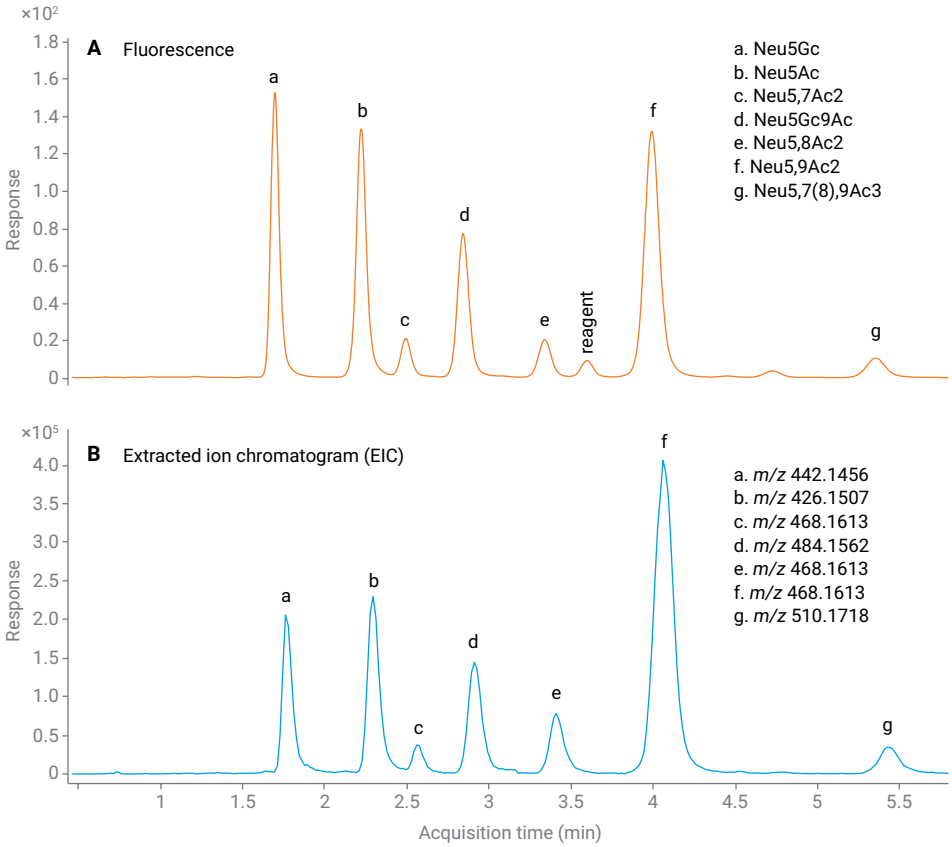

Figure 2. UHPLC chromatogram of DMB-labeled SARP. (A) fluorescence; (B) extracted ion chromatogram of DMB-labeled sialic acid species, [M+H]1+.

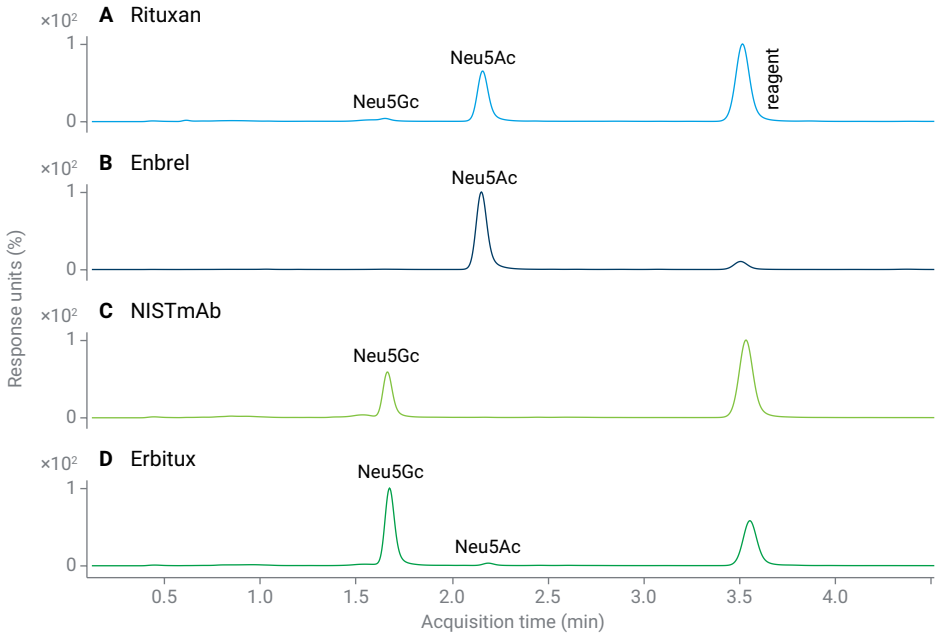

Figure 3. UHPLC fluorescence profiles of DMB-labeled sialic acids from different glycoproteins (A) Rituxan; (B) Enbrel; (C) NISTmAb; and (D) Erbitux.

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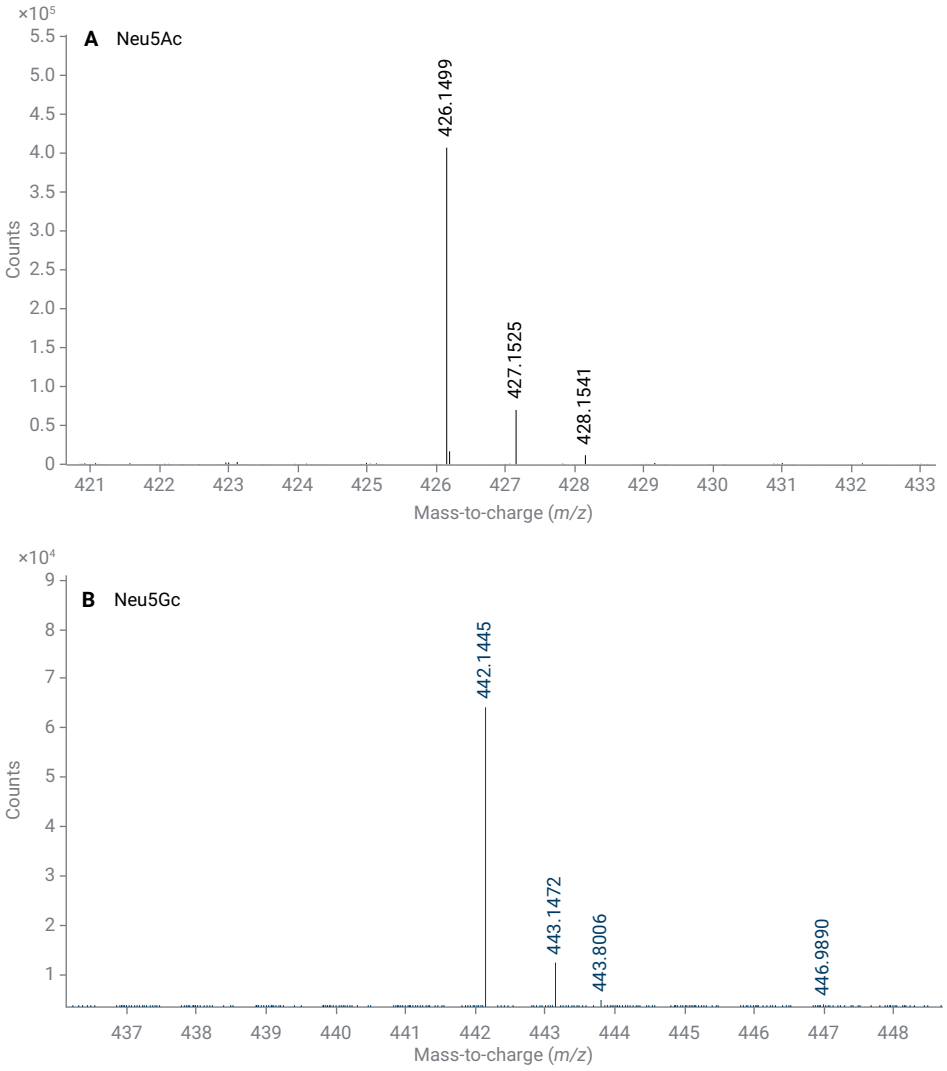
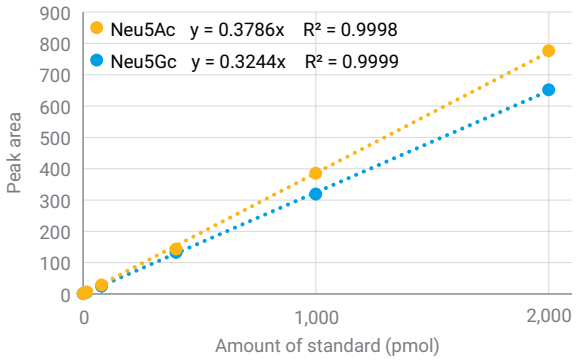


Figure 4. Mass spectrum of DMB-labeled sialic acid (A) Neu5Ac from Enbrel; and (B) Neu5Gc from Erbitux.



Sialic Acid	LOD (pmol)	LOQ (pmol)
Neu5Ac	0.016	0.053
Neu5Gc	0.0162	0.040

Figure 5. Neu5Ac and Neu5Gc calibration curves, n = 2. LOD and LOQ for Neu5Ac and Neu5Gc are shown in the table.

Table 3. Average pmol/µg of Neu5Ac and Neu5Gc for each glycoprotein is shown in the table, n = 3. ND = not detected.

Column	Concentration (mg/ml)	RSample Mass (µg)	Neu5Ac (pmol/µg)	%CV	Neu5Gc (pmol/µg)	%CV
Rituxan	10	200	0.60	4.2%	0.02	1.8%
Enbrel	0.25	5	228	6.9%	ND	-
NIST mAb	10	200	ND	-	0.36	1.8%
Erbitux	2	40	0.12	10.9%	3.72	7.1%

Table 4. Total sialic acid (Neu5Ac and Neu5Gc) with the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Agilent AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ), n = 3.

		GKK-407		Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit	
Glycoprotein	Sialic acid	pmol/µg	%CV	pmol/µg	%CV
Rituxan	Neu5Gc	ND	-	40.02	1.75%
	Neu5Ac	0.58	1.12%	0.60	4.25%
Enbrel	Neu5Ac	226	3.57%	223	2.92%
Erbitux	Neu5Gc	ND	-	3.68	1.02%
	Neu5Ac	ND	-	0.12	4.46%
Fetuin	Neu5Gc	ND	-	10.9%	3.72
	Neu5Ac	201	1.47%	222	4.44%

Table 5. Quantitation of Neu5Ac and Neu5Gc (pmol/µg) using the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Signal DMB Sialic Acid labeling kit (p/n GKK-407), n = 3. ND = not detected

	Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit		Agilent AdvanceBio Total Sialic Acid Quantitation Kit	
Peak	(pmol/µg)	%CV	(pmol/µg)	%CV
Rituxan	0.62	4.17%	0.47	5.04%
Enbrel	220	1.65%	210	12.34%
Erbitux	3.80	7.26%	3.49	0.69%
Fetuin	226	4.45%	232	7.39%

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Conclusions

The AdvanceBio Sialic Acid profiling and quantitation kit offers improved sensitivity for proteins with low levels of sialylation such as monoclonal antibodies with a single N-glycosylation site in the Fc region. The updated DMB labeling workflow eliminates the dry down step of samples, decreasing sample preparation time.

This workflow provides a method to determine both absolute and relative quantities of Neu5Ac and Neu5Gc present in biotherapeutics. Sample preparation uses a 96-well plate format for high-throughput sample preparation and is highly reproducible. Quantitative data is comparable to older DMB labeling workflows (GKK-407) and AdvanceBio Total Sialic Acid quantitation kit (GS48-SAQ) results.

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Application Note

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

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Introduction

This application note describes use of the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) to profile and perform absolute quantitation of sialic acids present in biotherapeutic glycoproteins as well as the NISTmAb. This kit uses a new and improved high-throughput workflow for the preparation, separation, and detection of sialic acids labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of biotherapeutic glycoproteins.[1]

The workflow described here demonstrates the AdvanceBio Sialic Acid profiling and quantitation kit for release of terminal sialic acid by acid hydrolysis, followed by DMB labeling and both qualitative and quantitative analysis. DMB-labeled sialic acids from samples and standards are separated by reversed-phase liquid chromatography (LC) and quantitated using fluorescence detection (FLD) and structurally confirmed by mass spectrometry (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.

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.

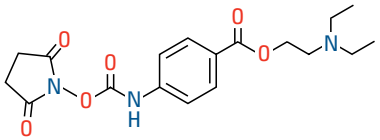

Figure 1. Diagram of InstantPC (ProZyme, Inc), an amine reactive instant label for fluorescence and MS detection of glycans.

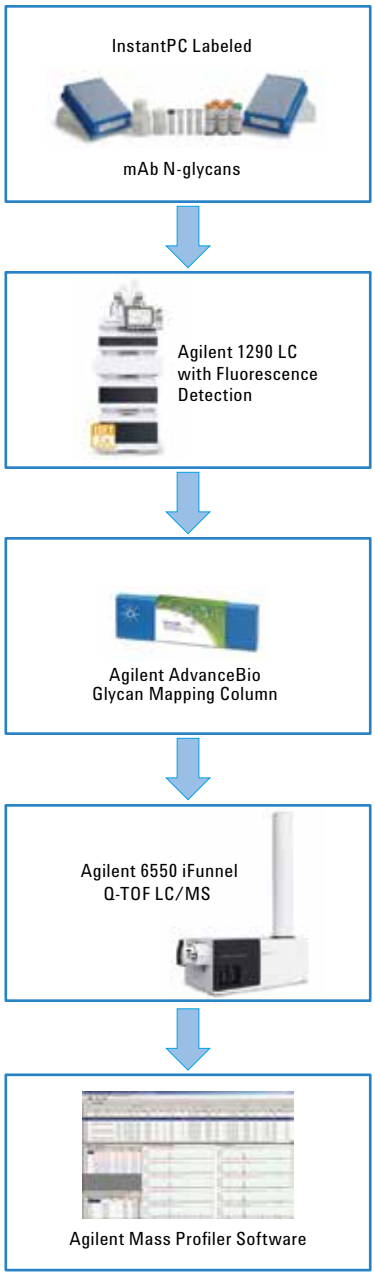

Figure 2. Workflow used for identification and quantification of InstantPC-labeled N-glycans from mAbs.

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

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.

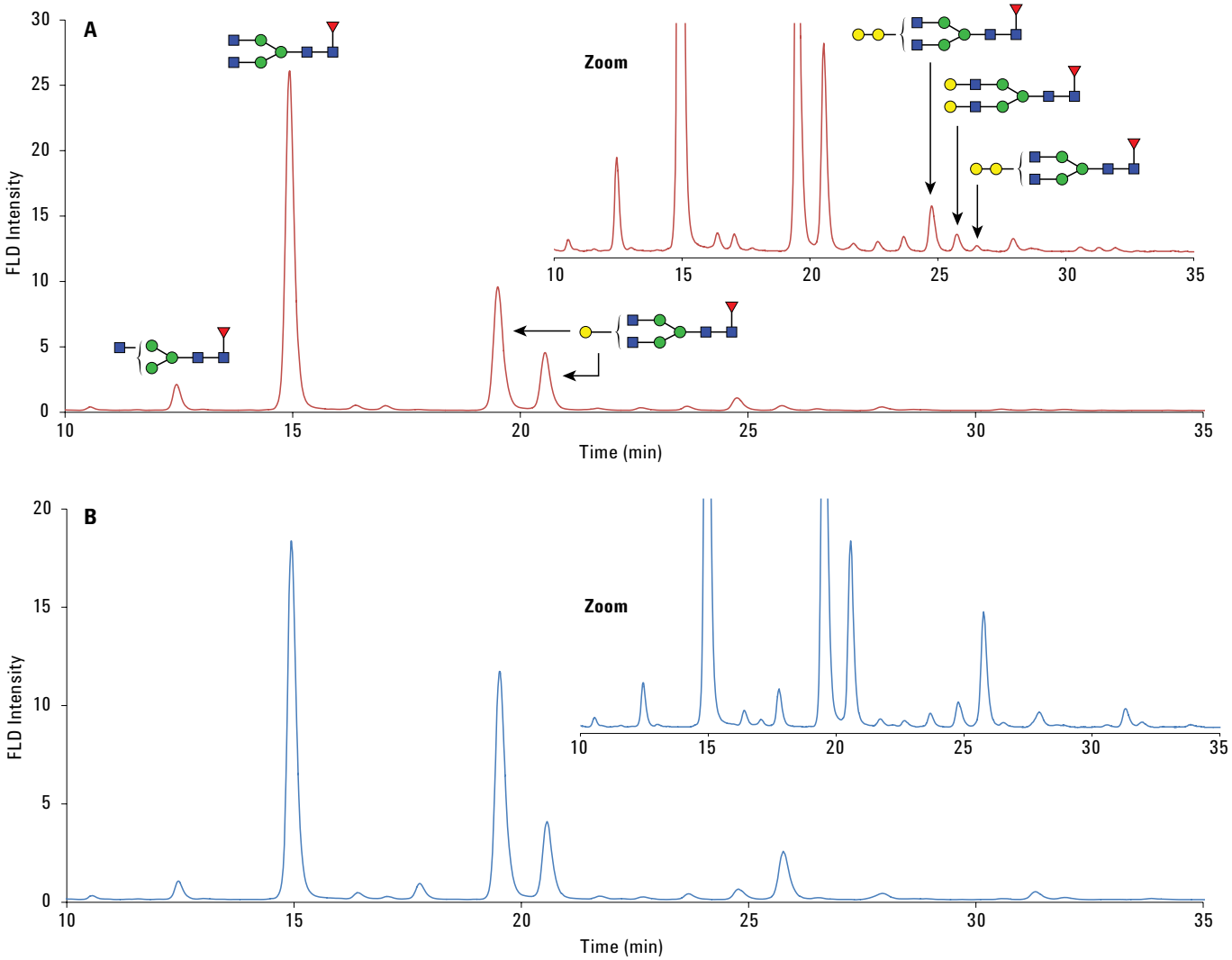

Figure 3. FLD chromatograms of InstantPC-labeled N-glycans released from mAb 1 and mAb 2. A) FLD chromatogram for mAb 1. B) FLD chromatogram for mAb 2.

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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.

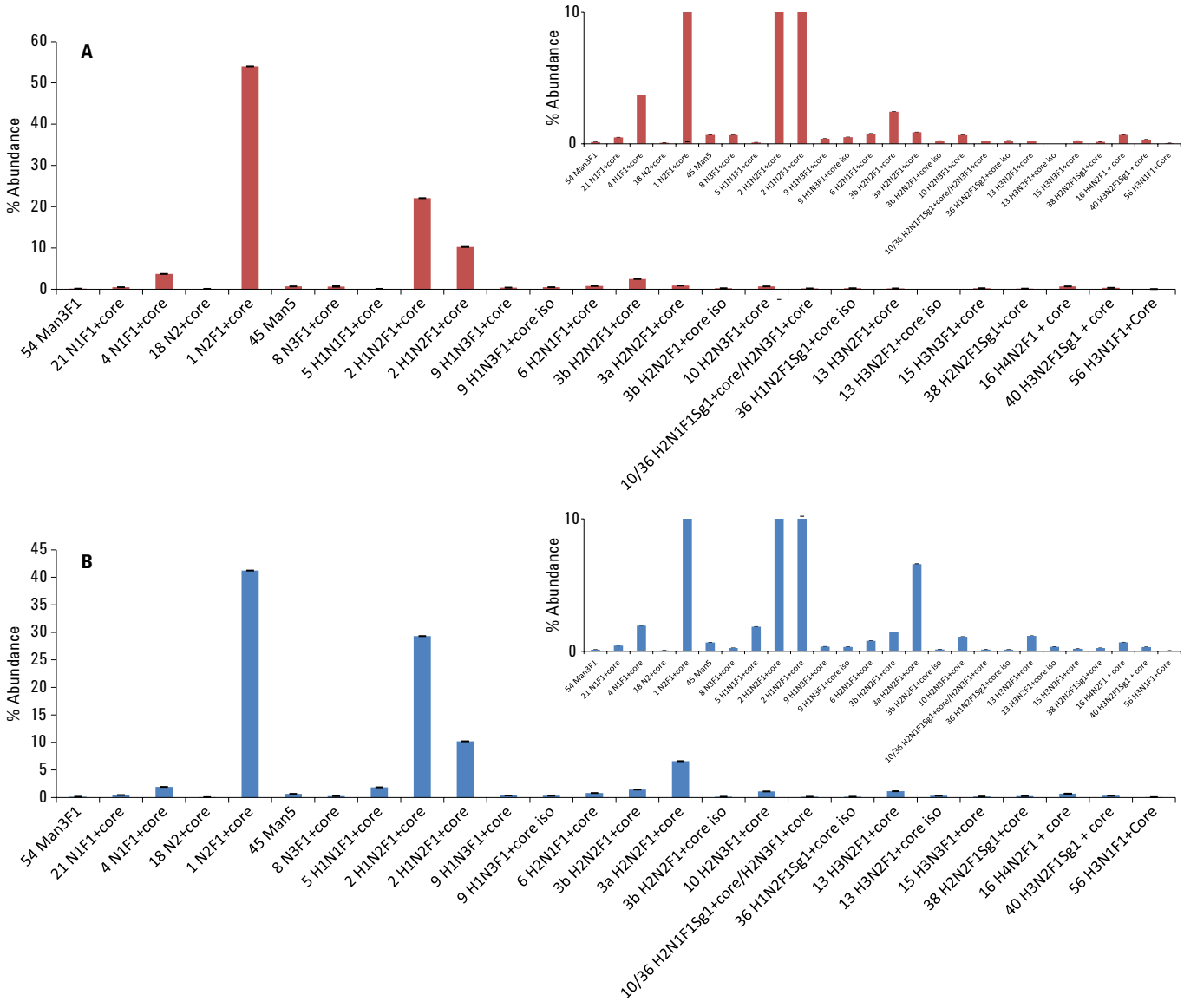

Figure 4. A) Relative FLD quantification of mAb 1 glycans. B) Relative FLD quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Integration of FLD signals was performed using Agilent MassHunter Qualitative Analysis Software. 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.

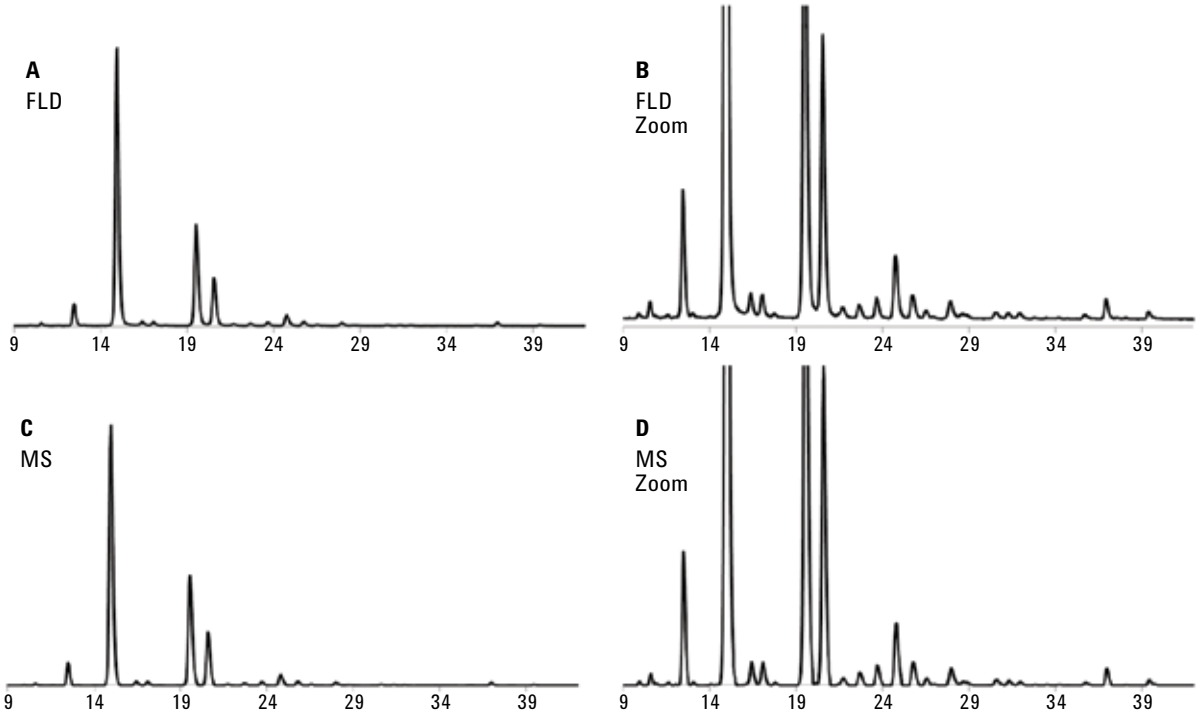

Figure 5. Comparison of FLD and MS chromatograms for mAb 1. A) FLD chromatogram of mAb 1 glycans. B) Zoom of FLD chromatogram of mAb 1 glycans. C) MS chromatogram of mAb 1 glycans. D) Zoom of MS chromatogram of mAb 1

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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.

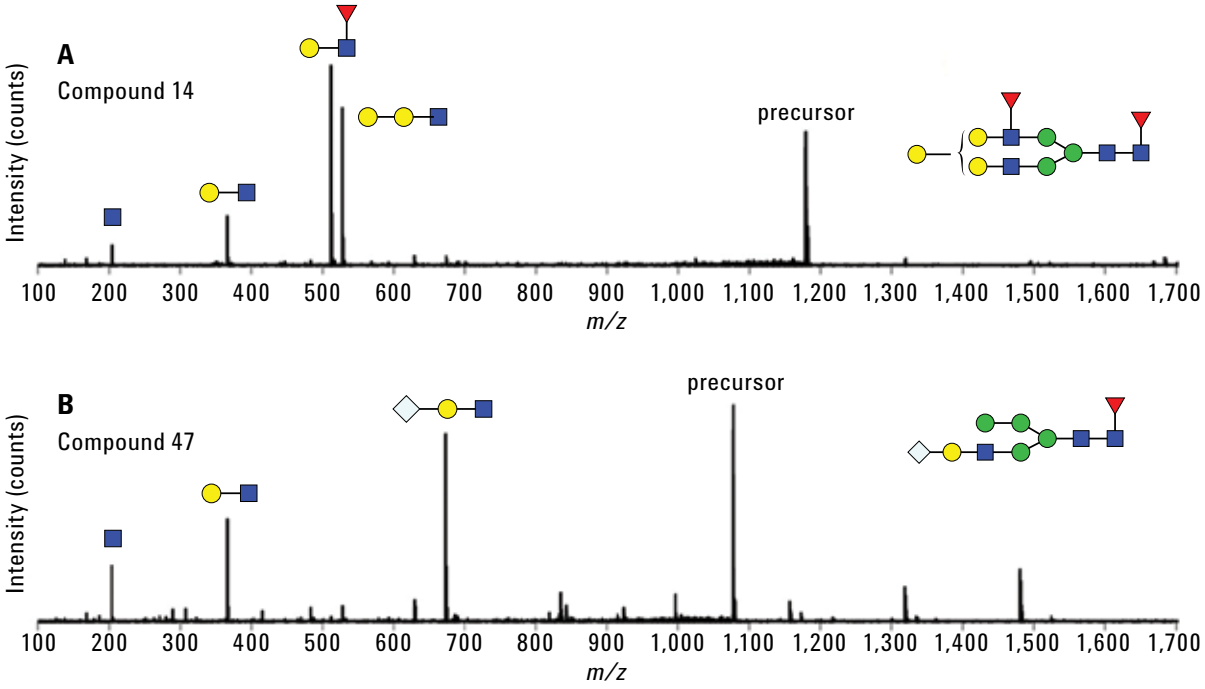

Figure 6. Tandem MS data were acquired for all glycans. MS/MS aided in compound identification when accurate mass was insufficient. The two examples above are consistent with gal-gal and outer arm fucose (A) and NeuGC (B) modifications.

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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.

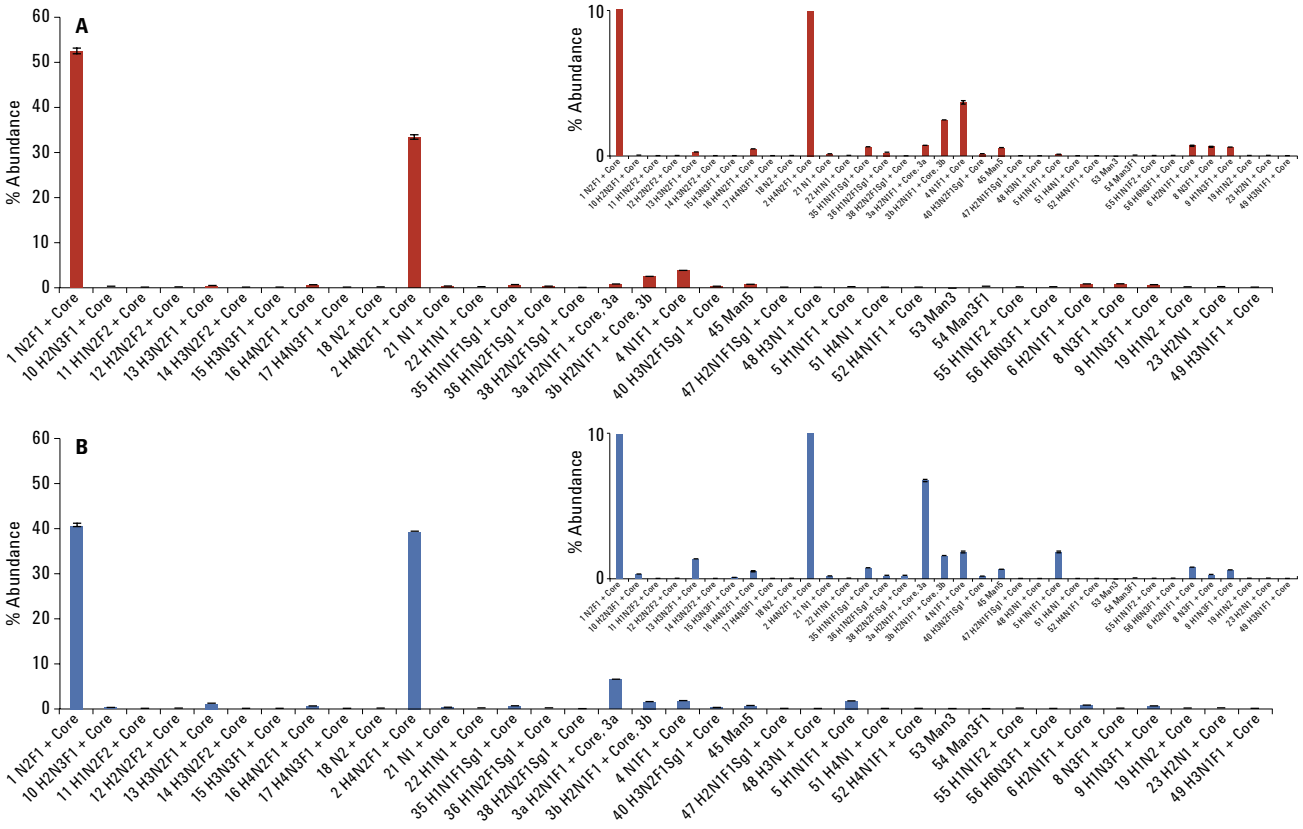

Figure 7. Relative MS quantification of InstantPC labeled N-glycans released from mAb 1 and mAb 2. A) Relative MS quantification of mAb 1 glycans. B) Relative MS quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Insets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

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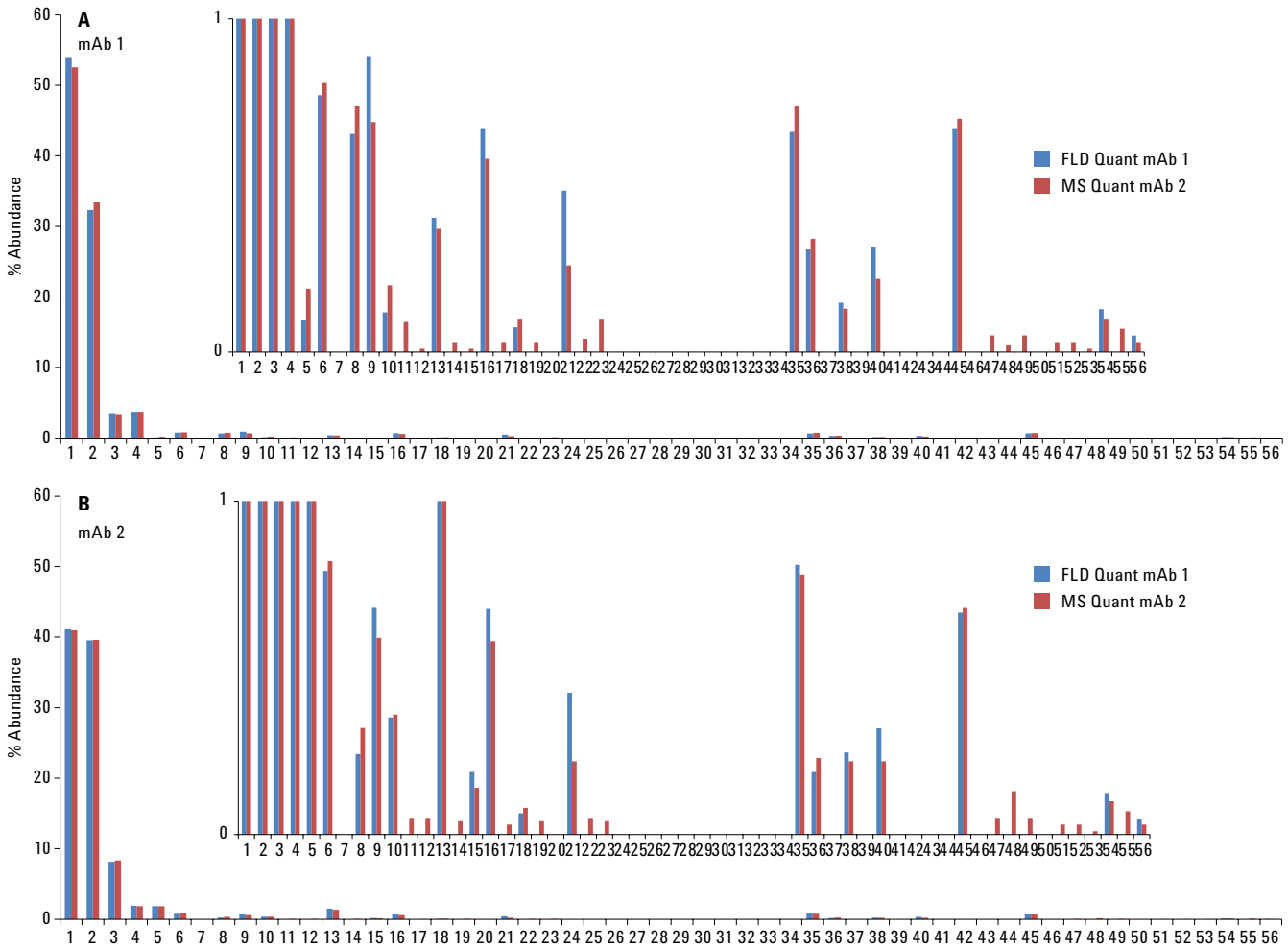

Figure 8. Comparison of fluorescence and relative MS abundance (area sum percentage) of InstantPC-labeled N-glycans from mAb 1 and mAb 2. The X-axis represents individual glycan compositions quantified in the study.

Conclusions

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.

References

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

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

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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.

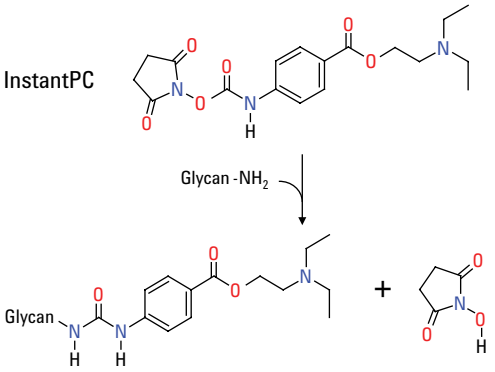

Figure 1. Diagram of InstantPC-labeled N-glycans released from an mAb.

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

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.

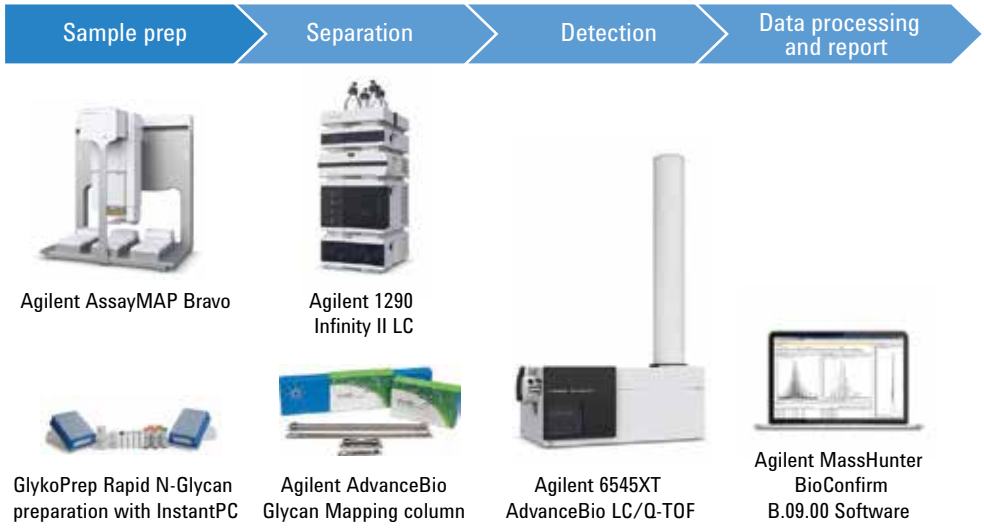

Figure 2. mAb Glycan characterization workflow.

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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 λEx = 285 nm, λEm = 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

Conditions, Agilent 1290 Infinity II LC System

Parameter	Value	
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm	
Column Temp:	40 °C	
Thermostat:	4 °C	
Solvent:	A) 50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide B) Acetonitrile	
Gradient:	Time (min)	% B
	0-0.5	75-71
	0.5-1.6	71-67.5
	1-22	67.5-60
	22-22.5	60-40
	22.5-23.5	40 (0.7 mL/min)
	23.5-24	40-75 (0.7 mL/min)
	24-30	75 (0.9 mL/min)
Flow rate:	0.4 mL/min	
Injection volume:	2.0 µL	

Agilent 1260 Infinity Fluorescence Detector (G1321B) was used.
The detector was set to λEx = 285 nm, λEm = 345 nm, with PMT gain = 10.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Acquisition mode	Low mass range, HiRes (4 GHz)
Mass Range (MS)	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).

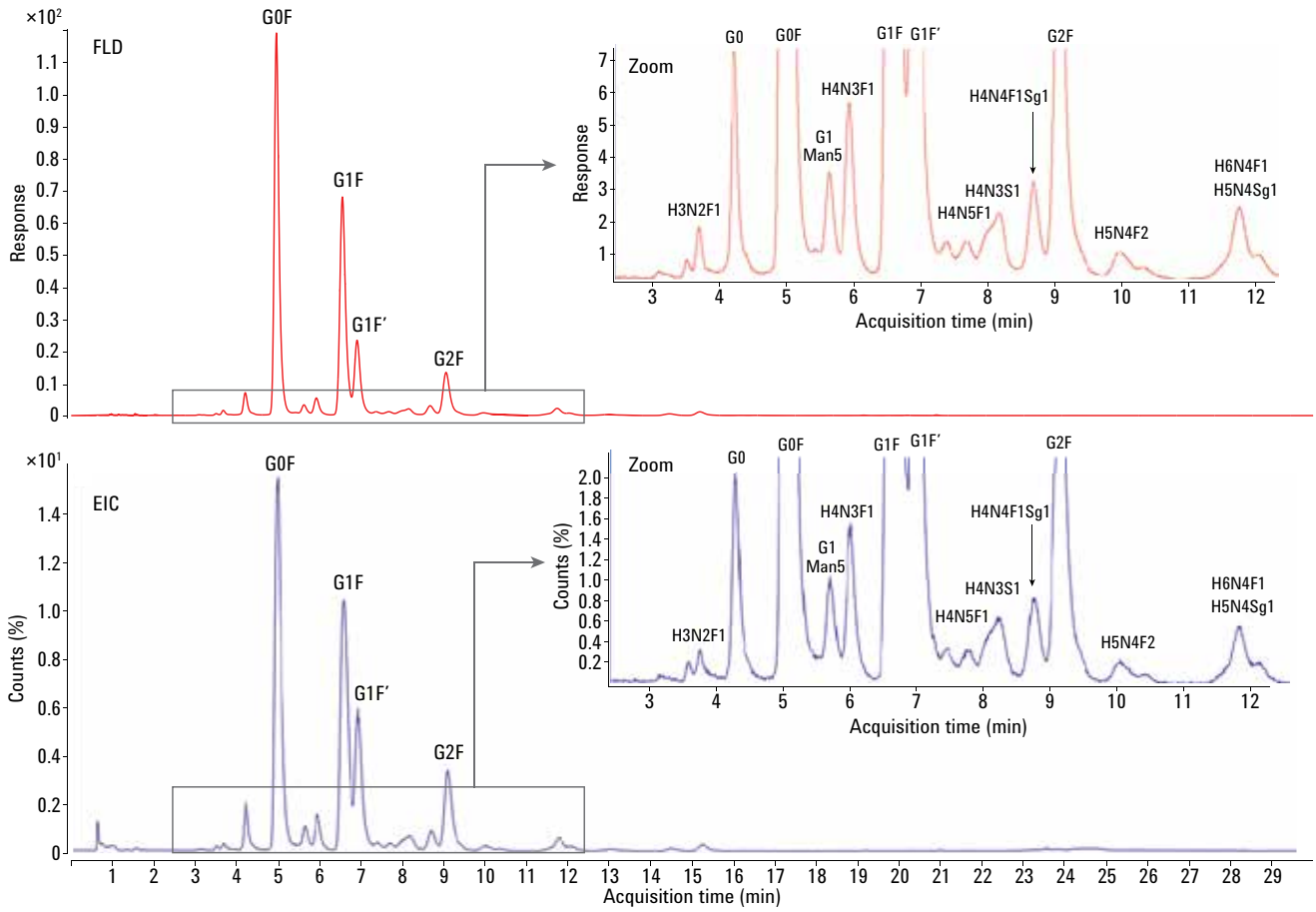

Figure 3. FLD chromatogram and mass spectra (EIC) of InstantPC-labeled N-glycans from NISTmAb

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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).

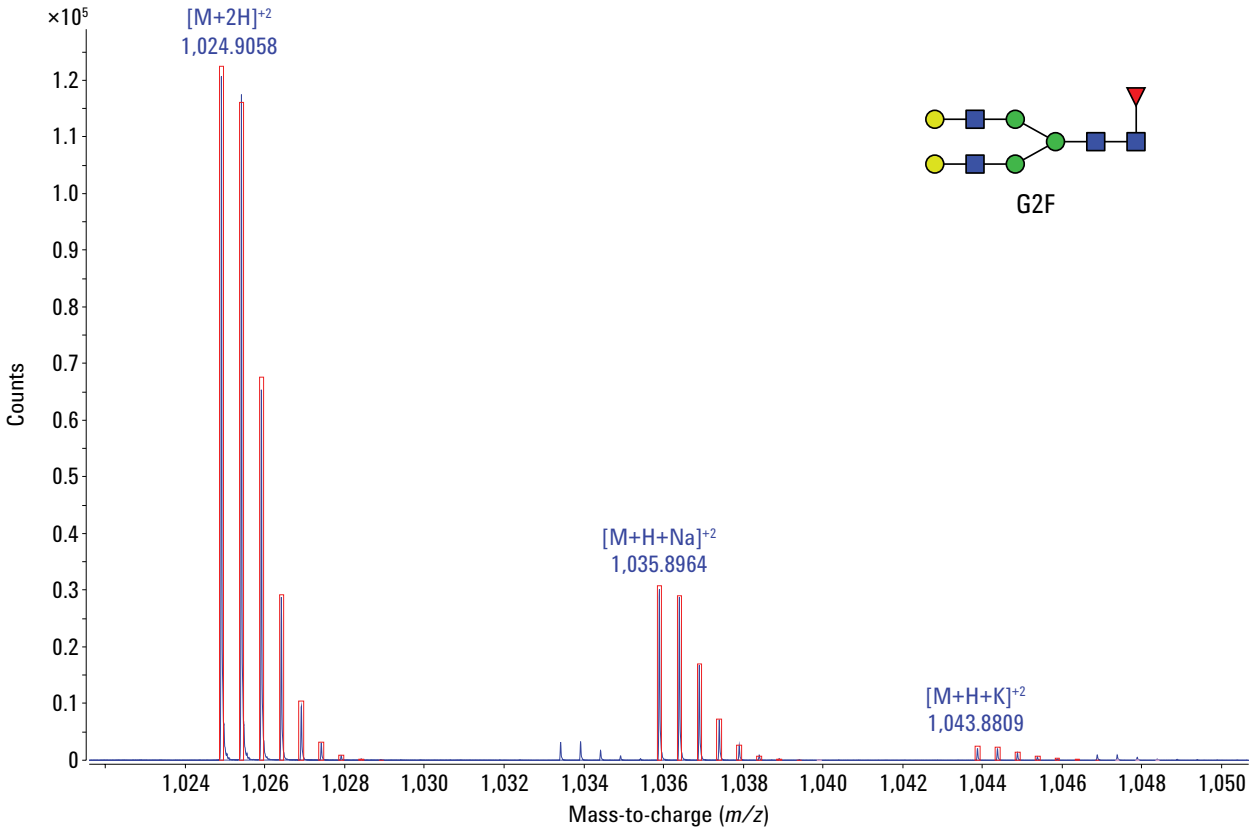

Figure 4. Representative spectrum of an InstantPC-labeled N-glycan (G2F). Excellent isotopic fidelity of the charge states of the InstantPC-labeled G2F glycan and its adducts. The red boxes represent the theoretical isotopic pattern, and the blue lines represent the actual raw MS spectrum.

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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 analysis4. 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.

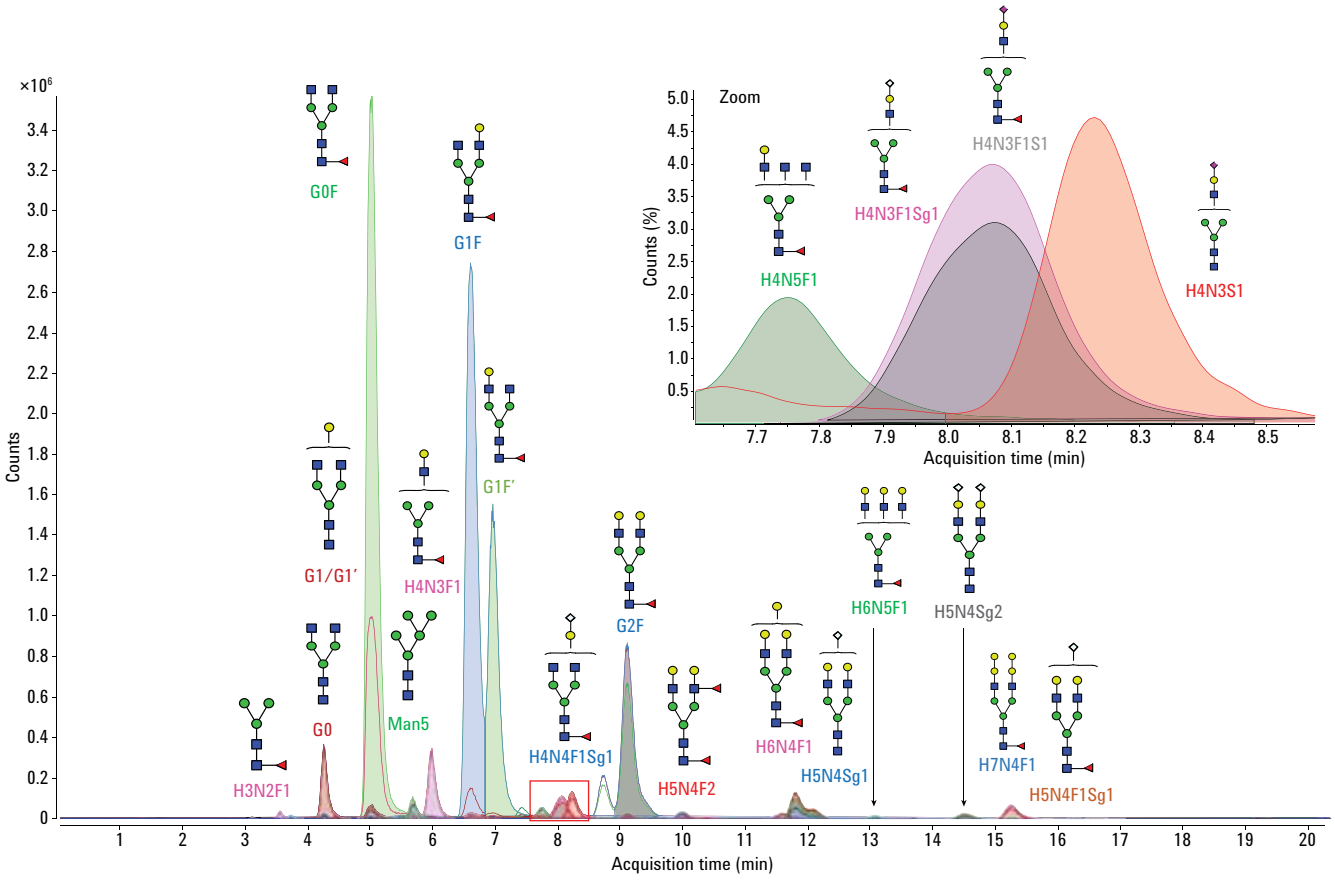

Figure 5. Extracted ion chromatograms of the identified glycans from NISTmAb. Inset: zoom of EICs of identified glycans eluted in the retention time range of 7.6–8.6 minutes.

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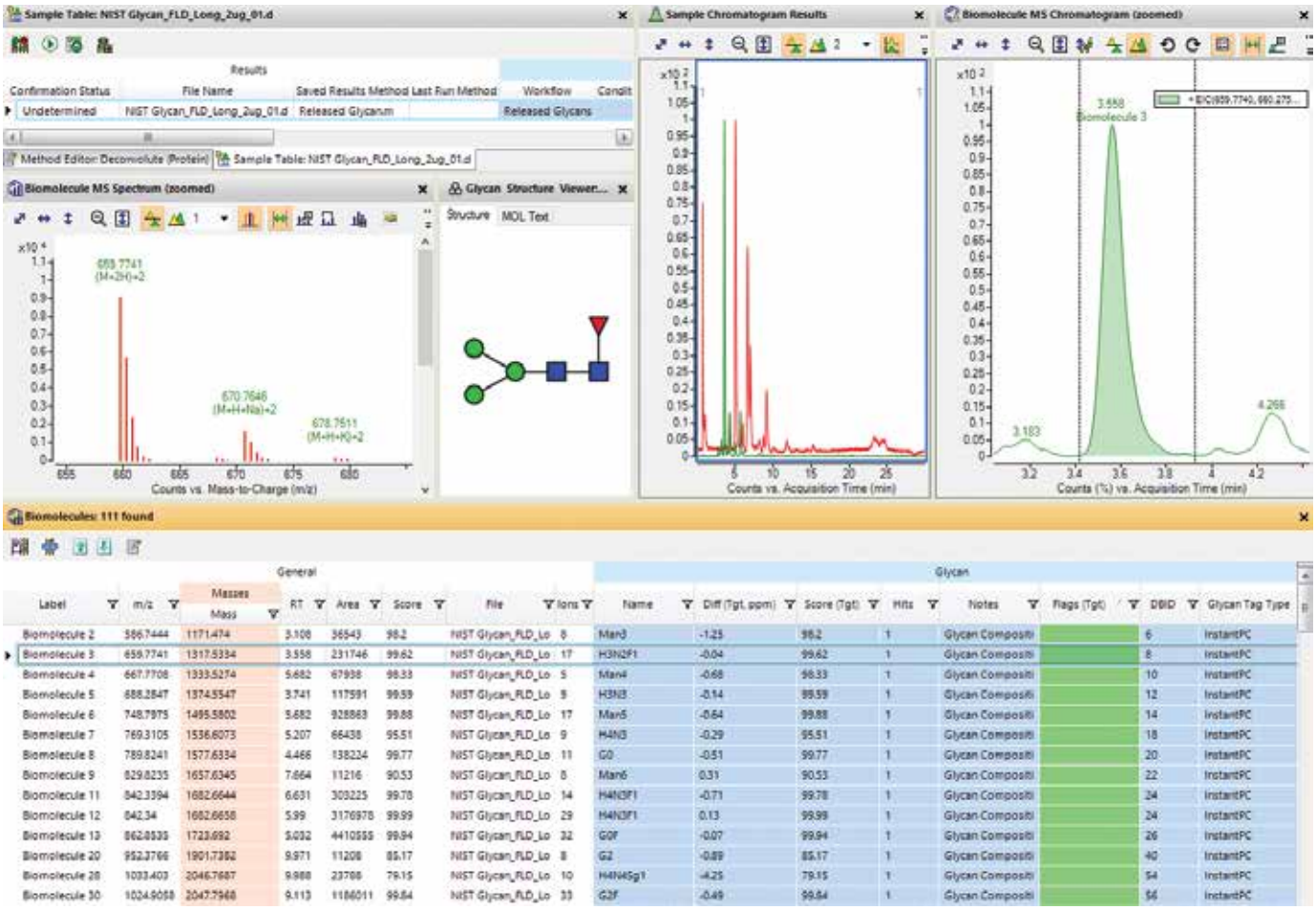

Figure 6. Screenshot of Agilent MassHunter BioConfirm B.09.00 software with representative glycan profiling results.

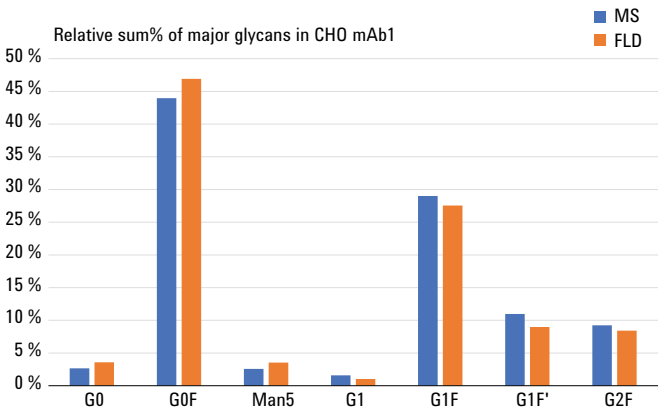

Figure 7. Relative sum % of the major N-glycans in the CHO mAb1 (0.5 µg), comparing results from MS-based quantitation (blue) with FLD-based quantitation (orange).

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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.

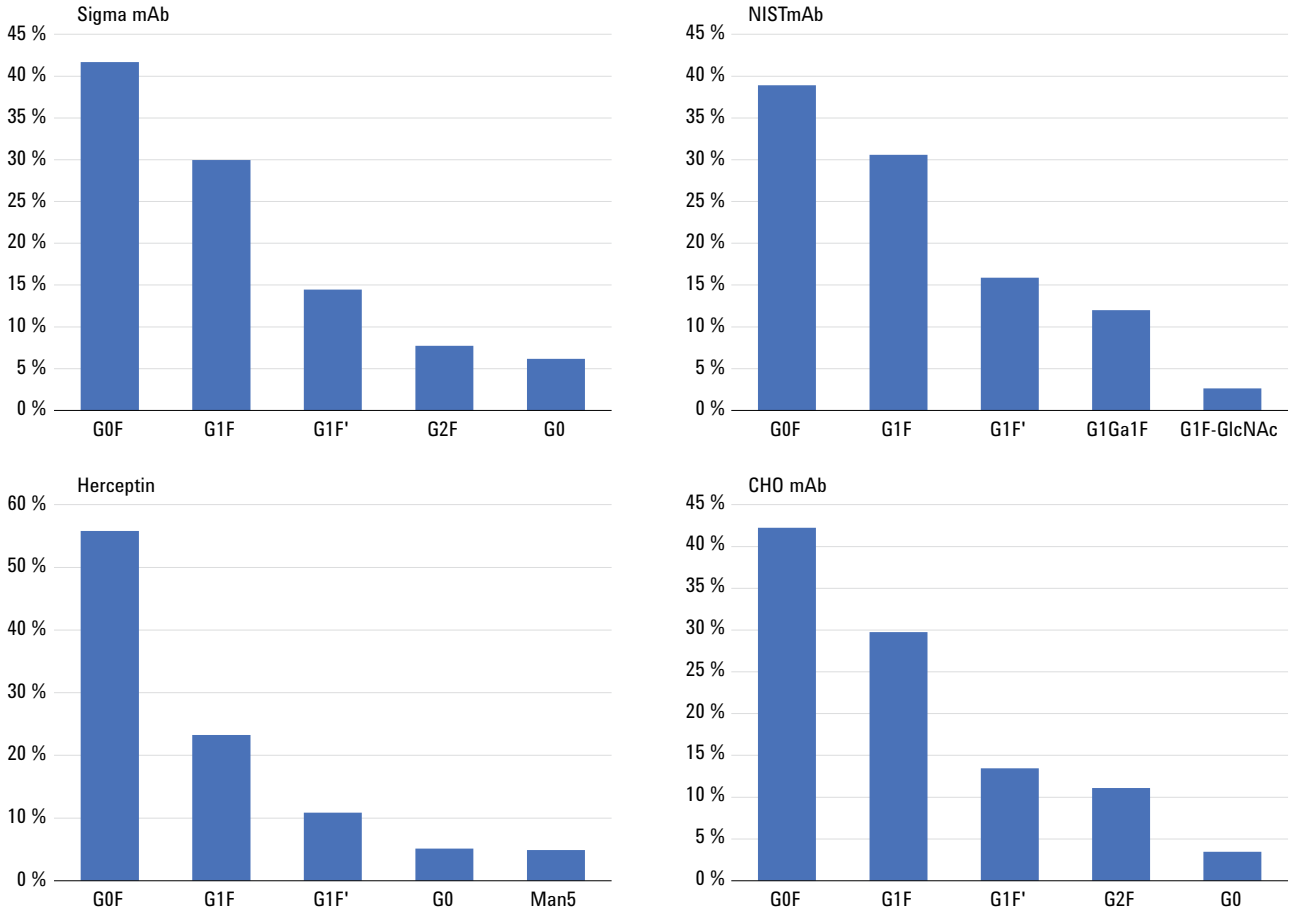

Figure 8. Relative sum % of the top five N-glycans in each of the four mAb samples.
Note: The NISTmAb contained a structure suspected to be G1F with an additional alpha-1,3-galactose, and this was labeled as G1Ga1F.

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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.

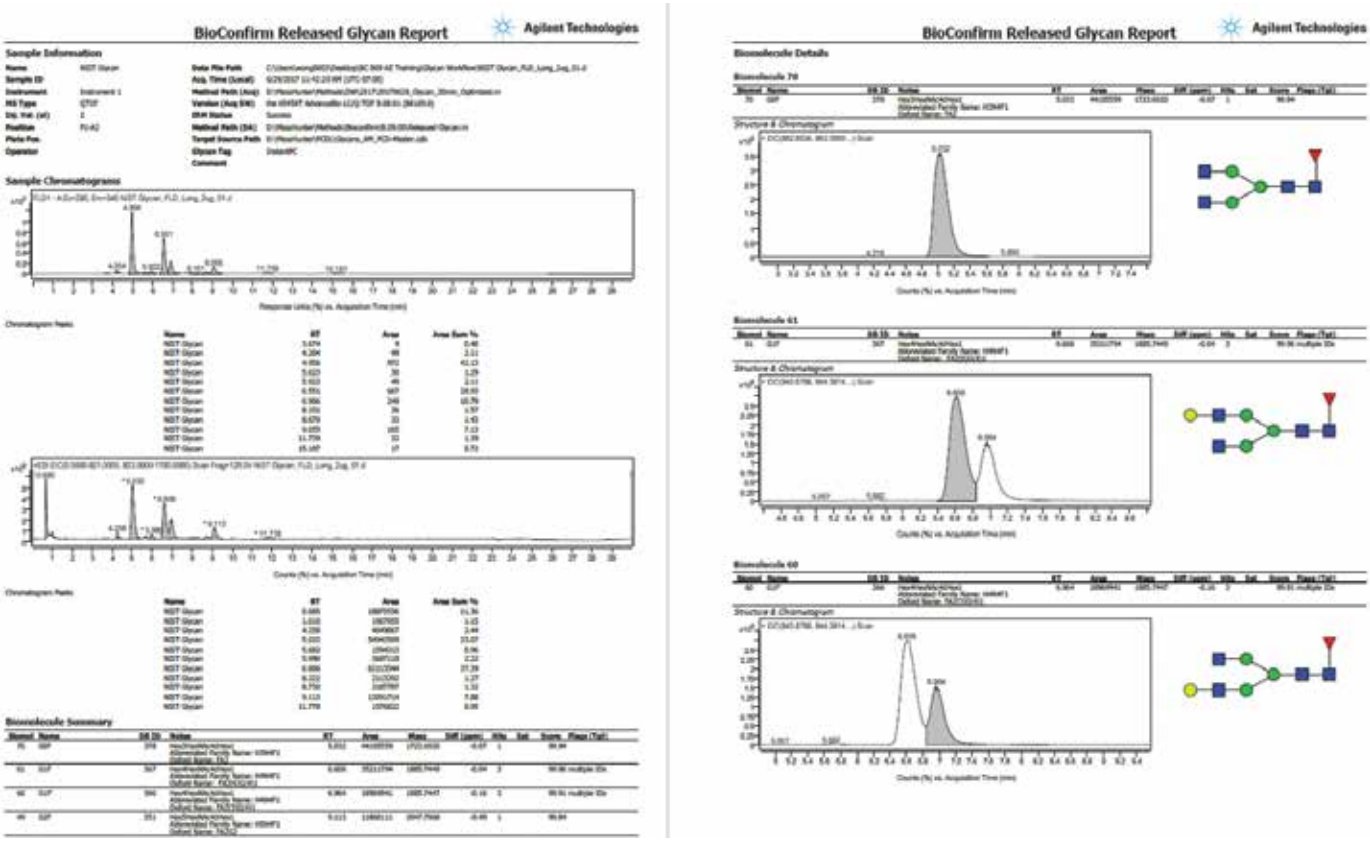

Figure 9. Agilent MassHunter BioConfirm B.09.00 Software – Released Glycan Report.

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Conclusions

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.

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3. N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection, *Agilent Technologies*, publication number 5991-5253EN.
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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

Glycan Analysis

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Fast and Efficient HILIC Methods for Improved Analysis of Complex Glycan Structures

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and Jia Liu
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Introduction

N-linked glycosylation is a critically important and very complex post-translational modification. It therefore needs to be controlled and monitored throughout development, processing, and manufacture of drug glycoproteins. Therapeutic protein characteristics, including safety, efficacy, and serum half-life, can be affected by differences in their glycosylation pattern, and so the analysis of these patterns is an important part of the characterization of therapeutic glycoproteins, particularly mAbs. Separation using HILIC with fluorescence detection is a robust method for glycan analysis, whereas HILIC/LC can also be coupled to mass spectrometry to obtain important mass and structure information.

One of the growing challenges in HILIC/LC, however, is achieving high-resolution separations with fast analysis times. With ever-increasing demands placed on biocharacterization for higher throughput, researchers are looking for improved separation (HILIC) methods, but not at the cost of lost separation performance. Since glycans include many closely related structures, it is critical to achieve the highest resolution possible, and preferably during a fast analysis time.

In this work, we used a sub-2 µm UHPLC HILIC column with amide chemistry for high-throughput glycosylation profiling. Specifically, we profiled 2-AB labeled human IgG and bovine fetuin N-linked glycans using a 1.8 µm, 2.1 × 150 mm column with fluorescence detection. Rapid, sensitive and selective separations were achieved to provide ultra-high resolution of these complex glycans in run times as short as 9 minutes. In a run time comparison to a currently available UHPLC glycan column, we observed a 40 % reduction in analysis speed for human IgG N-linked glycans under identical conditions.

Materials and Methods

Conditions, recombinant human IgG1

Parameter	Value
Sample:	Agilent 2-AB labeled IgG N-linked glycan library, 200 pmol (p/n5190-6996)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 µL in 70/30 ACN:water
Column temp:	55 °C
Sample thermostat:	10 °C
Detection:	Fluorescence, excitation 260 nm, emission 430 nm
Instrument:	Agilent 1290 Infinity LC with 1260 Fluorescence Detector

Conditions, bovine fetuin

Parameter	Value
Sample:	AdvanceBio 2-AB bovine fetuin N-linked library (p/n GKSB-002)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	5 µL (20 pmol) in 70/30 ACN:water
Column temp:	55 °C
	Other conditions as above

Conditions, dextran ladder

Parameter	Value
Sample:	Agilent 2-AB labeled dextran ladder standard GKSB-503
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 L 75:25 ACN:water (10 pmol total glycan)
	Other conditions as above

Conditions, mass spectrometry, recombinant human IgG1

Parameter	Value
Instrument:	Agilent 6550 iFunnel Q-TOF LC/MS
Source:	Agilent Dual JetStream
Drying gas temp:	200 °C
Drying gas flow:	12 L/min
Sheath gas temp:	250 °C
Sheath gas flow:	12 L/min
Nebulizer pressure:	25 psi
Capillary voltage:	3,500 V
Nozzle voltage:	500 V
Fragmentor voltage:	250 V
Skimmer voltage:	45 V
Octupole RF Vpp:	550 V
MS range:	100 to 1,700 m/z
MS scan rate:	2 spectra/s

The workflow is shown in Figure 1.

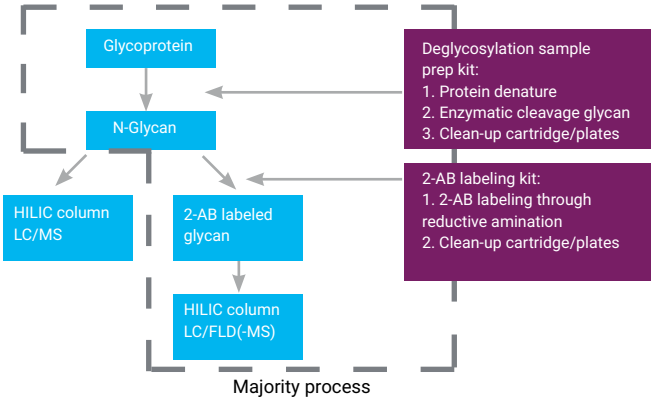

Figure 1. Total workflow solution used in an investigation of 2-AB-labeled antennary glycans using an AdvanceBio Glycan Mapping HILIC column with fluorescence detection.

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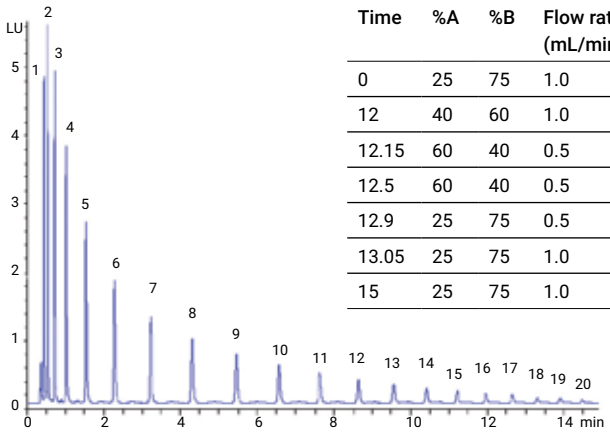
Results and Discussion

Dextran ladder

Figure 2 shows the separation of a 2-AB labeled dextran ladder. In this separation, a homopolymeric series of 20 glucose oligomers were efficiently baseline resolved in less than 15 minutes.



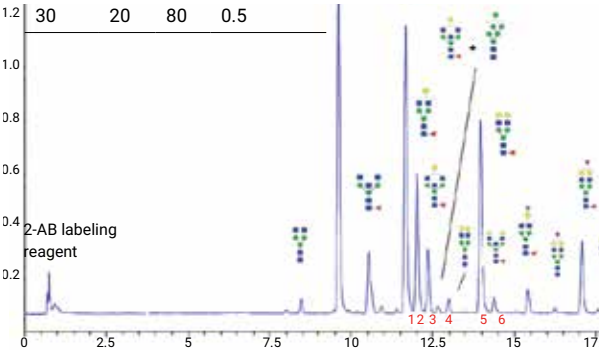
Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

Figure 2. The Agilent AdvanceBio Glycan Mapping column separates a 2-AB labeled dextran ladder to baseline in less than 15 minutes

Human IgG glycans

Figure 3 demonstrates an ultra-high resolution separation of 2-AB labeled N-linked human IgG glycans.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5

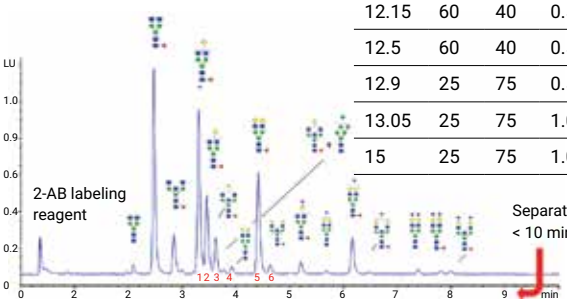


Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
12.7	2.60	2.90	5.43	2.81	0.0741	298	221

Figure 3. Very high resolution of 2-AB labeled N-linked human IgG glycans on the Agilent AdvanceBio Glycan Mapping column.

Fast separation in less than 10 minutes was also achieved, as shown in Figure 4.

Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0



Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
3.93	1.63	1.70	3.05	2.00	0.059	298	135

Figure 4. The Agilent AdvanceBio Glycan Mapping column separates 2-AB labeled N-linked human IgG glycans in less than 10 minutes.

MS N-linked glycans

Figure 5 shows eight representative mass spectra from the ultra-high resolution separation of human IgG glycans. The spectra were generated by Q-TOF analysis (experimental). All spectra matched theoretical masses to within 6 ppm.

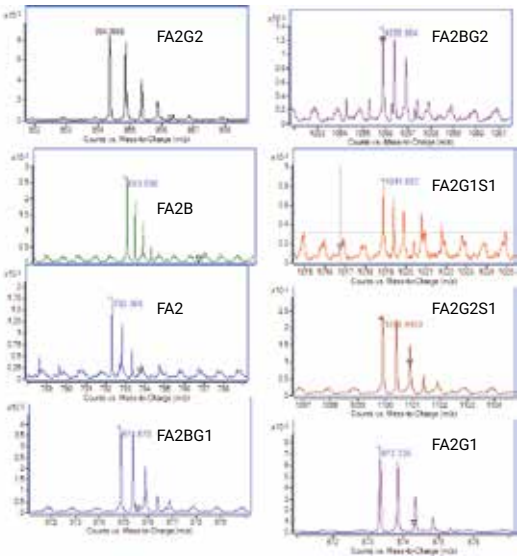


Figure 5. Eight representative mass spectra from the ultra-high resolution separation of human IgG glycans (fluorescence detector separations are displayed on the panel to the left).

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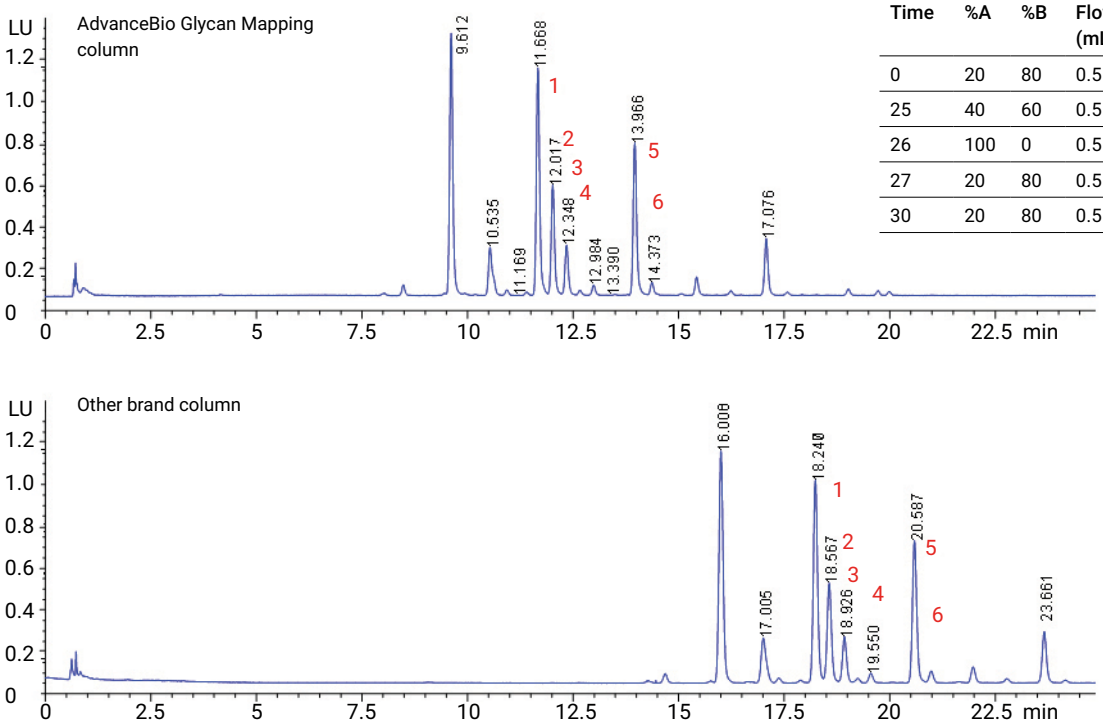
Sub-2 µm HILIC comparison

Figure 6 shows the results of a comparison of glycan amide columns. Using the same chromatographic conditions, the AdvanceBio Glycan Mapping column delivered better resolution and narrower bands, with higher peak capacity, at a 40 % faster separation time than another brand of sub-2 µm HILIC column in a 2.1 × 150 mm configuration.



Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5

Column	Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
Agilent AdvanceBio Glycan Mapping	12.7	2.60	2.90	5.43	2.81	0.0741	298	221
Other brand	20.2	1.77	1.94	3.39	2.10	0.1085	349	214

Figure 6. The AdvanceBio Glycan Mapping column delivers better resolution and narrower bands, with higher peak capacity at a 40 % faster separation time.

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Fetuin glycans

Finally, we revealed the fast and highly efficient performance of the AdvanceBio Glycan Mapping column in a separation of 2-AB labeled bovine fetuin N-linked glycans (Figure 7).

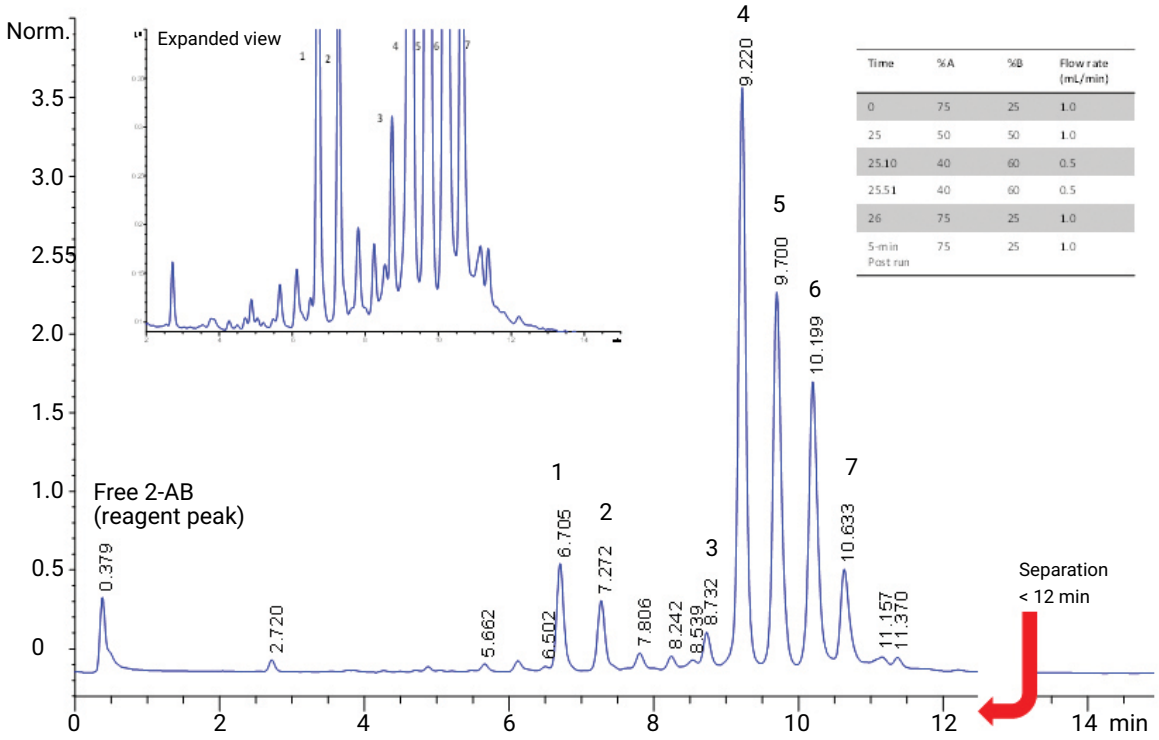

Figure 7. Fast and highly efficient separation of 2-AB labeled bovine fetuin N-linked glycans, 2.1 × 150 mm 1.8 µm AdvanceBio Glycan Mapping column.

Table 1. Glycan nomenclature and structure assignments for the optimized rapid separation of bovine fetuin 2-AB labeled N-linked glycans displayed in top chromatogram of Figure 7.

Peak	Retention	GU value	Glycan structure	Structures
1	6.70	9.4	A2G2S2	
2	7.27	9.8	A2G2S2	
3	8.73	10.8	A3G3S3, A3G3S2 (trace)	
4	9.22	11.2	A3G3S3, A3G3S2 (trace)	
5	9.70	11.6	A3G3S3, A3G3S4 (trace)	
6	10.20	12	A3G3S4, A3G3S3	
7	10.63	12.4	A3G3S4	

Galactose
N-acetylglucosamine
Fucose
Mannose
N-acetylneuramic acid

Conclusions

The Agilent 1.8 µm HILIC amide AdvanceBio Glycan Mapping column provided separation of N-linked glycans with high speed, excellent resolution and increased efficiency. In a 2.1 × 150 mm configuration and under identical chromatographic conditions, the column enabled a well-resolved separation of 2-AB labeled IgG N-linked glycans, with a 40 % reduction in elution time compared to another brand of sub-2 µm HILIC column. A separation of 2-AB labeled bovine fetuin N-linked glycans demonstrated the column's excellent analytical selectivity and resolving power for separating these complex biantennary and triantennary glycans.

Acknowledgement

This work was presented in a poster session at the 62nd ASMS Conference on Mass Spectrometry and Allied Topics, 15–19 June, 2014, Baltimore.

Application Note

Glycan Analysis

N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection

The Agilent 1290 Infinity Binary LC System with the Agilent AdvanceBio Glycan Mapping Column

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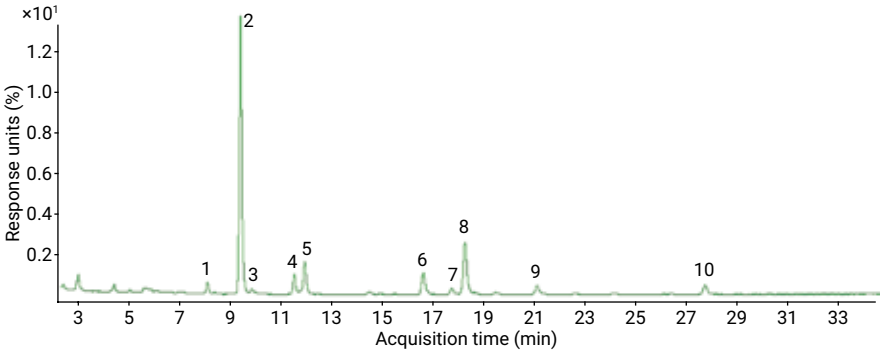
Abstract

This Application Note describes the analysis of N-linked glycans with hydrophilic interaction chromatography (HILIC) using the Agilent 1290 Infinity Binary LC together with the Agilent 1260 Infinity Fluorescence Detector and the Agilent 6530 Accurate-Mass Q-TOF LC/MS. Enzymatic glycan release with PNGase F followed by derivatization with 2-aminobenzamide (2-AB) was conducted on monoclonal antibodies (mAbs) and two other glycoproteins, fetuin and ovalbumin. The excellent resolution provided by the Agilent AdvanceBio Glycan Mapping column allowed detection and identification of all major N-glycans in the mAb sample. Furthermore, the highly complex N-glycans released from fetuin and ovalbumin were well resolved.



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Introduction

Glycosylation is one of the most frequently observed post translational modifications. Mammalian glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors, which consist of one or more monosaccharide units. A single glycosylation site can generate considerable heterogeneity of the mass and charge of glycoproteins. These oligosaccharides are involved in many biological regulation and recognition processes, for example, protein sorting, immune and receptor recognition, inflammation, pathogenicity, metastasis, and other cellular processes[1,2]. In addition, properties such as safety, efficacy, and the serum half-life of therapeutic proteins can be affected by their glycosylation pattern.

Recombinant monoclonal antibody therapeutics (mAbs) represent the largest group of therapeutic proteins. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern of the mAbs and, so far, all licensed therapeutic mAbs are immunoglobulins G (IgGs)[3]. Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn2974 (Figure 1), resulting in the presence of two N-glycans per IgG. This typically consists of a handful of major structures and numerous minor structures[5]. The combination of glycans at each of the two glycosylation sites on the Fc region leads to large numbers of different glycoforms in each batch of mAb production.

The glycan structure plays a critical role in complement activation and receptor affinity[6], which affect the efficacy of therapeutic mAbs. Moreover, non-human glycans are a safety issue due to induced immune responses. Therefore, analysis of the glycan pattern is an important part of the characterization of therapeutic glycoproteins, especially mAbs.

This Application Note uses symbolic glycan structures according to the Consortium for Functional Glycomics (CFG), as shown in Figure 2. Assigned glycans are also described by the Oxford glycan nomenclature and by another style of nomenclature, which is popular for mAb glycans, shown here in italics.

Figure 2A shows the general nomenclature used to describe sugar residues of different glycan structures on proteins. Figure 2B shows some predominant glycan structures present on the Asn-297 site in human IgG. In general, N-glycans have a core structure, containing two b-D-N-acetylglucosamine (GlcNac) and three mannose (Man) units. IgG Fc N-glycans are predominantly biantennary complex-type structures, partially core-fucosylated (for example, FA2 or G0F).

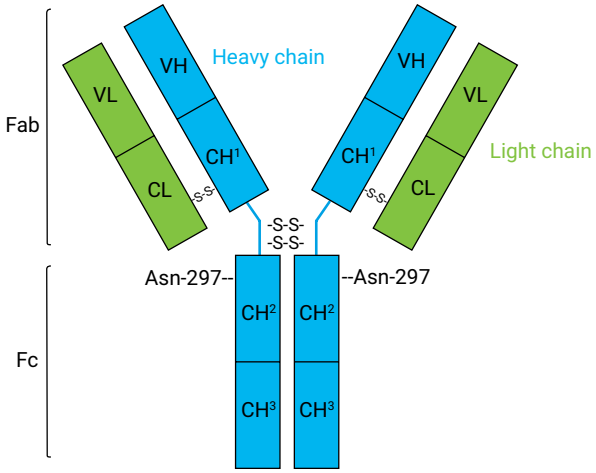

Figure 1. IgG antibody structure.

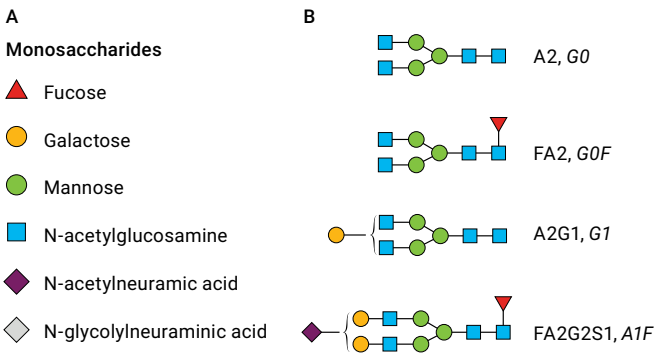

Figure 2. Glycan structure and isoforms. A) Monosaccharide description after the Consortium for Functional Glycomics, B) predominant glycan structures of human IgGs.

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Different strategies for the analysis of N-glycans have been described. Many methods are based on enzymatic release of N-glycans from the protein by PNGase F. Due to the lack of intrinsic chromophores, it is also common to derivatize the glycans with a fluorescent label prior to analysis[7]. Each N-glycan contains a single reducing end site that can be reacted with an excess of fluorescent label, such that each N-glycan will be attached to one fluorophore. The processed sample is, therefore, appropriate for relative quantification by separation with fluorescence detection without the need for any quantitation standards or calibration. 2-AB is a stable, neutral label that is popular for N-glycan analysis[7,8,9]. Figure 3 illustrates 2-AB labeling by reductive amination (Schiff's base intermediate not shown).

Subsequent purification using hydrophilic interaction chromatography/solid phase extraction (HILIC/SPE) is performed to remove the large excess of 2-AB so that it does not interfere with the HILIC/FLD analysis.

Here, we show enzymatic release of N-glycans using PNGase F with subsequent derivatization with 2-AB prior to separation by HILIC UHPLC, with fluorescence detection and identification by on-line quadrupole time-of-flight mass spectrometry (Q-TOF/MS).

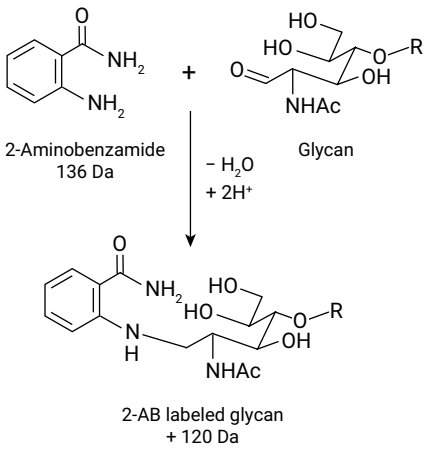

Figure 3. Labeling of a glycan with 2-aminobenzamide (2-AB).

Experimental

Reagents, samples, and materials

The Agilent 1290 Infinity Binary LC System consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A) with 35 µL Jet Weaver
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B) with standard flow cell

Reagents, samples, and materials

Agilent 6530 Accurate-Mass Q-TOF LC/MS

Column

Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems Rev. C.01.05 [38] and Agilent MassHunter Workstation Software, Version B.05.01, Build 4.0.479.0. Glycan structures were created with GlycoWorkbench, Version 2.1, stable (146).

Sample preparations

Deglycosylation procedure:

N-glycans were released from a monoclonal antibody, fetuin, and ovalbumin using PNGase F. This enzyme cleaves asparagine-linked high mannose as well as hybrid and complex oligosaccharides from the glycoproteins and leaves the glycans intact. Fetuin has three N-glycosylation sites (Asn-81, Asn-138, and Asn-158) and four O-linked sites (Ser-253, Thr-262, Ser-264, and Ser-323)11. Ovalbumin has only one glycosylation site, whereas the mAb contains two glycosylation sites. The amount of PNGase F was adjusted to the amount of N-glycosylation sites. The proteins were deglycosylated according to instructions for 3 hours at 37 °C. The reaction was then stopped, and the sample was vacuum-dried for further processing.

2-AB-labeling for fluorescence detection and sample cleanup

The dried glycan samples were labeled with 2-aminobenzamide according to the protocol for 3 hours at 65 °C. After the labeling procedure, the samples were purified using the HILIC cleanup cartridges according to the instruction manual. After the cleanup procedure, the samples were vacuum-dried and reconstituted in ultrapure water:acetonitrile 30:70 (v/v) for analysis.

Solvents and samples

Buffer A was 100 mM ammonium formate in water, pH 4.5 and buffer B was acetonitrile. All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). The monoclonal antibody was RAT Anti-DYKDDDDK Tag Antibody. Ammonium formate, fetuin and ovalbumin, PNGase F from Elizabethkingia miricola, GlycoProfil 2-AB Labeling Kit, and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich Corp., St. Louis, USA.

Instrumental conditions

	Antibody standard gradient	Fetuin gradient	Ovalbumin gradient
Starting flow rate	0.5 mL/min	0.5 mL/min	0.5 mL/min
Gradient	0 minutes 85 % B	0 minutes 75 % B	0 to 6 minutes 85 % B
	5 minutes 75 % B	45 minutes 50 % B	10 minutes 80 % B
	35 minutes 64 % B	47 minutes 40 % B, flow 0.5 mL/min	60 minutes 70 % B
	40 minutes 50 % B	47.01 minutes, flow 0.25 mL/min	65 minutes 50 % B, flow 0.5 mL/min
	42 minutes, flow 0.5 mL/min 42.01 minutes, flow 0.25 mL/min	49 minutes 0 % B	65.01 minutes, flow 0.25 mL/min
	43 minutes 0 % B	51 minutes 0 % B	68 minutes 0 % B
	48 minutes 0 % B	51.01 minutes 75 % B, flow 0.25 mL/min	73 minutes 0 % B
	50 minutes 85 % B 50.01 minutes, flow 0.25 mL/min	52.00 minutes, flow 0.5 mL/min	74 minutes 85 % B, flow 0.25 mL/min
	51 minutes, flow 0.5 mL/min		75.00 minutes, flow 0.5 mL/min
Stop time	51 minutes	52 minutes	75 minutes
Post time	20 minutes	20 minutes	20 minutes
Injection volume	5 µL	1 µL	1 µL
Thermostat autosampler	5 °C		
Column temperature	60 °C		
FLD	Ex. 260 nm, em. 430 nm		
Peak width	> 0.013 minutes (0.25 seconds resp. time) (37.04 Hz)		

MS parameters	
Gas temperature	250 °C
Sheath gas temperature	250 °C
Gas flow	8 L/min
Sheath gas flow	8 L/min
Nebulizer	25 psi
Vcap	3,500 V
Nozzle	1,000 V
Fragmentor	200 V
Skimmer	45 V
Oct 1 RF Vpp	550
Collision energies	15 and 30 V
Mode	MS and targeted MS/MS

Results and Discussion

Analysis of N-glycans from monoclonal antibodies

Figure 4 shows the separation of the mAb N-glycans. The mAb glycan pattern was optimally resolved, allowing separation and integration of all major N-glycans. Relative quantification was made based on the calculation of the peak area percentage. High intensity of the labeled glycans was achieved by setting the optimal wavelengths for glycan detection on the Agilent 1260 Infinity Fluorescence Detector, using 260 nm as excitation wavelength and 430 nm as emission wavelength[10].

The resulting HILIC glycan profile was assigned to the corresponding glycan structures based on the parent ion mass observed and the related MS/MS spectra. The parent masses were entered into the GlycoMod tool from Expasy to find related glycan structures.

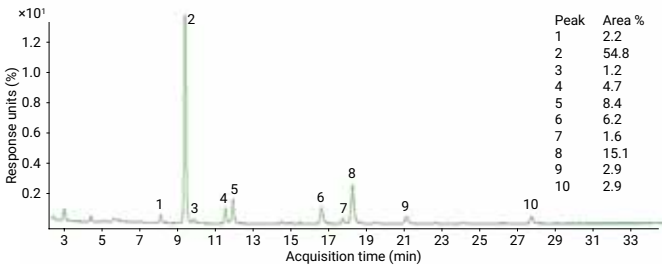

Figure 4. Separation of mAb N-glycans with fluorescence detection with 260 nm as the excitation wavelength.

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GlycoMod predicts possible glycan structures (labeled or unlabeled) from the experimentally determined masses (http://web.expasy.org/glycomod/). Another helpful tool for glycan assignment and glycan structure design is GlycoWorkbench15, which was used in this work to prepare glycan structure cartoons.

As an example of the workflow, the N-glycan FA2G1Sg1 with a parent mass of 1026.88 [z = 2] (peak 7 and 8) was chosen. The glycan databases revealed the two most likely glycan structures for this mass (Figure 5).

MS/MS data was then used to distinguish between these two potential structures. Figure 6 shows the collision-induced dissociation (CID) MS/MS spectrum of the N-glycan FA2G1Sg1. The MS/MS data confirm the presence of a type of sialic acid, N-glycolylneuraminic acid (NeuGc), which results in strong signals for fragment ions at m/z 308 (NeuGc) and m/z 673 (NeuGc attached to galactose and N-acetylglucosamine). Meanwhile, there are no signals at m/z 292 or m/z 657, which would have indicated the presence of N-acetylneuraminic acid (NeuAc). Therefore, these results provided strong evidence that the structure was FA2G1Sg1 (containing NeuGc) rather than A2G2S1 (which contains NeuAc). The decision to assign a structure with a core fucose was also supported by the MS/MS data, based on the lack of strong fragment ion signals at m/z 512 or m/z 350, which would have been present if the fucose had instead been attached in the outer arm region.

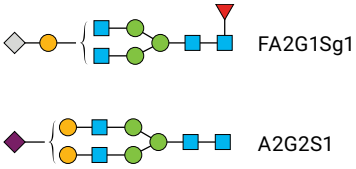

Figure 5. Two most likely glycan structures for the parent mass of 1,026.88 [z = 2], FA2G1Sg1 and A2G2S1.

All other peaks were similarly assigned using their MS and MS/MS spectra. The assigned structures are given in Table 1. The results show that the mAb mainly contains core fucosylated complex glycans, including several structures with NeuGc. These findings are typical for an IgG antibody produced by rat cells. NeuGc does not normally occur in human glycoproteins and is undesirable in therapeutic proteins[12]. The sialic acids occurring in human glycoproteins are typically N-acetylneuramic acids.

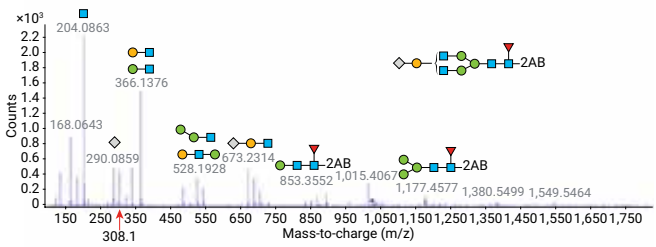

Figure 6. MS/MS spectra of FA2G1Sg1 - 1026.88 [z = 2] - 1931.6876 Da.

Table 1. Overview of masses and assigned 2AB- glycan structures of rat monoclonal antibody.

Peak	Oxford	Biopharma mAb style	Mass detector (Da)	Structure
1	FA1	G0-GlcNAc	A2G2S2	
2	FA2	G0F	792.3130 [z = 2]	
3	M5	Man5	1,355.5 [z = 1]	
4,5	FA2G1	G1F	873.34 [z = 2]	
6	FA1G1Sg1	G1FSg1-GlcNAc	925.34 [z = 2]	
7,8	FA2G1Sg1	G1FSg1	1,026.88 [z = 2]	
9	FA2G2Sg1	Ag1F	1,107.9135 [z = 2]	
10	FA2G2Sg2	Ag2F	1,261.446 [z = 2]	

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Analysis of antibodies from fetuin and ovalbumin

N-glycans from two more proteins, fetuin and ovalbumin, were released by PNGase F, derivatized with 2-AB and analyzed using HILIC/UHPLC with online MS. Figure 7 shows the separation of bovine fetuin N-glycans. This glycosylation profile was dominated by complex non-fucosylated biantennary and triantennary glycans containing NeuAc. Nine major peaks could be assigned using Q-TOF/MS detection. Table 2 shows the assigned glycan structures.

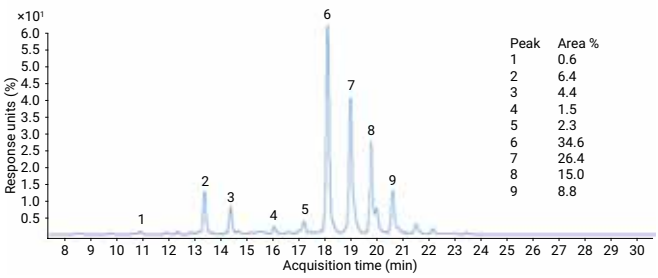

Figure 7. Separation of 2-AB-labeled fetuin.

Table 2. Detailed information of N-glycan ovalbumins.

Peak	Oxford	Structure
1	A2G2S1	
2,3	A2G2S2	
4	A3G3S2	
5	A3G3S3, A3G3S2 (trace)	
6	A3G3S3, A3G3S2 (trace)	
7	A3G3S3, A3G3S4 (trace)	
8	FA3G3S4, A3G3S3	
9	A3G3S4	

GlycoMod Figure 8 shows the separation of ovalbumin glycans. Ovalbumin is N-glycosylated only at one site (Asn292), but a complex glycosylation pattern can be associated to this site[13]. Due to the complexity of the glycan profile, the gradient had to be adjusted to a longer separation time to achieve higher resolution. The high performance of the AdvanceBio Glycan Mapping column allowed resolution of over 50 peaks with a good signal-to-noise (S/N) ratio. Twenty major peaks were assigned based on the parent ion-mass data (Table 3). Detailed structural conclusions were not achievable due to the high chance of isobaric structures occurring, several of which cannot necessarily be distinguished from the MS/MS data. Instead, the N-glycans are described in terms of their monosaccharide composition.

Compared to the relatively simple glycan pattern of the mAb, the two other glycoproteins had a greater variety of glycan structures. No fucosylated glycans were detected in ovalbumin in contrast to the mAb glycans, which matches previously reported findings that avian egg glycoproteins are non-fucosylated[14].

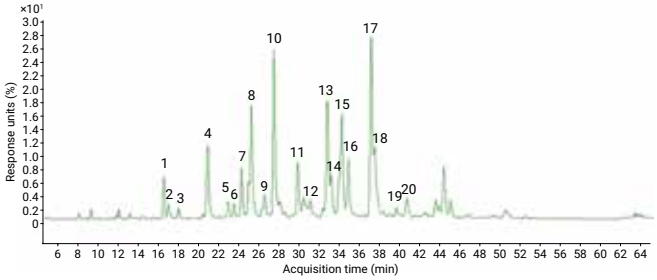

Figure 8. Separation of N-glycans released from ovalbumin.

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Table 3. Assigned masses and monosaccharide composition of ovalbumin N-glycans; H = hexoses, i.e. galactose or mannose; N = N-acetylglucosamine.

Peak	Mass + 2AB (Da)	Calculated mass (Da)	PComposition (short form)	PComposition (long form)
1,2	1,234.48 [M]	1,114.48	H3N3	$(HexNAc)_1+(Man)_3(GlcNAc)_2$
3	1,193.45 [M]	1,073.45	H4N2	$(Hex)_4(HexNAc)_2$
4	1,437.56 [M]	1,317.56	H3N4	$(HexNAc)_2+(Man)_3(GlcNAc)_2$
5	1,396.53 [M]	1,276.53	H4N3	$(Hex)_1(HexNAc)_1+(Man)_3(GlcNAc)_2$
6, 7	1,640.64 [M]	1,520.64	H3N5	$(HexNAc)_3+(Man)_3(GlcNAc)_2$
8	1,355.506 [M]	1,235.51	H3N5	$(Hex)_2+(Man)_3(GlcNAc)_2$
9	1,599.61 [M]	1,479.61	H4N4	$(Hex)_1(HexNAc)_2+(Man)_3(GlcNAc)_2$
10	1,843.73 [M]	1,723.73	H3N6	$(HexNAc)_4+(Man)_3(GlcNAc)_2$
11	1,802.74 [M]	1,682.74	H4N5	$(Hex)_1(HexNAc)_3+(Man)_3(GlcNAc)_2$
12, 14	2,046.884 [M]	1,926.88	H3N7	$(HexNAc)_5+(Man)_3(GlcNAc)_2$
13	1,517.56 [M]	1,397.56	H6N2	$(Hex)_3+(Man)_3(GlcNAc)_2$
15	1,761.6574 [M]	1,641.66	H5N4	$(Hex)_2(HexNAc)_2+(Man)_3(GlcNAc)_2$
16	2,005.8098 [M]	1,885.81	H4N6	$(Hex)_1(HexNAc)_4+(Man)_3(GlcNAc)_2$
17	2,249.9728 [M]	2,129.97	H3N8	$(HexNAc)_6+(Man)_3(GlcNAc)_2$
18	1,964.82 [M]	1,844.82	H5N5	$(Hex)_2(HexNAc)_3+(Man)_3(GlcNAc)_2$
19, 20	2,208.87 [M]	2,088.87	H4N7	$(Hex)_1(HexNAc)_5+(Man)_3(GlcNAc)_2$

Conclusions

The Agilent 1290 Infinity Binary LC System, together with the Agilent 1260 Infinity Fluorescence Detector and Agilent 6530 Accurate-Mass Q-TOFLC/MS, was an ideal combination for the analysis of released N-glycans that were derivatized with 2-aminobenzamide. Sample preparation using PNGase F for the release of N-linked glycans followed by 2-AB derivatization with subsequent HILIC sample cleanup was demonstrated for one monoclonal antibody and two other glycoproteins.

The Agilent AdvanceBio Glycan Mapping column demonstrated excellent resolving power, allowing separation and identification of all major N-glycans in a rat mAb sample. Complex biantennary and triantennary N-glycans from fetuin and ovalbumin were also analyzed with very high resolution. Optimized fluorescence excitation and emission wavelengths of 260 and 430 nm provided better S/N ratios. Electrospray ionization Q-TOF MS analysis allowed assignment of different glycan structures or monosaccharide compositions, depending on the complexity of the sample.

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Application Note

Glycan Analysis

Agilent

Trusted Answers

Agilent Solutions for High-throughput N-linked Glycan Profiling from Biotherapeutics

Authors

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Abstract

This Application Note presents an automated high-throughput sample preparation for 2-AB labeled N-linked glycans from innovator and biosimilar monoclonal antibody (mAb) followed by liquid chromatography (LC) analysis.
Agilent AssayMAP Bravo liquid handling platform was used for the automated sample preparation involving glycan cleavage and release from mAb, 2-AB labeling, and purification. The downstream ready samples were then analyzed using an Agilent 1290 Infinity LC system with Agilent AdvanceBio Glycan Mapping columns. The glycan distribution and heterogeneity between the samples were deduced by comparing the chromatogram from both innovator and biosimilar mAb.

The study highlights the high-throughput application of the AssayMAP Bravo platform for automated and reproducible sample preparation for glycan profiling, followed by fast chromatographic separation using a 1290 Infinity LC system.

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Introduction

Monoclonal antibodies (mAbs) are leading the race in biotherapeutics, and have revolutionized the way diseases are treated and intervened. Patents for most of the 20 clinically approved[1] first-generation mAbs have either expired, or are about to expire. This has increased the opportunity for generating generic versions, referred to as biosimilars. Regulatory bodies such as the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) have published guidelines for the characterization of biosimilar protein therapeutics including post-translation modifications[2]. Glycosylation modification results in structural heterogeneity that changes an mAb's target binding capacity, stability, charge, and mass[3]. During the course of developing the antibodies from the cell line, the glycans are monitored for cell line alteration and culture optimization. This requires a high-throughput sample preparation to screen several cell culture conditions in parallel. The Agilent Bravo Automated Liquid Handling Platform is a precise liquid-handling system designed for high-throughput applications like glycan profiling. Agilent Bravo with Agilent AssayMAP technology combines the automation with miniature 5 µL pack bed cartridges for sample preparations, enabling high-throughput chromatography and sample preparations in a shorter time[4].

This Application Note presents a workflow solution for profiling N-glycans from mAb using an Agilent AssayMAP Bravo platform. The system automates the N-glycan purification and derivatization using 2-aminobenzamide (2-AB) dye, which is then followed by Agilent HPLC analysis using Agilent Glycan Mapping columns. We showcase the ease-of-use of an AssayMAP Bravo for the automated sample preparation required for high-throughput profiling studies.

Experimental

Materials

Agilent's AdvanceBio GlykoPrep-plus Rapid N-Glycan Sample Preparation with 2-AB kit, and 2-AB labeled Oligomannose 5 and 6 were used to prepare and analyze the samples. An Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913), and Agilent 2-AB labeled human IgG N-linked glycans standards (p/n GKSB-005) were purchased from Agilent Technologies. Innovator rituximab and biosimilar product were purchased commercially from a local pharmacy. All other chemicals were procured as HPLC grade from Sigma.

Sample preparation

Reagents from an Agilent AdvanceBio GlykoPrep-plus kit with an AssayMAP protocol were used for the sample preparation. An innovator and biosimilar sample was diluted to 1 mg/mL, then loaded onto three columns of the 96-well plate (24 replicates each). After placing the sample plates and reagents plates as specified in the guideline[5], the samples were processed by launching the N-Glycan Sample Prep: RX digestion & 2-AB labeling module from Agilent VWorks software. The protocol consists of five modules that are performed in sequential order to immobilize the samples, digest the glycans, elute, label with 2-AB, and complete a final cleanup to remove the excess dyes. The final purified labeled glycan from each well were then transferred to HPLC vials and analyzed immediately, or stored at –80 °C.

Figure 1 presents a schematic diagram of the complete workflow.

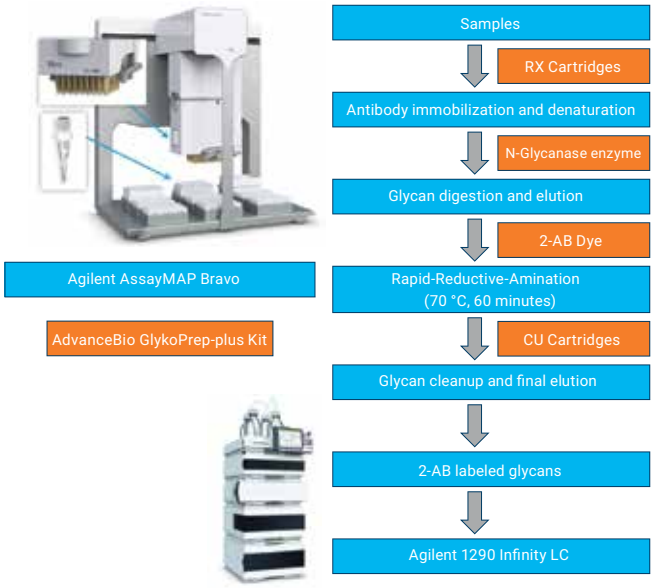

Figure 1. Schematic workflow for the glycan isolation, labelling, purification followed by LC analysis using Agilent solutions. Components of the Agilent AssayMAP steps are shown in blue; the AdvanceBio kits are shown in orange.

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Instrumentation

Agilent 1290 Infinity LC System including:

- Agilent 1290 Infinity Binary Pump G4220A
- Agilent 1290 Infinity Autosampler G4226A
- Agilent 1290 Infinity TCC G1316 C
- Agilent 1260 Fluorescence Detector G1321 B

The LC method described earlier was adopted for this study[6]. Every sample was analyzed in quadruplet injections, followed by a blank injection.

Software

- Agilent VWorks Automation Control 11.4.0.1233
- Agilent AssayMAP Launch Pad 3.0
- Agilent N-Glycan Sample Prep: RX digestion & 2-AB labeling protocols 1.0
- Agilent ChemStation C.01.06

Results and Discussion

Agilent AssayMAP sample preparation

The samples were processed following a protocol consisting of five modules, as presented in Figure 2. The software suite consists of a deck layout, labware table, and application settings tab for each module to be performed (Figure 3). The user was prompted to place the appropriate consumables and reagents listed in the labware table in the specified deck positions. After setting up all labware, the protocol was executed, and AssayMAP Bravo completed the protocol, and a confirmation message was displayed to proceed to the next module.

The final Cleanup Protocol module eluted the labeled and purified glycans in an aqueous buffer into a clean 96-well plate. The samples were then analyzed in quadruplets, along with blanks, using the Agilent AdvanceBio Glycan Mapping column for the downstream LC analysis.

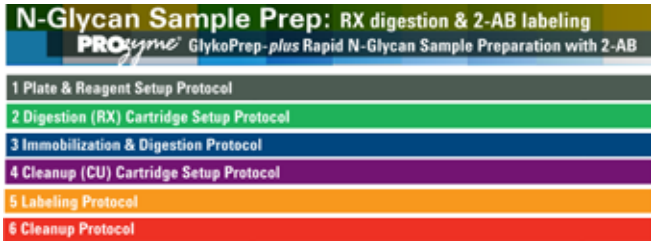

Figure 2. Agilent VWorks modules of an Agilent AssayMAP N-Glycan Sample Preparation.

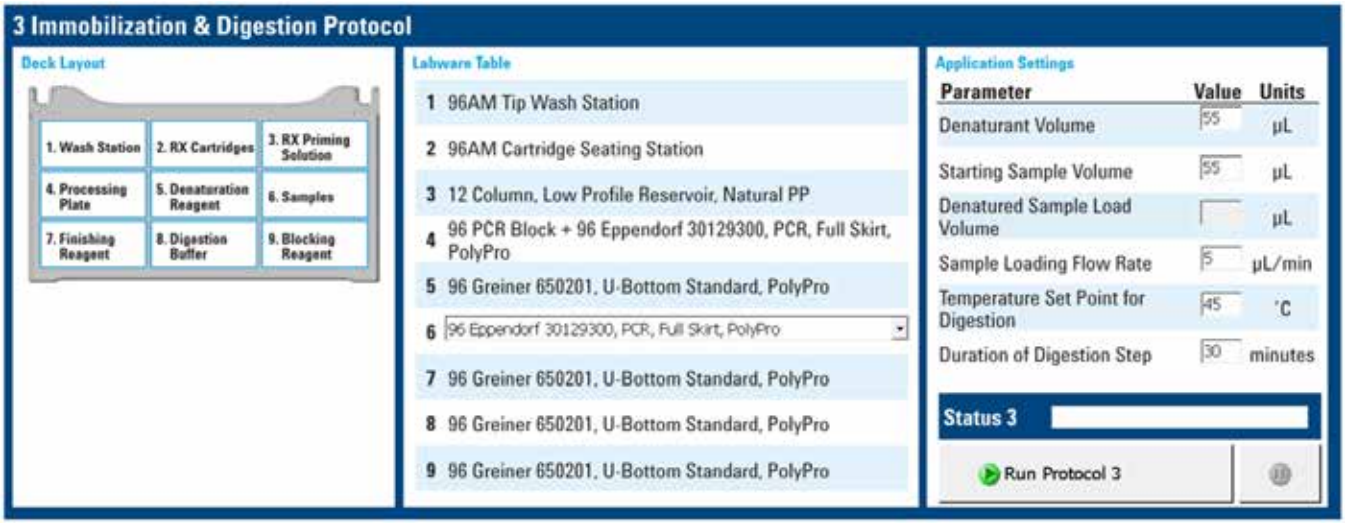

Figure 3. The deck layout, labware table, and application settings of the Immobilization and Digestion Protocol, one of the five sample preparation modules in Agilent VWorks software.

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HPLC Analysis

The N-glycan profiles were compared between the innovator and biosimilar mAbs using a fast analysis method with the Agilent 1290 Infinity system. The Agilent 2-AB labeled IgG N-linked glycan library was used as the standard to assess the column performance, and to annotate the peaks in mAb samples. The 1290 Infinity system with high backpressure enabled the analysis of the samples in less than 6 minutes, saving analysis time per sample, and increasing sample throughput.

Each sample was HPLC analyzed in replicates of four. Figure 4 presents the peak area of four major glycans species from 96 replicates, demonstrating the excellent column-to-column reproducibility of the AssayMAP micro chromatography pipette tips. The Reproducibility Standard Deviation (RSD) calculated for peak area and peak height showed a coefficient of variation (CV) of less than 6 % for all glycan species. This demonstrates the very robust and reproducible sample preparation capability of the AssayMAP Bravo system.

Glycan profiling and comparison of innovator and biosimilar rituximab

The chromatograms of the innovator and biosimilar rituximab were compared with the standard N-linked IgG glycan library, and the peaks corresponding to glycans were annotated. Separate standards comprising 2-AB labeled oligomannose 5 and 6 were also used to annotate additional peaks. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the innovator and biosimilar product (Figure 5). Small differences in the low abundant glycans were observed; the biosimilar sample contained lower amounts of mannose (Man5), as shown in the zoomed view of Figure 6. Despite minor differences in some low abundant glycans, the glycan profile of the innovator and biosimilar rituximab can be concluded to be comparable.

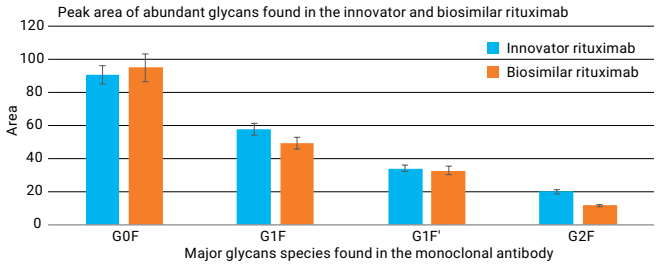

Figure 4. Peak area of major glycan species from 96 replicates for each innovator and biosimilar sample.

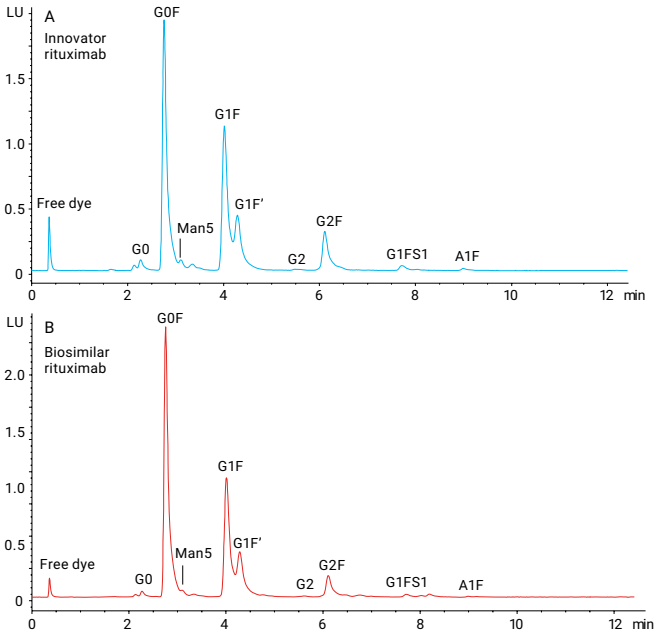

Figure 5. Glycosylation pattern of innovator and biosimilar rituximab.

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The area percentage for each N-glycan core was calculated and compared between both samples. Table 1 shows the ratio of N-glycans between the innovator and biosimilar rituximab. The table shows a similar N-glycan fingerprint profile for both innovator and biosimilar.

Both samples showed a similar trend in the distribution of N-glycans, with G0F as the major glycoform followed by G1F, G1F', and G2F. Apart from the annotated glycans, there were a few unknown glycan forms that may be assigned with an orthogonal detection system. A correlation graph (Figure 7) plotted for the area percentage of both samples shows high similarity, with an R2 of 0.973.

Table 1. Glycan area percentage between innovator and biosimilar mAbs.

Percentage of N-glycans		
N-glycans	**Innovator**	**Biosimilar**
G0	1.5	0.8
G0F	42.2	50.5
Man5	1.7	1.2
G1F	28.6	26.7
G1F'	13.0	11.6
G2	0.3	0.2
G2F	8.7	5.5
G1FS1	0.9	0.6
A1F	0.2	0.5

Conclusions

This study highlights the versatility of the Agilent AssayMAP Bravo system for a high-throughput sample preparation.

- The Agilent VWorks Automation Control software suite simplifies the sample preparation with ready-to-go protocols, resulting in downstream-compatible samples with minimal hands-on operation.
- Monoclonal antibody samples from a 96-well plate were processed, in parallel, for enzymatic glycan cleavage, separation, derivatization with 2-AB, and purification.
- The AssayMAP demonstrated excellent reproducibility in the glycan purification, and performed robustly.
- The purified samples were then analyzed using an Agilent 1290 Infinity LC system with the Glycan Mapping column.
- The glycan species were well-resolved in a shorter time, and were annotated using standards.
- The distribution of the glycan species between the innovator and biosimilar were assessed, and the data suggest comparable glycan profiles for the innovator and biosimilar rituximab used in this study.

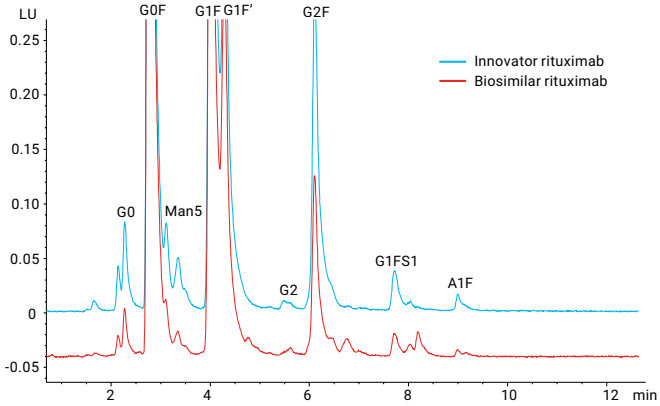

Figure 6. Zoomed view of Figure 5: the glycosylation pattern of innovator and biosimilar rituximab, showing minor differences in the low abundant glycans.

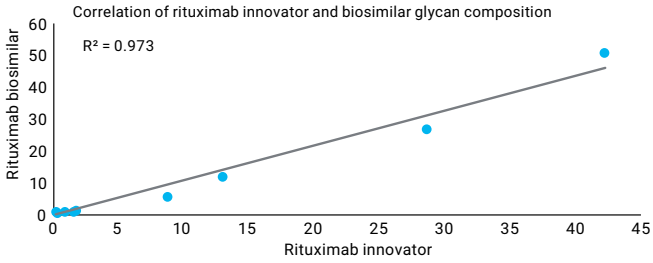

Figure 7. Linear graph showing the comparison of the area % of glycans released from innovator and biosimilar rituximab.

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Additional Application Notes

Part Number	Title
5991-4801EN	Sensitive and Reproducible Glycan Analysis of Human Immunoglobulin G
5991-4886EN	A Novel HILIC Column for High Speed N-linked Glycan Analysis
5991-7024EN	Analysis of N-Linked Glycans from Antibody-Drug Conjugate (ADC) Using the Agilent AssayMAP Automated Sample Preparation and Agilent 1290 Infinity LC System
5991-8071EN	Analysis of Monoclonal Antibody N-Glycans by Fluorescence Detection and Robust Mass Selective Detection using the Agilent LC/MSD XT
5991-8796EN	Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Additional Information

Glycan sample preparation kits are available, containing supplies and reagents for deglycosylation, glycan labeling, and sample clean-up. Detailed protocols may be found in the user guides. 5991-9561EN and 5991-9560EN.

Glycan standards are available to assist with method development, data analysis, and system checks.
See pages 12-15, or download 5994-2202EN.

For more information on the glycan mapping workflow, visit www.agilent.com/chem/glycoscience

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