Application Note

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Automated Workflow for Monoclonal Antibody N-Linked Glycan Analysis

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Abstract

This application note describes an automated workflow for the release, labeling, and cleanup of N-glycans using the Agilent AssayMAP Bravo and Agilent GlykoPrep InstantPC kit. A total of 24 antibody were analyzed in a single experiment, with N-glycan identification using the Agilent 6545XT AdvanceBio LC/Q-TOF.

Introduction

Monoclonal antibodies (mAbs) are critical entities that have been implemented in cancer immunotherapy and gained much development in recent years. Glycosylation plays an important role in maintaining antibody stability and efficacy.[1] Therefore, glycans are carefully monitored throughout the antibody development process and manufacturing. High-throughput glycan sample preparation is essential during the clone selection and cell culture optimization process. Agilent has developed an automated workflow using the AssayMAP Bravo and GlykoPrep InstantPC kit to release, label, and purify N-glycans.

In this workflow, automation protocols incorporate a GlykoPrep InstantPC kit with AssayMAP Bravo (Figure 1). IgG samples were immobilized and denatured on RX cartridges followed by on-cartridge N-glycan release using PNGase F. The released N-glycans were eluted into InstantPC Dye for instant labeling. The final sample preparation step includes an N-glycan cleanup procedure using a CU cartridge. With a cartridge-based platform, the method fully integrates the InstantPC dye and the automated liquid handler to streamline N-glycan sample preparation. High-throughput sample preparation were achieved in about three hours. The InstantPC-labeled N-glycans generate enhanced signals in both fluorescence detection (FLD) and mass spectrometry (MS) measurements.

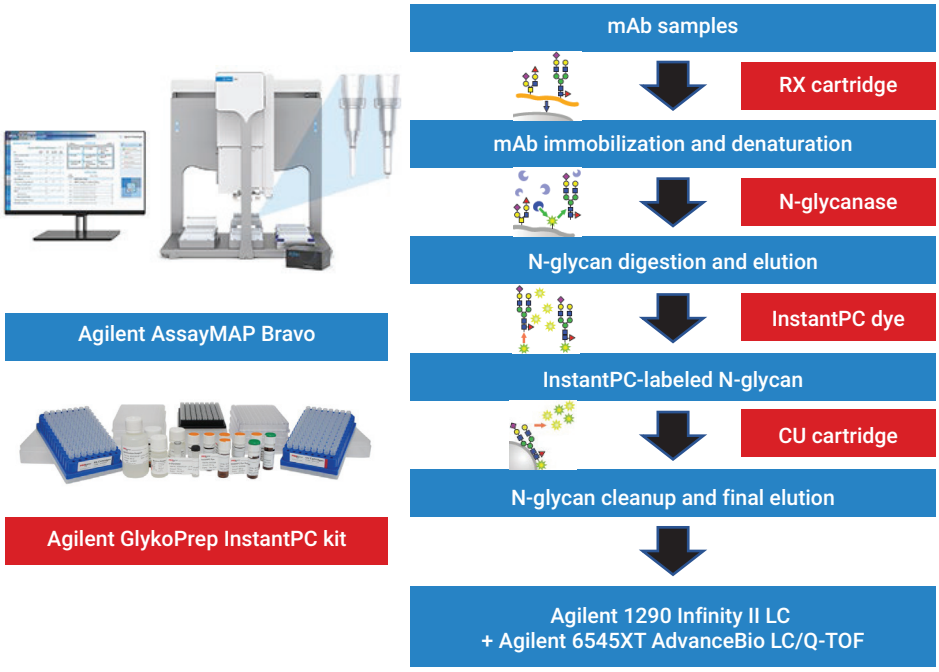

Figure 1. Automated N-glycan sample preparation using the Agilent AssayMAP Bravo and Agilent GlykoPrep InstantPC kit.

Experimental

Materials

GlykoPrep-plus N-glycan prep with InstantPC kit, 96-ct was from Agilent (formerly ProZyme) (part number GPPPNG-PC), including the following modules: GlykoPrep-plus digestion module, 96-ct (part number GSP-RX); InstantPC labeling module (part number GSP-PC); GlykoPrep cleanup module, 96-ct (part number GSP-CU); GlykoPrep-plus labware set (part number AM96-NG).

Agilent CHO mAb was formulated at Agilent.

- Herceptin was purchased from Genentech (NDC 50242-134-68)
- Human IgG was formulated at ProZyme
- NISTmAb was purchased from NIST (RM 8671)
- All other chemicals were ordered from Sigma-Aldrich (St. Louis, MO, USA)

Automated N-glycan sample preparation

The Agilent Protein Sample Prep Workbench software includes a preloaded workflow for automating the GlykoPrep InstantPC kit using an AssayMAP Bravo platform. The "RX Digestion – InstantPC Labeling" workflow can be found within the Workflow Library under the N-glycan Sample Prep category. This workflow includes five protocols for semi-automated N-glycan sample preparation.[2] Briefly, protocol 1 enables the user to set up reagent plates, protocols 2 and 4 enable the user to define the microchomatography cartridge layout, and protocols 3 and 5 perform the sampler processing.

The first step uses the "Plate & Reagent Setup Protocol" to prepare reagent plates from the InstantPC kit. A dedicated "Reagent Volume Calculator" was designed for this step. Based on the columns and number of samples to be processed, the calculator provides exact volumes to prepare for each reagent, and instructions for where to dispense these reagents in the reagent source plate. In the experiment, there are three full columns with a total of 24 samples and 50 µg of each mAb. Once a certain volume of reagents was transferred from kit to reagent source plate according to the calculator, different labware could be set up on the deck according to Figure 2 (protocol 1, Deck Layout). At deck location 7, the four U-bottom plates should be labeled and stacked from Digestion Buffer (top), Blocking Reagent, Denaturation Reagent, to InstantPC Solution (bottom). After the protocol was started, AssayMAP Bravo aliquoted different reagents with the desired volume. This is a critical step to prepare the reagent plates for the following reaction and glycan labeling. The calculator also calculates the RX cartridge priming solution volume to be used in protocol 3 and the volume of all the buffer solutions to be used in protocol 5.

The second step in this application is only an RX cartridge transfer protocol where the user can set up the deck for the following reaction and transfer the cartridges to deck location 2 without leaving the application page. Three full columns of cartridges were transferred in the experiment starting at column 1.

Six different antibody samples including control human IgG, Agilent CHO mAb, Herceptin, NISTmAb, rituximab, and SILuMAb were normalized to 1 µg/µL and located on the original sample plate as shown in Figure 3. N-glycan release and labeling were performed in protocol 3. 50 µL mAb samples (four replicates for each mAb) were denatured using the denaturation

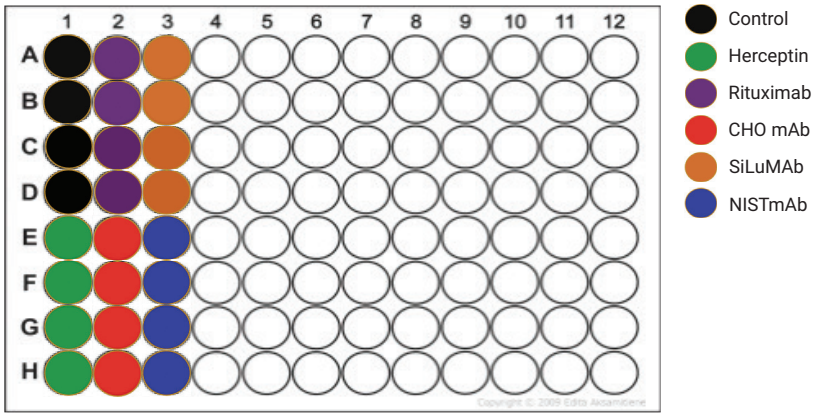

Figure 3. Antibody sample location on the 96-well plate.

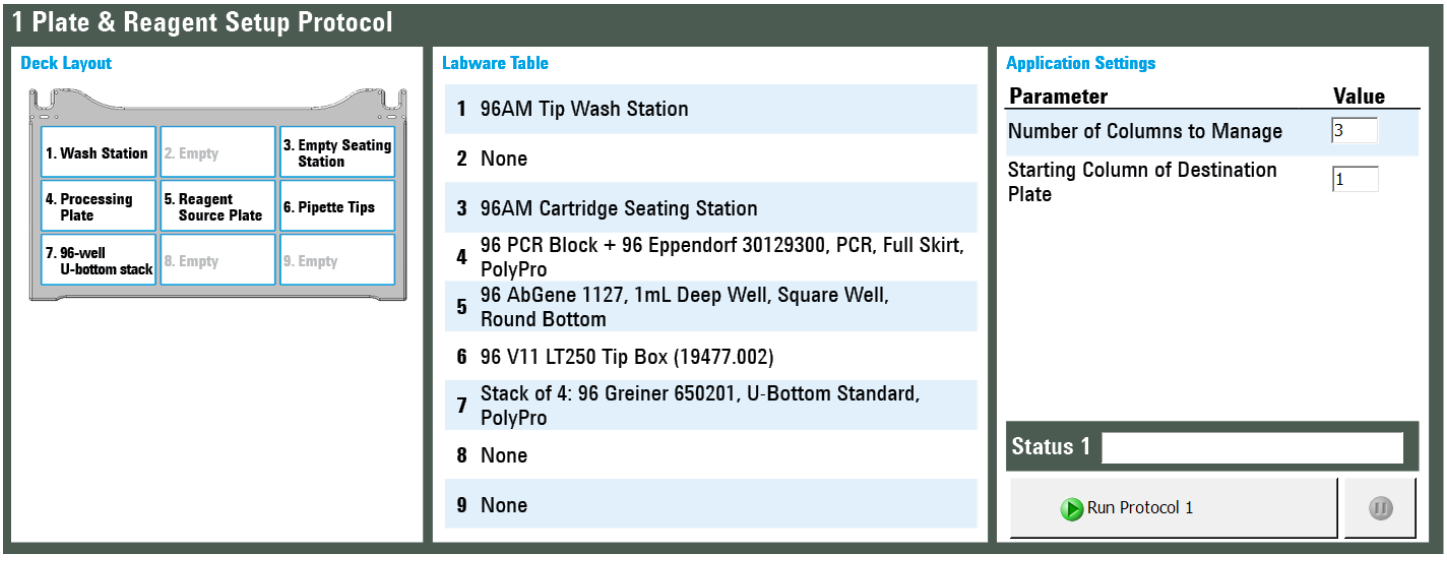

Figure 2. Reagent setup protocol in Agilent InstantPC software.

reagent. mAbs were immobilized in parallel onto 24 RX cartridges. A blocking step was used afterwards to block the remaining active binding sites on the RX cartridges. N-glycans were released from immobilized proteins by aspirating PNGase F into the cartridge at 45 °C and mAbs were incubated for 30 minutes.

At the end of the reaction, released N-glycans were eluted into the plate with InstantPC solution and N-glycans were labeled instantly (Figure 4, Deck location 7).

The cleanup (CU) cartridges were transferred in protocol 4. Once the deck layout was set as in Figure 5, the InstantPC labeled N-glycan samples were cleaned by protocol 5 to remove excess free InstantPC. The final InstantPC-labeled glycan samples were eluted with a 50 µL final elution volume.

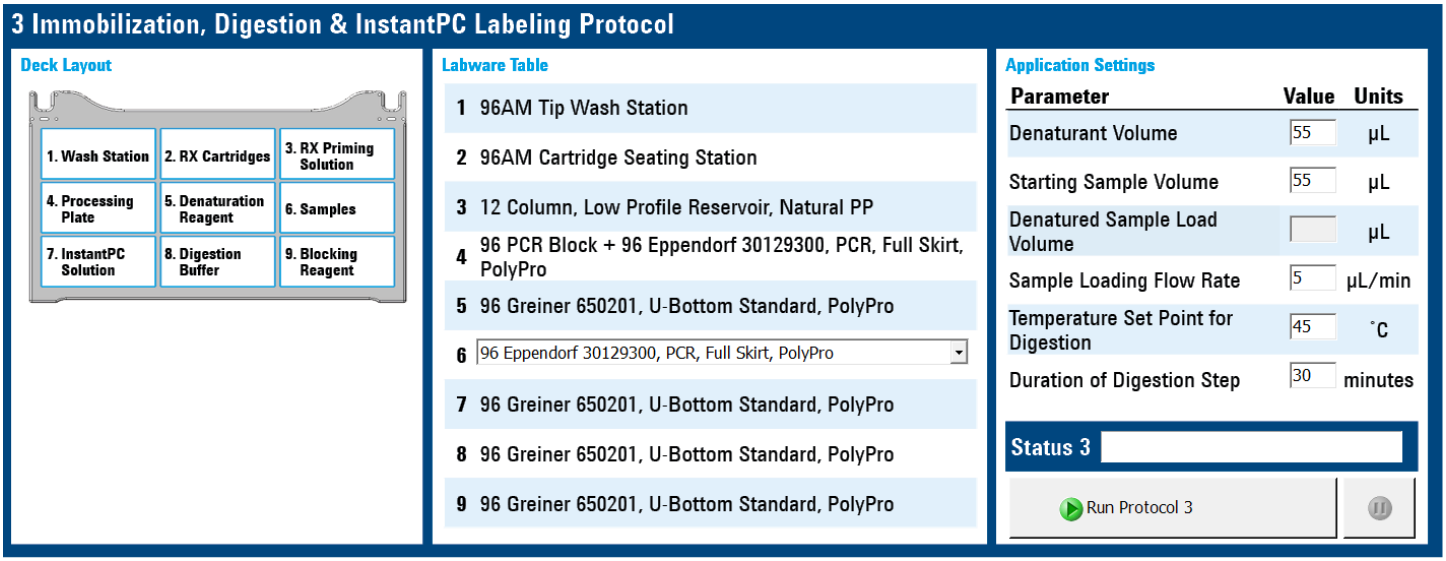

Figure 4. N-glycan release and labeling protocol in Agilent InstantPC software.

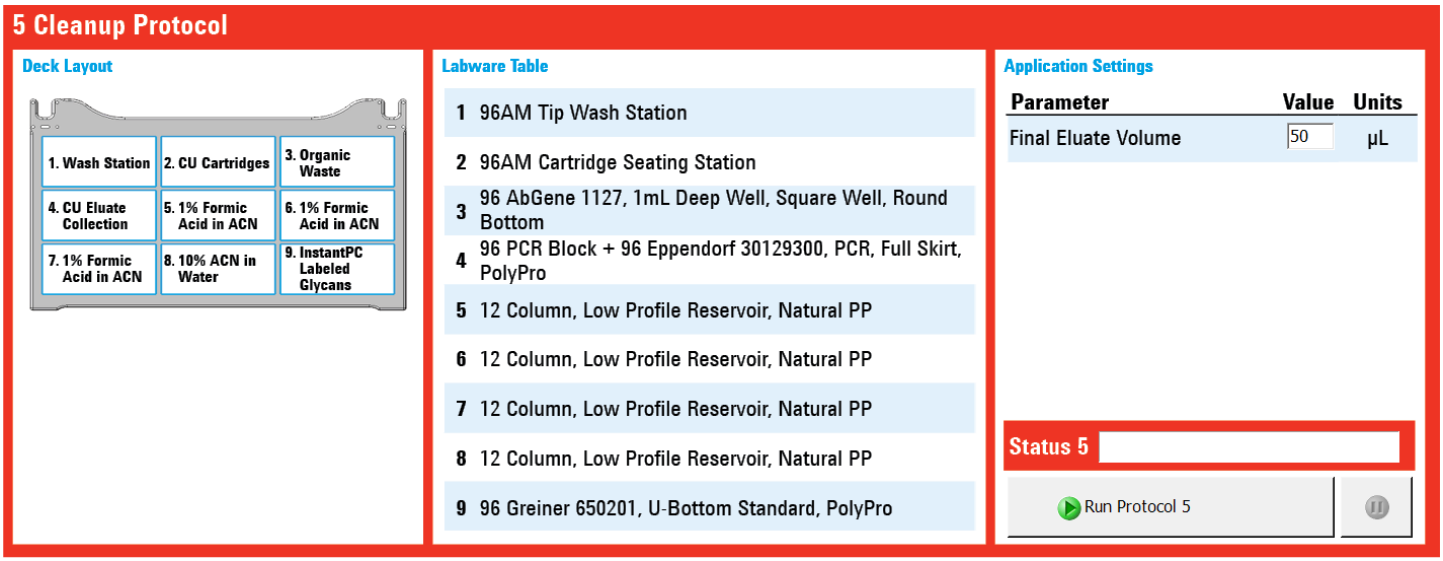

Figure 5. N-glycan cleanup protocol in Agilent InstantPC software.

N-glycan analysis with LC/MS

The cleaned N-glycan samples were injected on an LC/Q-TOF system without further purification. Since the volume of final N-glycan sample is the same as the original mAb sample, 1 µL of final sample represents how much N-glycan is prepared from 1 µg of mAb. An Agilent 1290 Infinity II LC was equipped with an Agilent 1260 Infinity FLD and coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF MS with a dual Agilent Jet Stream (AJS) source. N-glycan samples were separated by hydrophilic interaction liquid chromatography (HILIC) with an Agilent AdvanceBio glycan mapping column, 2.1 × 150 mm, 1.8 µm (part number 859700-913) with column temperature at 40 °C (Figure 6). Since the separation conditions were carefully developed previously, the same gradient was implemented in this study with a 28-minute gradient in a total run time of 35 minutes.[3] LC conditions were listed in Table 1. The FLD was set to λ_{Ex} = 285 nm and λ_{Em} = 345 nm, with PMT gain = 10.

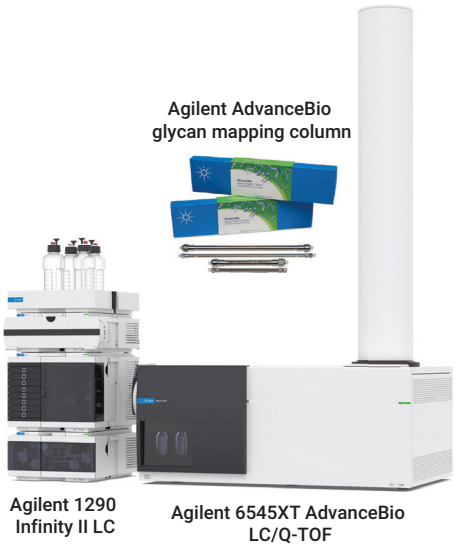

Figure 6. N-glycan analysis using an Agilent 1290 Infinity II LC and Agilent 6545XT AdvanceBio LC/Q-TOF with an Agilent AdvanceBio glycan mapping column.

With a dual AJS source, N-glycan samples were analyzed in an *m/z* 300 to 1,700 mass range with 2 spectra/sec scan rate in high-resolution (4 GHz) mode with detailed Q-TOF settings listed in Table 2.

Table 1. UHPLC HILIC/FLD conditions.

Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio glycan mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
Solvent A	50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	Acetonitrile
Gradient	Time (min) B (%) Flow rate (mL/min) 0.0 78.0 0.6 0.5 74.0 0.6 13.0 72.5 0.6 28.0 61.0 0.6 28.5 50.0 0.5 28.6 50.0 0.4 28.8 78.0 0.4 31.0 78.0 0.5 31.5 78.0 0.55 33.5 78.0 0.6 Stop time = 35.0 min
Column Temperature	40 °C
Injection Volume	1 µL
FLD	λ_{Ex} = 285 nm λ_{Em} = 345 nm

Table 2. LC/Q-TOF parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Source	Dual Agilent Jet Stream
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* 300 to 1,700
Scan Rate	2 spectra/sec
Acquisition Mode	High resolution (4GHz)

Results and discussion

Qualitative analysis of InstantPC-labeled N-glycans

The control human IgG contained 17 well characterized N-glycans, which can be used to identify N-glycan by comparing retention time and accurate mass with these glycans. In Figure 7A and Figure 8A, all 17 glycans were annotated. The same glycan with the same retention time in Figures 7B to 7F were not labeled. In addition, five other common N-glycans (labeled in Figures 7B, 7C, 7D, 8B, 8C, and 8D) were identified by accurate mass and monosaccharide composition. FLD results showed the same retention time and similar relative peak abundance for these glycans (Figure 8).

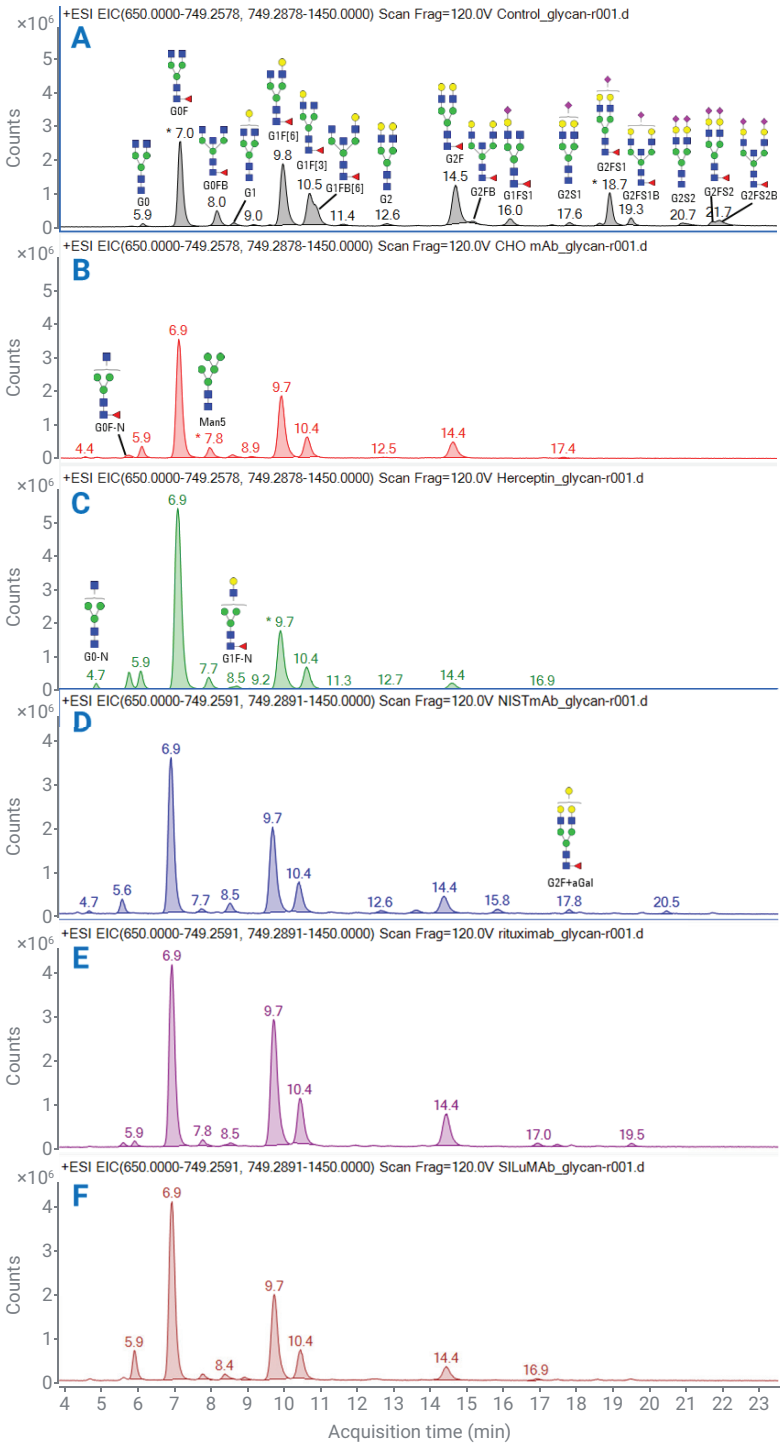

Figure 7. LC/Q-TOF analysis of InstantPC-labeled N-glycans from all samples. (A) Control human IgG; (B) CHO mAb; (C) Herceptin; (D) NISTmAb; (E) Rituximab; (F) SILuMAb.

Figure 8. LC/FLD analysis of InstantPC-labeled N-glycans from all samples. (A) Control human IgG; (B) CHO mAb; (C) Herceptin; (D) NISTmAb; (E) Rituximab; (F) SILuMAb.

Table 3 listed the detailed information for the 22 N-glycans found in this study. The glycans were listed based on their retention time, theoretical mass, monosaccharide composition, structure, and common name.

Table 3. A summary of 22 N-glycans identified from all the samples.

Peak number	R.T. (min)	Theoretical $[M+2H]^{2+}$	Composition	Structure	Common name
1	4.7	688.2856	Hex3HexNAc3		G0-GlcNAc
2	5.6	761.3130	Hex3HexNAc3dHex1		G0F-GlcNAc
3	5.9	789.8244	Hex3HexNAc4		G0
4	7.0	862.8534	Hex3HexNAc4dHex1		G0F
5	7.8	748.7987	Hex5HexNAc2		Man5
6	8.0	964.3931	Hex3HexNAc5dHex1		G0FB
7	8.4	870.8508	Hex4HexNAc4		G1
8	8.5	842.3410	Hex4HexNAc3dHex1		G1F-GlcNAc
9	9.8	943.8798	Hex4HexNAc4dHex1		G1F[6]
10	10.5	943.8798	Hex4HexNAc4dHex1		G1F[3]
11	10.7	1045.4195	Hex4HexNAc5dHex1		G1FB[6]
12	12.6	951.8772	Hex5HexNAc4		G2
13	14.5	1024.9062	Hex5HexNAc4dHex1		G2F
14	14.9	1126.4459	Hex5HexNAc5dHex1		G2FB
15	16.0	1089.4275	Hex4HexNAc4dHex1NeuAc1		G1FS1[3]
16	17.6	1097.4249	Hex5HexNAc4NeuAc1		G2S1
17	17.8	1105.9325	Hex6HexNAc4dHex1		G2F+aGal
18	18.7	1170.4539	Hex5HexNAc4dHex1NeuAc1		G2FS1
19	19.3	1271.9936	Hex5HexNAc5dHex1NeuAc1		G2FS1B
20	20.7	1242.9726	Hex5HexNAc4NeuAc2		G2S2
21	21.5	1316.0016	Hex5HexNAc4dHex1NeuAc2		G2FS2
22	21.7	1417.5413	Hex5HexNAc5dHex1NeuAc2		G2FS2B

Quantitative analysis of InstantPC-labeled N-glycans with MS

The InstantPC-labeled N-glycan samples were also analyzed using Agilent MassHunter BioConfirm 10.0 software using the Released Glycans workflow. The software allows the user to set matching criteria including mass error, retention time window, glycan ion species, and tags (2-AB and InstantPC), and validates results with its own scoring system. N-glycans were observed mostly as doubly charged ions $[M+2H]^{2+}$, with some other species including $[M+NH_4+H]^{2+}$, $[M+H+Na]^{2+}$, and $[M+H+K]^{2+}$.[4] InstantPC added a monoisotopic mass of 261.1477 Da to the reducing end. An internal glycan database was implemented in this study to quickly identify N-glycans with high mass accuracy (<2 ppm) in all the mAbs. Using an internal glycan database, BioConfirm quickly identified and integrated 10 labeled N-glycans from the 24 samples based on retention time and accurate mass. These 10 glycans are the top 10 most abundant N-glycans identified in all mAbs.

Figure 9A was generated from MS measurement, which represented the relative ratio of 10 N-glycans from each mAb. The 10 glycans were listed based on their retention time order from the LC chromatogram. The relative abundance analysis is mostly to compare different N-glycan ratios within the same mAb. For example, rituximab G0F, which shows a ratio of 40.85%, has a lower relative ratio than CHO mAb (44.58%) and NISTmAb (44.67%). Fucosylated glycans (G0F, G1F, and G2F) were the most abundant glycans, representing more than 90% of the 10 glycans. The average value of the relative percentage of each N-glycan was listed in Table 4 with the CV% (n = 4). After multiple steps, including N-glycan release, InstantPC labeling, and cleanup, almost all CV% were less than 10%, showing the high reproducibility of the automated workflow.

Quantitative analysis of InstantPC-labeled N-glycans with FLD

Since FLD was used as an analytical method, the quantitation result was also evaluated based on FLD measurement.

Figure 9B summarized FLD results from the 24 samples. As in Figure 9A, the relative abundance was calculated and annotated for the top 10 most abundant N-glycans. Fucosylated glycans (G0F, G1F, and G2F) comprised more than 90% of the 10 glycans in abundance. The average relative percentage areas of N-glycans were listed in Table 5 with CV% (n = 4) measured by FLD.

By comparing the quantitation result between MS and FLD, the relative abundance for each glycan showed very similar results, with less than 5% difference for all glycans and less than 2% difference for most glycans.

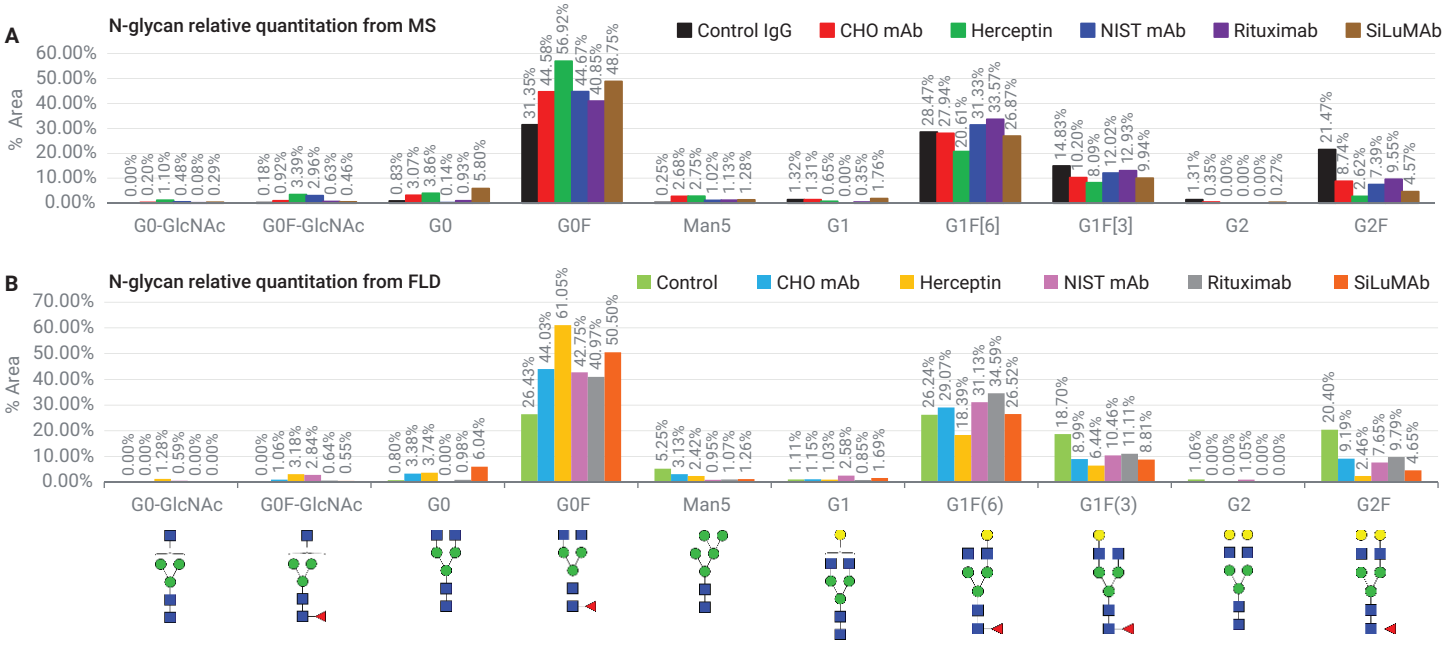

Figure 9A. N-glycan relative abundance measured by MS. 9B. N-glycan relative abundance measured by FLD.

Table 4. The average relative % area of 10 major N-glycan from six IgG samples with CV% measured by MS.

Common Name	Control IgG	CV%	CHO mAb	CV%	Herceptin	CV%	NIST mAb	CV%	Rituximab	CV%	SiLuMAb	CV%
G0-GlcNAc	0.00%	0.00%	0.20%	7.60%	1.10%	1.19%	0.48%	7.47%	0.08%	6.84%	0.29%	8.14%
G0F-GlcNAc	0.18%	5.31%	0.92%	13.10%	3.39%	1.85%	2.96%	7.88%	0.63%	4.51%	0.46%	8.82%
G0	0.83%	6.81%	3.07%	10.45%	3.86%	3.11%	0.14%	6.70%	0.93%	4.67%	5.80%	8.49%
G0F	31.35%	6.11%	44.58%	7.22%	56.92%	2.16%	44.67%	5.91%	40.85%	3.72%	48.75%	6.20%
Man5	0.25%	8.27%	2.68%	9.75%	2.75%	3.10%	1.02%	8.21%	1.13%	3.74%	1.28%	9.19%
G1	1.32%	6.56%	1.31%	9.14%	0.65%	3.25%	0.00%	0.00%	0.35%	2.54%	1.76%	8.56%
G1F[6]	28.47%	6.15%	27.94%	6.06%	20.61%	3.17%	31.33%	8.92%	33.57%	3.85%	26.87%	6.76%
G1F[3]	14.83%	6.30%	10.20%	9.22%	8.09%	3.09%	12.02%	11.05%	12.93%	5.63%	9.94%	7.60%
G2	1.31%	9.30%	0.35%	9.26%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.27%	6.77%
G2F	21.47%	7.54%	8.74%	8.27%	2.62%	2.95%	7.39%	8.03%	9.55%	5.96%	4.57%	6.07%

Table 5. The average relative % area of 10 major N-glycan from six IgG samples with CV% measured by FLD.

Common Name	Control IgG	CV%	CHO mAb	CV%	Herceptin	CV%	NIST mAb	CV%	Rituximab	CV%	SiLuMAb	CV%
G0-GlcNAc	0.00%	0.00%	0.00%	0.00%	1.28%	2.66%	0.59%	10.92%	0.00%	0.00%	0.00%	0.00%
G0F-GlcNAc	0.00%	0.00%	1.06%	8.21%	3.18%	2.43%	2.84%	7.18%	0.64%	4.12%	0.55%	9.22%
G0	0.80%	6.03%	3.38%	8.95%	3.74%	3.05%	0.00%	0.00%	0.98%	5.90%	6.04%	9.23%
G0F	26.43%	5.54%	44.03%	9.38%	61.05%	3.43%	42.75%	7.89%	40.97%	4.16%	50.50%	8.79%
Man5	5.25%	5.85%	3.13%	9.58%	2.42%	14.36%	0.95%	11.55%	1.07%	5.96%	1.26%	10.77%
G1	1.11%	6.11%	1.15%	9.11%	1.03%	5.05%	2.58%	11.34%	0.85%	8.62%	1.69%	8.86%
G1F(6)	26.24%	7.09%	29.07%	9.08%	18.39%	2.99%	31.13%	9.62%	34.59%	3.96%	26.52%	7.43%
G1F(3)	18.70%	7.66%	8.99%	13.25%	6.44%	3.06%	10.46%	10.26%	11.11%	1.89%	8.81%	7.10%
G2	1.06%	8.33%	0.00%	0.00%	0.00%	0.00%	1.05%	11.10%	0.00%	0.00%	0.00%	0.00%
G2F	20.40%	7.67%	9.19%	8.44%	2.46%	2.96%	7.65%	9.75%	9.79%	6.59%	4.65%	5.35%

Conclusion

An automated N-glycan releasing, labeling, and cleanup workflow was implemented using the Agilent AssayMAP Bravo and Agilent GlykoPrep InstantPC Kit. Twenty-four antibody samples were processed in one experiment. AssayMAP enables high-throughput and highly reproducible sample preparation, which increases precision and walk-away time. InstantPC-labeled N-glycan showed enhanced signal in both MS and FLD. The Agilent AdvanceBio glycan mapping column provides excellent separation of N-glycans. The Agilent 6545XT AdvanceBio LC/Q-TOF provides highly sensitive and accurate mass measurement to enable N-glycan identification.

With an internal N-glycan database, Agilent MassHunter BioConfirm software enables fast identification by matching the retention time and accurate mass (<2 ppm). BioConfirm also allows batch analysis for N-glycan relative quantitation and automatic plotting of N-glycan content. Fucosylated N-glycans (G0F, G1F, and G2F) are the most abundant glycan in all five mAbs, with a combined abundance of more than 90%. Using automated sample preparation, the CV% of each glycan is within 10%, showing the high reproducibility of the workflow.

FLD quantitation of InstantPC-labeled N-glycan samples showed comparable results to MS quantitation. The differences between the two methods are within 2% for most glycans and within 5% for all glycans. This indicates whether, if glycans are baseline separated by HPLC, MS quantitation is within an acceptable range and relatively accurate compared to quantitation using FLD. This is enabled by the strong MS response of the InstantPC glycan label, particularly when compared with traditional labels such as 2-aminobenzamide (2-AA) and 2-aminobenzoic acid (2-AB).[5]

Alongside manual and automated GlykoPrep N-glycan sample preparation kits, Agilent provides next-generation AdvanceBio Gly-X N-Glycan preparation with InstantPC, which consists of three modules including a deglycosylation module, InstantPC labeling module, and cleanup module with either 24-ct or 96-ct options.6 The Gly-X InstantPC kit offers a shortened manual sample preparation time of under 1 hour by using a 5-minute PNGase F in-solution digestion and labeling with InstantPC dye[7], and also has a 2-AB version using a no-drydown, on-matrix labeling procedure.[8] The future goal is to develop protocols to fully integrate Gly-X sample preparation on the Bravo platform with an automated N-glycan sample preparation.

References

1. Arnold, J. N. *et al.* The Impact of Glycosylation on the Biological Function and Structure of Human Immunoglobulins. *Annu. Rev. Immunol.* **2007**, *25*, 21–50.
2. GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC Automated on AssayMAP Technology, part number GPPNG-PC, Instruction Manual.
3. Oscar, P. *et al.* Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT. *Agilent Technologies application note*, publication number 5991-8071EN, **2017**.
4. Wong, D. L. *et al.* A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis. *Agilent Technologies application note*, publication number 5991-8550EN, **2017**.
5. Yan, J. *et al.* Comparison of Common Fluorescent Labels for LC/MS Analysis of Released N-Linked Glycans. *Agilent Technologies application note*, publication number 5994-0942EN, **2019**.
6. N-Glycan Analysis: Better Together. *Agilent Technologies brochure*, publication number 5994-1647EN, **2020**.
7. Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC kit: Simplified Workflow for Rapid FLD/MS Glycan Analysis. *Agilent Technologies brochure*, publication number 5994-0918EN, **2019**.
8. Yan, J.; Jones, A. Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS. *Agilent Technologies application note*, publication number 5994-1348EN, **2019**.

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