Application Note

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High-Throughput Human Serum and Immunoglobulin G N-Glycome Profiling with the Agilent AdvanceBio Gly-X InstantPC Technology for Biomarker Discovery

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Abstract

Protein glycosylation is an intracellular process resulting in a glycan profile that is dependent upon the host cell and the translated protein. The resultant glycan profile can change with any alteration of the cellular environment. Quantitative glycomic profiling of human plasma or serum yields biological information about the levels and patterns of glycosylation, which are critical for disease diagnostic biomarkers and drug targets. This application note describes the use of the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) for high-throughput N-glycome profiling from human serum and purified immunoglobulin G (IgG) fraction. The actual reaction steps take only 10 minutes, with only 1 µL of human serum or 40 µg of IgG required. This workflow enables high-throughput efficiency and reproducibility in sample preparation for obtaining high-sensitivity N-glycome profiling, and holds great potential for the discovery of disease diagnostic biomarkers.

Introduction

Protein glycosylation is involved in many biological processes, such as receptor interaction, immune response, protein secretion and transport. It also plays a critical role in a wide range of physiological and pathological processes, including cancer; neurodegenerative, liver, infectious, and inflammatory diseases, and other eye, kidney, or arthritis-related disorders.[1-3] Therefore, disease development is usually associated with changes in protein glycosylation. Quantitative glycomic profiling of body fluids or tissues of interest can yield a vast amount of biological information about the levels and patterns of glycosylation present in these samples.

Apart from the nonglycosylated albumin, most of the proteins in human plasma or serum are heavily glycosylated, making them a potential reservoir of glycans, which reflect the body's physiological and pathological states.[4,5] Immunoglobulin G (IgG) is the most abundant glycoprotein in human plasma or serum, and is a major component of the adaptive immune system.[6] Also, recent studies have illustrated the association of altered glycosylation patterns of IgG with various pathological processes.[7-9] However, the continuous development of fast and sensitive strategies is critical for profiling N-glycomes from human serum glycoproteins or purified IgG fraction, for identifying potential disease diagnostic biomarkers.

Most of the commonly used glycan preparation procedures use 2-aminobenzamide (2-AB) or other fluorescent dyes, which are relatively labor-intensive and time-consuming, and deliver low sensitivity and poor reproducibility.[10,11] This application note presents an innovative, streamlined workflow for N-glycan sample preparation from human serum and IgG, followed by hydrophilic interaction liquid chromatography coupled with fluorescence detection (HILIC/FLD). The whole sample preparation process takes under one hour, with as little as 1 µL of human serum or 40 µg of IgG required. All are in a 96-well plate format and are ready for automation.[12] This workflow enables high-throughput and reproducible sample preparation for high-sensitivity N-glycome profiling, and is believed to hold great potential for the discovery of disease diagnostic biomarkers.

Experimental

Materials

The AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) consists of three modules, including Gly-X deglycosylation module (GX96-100), Gly-X InstantPC labeling module (GX96-101), and Gly-X InstantPC cleanup module (GX96-102). Agilent AdvanceBio InstantPC maltodextrin ladder (GKPC-503). Human serum (H4522-100ml) was purchased from Sigma-Aldrich. IgGs were purified from human serum (H4522-100ml) with protein A affinity chromatography using an AKTA Avant system (GE Healthcare) as described previously.[12] HPLC-grade acetonitrile was purchased from Sigma, and Milli-Q water was used in all preparations. All the common chemicals were purchased from Sigma-Aldrich.

Sample preparation deglycosylation

The in-solution enzymatic deglycosylation of human serum or IgG was carried out according to the instruction of the AdvanceBio Gly-X N-glycan prep with InstantPC kit (GX96-IPC).

1. Dilute human serum (1 µL) or IgG (40 µg) with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) to make a final volume of 20 µL.
2. Add 2 µL of Gly-X denaturant to the 20 µL of human serum or IgG solution, and mix thoroughly.
3. Incubate at 90 °C for 3 minutes, leave at room temperature for 2 minutes.
4. Add 2 µL of N-glycanase working solution, and mix thoroughly.
5. Incubate at 50 °C for 5 minutes.

Fluorescent derivatization with InstantPC

6. Prepare InstantPC dye solution by dissolving one vial of InstantPC dye with 150 µL of the accompanying solvent, and mix well.
7. Add 5 µL of the InstantPC dye solution to the above-prepared sample, and mix thoroughly.
8. Incubate at 50 °C for 1 minute.

InstantPC-labeled glycan purification

9. Add 150 µL of the Load/Wash solution (2.5% formic acid/97.5% acetonitrile) to each sample.
10. Transfer the entire sample (179 µL) to each well of the Gly-X cleanup plate containing 400 µL of Load/Wash solution.
11. Wash samples three times with 600 µL of Load/Wash solution after passing the solution through the cleanup plate by applying a vacuum.

12. Elute the InstantPC-labeled N-glycans with 100 µL of Gly-X InstantPC eluent (160 mM ammonium formate/10% (v/v) acetonitrile, pH 4.4).
13. Analyze the collected N-glycan solutions immediately without further treatment, or alternatively, store at −20 °C for future analysis.

HILIC/FLD analysis of InstantPC-labeled N-glycans

The profiles of InstantPC-labeled N-glycans from human serum or IgG were determined by HILIC/FLD using the Agilent 1260 Infinity II LC system equipped with Agilent AdvanceBio Glycan Mapping column (120 Å, 2.1 × 150 mm, 2.7 µm (part number 683775-913)) controlled by the Agilent OpenLab ChemStation software. The system consists of a quaternary solvent pump, an autosampler, and a fluorescence detector. The detector was set with excitation and emission wavelengths at 285 and 345 nm, respectively, for InstantPC. The InstantPC-labeled glycan samples, without any further treatment, were injected at a volume of 1 µL. The N-glycans were separated with 50 mM ammonium formate (pH 4.4) as solvent A, and acetonitrile as solvent B. The HPLC system was equilibrated with 50 mM ammonium formate (pH 4.4) and acetonitrile (27/73, v/v) for 1.5 minutes, at a flow rate of 0.5 mL/min. After, the separation was carried out by a linear gradient of 73 to 62% acetonitrile (v/v) in a 35-minute analytical run, at a flow rate of 0.5 mL/min. Samples were maintained at 5 °C before injection and the column temperature was set to 60 °C. The system was calibrated using the AdvanceBio InstantPC maltodextrin ladder (GKPC-503). The glucose unit (GU) value and retention time T (minutes) data were fitted to a fifth-order polynomial curve to obtain the standard curve.

Results and discussion

AdvanceBio Gly-X technology for express glycan preparation

As demonstrated in Figure 1, the AdvanceBio Gly-X N-glycan prep with InstantPC kit used in this study enables streamlined N-glycan sample preparation in an innovative 96-well plate format workflow. The kit features a three-minute glycoprotein denaturation at 90 °C, followed by a five-minute deglycosylation at 50 °C. This enables the complete release of glycans from the targeting glycoproteins in an efficient way.

Also, with the introduction of the InstantPC fluorescent label, an active form of procaine, the released glycosylamine intermediates are attached to InstantPC via activated carbamate chemistry to form a stable urea linkage. This innovative glycan preparation workflow takes only 10 minutes from glycoprotein preparation to glycan profiling, and uses as little as 1 µL of human serum or 40 µg of IgG. Therefore, it ensures a complete qualitative and quantitative glycomic analysis.

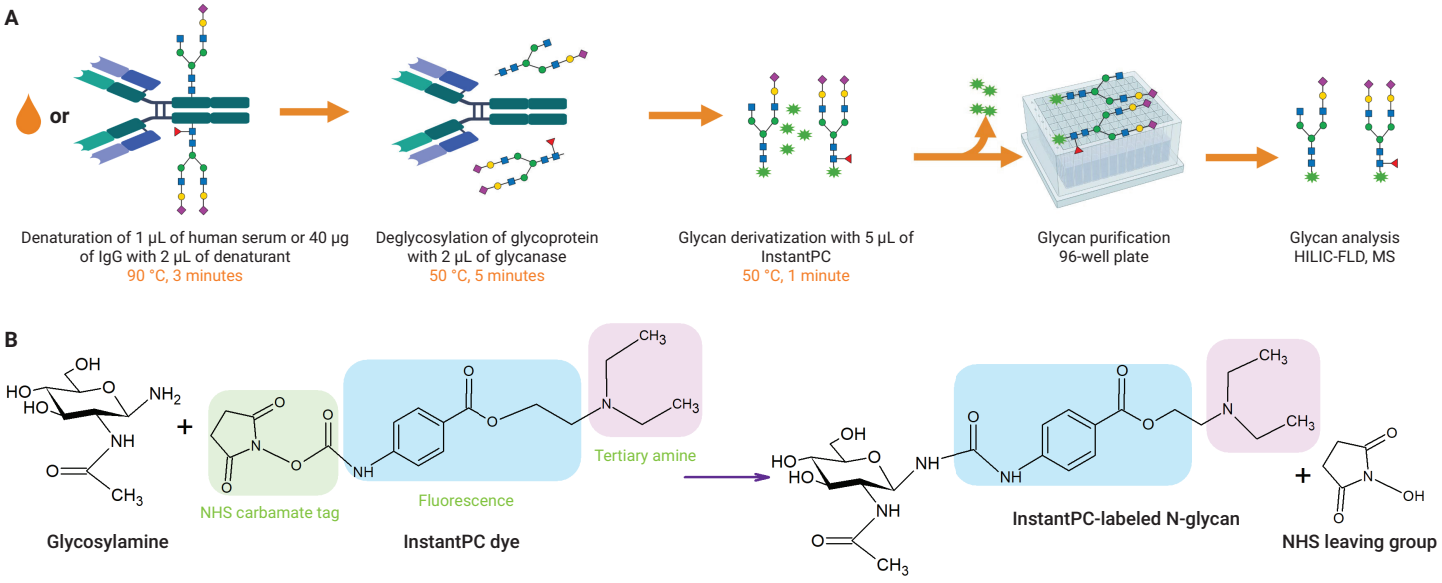


Figure 1. Agilent AdvanceBio Gly-X technology for glycosylamine release and InstantPC derivatization. (A) Workflow for glycosylamine release from human serum or IgG, InstantPC derivatization, and purification. (B) Activated carbamate chemistry based InstantPC labeling of glycosylamine. Created with ACD/ChemSketch and BioRender.com.

Human serum IgG InstantPC-labeled glycan profiling by HILIC/FLD

The N-glycomic profiles of human serum and IgG have been studied extensively as it pertains to disease biomarker discovery. In this application note, the released glycosylamine intermediates from human serum and IgG were labeled with InstantPC for qualitative and quantitative analysis by HILIC/FLD. Under the developed chromatographic conditions (Table 1), the InstantPC-labeled N-glycans from both IgG and human serum resulted in well-resolved peaks for all major N-glycan species (Figures 2 and 3). The developed HILIC/FLD method for glycan profiling shows good reproducibility (data not shown).

As shown in Figure 2 and Table 2, human serum IgG possesses a variety of N-glycan species. These predominantly consist of high-fucose glycans, sialic acid glycans, GlcNAc-bisected glycans, and some neutral galactose and GlcNAc-containing glycans. The most abundant glycan is FA2G1 (24.95%), followed by FA2 (18.36%), FA2G2 (13.01%), FA2G2S1 (11.13%), respectively.

Human serum InstantPC-labeled glycan profiling by HILIC/FLD

Figure 3 and Table 2 show a typical N-glycome profile for human serum, with A2G2S2 as the dominant glycan peak, accounting for 41.90% of the relative abundance. Also, there are three major glycan peaks existing in a lesser abundance, including A3G3S3 (7.65%), A2G2S1 (6.13%), and FA2G1 (5.35%). The other glycan peaks are assigned to a various N-glycan species, including fucose glycans, sialic acid glycans, GlcNAc-bisected glycans, and some neutral galactose and GlcNAc-containing glycans, with a relative abundance of less than 5%.

Table 1. HILIC/FLD conditions for InstantPC-labeled N-glycans profiling.

Parameter	Value
Instrument	Agilent 1260 Infinity II LC system
Column	Agilent AdvanceBio Glycan Mapping column, 120 Å, 2.1 × 150 mm, 2.7 µm (p/n 683775-913)
Column Temperature	60 °C
Mobile Phase	A) 50 mM ammonium formate (pH 4.4) B) Acetonitrile
Gradient Program	Time (min) A (%) B (%) Flow rate (mL/min) 0 27 73 0.5 1.5 27 73 0.5 36.5 38 62 0.5 45.0 47 53 0.5 46 70 30 0.4 47 27 73 0.25 48 27 73 0.5 60 27 73 0.5
Injection Volume	1 µL (equivalent to glycans from 0.01 µL human serum or 0.4 µg IgG)
Detection	Agilent 1260 Infinity II LC system FLD λEx 285 and λEm 345 nm

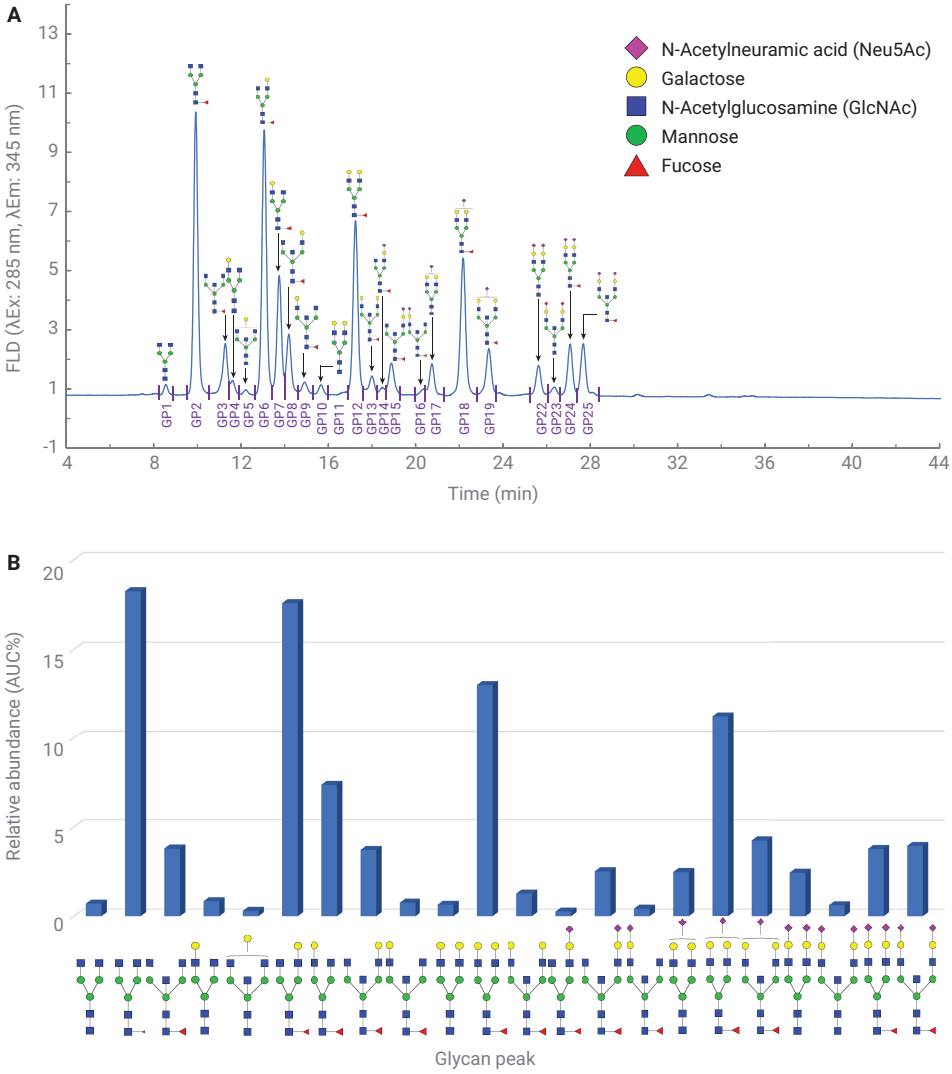


Figure 2. HILIC/FLD chromatograms of InstantPC-labeled IgG N-glycans (A), and relative abundance of major N-glycan species (B). Only major glycan peaks are displayed.

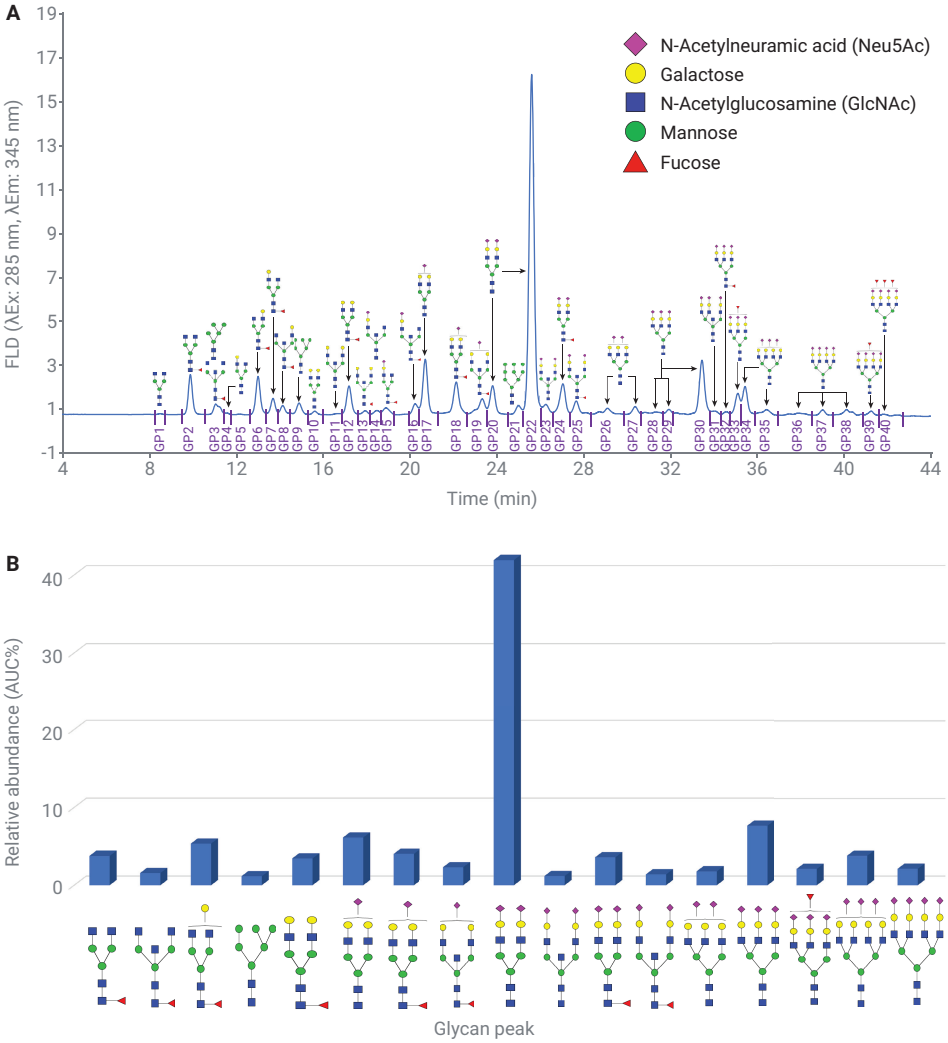
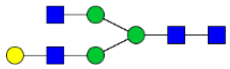
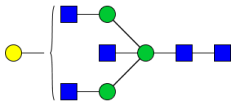
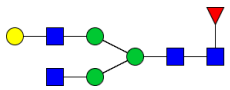
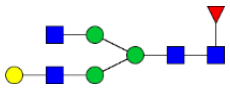
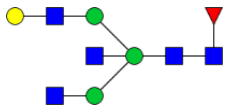
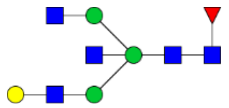
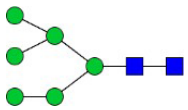
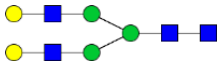
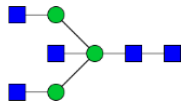
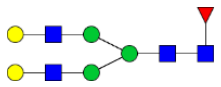
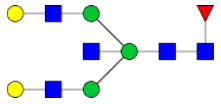
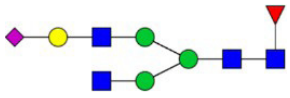
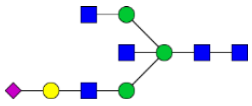
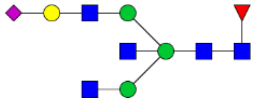
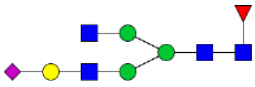
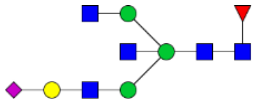
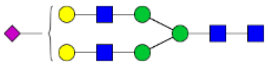
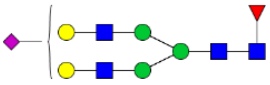
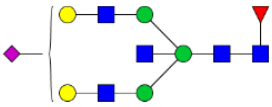
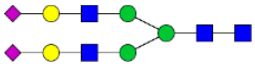
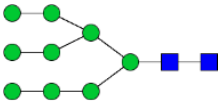
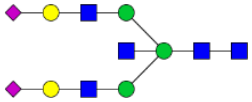
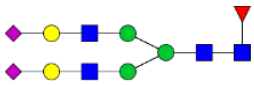
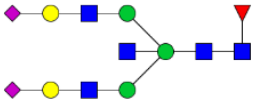
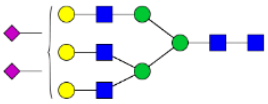
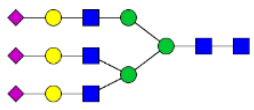
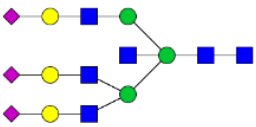
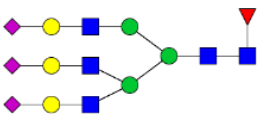
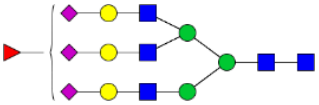


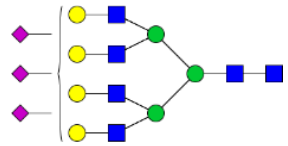
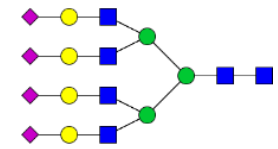
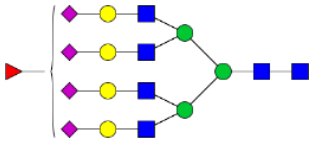
Figure 3. HILIC/FLD chromatograms of InstantPC-labeled human serum N-glycomes (A), and relative abundance of major N-glycan species (B). Only major glycan peaks are displayed.

Table 2. Human serum and IgG N-glycome profiling labeled with InstantPC.

Label	Oxford Notation Name	GU	N-Glycan Structure	IgG		Human Serum	
				AUC	AUC (%)	AUC	AUC (%)
GP1	A2	5.441		5.008	0.690	0.566	0.086
GP2	FA2	5.946		133.363	18.362	24.686	3.766
GP3	FA2B	6.368		28.313	3.898	10.262	1.565
	M5	6.368		–	–		

Label	Oxford Notation Name	GU	N-Glycan Structure	IgG		Human Serum	
				AUC	AUC (%)	AUC	AUC (%)
GP4	A2[3]G1	6.501		6.169	0.849	0.793	0.121
GP5	A2BG1	6.690		2.128	0.293	0.356	0.054
GP6	FA2[6]G1	6.951		127.747	17.589	25.302	3.859
GP7	FA2[3]G1	7.159		53.461	7.361	9.759	1.488
GP8	FA2[6]BG1	7.291		26.905	3.705	5.334	0.813
GP9	FA2[3]BG1	7.510		5.401	0.744	7.613	1.161
	M6	7.510		–	–		
GP10	A2G2	7.729		4.616	0.636	2.182	0.333
GP11	A2BG2	7.973		–	–	0.200	0.031
GP12	FA2G2	8.196		94.452	13.005	22.588	3.446
GP13	FA2BG2	8.420		9.196	1.266	3.331	0.508
GP14	FA2[6]G1S1	8.570		1.833	0.252	3.541	0.540
	A2[3]BG1S1	8.570		–	–		
GP15	FA2[6]BG1S1	8.695		18.217	2.508	6.925	1.056
	FA2[3]G1S1	8.695		–	–		

Label	Oxford Notation Name	GU	N-Glycan Structure	IgG		Human Serum	
				AUC	AUC (%)	AUC	AUC (%)
GP16	FA2[3]BG1S1	9.126		3.007	0.414	7.325	1.117
GP17	A2G2S1	9.252		17.917	2.467	40.173	6.128
GP18	FA2G2S1	9.692		80.837	11.130	26.382	4.024
GP19	FA2BG2S1	10.064		29.300	4.035	15.114	2.306
GP20	A2G2S2	10.217		–	–	274.665	41.898
GP22		10.773		17.728	2.441		
GP21	M9	10.588		–	–	6.234	0.951
GP23	AA2BG2S2	10.988		4.526	0.623	7.718	1.177
GP24	FA2G2S2	11.222		27.512	3.788	23.509	3.586
GP25	FA2BG2S2	11.410		28.651	3.945	9.084	1.386
GP26	A3G3S2	11.863		–	–	11.642	1.776
GP27	12.256						
GP28	A3G3S3	12.550		–	–	50.139	7.648
GP29		12.739					
GP30		13.218					
GP31	A3BG3S3	13.406		–	–	2.779	0.424
GP32	FA3G3S3	13.581		–	–	1.165	0.178
GP33	A3F1G3S3	13.760		–	–	13.765	2.100

Label	Oxford Notation Name	GU	N-Glycan Structure	IgG		Human Serum	
				AUC	AUC (%)	AUC	AUC (%)
GP34	A4G4S3	13.873		–	–	24.941	3.805
GP35		14.220					
GP36	A4G4S4	14.770		–	–	13.857	2.113
GP37		15.232					
GP38		15.734					
GP39	A4F1G4S4	16.315		–	–	3.631	0.554

1. InstantPC–labeled maltodextrin ladder standard curve: $GU = -0.0215 + 1.109T - 0.07722T^2 + 0.003524T^3 - 0.00007714T^4 + 0.0000006543T^5$.
2. The relative abundance of each N–glycan was calculated with the following equation: $\text{FLR AUC}(\%) = \text{FLR AUC}_{\text{glycan i}} / \text{sum}(\text{FLR AUC}_{\text{glycans}}) \times 100$
3. The GU, area under the curve (AUC), relative abundance (AUC%) were calculated by averaging two different measurements.
4. Only the most abundant glycan peaks are displayed.

Conclusion

Protein glycosylation is a posttranslational intracellular process that may change during disease development and result in alterations in glycan profiles. Glycomic profiling of human serum and immunoglobulin G (IgG) yields biological information about the relative levels and patterns of glycosylation, which could eventually be used as diagnostic biomarkers for disease progression. This application note showed that the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit can achieve high-throughput glycan preparation for human serum and IgG N-glycome profiling. The reaction steps take approximately 10 minutes and the innovative fluorescent dye, InstantPC, labels the glycosylamine intermediates in an “instant” manner and with high fluorescence signal. Furthermore, the Gly-X N-Glycan Prep with InstantPC kit can use as little as 1 µL of human serum to prepare labeled N-glycans for HILIC separation. The streamlined 96-well plate-based format for glycan sample preparation is reliable and robust. Finally, the developed HILIC/FLD method separates InstantPC-labeled N-glycans from human serum and IgG into well-resolved peaks.

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