Application Note

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High-Throughput N-Glycans Profiling of Monoclonal Antibodies EG2-hFc and Rituximab Using the Agilent AdvanceBio Gly-X N-Glycan Prep with InstantPC Kit

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Abstract

Protein glycosylation is one of the main sources of heterogeneity of therapeutic glycoproteins, with potential impact on safety and efficacy, and is therefore considered a critical quality attribute (CQA). Detailed characterization and quantification of glycan profiles of the therapeutics should be performed routinely to ensure desired product quality. However, glycosylation can introduce considerable complexity and heterogeneity to therapeutic drugs, which can significantly hamper the progress in biopharmaceuticals glycan characterization and quality control. This application note describes use of the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) for high-throughput, high-sensitivity glycan profiling. This kit allows rapid glycoprotein denaturation, deglycosylation, glycan derivatization, purification, and detection of InstantPC-labeled N-glycans from two monoclonal antibodies of EG2-hFc and rituximab with different molecular sizes. The total glycan preparation procedure has been shortened significantly to less than one hour, accompanied with a highly sensitive fluorescence detection. Therefore, this type of workflow holds great potential for reliable and reproducible sample preparation for high-throughput glycan profiling and quantitation. It can also easily be adapted to any analytical laboratory requirements.

Introduction

Glycosylation is one of the most common and complex post-translational modifications of therapeutic proteins.[1-3] Glycosylation of biopharmaceutical drugs plays a critical role in their safety and efficacy by modulating many properties, such as protein folding, stability, pharmacokinetics, immunogenicity, and effector functions.[4-6] Therefore, glycosylation is often deemed a CQA of therapeutic glycoproteins[7], and a thorough characterization and quantification of the glycan profiles of the therapeutics is necessary to ensure consistent desired product quality. However, many factors such as mammalian host cell lines, culture media, the bioprocess conditions, and the downstream purification strategies affect glycosylation profiles.[8-10] As a result, glycosylation introduces considerable complexity and heterogeneity to therapeutic drugs, varying in monosaccharide composition, glycosidic linkages, and glycan branching.[11] This significantly hampers the progress in biopharmaceutical glycan characterization and quality control.

There are a wide range of techniques being developed for glycan profiling, including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), nuclear magnetic resonance (NMR), and microarray-based technology.[12] However, hydrophilic interaction liquid chromatography coupled with fluorescence detection (HILIC/FLD) serves as the standard choice of method, postderivatization with 2-aminobenzamide (2-AB).[13] A major drawback to glycan profiling is the time-consuming sample preparation, which results in poor reproducibility and low sensitivity. Previously, we have developed and streamlined workflow featuring a 96-well plate-based platform for high-throughput and high-sensitivity glycan profiling, with potential application for biopharmaceutical development and disease biomarker discovery.[14]

Monoclonal antibodies (mAbs) represent the major category of glycoprotein-based therapeutic drugs.[15] This application note used the mAbs of EG2-hFc and rituximab to demonstrate the feasibility of this innovative workflow. EG2-hFc is a humanized camelid-type antibody displaying a devoid of light chains and reduced molecular size of approximately 80 kDa. EG2-hFc targets the epidermal growth factor receptor (EGFR) that is most commonly overexpressed in nonsmall cell lung cancer (NSCLC).[16] Rituximab is the world's first oncology-relevant monoclonal antibody therapy. It is genetically engineered to contain murine light and heavy chain variable region sequences, along with the human constant region sequences, and has a molecular size of approximately 145 kDa. Rituximab targets and binds to the CD20 antigen, which is primarily found on the surface of both normal and malignant immune system B cells. It is routinely used to treat adults with non-Hodgkin's lymphoma (NHL) or chronic lymphocytic leukemia (CLL).[17,18] By using "instant" glycoprotein deglycosylation and fluorescent derivatization from the workflow, the total glycan preparation shortens significantly to less than 10 minutes, with high sensitivity for fluorescence detection. Therefore, the developed, robust workflow holds great potential for reliable and reproducible sample preparation for high-throughput glycan profiling and quantitation of mAbs, and can be adapted for any other glycoprotein-based therapeutic drug with minimal modification.

Experimental

Materials

The AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) consists of three modules:

- Gly-X deglycosylation module (GX96-100)
- Gly-X InstantPC labeling module (GX96-101)
- Gly-X InstantPC clean up module (GX96-102).

Agilent AdvanceBio InstantPC maltodextrin ladder (GKPC-503)

Monoclonal antibodies of EG2-hFc and rituximab were produced from Chinese hamster ovary (CHO) cells and purified with protein A affinity chromatography using an AKTA Avant system (GE Healthcare) as described previously.[14] 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 monoclonal antibodies, EG2-hFc and rituximab, were carried out according to the instructions of the AdvanceBio Gly-X N-glycan prep with InstantPC kit (GX96-IPC).

1. Dilute monoclonal antibodies, EG2-hFc and rituximab (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 EG2-hFc and rituximab solution, 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, 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, mix well.
7. Add 5 µL of the InstantPC dye solution to the sample prepared above, 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 with 600 µL of Load/Wash solution three times after passing the solution through the cleanup plate by applying 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 EG2-hFc and rituximab were determined by HILIC/FLD using the Agilent 1260 Infinity system equipped with Agilent AdvanceBio Glycan Mapping column (120 Å, 2.1 × 150 mm, 2.7 µm (part number 683775-913)) and the Agilent OpenLab ChemStation software. The system consists of a quaternary solvent pump, 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 were injected at a volume of 1 µL without any further treatment before injection.

The N-glycans were separated using 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 done using a linear gradient of 73 to 62% acetonitrile (v/v) in 30 minutes. Samples were stored 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 a 96-well plate format workflow. The kit features a three minute glycoprotein

Figure 1. Agilent AdvanceBio Gly-X technology for glycosylamine release and InstantPC derivatization. (A) Workflow for in-solution glycoprotein deglycosylation, InstantPC derivatization, and on-matrix clean up. (B) Reaction scheme for activated carbamate chemistry based InstantPC derivatization of released glycosylamine.

denaturation at 90 °C, followed by a five minute deglycosylation at 50 °C, which enables the release of glycans from the targeting glycoproteins in an efficient way. Also, with the introduction of 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. By taking advantage of this innovative workflow, it only takes one hour from glycan preparation to glycan profiling, to obtain a complete qualitative and quantitative glycan analysis.

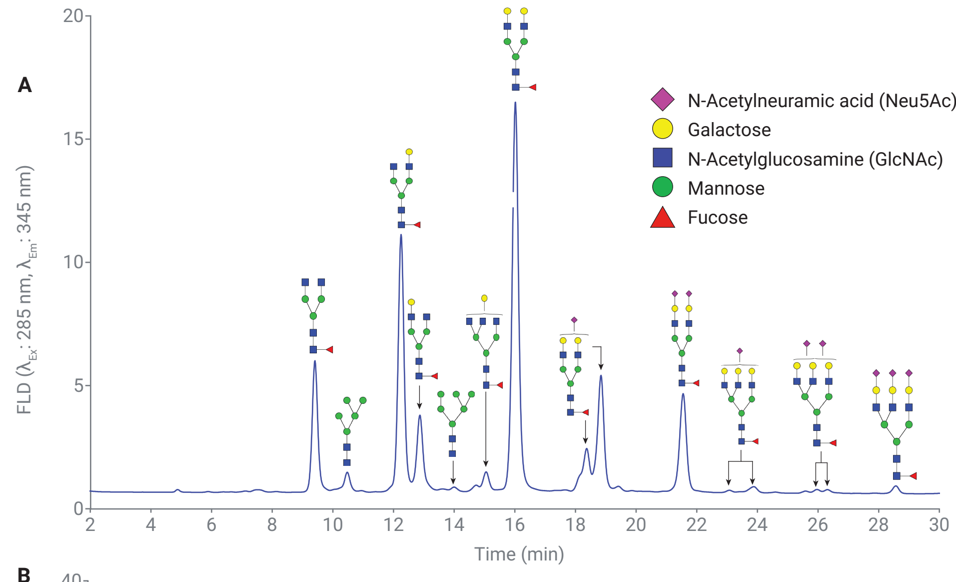
InstantPC-labeled glycan profiling of monoclonal antibodies by HILIC/FLD

Monoclonal antibodies of EG2-hFc and rituximab were selected as model glycoproteins. Their released glycosylamine intermediates 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 EG2-hFc and rituximab 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 2A, the glycan profiles of EG2-hFc are typical for mAbs produced from CHO cells. They consist predominantly of neutral biantennary complex N-glycans with core fucosylation, and a relatively low proportion of sialylated N-glycans, with GlcNAc-bisected glycans being absent. As shown in Figure 2B and Table 2, the most abundant glycan is FA2G2 (33.138%), followed by FA2G1 (27.200%), FA2G2S1 (14.324%), FA2 (9.878%), and FA2G2S2 (9.249%), respectively. Similarly, in Figure 3A, rituximab also possesses typical N-glycan profiles for mAbs produced from CHO cells like its counterpart, EG2-hFc. However, a

Table 1. HILIC/FLD conditions for InstantPC-labeled N-glycan 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 31.5 / 38 / 62 / 0.5 33 / 70 / 30 / 0.4 34 / 27 / 73 / 0.25 35 / 27 / 73 / 0.5 50 / 27 / 73 / 0.5
Injection Volume	1 µL (equivalent to glycans from 0.4 µg of glycoprotein)
Detection	Agilent 1260 Infinity II LC system FLD λ_{Ex} 285 and λ_{Em} 345 nm



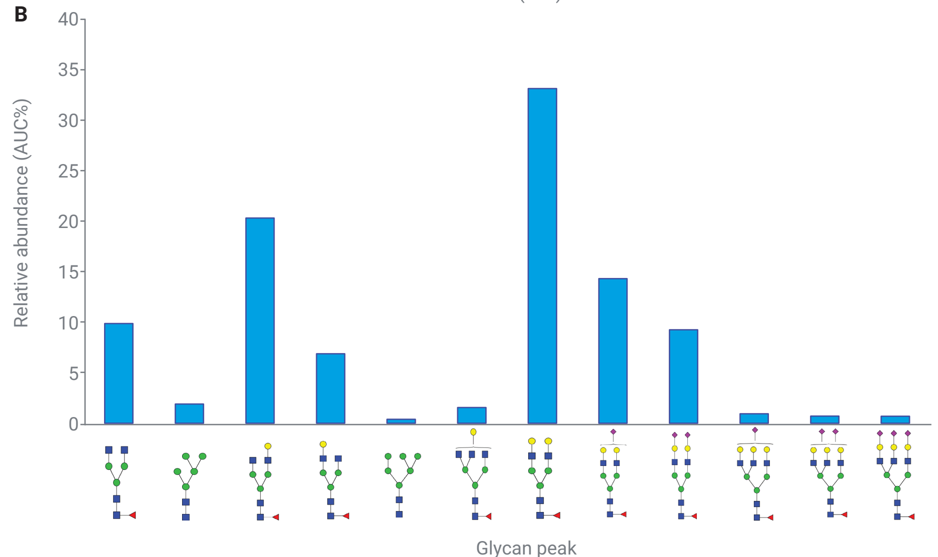


Figure 2. (A) HILIC/FLD chromatograms of InstantPC-labeled N-glycans from EG2-hFc. (B) Relative abundance of each N-glycan species.

noticeable difference was observed in the relative abundance of each N-glycan peak. As shown in Figure 3B and Table 2, the most abundant glycan is FA2 (40.208%), followed by FA2G1 (35.672%), and FA2G2 (10.734%), respectively. Low level of high-mannose glycan species were also detected, M5 (6.173%), M6 (0.941%), and M7 (0.440%), respectively. Regarding sialylated glycan species, the relative abundance was negligible, FA2G2S1 (1.757%), FA2G2S2 (0.828%), and FA1G1S1 (0.431%), respectively. The average area under the curve (%AUC) values agreed with the previously obtained values for rituximab N-glycans labeled with InstantPC and analyzed using Agilent LC/FLD/MS instrumentation.[19] However, a larger than usual abundance for M5 species was observed, which could be attributed to the expression of rituximab in CHO cells in-house and the resulting batch-to-batch variations that arise between glycan species that are present in low abundance.[20]

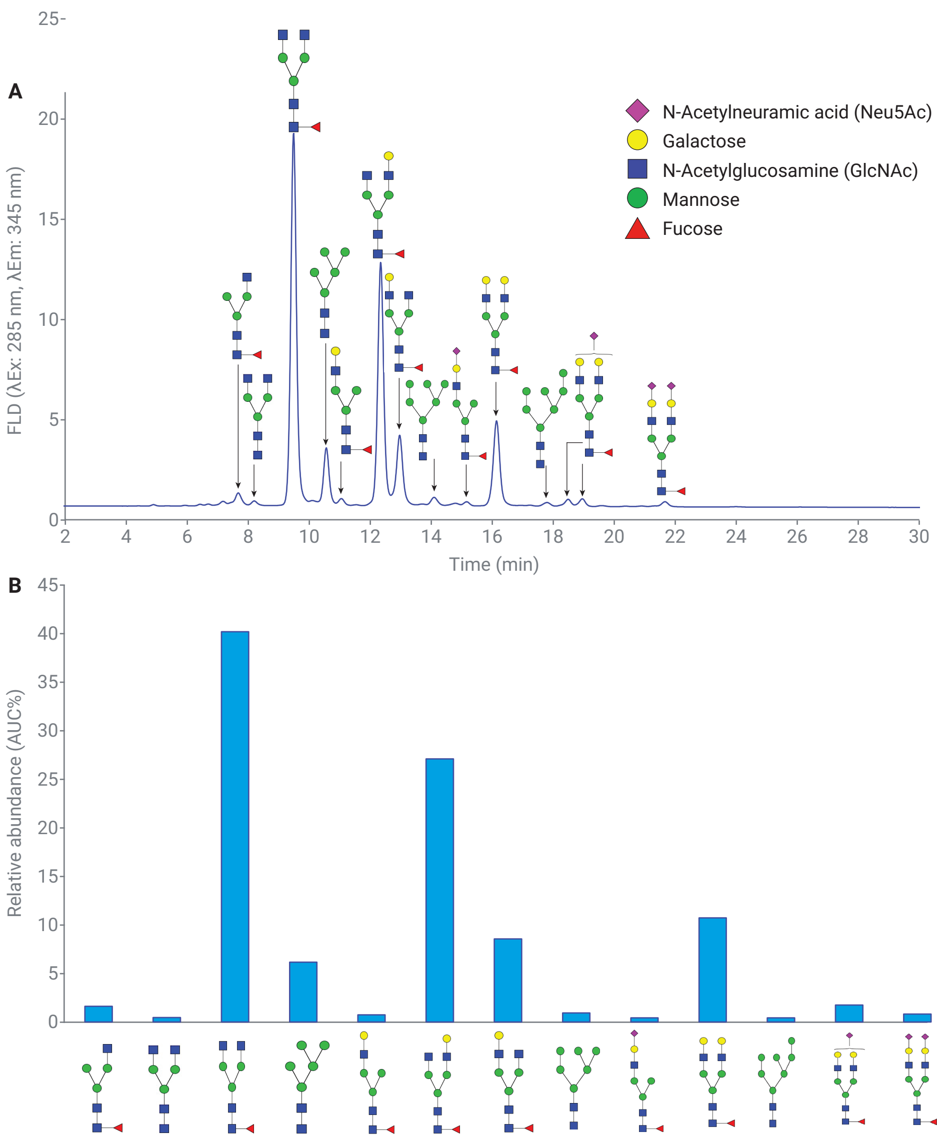


Figure 3. (A) HILIC-FLD chromatograms of InstantPC-labeled N-glycans from rituximab. (B) Relative abundance of each N-glycan species.

Table 2. Monoclonal antibodies of EG2-hFc and rituximab N-glycan profiling labeled with InstantPC.

Oxford Notation Name	N-glycan Structure	GU	EG2-hFc		Rituximab	
			AUC	AUC (%)	AUC	AUC (%)
FA1[6]		5.202	–	–	9.848	1.616
A2		5.425	–	–	2.839	0.465
FA2		5.922	71.368	9.878	244.882	40.208
M5		6.320	13.735	1.901	37.594	6.173
FA1[3]G1		6.515	–	–	4.487	0.737
FA2[6]G1		6.942	146.760	20.313	165.128	27.113
FA2[3]G1		7.152	49.752	6.886	52.128	8.559
M6		7.526	2.885	0.399	5.730	0.941
FA3G1		7.859	11.298	1.563	–	–
FA1[3]G1S1		7.891	–	–	2.625	0.431
FA2G2		8.194	239.420	33.138	65.368	10.734
M7		8.754	–	–	2.679	0.440
FA2G2S1		8.968 9.124	103.495	14.324	10.699	1.757
FA2G2S2		10.048	66.820	9.249	5.042	0.828
FA3G3S1		10.564 10.846	6.799	0.941	–	–

Oxford Notation Name	N-glycan Structure	GU	EG2-hFc		Rituximab	
			AUC	AUC (%)	AUC	AUC (%)
FA3G3S2		11.594 11.720	5.124	0.709	–	–
FA3G3S3		12.553	5.045	0.698	–	–

1. InstantPC–labeled maltodextrin ladder standard curve: $GU = 0.007522 + 1.037T - 0.06697T^2 + 0.003039T^3 - 0.00006653T^4 + 0.0000005791T^5$.
2. The relative abundance of each N–glycan was calculated with the following equation: $FLR\ AUC(\%) = FLR\ AUC_{glycan\ i}/sum(FLR\ AUC_{glycans}) \times 100$
3. The glucose unit (GU), area under the curve (AUC), and relative abundance (AUC%) were calculated by averaging different measurements.
4. "–" Not detected.

Conclusion

Protein glycosylation plays a critical role on the safety and efficacy of therapeutic glycoproteins and is deemed as a critical quality attribute. This application note showed that the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit can achieve high-throughput glycan preparation for biotherapeutics glycan profiling, with the whole process taking less than one hour. InstantPC is an innovative fluorescent dye, which labels the glycosylamine intermediates in an "instant" manner with high FLD sensitivity. Moreover, the developed HILIC/FLD method separates InstantPC-labeled N-glycans from monoclonal antibodies EG2-hFc and rituximab into well-resolved peaks. Finally, the streamlined 96-well plate-based format for glycan sample preparation is reliable and robust, with good reproducibility.

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