Application Note

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High-Throughput N-Glycan Analysis with Agilent AdvanceBio InstantPC Kit

Sample preparation using a liquid handling workstation

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Abstract

This application note describes a fully automated workflow for high-throughput N-glycan analysis of therapeutic antibodies using the Tecan Freedom EVO liquid handling workstation in combination with the Agilent AdvanceBio Gly-X InstantPC labeling kit and UHPLC-HILIC separation. Comparative analysis demonstrated that the glycoform distribution in samples prepared using the automated method was consistent with those prepared manually. This robust, scalable solution enhances analytical efficiency and consistency, supporting the streamlined characterization of critical quality attributes during biopharmaceutical development.

Introduction

The development and manufacturing of therapeutic antibodies require frequent monitoring of multiple product quality attributes. Numerous samples are often required to be analyzed during clone selection and throughout process development to ensure a successful final product, which may slow down the development of new therapeutics. Rapid and high-throughput screening of therapeutic antibodies can accelerate the development process, improve the overall quality of biopharmaceutical products, and reduce manufacturing costs. N-glycosylation of therapeutic antibodies starts as a cotranslational step followed by a set of post-translational modifications (PTM) and is considered a critical quality attribute due to its impact on biological functions as well as therapy outcome.[1]

N-glycan analysis is a more complex tool compared with other liquid chromatography or capillary electrophoresis analysis because of the relative higher cost and the high technical requirements for lab analysts due to the complexity of this test. Our fully automated N-glycan sample preparation platform effectively lowers the labor requirements for N-glycan detection. This application note presents an automated workflow for N-glycan analysis. A Tecan Freedom EVO was used for the automatic sample preparation. The AdvanceBio Gly-X InstantPC methods, along with the automation of sample pretreatment, can help achieve the high-efficiency analysis of N-glycan profiling.

Experimental

Materials

Analytical Reagent grade acetonitrile (ACN) and ammonium formate were purchased from Merck. Water was purified using a Milli-Q water purification system (Millipore). Glycoprotein samples included a monoclonal antibody (mAb), a bisepcific antibody, and an Fc fusion protein. Released and labeled N-glycans were prepared using AdvanceBio Gly-X N-glycan prep with InstantPC dye (part numbers GX96-IPC and GX24-IPC), using a modified protocol detailed in the following section. Labeled glycans were separated by hydrophilic interaction liquid chromatography (HILIC). UHPLC-HILIC separation was performed on an amide column (2.1 × 150 mm, 1.7 µm).

Sample preparation

Using a Tecan EVO 200 liquid handling workstation, a fully automated workflow was developed for sample preparation (Figure 1).

To treat samples of different concentrations from various processes, the first step involved the dilution of samples by adding diluent to a 96-well plate (reaction plate) for a total volume of 15 µL per well and a maximum of 40 µg of protein. The volume of diluent and protein added to each well was automatically calculated based on the protein concentration of the samples obtained from the database. The diluent was added using an independent eight-channel pipette

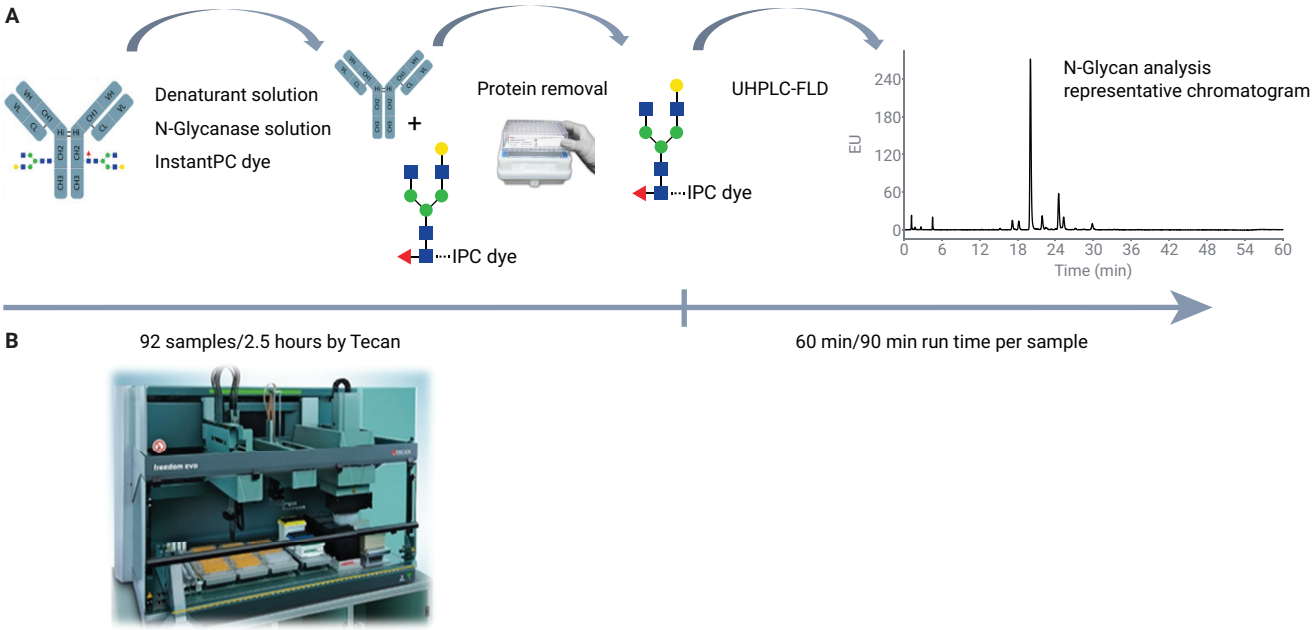

Figure 1. (A) Automated antibody sample preparation workflow with (B) the Tecan Freedom EVO for automation.

without changing the tips. This allowed any position, volume, and number of pipetting operations in the 96 wells to be completed within one minute.

In the denaturation, N-glycan release and labeling can automatically be finished in the same Inheco incubator-equipped Tecan workstation. A 3 µL sample of Digestion Buffer from the Agilent AdvanceBio Gly-X InstantPC kit was added to each well containing the samples to adjust the pH, followed by the addition of 5 µL of denaturant working solution (containing 2 µL of Denaturant from the Gly-X InstantPC kit and 3 µL of proprietary WuXi buffer). The plate was then transferred by a robotic arm to an incubator set to 400 rpm, 70 °C incubation temperature, and 80 °C lid temperature for five minutes of incubation. After removing the plate and cooling it at room temperature for one minute, 5 µL of N-Glycanase working solution (1 µL of N-Glycanase, 1 µL of Digestion Buffer, and 3 µL of proprietary WuXi buffer) were added to each well. The plate was then transferred by the robotic arm to an incubator set to 400 rpm, 50 °C incubation temperature, and 60 °C lid temperature for another five minutes of incubation. Then, 5 µL of labeling solution was added to each well, and the plate was returned to the same incubator for another five minutes. The final step on the 96-well plate was to add 150 µL of Load/Wash Solution (2.5% formic acid in ACN) to each well.

The sample was then mixed by pipetting up and down and transferred entirely to a purification plate well that was rinsed with 400 µL of load and washing buffer (2.5% formic acid in ACN) to perform the final purification step. The purification plate was first vacuumed until all the solution passed through. Then, 600 µL of load and washing buffer was added and vacuumed through the plate until all the solution passed through. This process was repeated twice. Next, the robotic arm placed the purification plate above a collection plate, and 100 µL of Gly-X InstantPC Eluent was added to each well. Vacuum was applied again to allow the liquid in the purification plate to flow into the collection plate. The collection plate was then directly transferred to the UHPLC for analysis.

Workflow overview

1. Import the sample information.
2. Add water and sample into the reaction plate for a total volume of 15 µL per well and a maximum of 40 µg of protein.
3. Add 3 µL of Digestion Buffer.
4. Add 5 µL of denaturing working solution (2 µL of denaturing solution and 3 µL of proprietary WuXi buffer per reaction).
5. Move the reaction plate to the heating block at 400 rpm and 70 °C for 5 minutes.
6. Remove the plate back and cool it at room temperature for one minute.
7. Add 5 µL of N-Glycanase working solution (1 µL of N-Glycanase, 1 µL of Digestion Buffer and 3 µL of proprietary WuXi buffer) per reaction.
8. Move the reaction plate to heating block at 400 rpm and 50 °C for five minutes.
9. Remove the plate back and add 5 µL of labeling solution.
10. Move the reaction plate to the heating block at 400 rpm and 50 °C for five minutes.
11. Add 150 µL of Load/Wash Solution to each well.
12. Equilibrate the purification plate with 400 µL of Load/Wash Solution.
13. Load the samples (approximately 180 µL) in the reaction plate to purification plate.
14. Apply vacuum through the purification plate until all the solution passes through.
15. Wash the purification plate twice by adding two 600 µL volumes of load/wash buffer and applying vacuum.
16. Transfer the purification plate over the collection plate.
17. Add 100 µL of Gly-X InstantPC Eluent into the purification plate.
18. Apply vacuum to allow the liquid in the purification plate to flow into the collection plate.
19. Move the collection plate directly to UHPLC for analysis.

UHPLC analysis

The UHPLC analysis of N-glycan was performed using either a 60- (for 1-2 glycosylation site proteins, e.g. monoclonal antibody) or 90-minute method (for highly glycosylated proteins, e.g. fusion protein) with 100 mM ammonium formate and acetonitrile mobile phase on a UHPLC system. The UHPLC method parameters and gradients used are shown in Table 1 and Table 2, respectively.

Table 1. UHPLC method parameters.

Parameter	Value
Detection	Fluorescence detector (FLD)
Wavelength	λEx 285 nm, λEm 345 nm
Column	Amide 1.7 µm, 2.1 × 150 mm, 130/300 Å
Column Temperature	60 ± 5 °C
Autosampler Temperature	8 ± 5 °C
Injection Volume	1 µL
Flow Rate	0.4 mL/min
Run Time	60/90 min
Mobile Phase	A) 100 mM Ammonium formate, pH 4.50 ± 0.05 B) 100% Acetonitrile

Table 2. Gradients used in the UHPLC method.

60-Minute Gradient Program			
Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**
0.0	0.4	20.0	80.0
2.0	0.4	20.0	80.0
2.5	0.4	25.0	75.0
50.0	0.4	38.0	62.0
52.0	0.4	60.0	40.0
53.5	0.4	60.0	40.0
55.0	0.4	20.0	80.0
60.0	0.4	20.0	80.0
90-Minute Gradient Program			
Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**
0.0	0.4	20.0	80.0
2.0	0.4	20.0	80.0
2.5	0.4	25.0	75.0
80.0	0.4	38.0	62.0
82.0	0.4	60.0	40.0
83.5	0.4	60.0	40.0
85.0	0.4	20.0	80.0
90.0	0.4	20.0	80.0

Results and discussion

To verify the reliability of automated sample preparation, manual (protocol detailed in Agilent publication 5994-1231EN) and automated sample preparation results in duplicate were compared. The signal and distributions of N-glycans were comparable in both sample preparation methods, and there was no significant difference in the five main glycoforms (Figure 2).

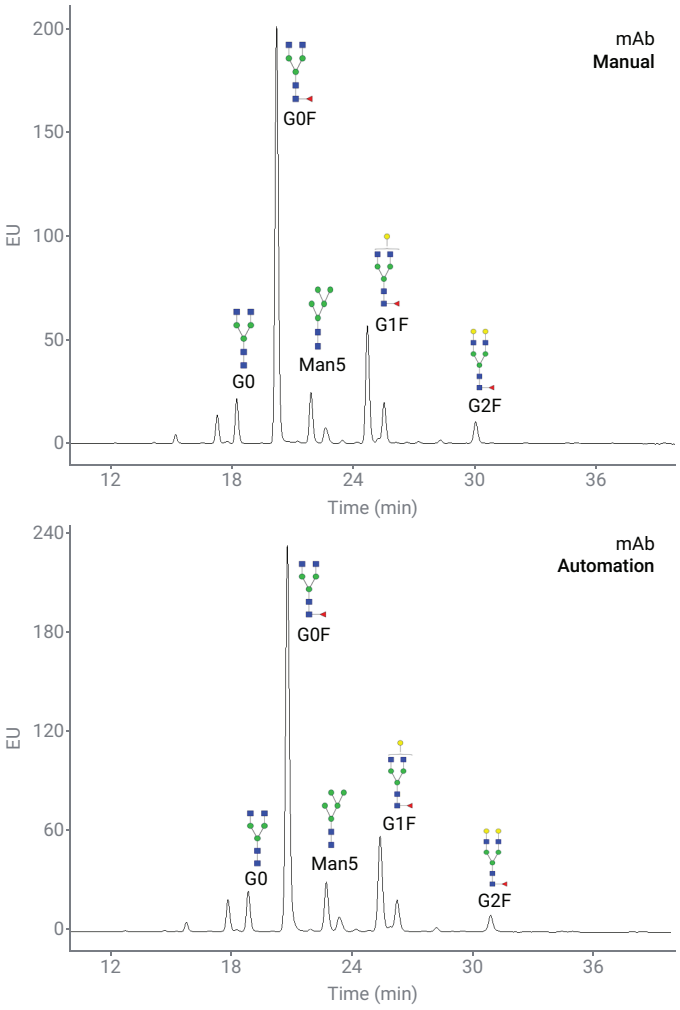

Figure 2. The representative chromatograms of N-glycan profiling by two sample preparation methods.

Automated sample preparation of different types of antibodies were conducted to compare their profiling and glycan distribution. Based on the average results of two replicates (Figure 3), the proportion of each glycoform in automated sample preparation was comparable to that in manual sample preparation.

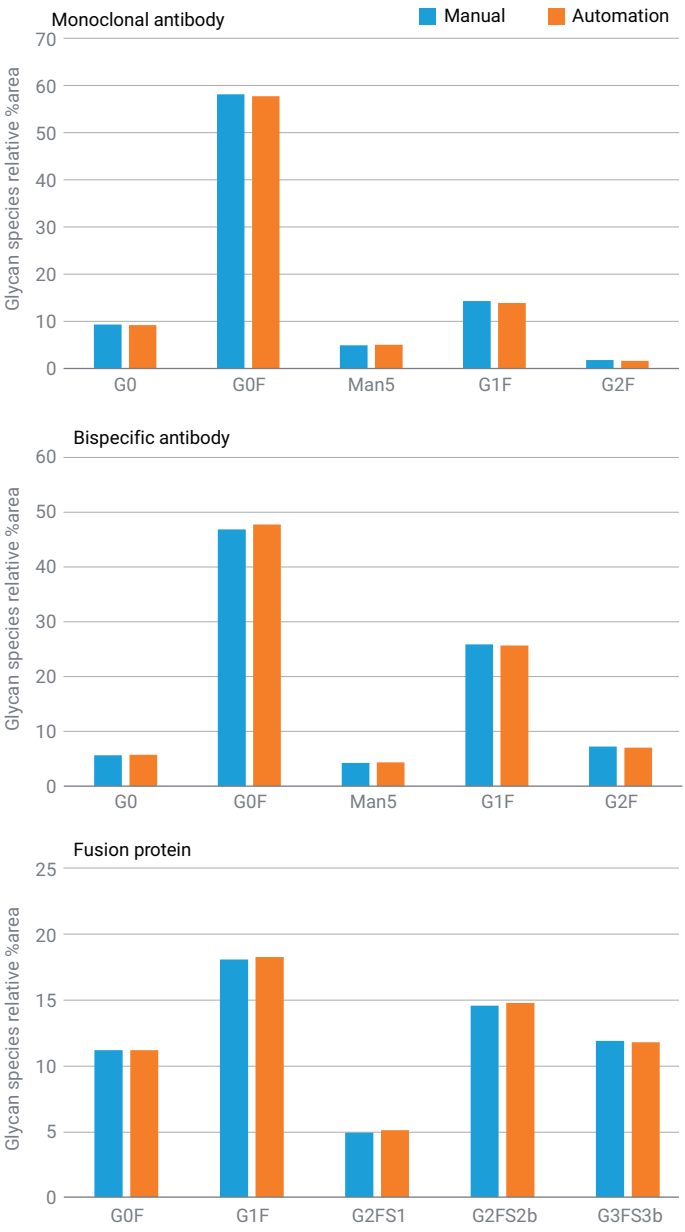

Figure 3. N-glycan profiling analysis comparison between manual and automated sample preparation in different therapeutic antibodies (average of duplicates).

Conclusion

This application note shows that with some modifications of the denaturant and glycanase buffer from the Agilent AdvanceBio Gly-X InstantPC kit, automated N-glycan sample preparation by a liquid handling workstation can be achieved.

With this fully automated N-glycan sample preparation platform, efficiency can be increased compared to traditional manual processing due to increased hands-off time, completing the preparation of 92 InstantPC-labeled N-glycan samples within 2.5 hours (92 samples is due to the liquid handling workstation configuration), including automated sample dilution and automated Gly-X preparation. This compares to 2.5 hours to manually process 20 to 25 samples (including sample dilution, and Gly-X preparation time of ~ 1 hour). Reagent preparation time for the manual and automated protocols were similar, but using a fully automated protocol, sample dilution and processing to generate labeled N-glycans is hands-off, allowing the user to focus on other tasks, in contrast to the manual process. Stable and reliable results for relative amounts of glycan species in each sample were generated with the automated protocol and were comparable to manual preparation. Furthermore, the ease of use of sample preparation on an automated workstation can reduce the retesting risk caused by user error. Finally, the combination of automated N-glycan sample preparation and an automatic data analysis system (the procedures of which are relatively complex) can achieve a fully automatic N-glycan analysis process from sample preparation to data analysis.

Reference

1. Shrivastava, A.; Joshi, S.; Guttman, A.; Rathore, A. S. N-Glycosylation of Monoclonal Antibody Therapeutics: A Comprehensive Review on Significance and Characterization. *Anal. Chim. Acta*. **2022**, *1209*, 339828. DOI: 10.1016/j.aca.2022.339828

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