Application Note

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Development of a Rapid APTS Sample Preparation Workflow for N-Glycan Release and Labeling

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Abstract

The N-glycan moieties of biotherapeutics can impact immunogenicity, pharmacokinetics, and pharmacodynamics, making N-glycan characterization an essential part of the development process. A common approach is to derivatize enzymatically released N-glycans with a fluorescent dye for separation and detection of the glycan mixture to assess relative distribution of glycan species. For separation by capillary electrophoresis (CE), the negatively charged dye 8-aminopyrene-1,3,6-trisulfonate (APTS) is frequently used to enable migration of neutral glycan species. Using traditional methods, preparation of APTS-labeled N-glycans often requires numerous hours or days to complete.

We present an APTS workflow as an addition to the Agilent AdvanceBio Gly-X N-Glycan Preparation platform (formerly ProZyme), where APTS-labeled N-glycans are ready for CE or UHPLC analysis in ~2.5 hours. Separations of APTS-labeled glycans from Rituxan and Enbrel are shown on two CE platforms and UHPLC-HILIC.

Introduction

More than 60% of biotherapeutics are glycosylated[1], including monoclonal antibodies (mAbs), Fc-fusion proteins, clotting factors, and cytokines such as erythropoietin. The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics, and pharmacodynamics[2,3]. This makes the characterization of N-glycans an essential part of the biotherapeutic development process. N-Glycans do not contain a chromophore or fluorophore suitable for online detection, so for separation by CE they are commonly derivatized with fluorescent tags such as APTS by reductive amination chemistry after enzymatic release with PNGase F. Traditional APTS labeling methods require multiple steps including drying released glycans prior to labeling and subsequent cleanup of excess APTS label that can take hours if not days to complete. GlykoPrep Rapid N-Glycan Prep with APTS streamlined the sample preparation process to four to five hours.[4] Agilent AdvanceBio Gly-X N-Glycan Prep with APTS Express (formerly ProZyme) is a simplified workflow where samples are typically ready for analysis in 2.5 hours (Figure 1). A five-minute in-solution deglycosylation with PNGase F in 96-well PCR plate format releases N-glycans and is enabled by a denaturation reagent that enhances exposure of N-glycosylation sites for rapid enzymatic cleavage at an elevated temperature.

Following rapid conversion of glycosylamines (-NH_2) to their free-reducing-end form (-OH), N-glycans are loaded onto a cleanup matrix in a 96-well vacuum filtration plate format. APTS Express labeling of N-glycans by reductive amination (one hour) is performed on the cleanup matrix, eliminating the need to dry down glycan samples prior to labeling as used in traditional APTS workflows. After labeling, free APTS label and other reaction components are washed from APTS-labeled glycans bound to the matrix, followed by elution of APTS-labeled N-glycan samples in water. The APTS workflow is supported by a range of APTS-labeled migration standards, individual N-glycans and N-glycan libraries[5], and exoglycosidases.

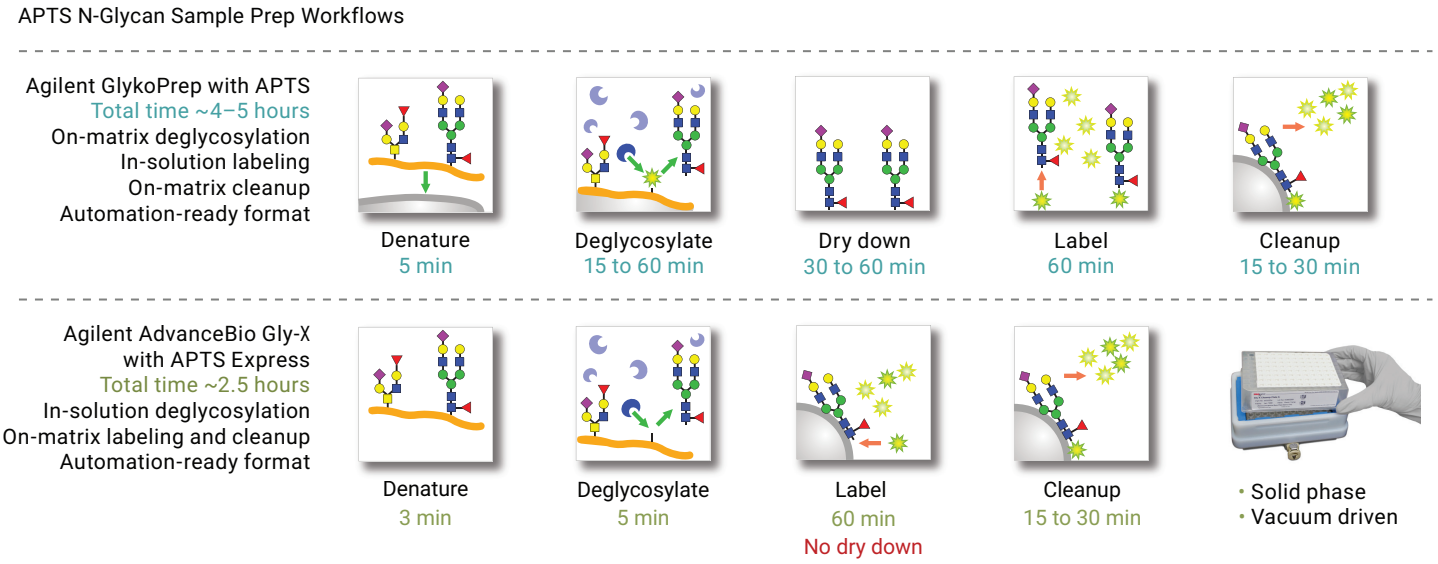

Figure 1. N-glycan sample preparation workflows: Agilent GlykoPrep with APTS, and next-generation Agilent AdvanceBio Gly-X with APTS Express.

Methods and materials

Sample preparation

N-Glycan samples from MabThera lot no. H0102B03 (rituximab) and Enbrel lot no. R170724 (etanercept) were prepared with Agilent AdvanceBio Gly-X N-Glycan Prep with APTS Express kit (GX96-APTS) and Agilent GlykoPrep Rapid N-Glycan Prep with APTS (GP96NG-APTS) following standard recommended protocols, using 40 µg of protein per Gly-X preparation, and 50 µg of protein for Agilent GlykoPrep.

CE Separations

APTS-labeled N-glycans were separated by:

- Agilent Gly-Q Glycan Analysis System (formerly ProZyme) with LED-induced fluorescence (LEDIF) detection, Ex/Em (nm) 310/>385 (product code GQ2100), with electrokinetic injection and two-minute separation.
- Sciex PA800-plus CE system with detection by laser-induced fluorescence (LIF), Ex/Em (nm) 488/520. N-CHO capillary, length to window: 50 cm; total length: 60 cm. Electrolyte: Carbohydrate Separation buffer. Sample injection pressure: 0.5 psi for 10 seconds. Separation voltage and time: 30 kV for 27 minutes, cartridge temperature: 20 °C.

UHPLC-HILIC

Hydrophilic interaction liquid chromatography (HILIC) separation of APTS-labeled glycans with fluorescence detection (FLD) was performed on an Amide column (2.1 x 150 mm, 1.7 µm) using a column temperature of 60 °C and a gradient of 30 to 60% 50 mM ammonium formate pH 4.4, between 2 and 50 minutes at a flow rate of 0.4 mL/min.

Labeled glycans were monitored by FLD, Ex/Em (nm) 473/520. Glycan assignments were made by comparison to existing Agilent data, and those in the literature.[6]

Sialidase A treatment of APTS Express glycans

In a PCR plate, 1 µL of Sialidase A (GK80040) was added to 75 µL of Gly-X APTS-labeled glycans and incubated at 50 °C for 10 minutes. Samples were allowed to cool and injected onto the Gly-Q CE system.

Results and discussion

APTS Express performance

APTS Express has a recommended sample input of 10 to 40 µg. Similar glycan profiles were observed in the range 1 to 200 µg for MabThera and 10 to 200 µg for Enbrel (data not shown).

APTS Express cleanup removes >99% of free dye (data not shown) and minimizes bias for individual glycans (Figure 2). The cleanup also preserves sialylated glycan species, as shown in Figure 3.

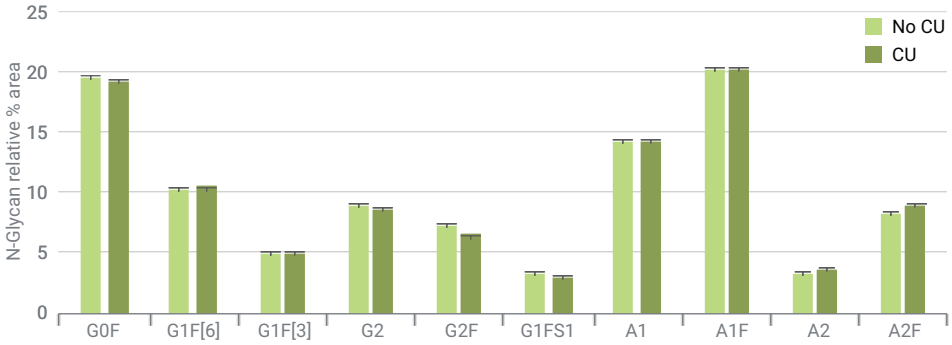

Figure 2. Glycan recovery. Next-generation Agilent AdvanceBio Gly-X with APTS Express N-glycan sample preparation workflow. Enbrel APTS N-glycans. No CU – no cleanup, CU – cleanup.

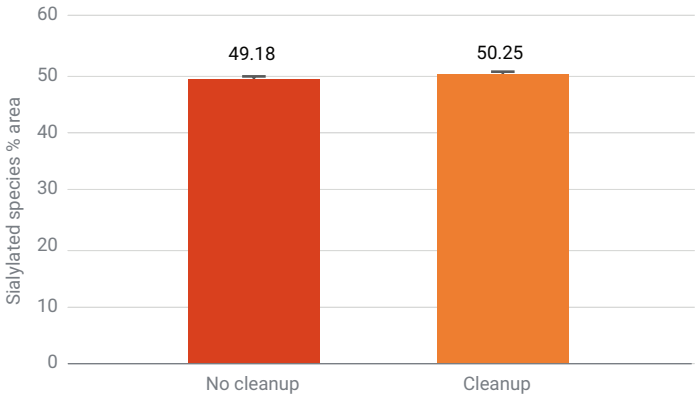

Figure 3. Preservation of sialylated species. Enbrel APTS N-glycans were measured by UHPLC, and the summed relative % area of sialylated species was compared with and without cleanup.

Reproducibility was assessed by preparing six replicates of MabThera and separating with the Agilent Gly-Q system (Figure 4). For the major glycan peaks, %CV was less than 3%.

CE separations

Agilent AdvanceBio Gly-X with APTS Express is an open format kit allowing for separation on a variety of CE systems.

N-Glycans from Rituxan and Enbrel were separated on the Agilent Gly-Q CE system (Figures 5A and 6A) and Sciex PA800 plus (Figures 5B and 6B).

AdvanceBio Gly-X APTS Cleanup Module removes >99% free dye enables a clean separation in the early part of the run.

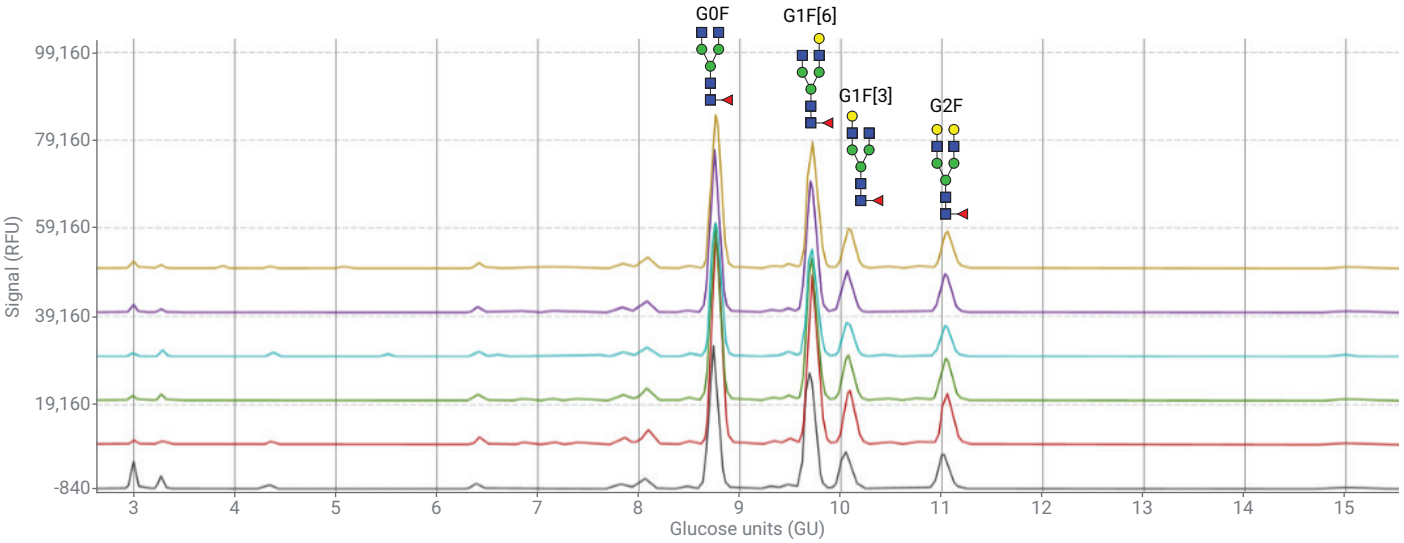

Figure 4. Reproducibility of Agilent Gly-X APTS Express. Six replicates of MabThera were prepared, and separated on the Agilent Gly-Q CE system, shown in (A). Relative % area and %CV for major glycan peaks is shown in (B).

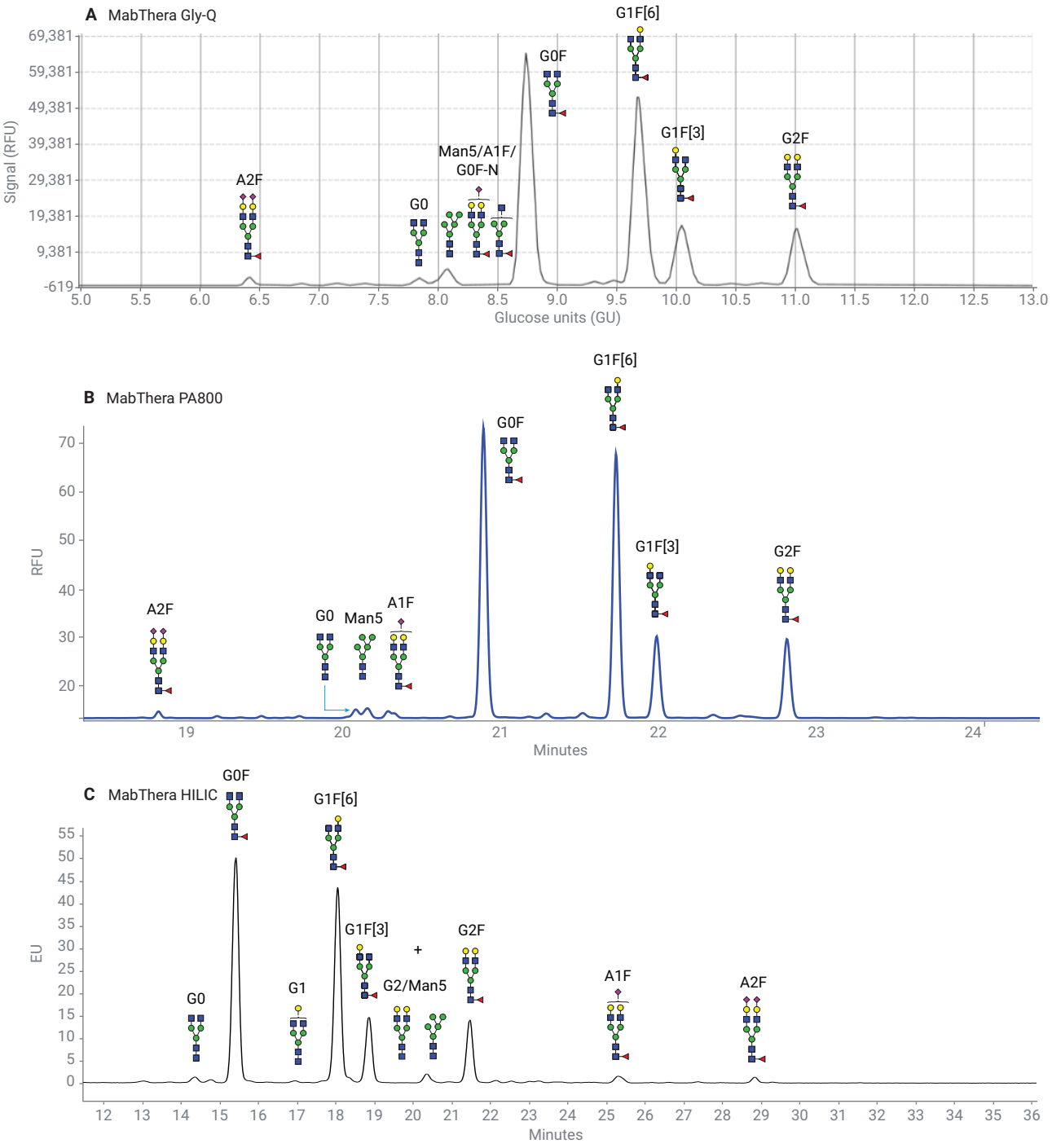

Figure 5. N-glycans from MabThera prepared with Agilent Gly-X APTS Express, separated by: A) CE on an Agilent Gly-Q system, B) CE on a PA800 plus, and C) UHPLC-HILIC.

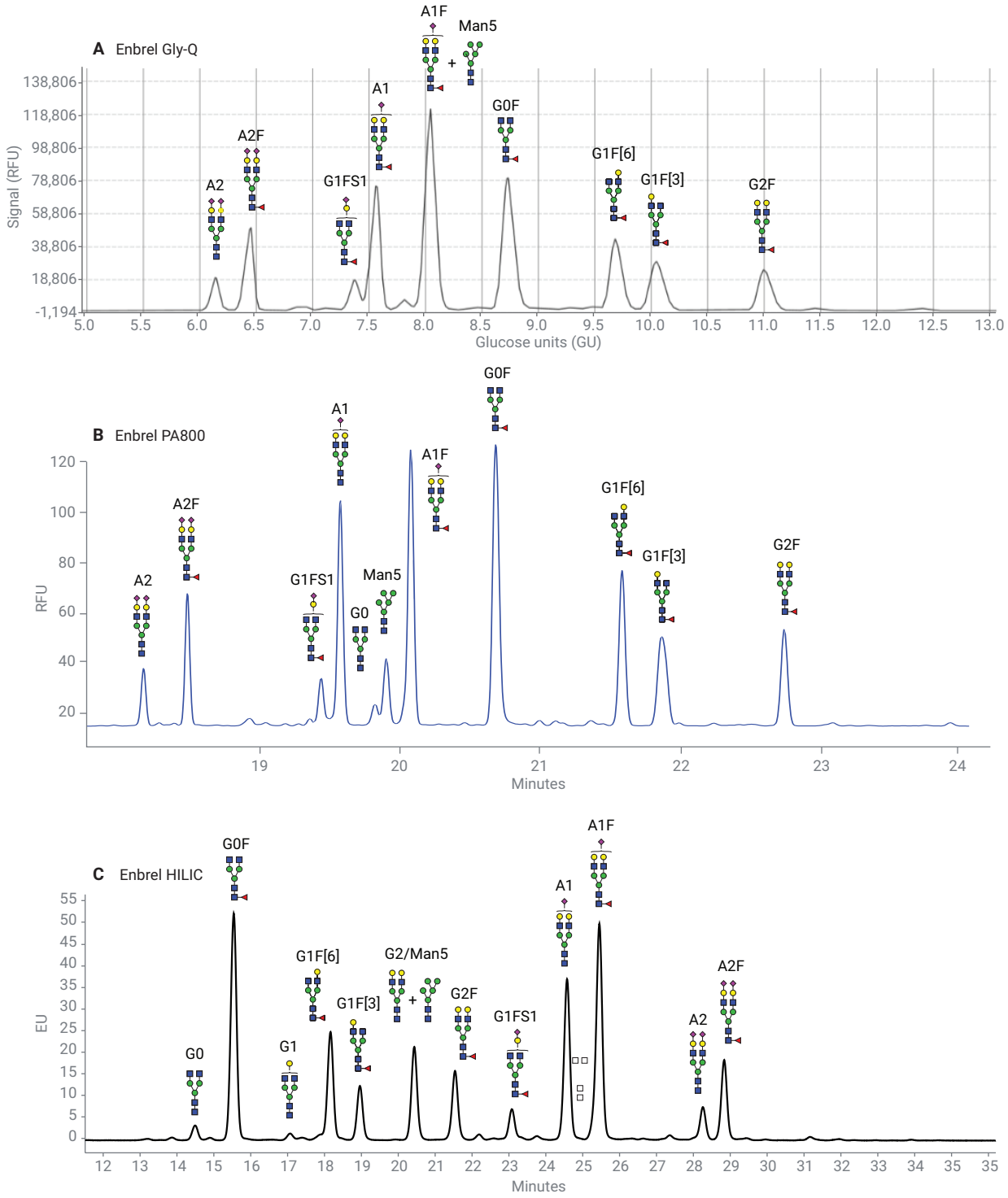

Figure 6. N-glycans from Enbrel prepared with Agilent Gly-X APTS Express, separated by: A) CE on an Agilent Gly-Q system, B) CE on a PA800 plus, and C) UHPLC-HILIC.

UHPLC-HILIC separation

Examples of N-glycans from MabThera and Enbrel labeled using AdvanceBio APTS Express and separated by HILIC are shown in Figures 5C and 6C, respectively.

APTS-labeled glycans can be separated by HILIC on UHPLC or HPLC instruments. However, APTS glycan peaks are broader than with other N-glycan dyes that are more regularly used for HILIC (for example, InstantPC, and 2-AB). A HILIC gradient starting at lower organic percent may be needed as APTS glycans are more polar and thus more retained during HILIC than 2-AB and other glycans.

Sialidase A treatment of APTS Express glycans

Sialidase A (GK80040) releases α(2,3)-, α(2,6)-, α(2,8)-, and α(2,9)-linked sialic acid from APTS-labeled glycans, and can aid in peak assignment.

A 10-minute sialidase A treatment of N-glycans from Enbrel causes sialylated peaks on the Gly-Q electropherogram to move to a neutral position (Figure 8). For example, A2F shifts to G2F, and A1F shifts to G1F.

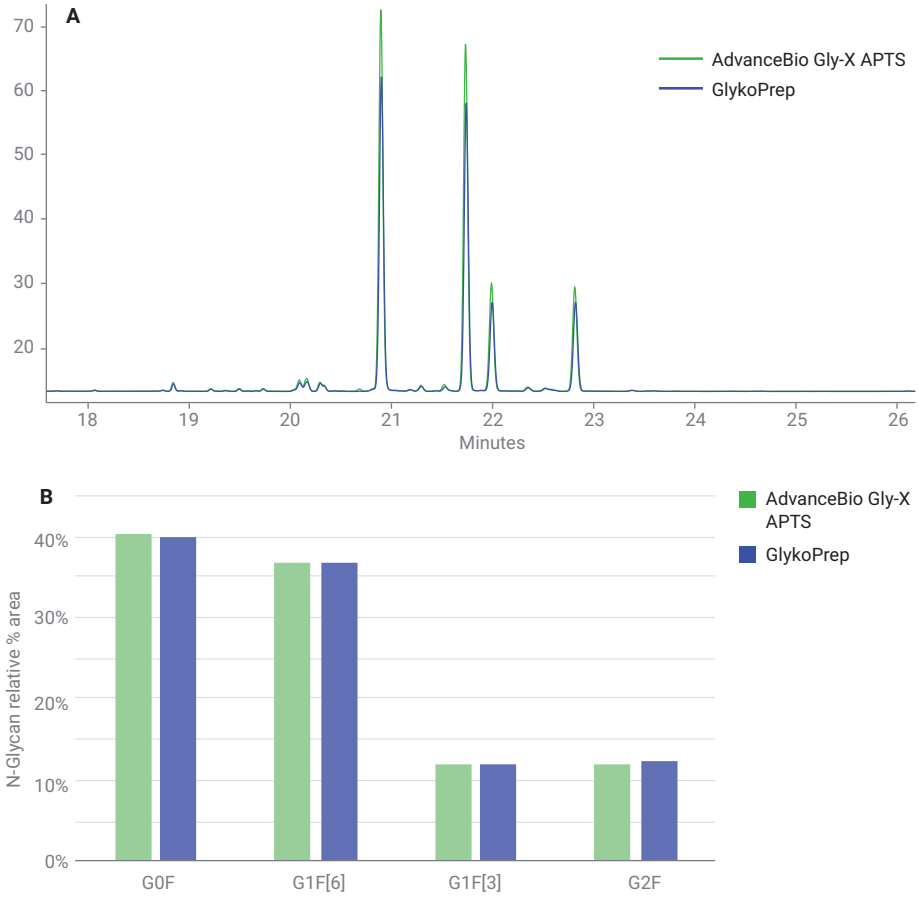

Figure 7. Comparison of Agilent AdvanceBio Gly-X APTS Express and Agilent GlykoPrep APTS labeled N-Glycans. Labeled N-glycans from MabThera were prepared and separated on a PA800 plus.

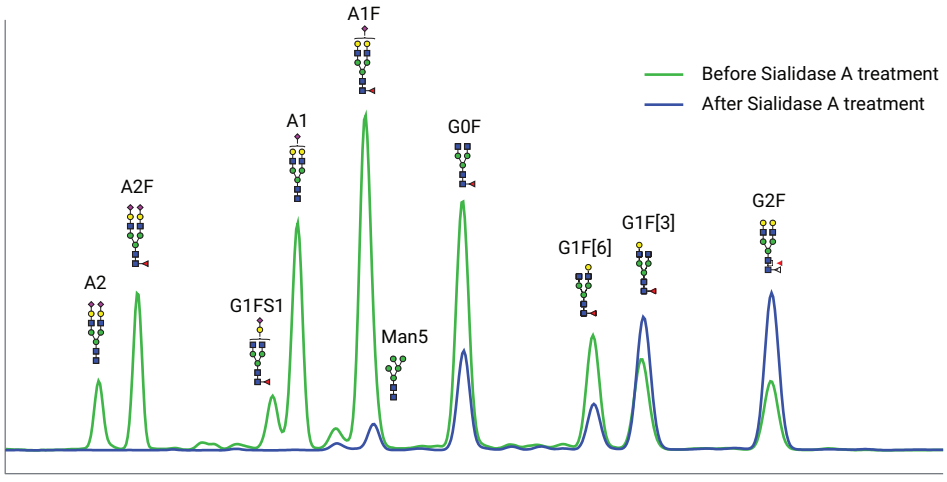

Figure 8. Agilent Gly-Q separation of APTS-labeled N-glycans from Enbrel before and after 10-minute sialidase A digestion.

Conclusion

Agilent AdvanceBio Gly-X APTS Express labeling of released N-glycans offers reproducible and reliable results that can be directly compared to historic data for example, Agilent GlykoPrep with APTS. The use of established APTS label ensures data continuity for ongoing projects.

AdvanceBio Gly-X with APTS Express offers a rapid workflow (~2.5 hours) with traditional APTS labeling without the need for lengthy dry down steps, shortening the time to results. Data from AdvanceBio Gly-X N-Glycan Prep with APTS Express is comparable to Agilent GlykoPrep Rapid N-Glycan Prep with APTS. The open format CE kit enables the next-generation AdvanceBio Gly-X APTS Express labeling solution on other CE instruments, and for separation by UHPLC-HILIC.

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