# Agilent Solutions for High-throughput N-linked Glycan Profiling from Biotherapeutics

## Application Note

Biotherapeutics and Biosimilars


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### Abstract

This Application Note presents an automated high-throughput sample preparation for 2-AB labeled N-linked glycans from innovator and biosimilar monoclonal antibody (mAb) followed by liquid chromatography (LC) analysis. Agilent AssayMAP Bravo liquid handling platform was used for the automated sample preparation involving glycan cleavage and release from mAb, 2-AB labeling, and purification. The downstream ready samples were then analyzed using an Agilent 1290 Infinity LC system with Agilent AdvanceBio Glycan Mapping columns. The glycan distribution and heterogeneity between the samples were deduced by comparing the chromatogram from both innovator and biosimilar mAb.

The study highlights the high-throughput application of the AssayMAP Bravo platform for automated and reproducible sample preparation for glycan profiling, followed by fast chromatographic separation using a 1290 Infinity LC system.



Agilent Technologies

## Introduction

Monoclonal antibodies (mAbs) are leading the race in biotherapeutics, and have revolutionized the way diseases are treated and intervened. Patents for most of the 20 clinically approved[1] first-generation mAbs have either expired, or are about to expire. This has increased the opportunity for generating generic versions, referred to as biosimilars. Regulatory bodies such as the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) have published guidelines for the characterization of biosimilar protein therapeutics including post-translation modifications[2]. Glycosylation modification results in structural heterogeneity that changes an mAb's target binding capacity, stability, charge, and mass[3]. During the course of developing the antibodies from the cell line, the glycans are monitored for cell line alteration and culture optimization. This requires a high-throughput sample preparation to screen several cell culture conditions in parallel. The Agilent Bravo Automated Liquid Handling Platform is a precise liquid-handling system designed for high-throughput applications like glycan profiling. Agilent Bravo with Agilent AssayMAP technology combines the automation with miniature 5-µL pack bed cartridges for sample preparations, enabling high-throughput chromatography and sample preparations in a shorter time[4].

This Application Note presents a workflow solution for profiling N-glycans from mAb using an Agilent AssayMAP Bravo platform. The system automates the N-glycan purification and derivatization using 2-aminobenzamide (2-AB) dye, which is then followed by Agilent HPLC analysis using Agilent Glycan Mapping columns. We showcase the ease-of-use of an AssayMAP Bravo for the automated sample preparation required for high-throughput profiling studies.

## Experimental

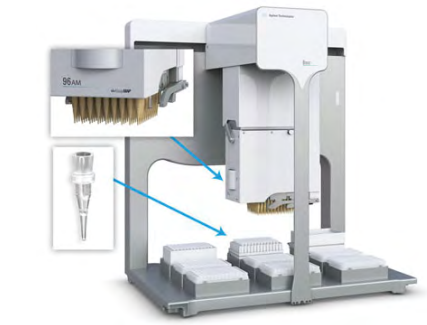
### Materials

ProZyme's GlykoPrep-plus Rapid N-Glycan Sample Preparation with 2-AB kit, and 2-AB labeled Oligomannose 5 and 6 were purchased from ProZyme. An Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913), and Agilent 2-AB labeled human IgG N-linked glycans standards (p/n 5190-6996) were purchased from Agilent Technologies. Innovator rituximab and biosimilar product were purchased commercially from a local pharmacy. All other chemicals were procured as HPLC grade from Sigma.

### Sample preparation

Reagents from a ProZyme GlykoPrep plus kit with an AssayMAP protocol were used for the sample preparation. An innovator and biosimilar sample was diluted to 1 mg/mL, then loaded onto three columns of the 96-well plate (24 replicates each). After placing the sample plates and reagents plates as specified in the guideline[5], the samples were processed by launching the N-Glycan Sample Prep: RX digestion & 2-AB labeling module from Agilent VWorks software. The protocol consists of five modules that are performed in sequential order to immobilize the samples, digest the glycans, elute, label with 2-AB, and complete a final cleanup to remove the excess dyes. The final purified labeled glycan from each well were then transferred to HPLC vials and analyzed immediately, or stored at –80 °C.

Figure 1 presents a schematic diagram of the complete workflow.



Agilent AssayMAP Bravo

ProZyme GlykoPrep-plus Kit

Samples

RX Cartridges

Antibody immobilization and denaturation

N-Glycanase enzyme

Glycan digestion and elution

2-AB Dye

Rapid-Reductive-Amination (70 °C, 60 minutes)

CU Cartridges

Glycan cleanup and final elution

2-AB labeled glycans

Agilent 1290 Infinity LC

Figure 1. Schematic workflow for the glycan isolation, labelling, purification followed by LC analysis using Agilent solutions. Components of the Agilent AssayMAP steps are shown in blue; the ProZyme kits are shown in orange.

### Instrumentation

Agilent 1290 Infinity LC System including:

- Agilent 1290 Infinity Binary Pump G4220A
- Agilent 1290 Infinity Autosampler G4226A
- Agilent 1290 Infinity TCC G1316 C
- Agilent 1260 Fluorescence Detector G1321 B

The LC method described earlier was adopted for this study[6]. Every sample was analyzed in quadruplet injections, followed by a blank injection.

### Software

- Agilent VWorks Automation Control 11.4.0.1233
- Agilent AssayMAP Launch Pad 3.0
- Agilent N-Glycan Sample Prep: RX digestion & 2-AB labeling protocols 1.0
- Agilent ChemStation C.01.06

## Results and Discussion

### Agilent AssayMAP sample preparation

The samples were processed following a protocol consisting of five modules, as presented in Figure 2. The software suite consists of a deck layout, labware table, and application settings tab for each module to be performed (Figure 3). The user was prompted to place the appropriate consumables and reagents listed in the labware table in the specified deck positions. After setting up all labware, the protocol was executed, and AssayMAP Bravo completed the protocol, and a confirmation message was displayed to proceed to the next module.

The final Cleanup Protocol module eluted the labeled and purified glycans in an aqueous buffer into a clean 96-well plate. The samples were then analyzed in quadruplets, along with blanks, using the Agilent AdvanceBio Glycan Mapping column for the downstream LC analysis.

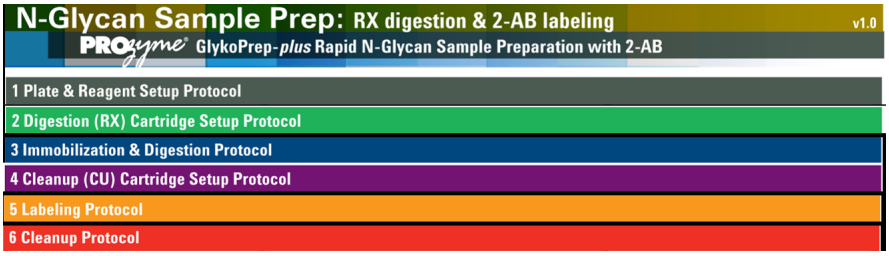



Figure 2. Agilent VWorks modules of an Agilent AssayMAP N-Glycan Sample Preparation.

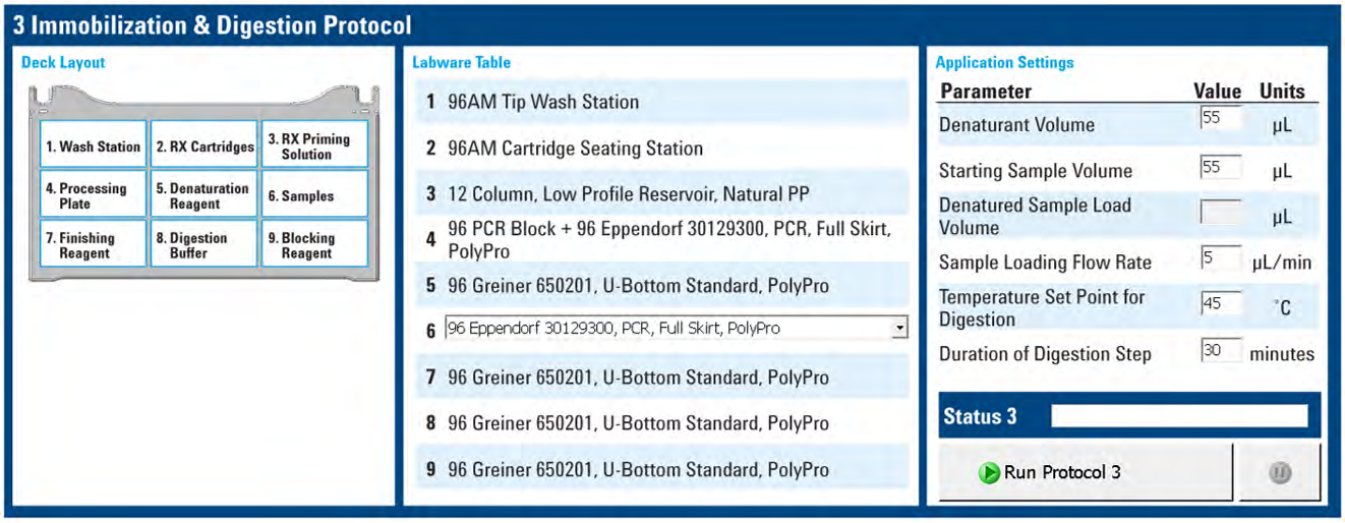



Figure 3. The deck layout, labware table, and application settings of the Immobilization and Digestion Protocol, one of the five sample preparation modules in Agilent VWorks software.

## HPLC Analysis

The N-glycan profiles were compared between the innovator and biosimilar mAbs using a fast analysis method with the Agilent 1290 Infinity system. The Agilent 2-AB labeled IgG N-linked glycan library was used as the standard to assess the column performance, and to annotate the peaks in mAb samples. The 1290 Infinity system with high backpressure enabled the analysis of the samples in less than 6 minutes, saving analysis time per sample, and increasing sample throughput.

Each sample was HPLC analyzed in replicates of four. Figure 4 presents the peak area of four major glycans species from 96 replicates, demonstrating the excellent column-to-column reproducibility of the AssayMAP micro chromatography pipette tips. The Reproducibility Standard Deviation (RSD) calculated for peak area and peak height showed a coefficient of variation (CV) of less than 6 % for all glycan species. This demonstrates the very robust and reproducible sample preparation capability of the AssayMAP Bravo system.

## Glycan profiling and comparison of innovator and biosimilar rituximab

The chromatograms of the innovator and biosimilar rituximab were compared with the standard N-linked IgG glycan library, and the peaks corresponding to glycans were annotated. Separate standards comprising 2-AB labeled oligomannose 5 and 6 were also used to annotate additional peaks. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the innovator and biosimilar product (Figure 5). Small differences in the low abundant glycans were observed; the biosimilar sample contained lower amounts of mannose (Man5), as shown in the zoomed view of Figure 6. Despite minor differences in some low abundant glycans, the glycan profile of the innovator and biosimilar rituximab can be concluded to be comparable.

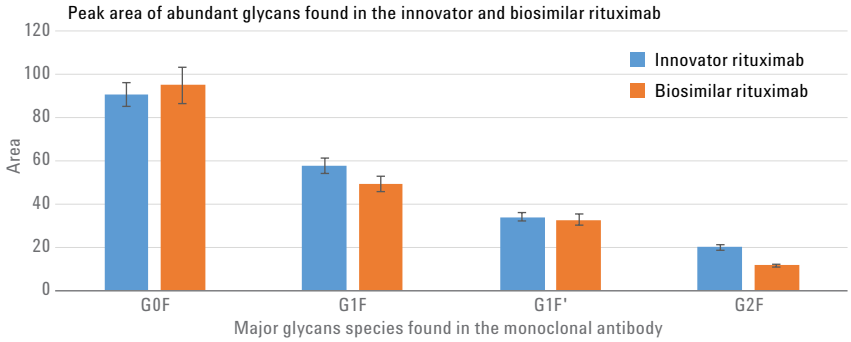



Figure 4. Peak area of major glycan species from 96 replicates for each innovator and biosimilar sample.

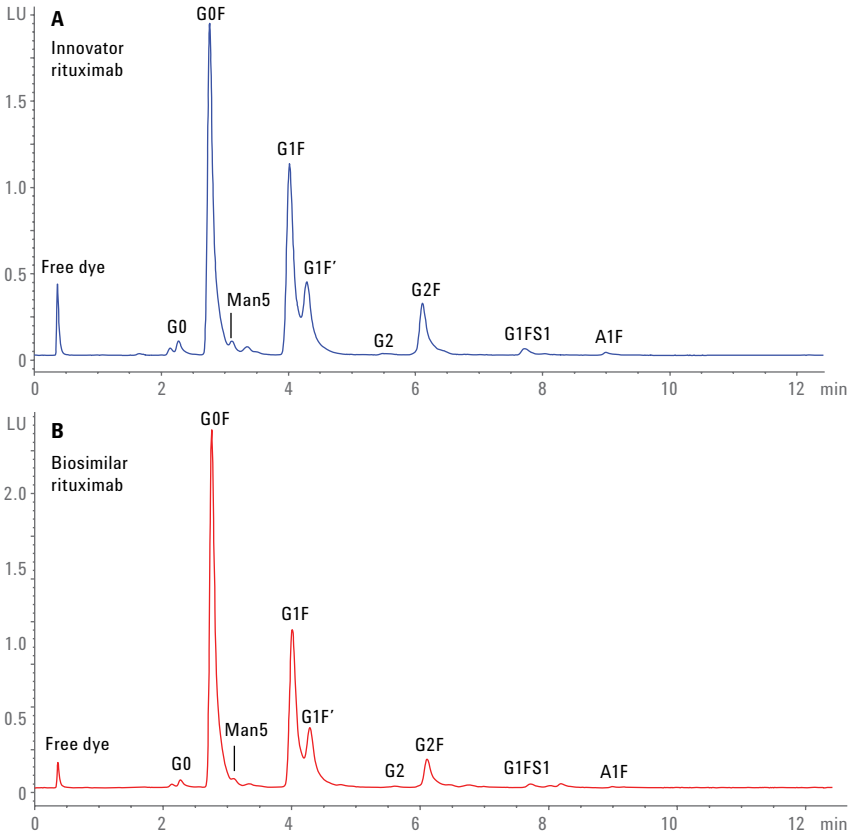



Figure 5. Glycosylation pattern of innovator and biosimilar rituximab.

The area percentage for each N-glycan core was calculated and compared between both samples. Table 1 shows the ratio of N-glycans between the innovator and biosimilar rituximab. The table shows a similar N-glycan fingerprint profile for both innovator and biosimilar.

Both samples showed a similar trend in the distribution of N-glycans, with G0F as the major glycoform followed by G1F, G1F′, and G2F. Apart from the annotated glycans, there were a few unknown glycan forms that may be assigned with an orthogonal detection system. A correlation graph (Figure 7) plotted for the area percentage of both samples shows high similarity, with an $R^2$ of 0.973.

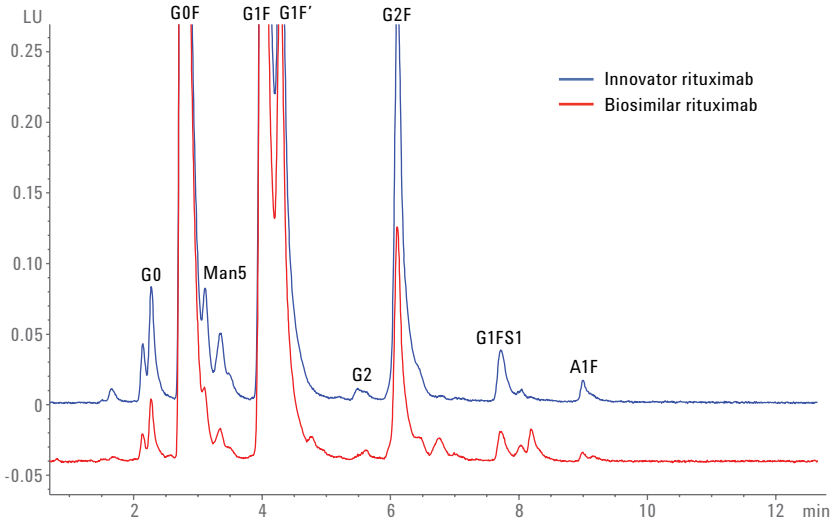



Figure 6. Zoomed view of Figure 5: the glycosylation pattern of innovator and biosimilar rituximab, showing minor differences in the low abundant glycans.

Table 1. Glycan area percentage between innovator and biosimilar mAbs.

| Percentage of N-glycans | | |
|---|---|---|
| N-glycans | Innovator | Biosimilar |
| G0 | 1.5 | 0.8 |
| G0F | 42.2 | 50.5 |
| Man5 | 1.7 | 1.2 |
| G1F | 28.6 | 26.7 |
| G1F′ | 13.0 | 11.6 |
| G2 | 0.3 | 0.2 |
| G2F | 8.7 | 5.5 |
| G1FS1 | 0.9 | 0.6 |
| A1F | 0.2 | 0.5 |

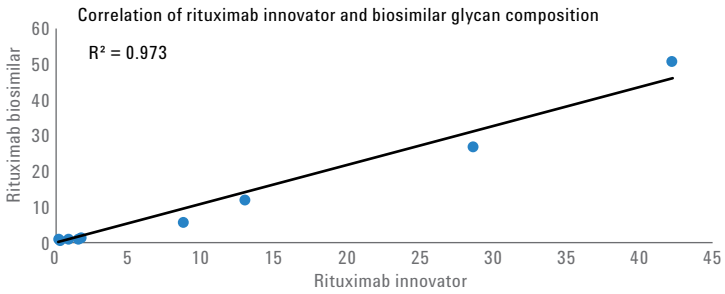



Figure 7. Linear graph showing the comparison of the area % of glycans released from innovator and biosimilar rituximab.

## Conclusions

- This study highlights the versatility of the Agilent AssayMAP Bravo system for a high-throughput sample preparation.
- The Agilent VWorks Automation Control software suite simplifies the sample preparation with ready-to-go protocols, resulting in downstream-compatible samples with minimal hands-on operation.
- Monoclonal antibody samples from a 96-well plate were processed, in parallel, for enzymatic glycan cleavage, separation, derivatization with 2-AB, and purification.
- The AssayMAP demonstrated excellent reproducibility in the glycan purification, and performed robustly.
- The purified samples were then analyzed using an Agilent 1290 Infinity LC system with the Glycan Mapping column.
- The glycan species were well-resolved in a shorter time, and were annotated using standards.
- The distribution of the glycan species between the innovator and biosimilar were assessed, and the data suggest comparable glycan profiles for the innovator and biosimilar rituximab used in this study.

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