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Nota de aplicación

Automated High-Throughput *N*-Glycan Labelling and LC-MS Analysis for Protein A Purified Monoclonal Antibodies

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Abstract

N-Glycans are routinely monitored during the development of biotherapeutics because they can affect the safety and efficacy of therapeutic proteins. Waters™ automated GlycoWorks™ *RapiFluor*-MS™ sample preparation protocol is a rapid and robust method for analyzing the glycan profile of glycoproteins, but it is limited to samples prepared in a narrow concentration range in non-nucleophilic buffers. Here, we present a complementary sample preparation and analysis method that provides a reproducible profile of *N*-glycans released from Protein A-purified monoclonal antibodies (mAbs) using the Andrew+™ pipetting robot and the BioAccord™ LC-MS System. This automated diafiltration protocol can be readily adapted for samples prepared in any buffer at any concentration.

Benefits

- Rapid automated sample preparation of released and labeled *N*-glycans from 48 samples in under 3 hours followed by BioAccord LC-MS analysis of all samples in less than 4 hours
- Reproducible *N*-glycan profiles from mAbs in a wide range of sample concentrations and buffers
- A method for the high-throughput glycan critical quality attribute (CQA) assessment of affinity-purified

bioreactor samples or downstream analytical samples

Introduction

N-linked glycosylation of biotherapeutics plays a key role in drug safety and efficacy due to its impact on immunogenicity, clearance rates, and effector function for antibodies.[1,2] As a result, glycosylation is closely monitored as a critical quality attribute (CQA) during drug development and production. *N*-Glycan analysis is particularly important in the development of biosimilars because the heterogeneity of different expression systems can render glycosylation difficult to control.[3] Rapid and robust methods for *N*-glycan analysis are therefore imperative for efficient development and approval of biotherapeutic drugs.

Glycosylation is typically assessed by releasing *N*-linked glycans from the protein backbone and labelling the released glycan with a chromophore for UV or fluorescence detection. Traditional methods for labelling released *N*-glycans are time-consuming, use hazardous labelling reagents, and produce labeled glycans with weak MS responses.[4] Waters GlycoWorks *RapiFluor*-MS *N*-Glycan labelling kit provides an alternative method capable of labelling released *N*-glycans within five minutes.[5] *RapiFluor*-MS label contains a quinoline fluorophore and a tertiary amine for enhanced fluorescence and MS detection. The rapid labelling is preceded by fast PNGase F deglycosylation and a quantitative HILIC-SPE cleanup procedure to facilitate immediate LC-FLR/MS analysis of released and labeled glycans.

The GlycoWorks *RapiFluor*-MS protocol has recently been adapted for automation using the Andrew+ pipetting robot and coupled to a five-minute UPLC-MS method to further increase sample throughput.[4] These high-throughput sample preparation and UPLC-MS methods were used to prepare and analyze released *N*-glycans from 48 infliximab (Remicade®) samples within eight hours. The analysis produced consistent results for critical and trace level high-mannose and sialylated glycoforms. This workflow is useful at various stages of drug development to improve throughput and productivity. However, its use for mAbs is limited to samples prepared within a narrow concentration range (0.5–3 mg/mL) in non-nucleophilic buffers due to the incompatibility of *RapiFluor*-MS with high concentrations of nucleophiles.[6]

The concentration and buffer limitations of the GlycoWorks *RapiFluor*-MS protocol can complicate its use in drug development. If, for example, Protein A affinity chromatography is used to purify a biotherapeutic mAb, buffer exchange is required prior to *N*-glycan analysis to ensure compatibility with *RapiFluor*-MS when nucleophiles

such as glycine and tris are used for elution and neutralization. To accommodate scenarios in which mAbs are prepared at low concentrations or in nucleophilic buffers, we incorporated a high-throughput diafiltration step into the automated GlycoWorks *RapiFluor*-MS protocol. The diafiltration step is compatible with both Vacuum+™ and Extraction+™ devices used with the Andrew+ liquid handler and can concentrate or buffer exchange up to 48 samples prior to *N*-glycan release and labelling.

After the automated sample preparation, a fast UPLC-MS method was optimized for high-throughput analysis using the BioAccord LC-MS System controlled by waters_connect™ informatics software. Figure 1 illustrates the entire analytical setup used for the high-throughput released glycan assay.

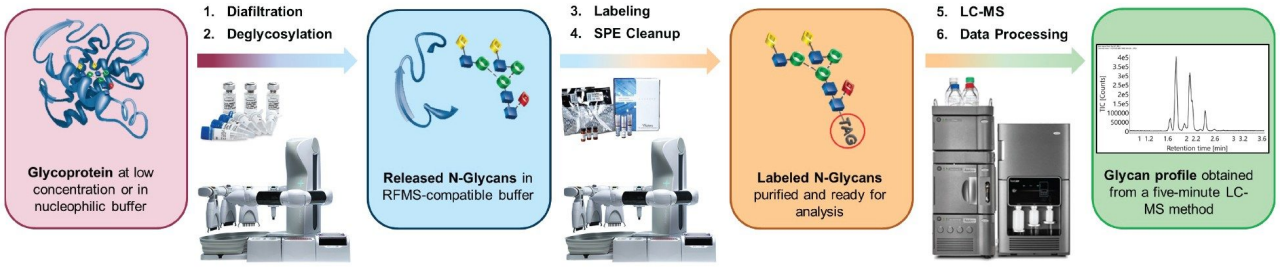

Figure 1. Workflow for the automated preparation of labeled N-glycans from samples at low concentration or in nucleophilic buffers using the Andrew+ pipetting robot and rapid sample analysis using a five-minute LC-MS method on the BioAccord LC-MS System.

Experimental

The GlycoWorks sample preparation protocol was adapted from the QC/Automation-friendly protocol in Application Note 720005506 to accommodate diafiltration and automation using the Andrew+ pipetting robot. Prior to automation, GlycoWorks reagents (p/n: 186008840 <https://www.waters.com/nextgen/global/shop/application-kits/186008840-glycoworks-rapid-deglycosylation-kit---4-x-24.html> and 186007989 <https://www.waters.com/nextgen/global/shop/application-kits/186007989-glycoworks-rapifluor-ms-label-96-sample.html>) were prepared as described below. Three vials of *Rapi*Gest™ Surfactant (10 mg) were reconstituted with 200 µL of GlycoWorks Rapid Buffer and 135 µL 18.2 MΩ water each,

combined, and diluted with 1 mL PBS (pH 7.4, 1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic, 155 mM sodium chloride; Thermo Fisher Scientific, p/n: 10010031). Four vials of GlycoWorks Rapid PNGase F enzyme were reconstituted with 270 µL 18.2 MΩ water each and combined. Three vials of GlycoWorks *RapiFluor*-MS (23 mg) were dissolved in 280 µL anhydrous DMSO each and combined. Additionally, 20 mL of 25 mM HEPES, 50 mM NaCl (pH 7.9) was prepared in 18.2 MΩ water. The Andrew+ pipetting robot equipped with Extraction+ was used to perform diafiltration, deglycosylation, labelling, and HILIC SPE cleanup (p/n: 186008747 <https://www.waters.com/nextgen/global/shop/application-kits/186008747-glycoworks-spe-reagents-automation.html> and 186002780 <https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186002780-glycoworks-hilic--elution-plate.html>). Diafiltration was carried out using a Pall AcroPrep™ Advance Omega 10 K MWCO Filter Plate. For this study, anhydrous DMSO (Thermo Fisher Scientific, p/n: D12345) was substituted for DMF in the GlycoWorks labelling protocol and ACS grade DMSO (Fisher Scientific, p/n: D128-500) was substituted for DMF in the cleanup protocol. DMSO was found to produce comparable results when used in place of DMF for these procedures in separate experiments (data not shown) while providing lower exposure risks.

The mAb used in this study was Kanjinti™ (trastuzumab-anns), a biosimilar of Herceptin™. Four samples of trastuzumab-anns were analyzed: 1 mg/mL prepared in PBS, 1 mg/mL prepared in 100 mM glycine, 100 mM tris buffer (pH 6.8) as a mock Protein A-purified sample, 1 mg/mL prepared in PBS and Protein A-purified, and 1 mg/mL spiked into clarified non-transfected CHO cell media (NTM) and Protein A-purified. The NTM was prepared by Syd Labs, Inc.. Briefly, 6 x 106 non-transfected CHO-K1 cells/mL were seeded in a spinner flask on day one and were incubated in 120 mL culture media. On days two through 15, 100 mL of spent media was collected from the flask and 0.2 µm filtered. All collected media, with an average cell viability of approximately 90%, was then pooled and stored at 4 °C.

All data were collected using the BioAccord LC-MS System under the control of waters_connect software. Data processing was performed using the embedded Accurate Mass Screening workflow in the software. A glycan database was used for glycan assignment; assignment was based on the molecular weight and retention time.

Reagent	Automated diafiltration protocol (new protocol)	QC/automation-friendly protocol (standard protocol)[7]
Diafiltration, deglycosylation, and labelling		
mAb	20 µL (1 mg/mL)	10 µL (1.5 mg/mL)
25 mM HEPES, 50 mM NaCl dilution buffer (pH 7.9)	200 µL	NA
*Rapi*Gest SF	20 µL (1:1 PBS:*Rapi*Gest SF)	10 µL
GlycoWorks Rapid PNGase F Enzyme	12 µL (5:1 GlycoWorks Rapid PNGase F Enzyme:H_2O)	10 µL
GlycoWorks *Rapi*Fluor-MS Solution in DMSO or DMF	10 µL in anhydrous DMSO (ThermoFisher Scientific, p/n D12345)	10 µL in anhydrous DMF (GlycoWorks Kit)
Cleanup		
Acetonitrile dilution	300 µL (2 × 150 µL)	300 µL (2 × 150 µL)
Water (condition)	200 µL	200 µL
Equilibration buffer (85:15 acetonitrile:water)	200 µL	200 µL
Wash buffer (1% formic acid in 9:1 acetonitrile:water)	4 × 290 µL	4 × 290 µL
Elution buffer (200 mM ammonium acetate in 95:5 acetonitrile:water)	90 µL (3 × 30 µL)	90 µL (3 × 30 µL)
Sample diluent (21:10 acetonitrile:DMSO or DMF)	310 µL (2 × 155 µL, DMSO, Fisher, p/n D128–500)	310 µL (2 × 155 µL, DMF)
Final volume	400 µL	400 µL

Table 1. Volumes for the automated and manual protocols used for this application note. Refer to experimental section above for more details on reagent preparation.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Sample collection:	Waters QuanRecovery™ 700 µL 96-well plate p/n: 186009184

Column:	ACQUITY UPLC Glycan BEH™ Amide Column p/n:186004742 (1.7 µm, 2.1 mm x 150 mm, 130 Å)
Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	15 µL
Mobile phase A:	50 mM Ammonium Formate, pH 4.4 (LC-MS grade, p/n:186007081)
Mobile phase B:	Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	1.0	25	75	Initial
3.50	1.0	42	58	6
3.55	1.0	60	40	6
3.75	1.0	60	40	6
3.80	1.0	25	75	6
5.00	1.0	25	75	6

ACQUITY RDa Detector Settings

Mass range:	400–7000 *m/z*
Mode:	ESI+

Sample rate:	10 Hz
Cone voltage:	45
Desolvation temperature:	300
Capillary voltage:	1.50 kV
Informatics:	Accurate Mass Screening using a glycan database

Data Management

Chromatography software:	waters_connect

Results and Discussion

The Automated Diafiltration GlycoWorks RapiFluor-MS Protocol

Initially, attempts were made to release and label *N*-glycans directly off Protein A-bound mAbs. mAbs were bound to Protein A magnetic beads, washed, and treated with PNGase F enzyme to release *N*-glycans directly off the surface-bound mAb. However, automation of this method yielded inconsistent results with low recovery. Instead, automated diafiltration using a 96-well 10 K molecular weight cutoff (MWCO) plate was used to buffer exchange Protein A-purified mAbs. *N*-Glycan release and labelling was then performed following a modified GlycoWorks *RapiFluor*-MS protocol with high recovery and precision (Figure 2).

For this procedure, the Andrew+ pipetting robot equipped with Extraction+ is placed inside a chemical hood (dimensions: 1.83 m W x 0.85 m D; 1.28 sq. m.; Model: HBBV6, Lab Crafters Inc.). Due to space constraints within the hood, a two-step protocol is employed wherein Step 1 executes diafiltration, deglycosylation, and labelling and Step 2 executes the purification. Figure 2 displays a flow diagram outlining the automated diafiltration GlycoWorks *Rapi*Fluor-MS protocol.

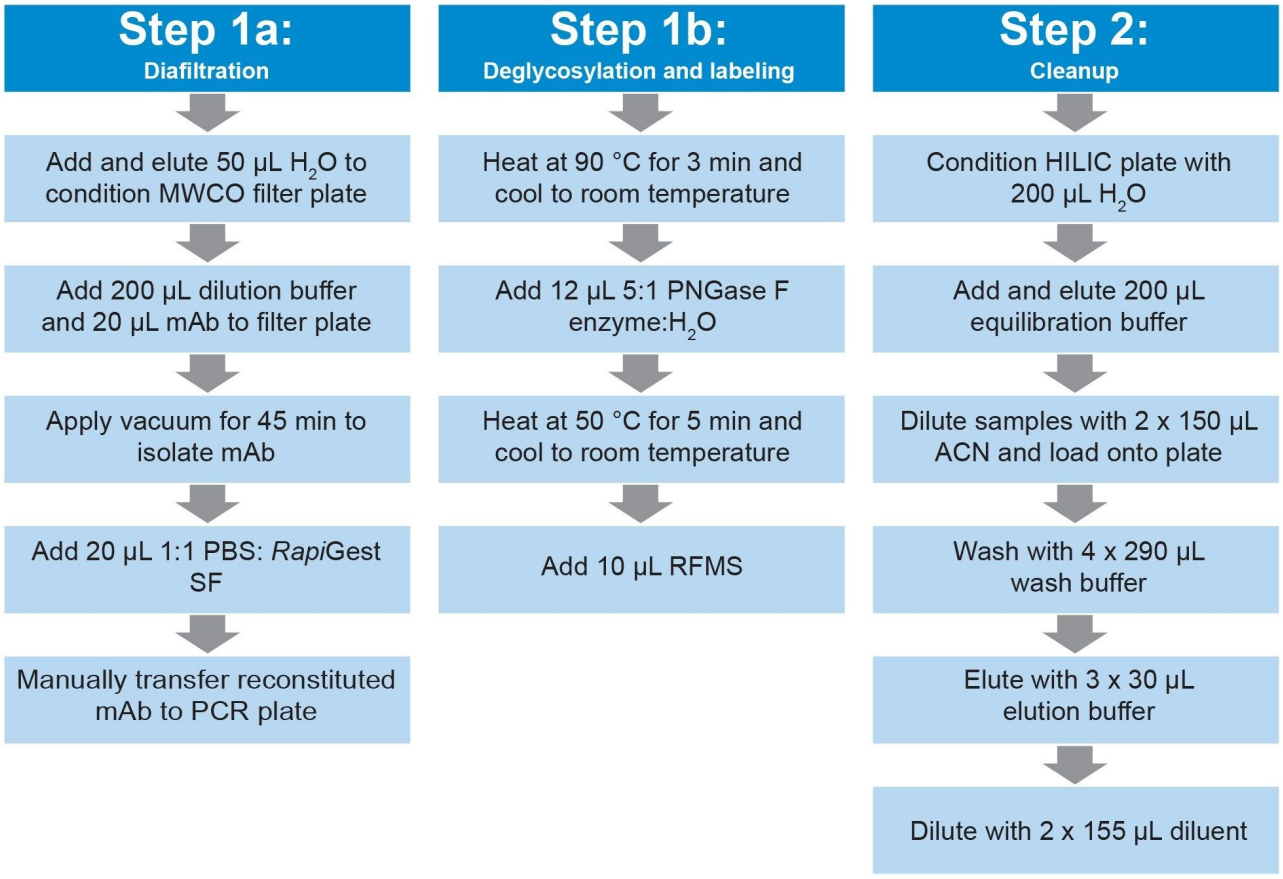

Figure 2. Flow diagram outlining the automated diafiltration GlycoWorks RapiFluor-MS protocol for rapid preparation of labeled N-glycans from mAb samples at low concentration or in nucleophilic buffer.

Step 1 begins with diafiltration; a HEPES dilution buffer is added to the MWCO plate followed by addition of mAb. In this procedure, the mAb load is increased relative to the standard QC/Automation-Friendly protocol to account for sample loss during diafiltration.[7] The volume of mAb can be adjusted to reach the desired sample load if lower or higher sample concentrations are used. Next, the Andrew+ pipetting robot isolates the sample on the MWCO plate via vacuum filtration and reconstitutes with a 1:1 PBS:*Rapi*Gest SF solution. Incorporation of PBS into the reconstitution solution is necessary to ensure complete sample digestion. Transfer between the MWCO plate and a PCR collection plate requires user intervention due to Andrew+ limitations. To circumvent arduous manual pipetting, the MWCO plate can be inverted over the PCR collection plate and centrifuged at 500 RPM for two minutes to transfer samples to the PCR collection plate for further workup. It is important to note

that this centrifugation procedure will reverse the order of the samples in their respective columns.

Digestion proceeds with heating for three minutes at 90 °C to denature the mAb. *N*-Glycans are then released by adding 5:1 GlycoWorks Rapid PNGase F Enzyme:H_2O and heating for five minutes at 50 °C. This procedure uses an increased volume of diluted PNGase F enzyme to account for solvent loss during the uncapped heating steps (Table 1). Finally, *N*-glycans are labeled with *Rapi*Fluor-MS (RFMS) and purified in Step 2 following the standard QC/Automation-Friendly protocol using DMSO in place of DMF.

Comparison of the Automated Diafiltration Protocol and the Standard Manual Protocol

To assess the efficacy of the new automated diafiltration GlycoWorks *RapiFluor*-MS protocol, *N*-glycans from the mock Protein A-purified mAb were released, labeled, and analyzed via UPLC-FLR. This sample mimics the solution of a Protein A-purified mAb where both tris and glycine are nucleophilic and will compromise the labelling step if their concentrations are not significantly reduced prior to analysis. A representative chromatogram of trastuzumab-anns *N*-glycans obtained from this procedure is shown in Figure 3. For comparison, the chromatogram of *N*-glycans manually released and labeled from trastuzumab-anns prepared in PBS using the standard QC/Automation-Friendly protocol is also shown in Figure 3. The *N*-glycan profile obtained from the automated diafiltration protocol exhibits minimal deviation from the *N*-glycan profile obtained from the manual protocol.

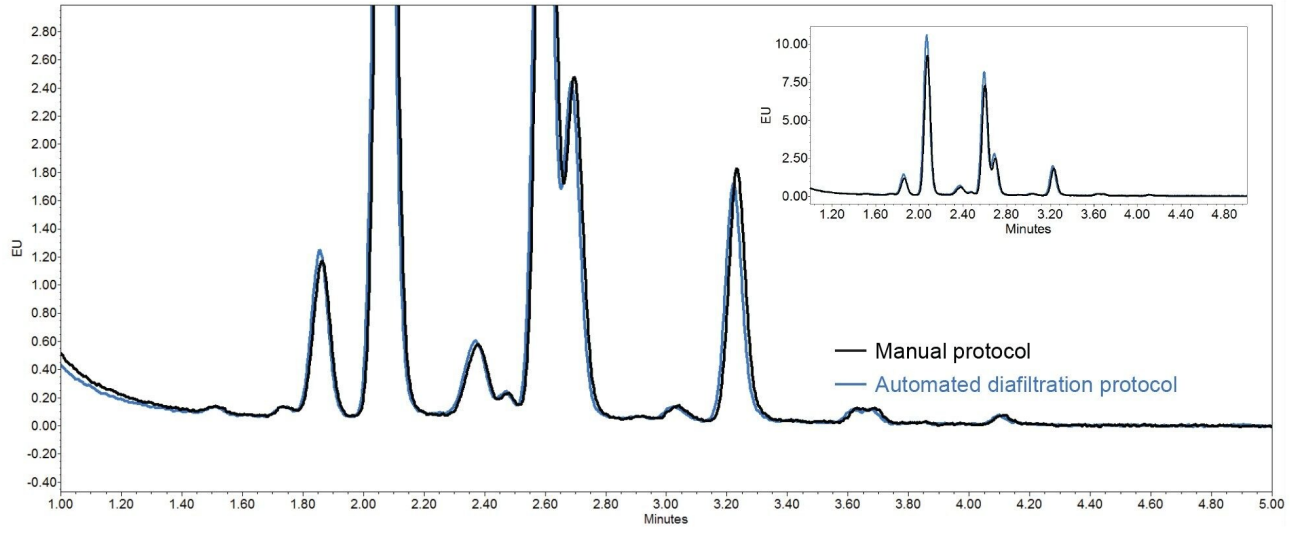

Figure 3. UPLC-FLR chromatograms of released and labeled N-glycans from trastuzumab-anns using the manual protocol (black trace) and the automated diafiltration protocol (blue trace). Zoomed chromatograms are y-axis normalized; full-scale chromatograms are absolute and shown in the inset.

In Figure 4, a comparison of the relative and total FLR peak abundances of selected *N*-glycans (FA2, FA2G1, FA2G2) released using the automated diafiltration protocol and the manual protocol (four samples were analyzed in each scenario) is shown. Both protocols yield comparable total and relative *N*-glycan abundances. The total *N*-glycan recoveries obtained from the manual protocol were higher than those obtained from the automated diafiltration protocol because the average recovery of the automated diafiltration protocol is 73%. Sample losses during the diafiltration process may be due to protein adsorption to the MWCO filter plate or incomplete liquid transfer during transfer to the PCR collection plate. As a result, a larger initial sample load is used in the automated diafiltration protocol (20 µg) relative to the manual protocol (15 µg). The final buffer exchanged sample prepared using the diafiltration protocol was comparable in concentration and produced comparable relative *N*-glycan abundances in comparison to the control sample prepared using the manual protocol.

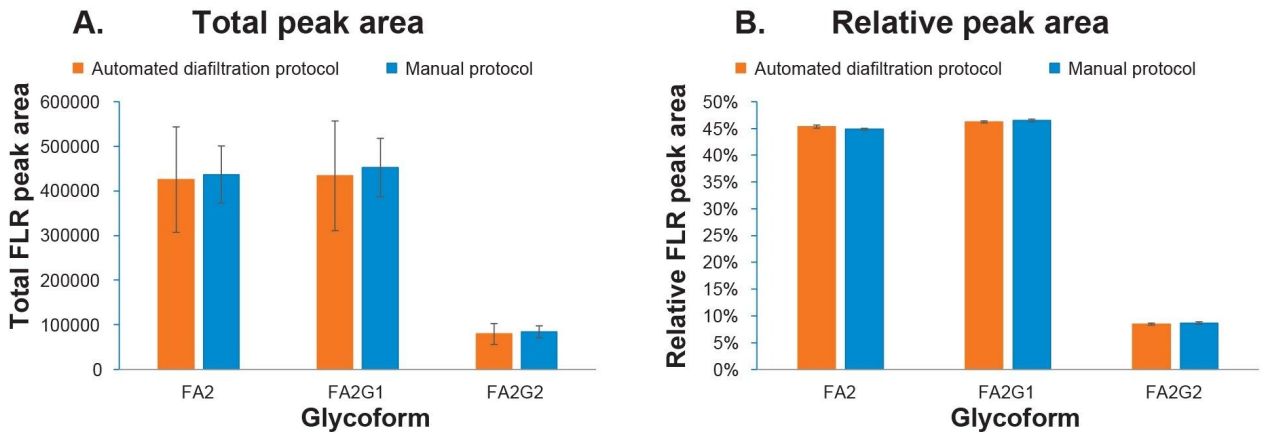

Figure 4. Comparison of UPLC-FLR relative and total N-glycan profiles of trastuzumab-anns using the automated diafiltration and manual protocols. Error bars show the 95% confidence interval (n=4). Both protocols yield comparable N-glycan profiles.

N-Glycan Analysis of Protein A-Purified mAb

The automated diafiltration protocol was used to release and label *N*-glycans from Protein A-purified trastuzumab-anns. Briefly, the 48-sample protocol was employed to release and label *N*-glycans and eight samples were selected for subsequent UPLC-MS analysis. Two sample types were used: mAb purified from PBS and mAb purified from NTM (non-transfected media). The released and labeled *N*-glycans were analyzed with a five-minute UPLC-MS method. Figure 5 displays the *N*-glycan profiles obtained from this procedure; both PBS and NTM samples yield similar chromatograms.

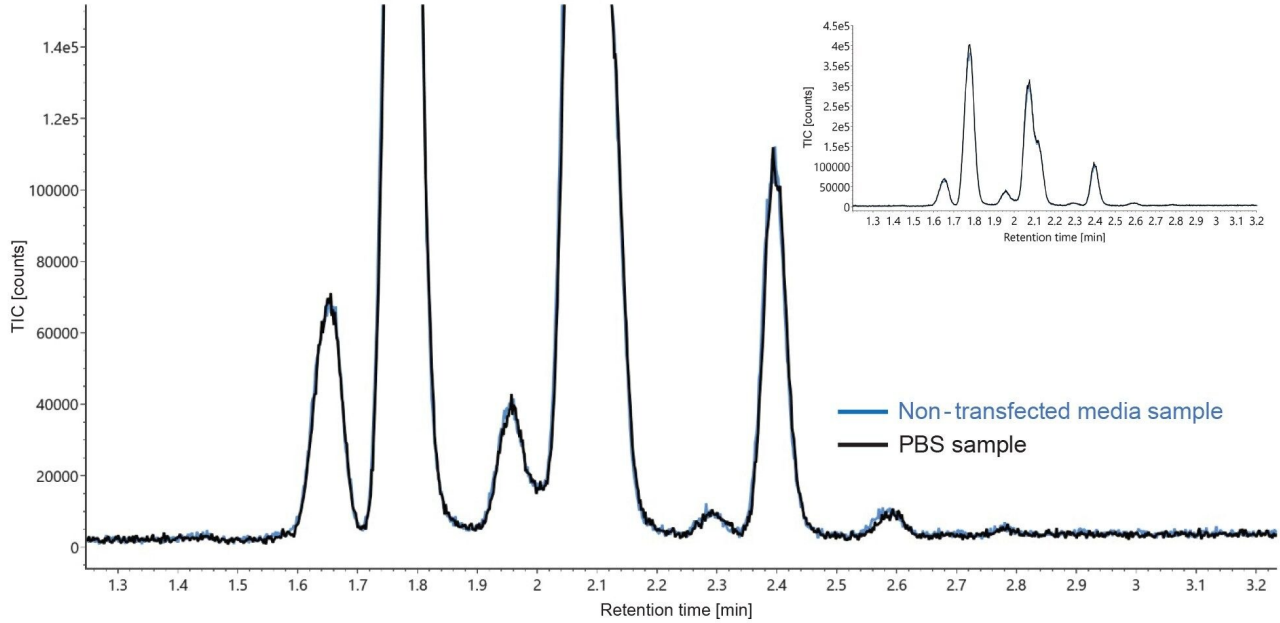

Figure 5. UPLC-MS total ion chromatograms of released and labeled N-glycans from trastuzumab-anns Protein A purified from PBS (black trace) and non-transfected media (blue trace) and prepared using the automated diafiltration protocol. Zoomed chromatograms are y-axis normalized; full-scale chromatograms are absolute and shown in the inset. The total run time is five minutes per sample.

The UPLC-MS data was analyzed in waters_connect using the Accurate Mass Screening workflow; 15 glycans of interest were imported from a Glycan Scientific Library to create a targeted list. This workflow enables facile analysis of highly abundant glycans and potentially immunogenic glycans that may be present in lower quantities (*i.e.* sialylated, high-mannose, or afucosylated glycans). The reproducibility of the protocol's glycan recovery was assessed using the percent relative standard deviation (%RSD) of the FA2 glycoform abundance across the eight analyzed samples. The %RSD was calculated to be 13%, demonstrating suitable repeatability of the diafiltration method.

The MS-derived *N*-glycan profiles of the mAb purified from PBS and NTM are shown in Figure 6. The PBS and NTM samples exhibit very similar amounts of the three most abundant glycans (FA2, FA2G1, and FA2G2) and a similar percentage of afucosylated glycans (Figure 6A). Three low-abundance glycans were targeted: high-mannose M5, sialylated FA2G2S1, and bisecting FA2BG1. The bisecting and sialylated glycoforms were present in similar abundances for both PBS and NTM samples. However, the M5 glycoform is more abundant in the NTM

samples than the PBS samples, as shown in Figure 6B and confirmed by the student's t-test (95% CI). The increase in M5 abundance in the NTM samples likely arises from trace amounts of host cell glycoproteins remaining in the samples after Protein A purification. Notably, this UPLC-MS screening method enables comparison of glycan profiles between multiple sample types, even for glycoforms present at very low abundances (<1%).

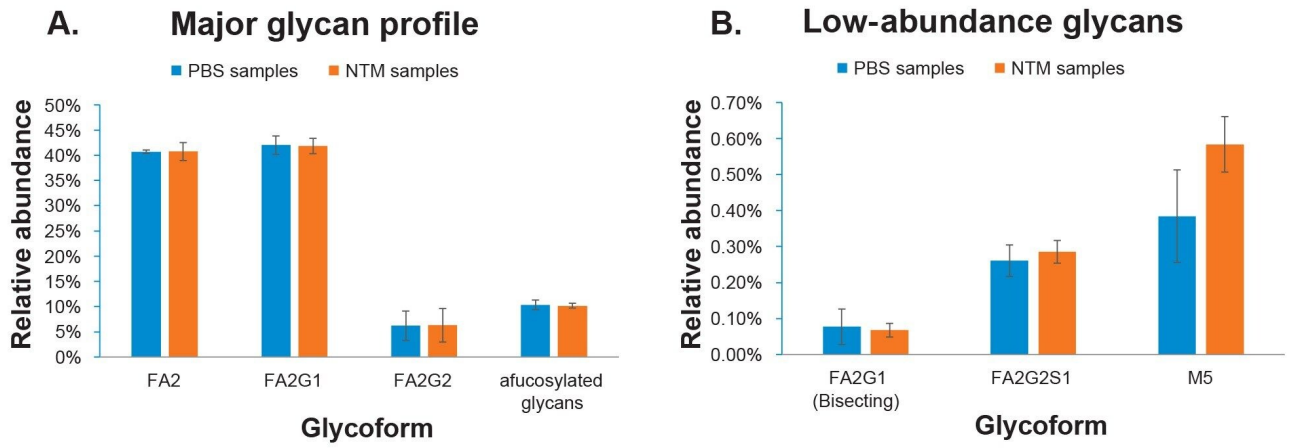

Figure 6. Comparison of UPLC-MS N-glycan profiles of trastuzumab-anns purified from PBS and NTM. Error bars show the 95% confidence interval (n=4). The method enables comparison of N-glycan profiles between multiple sample types for high- and low-abundance glycans.

Conclusion

Glycosylation is closely monitored as a CQA during drug development due to its impact on drug safety and efficacy. Rapid and robust methods for glycan analysis are invaluable in the research, development, and approval of biotherapeutic drugs. Here, we expand the capabilities of Waters' GlycoWorks *RapiFluor*-MS protocol to directly accommodate Protein A-purified mAbs. The automated diafiltration procedure enables facile buffer exchange or sample concentration with high recoveries in a high-throughput, automated format. In addition, the automated diafiltration protocol yields *N*-glycan profiles that are consistent with those obtained using the manual QC/Automation-Friendly GlycoWorks *RapiFluor*-MS protocol. When coupled with a five-minute UPLC-MS method, the protocol can prepare released and labeled *N*-glycans from 48 Protein A-purified samples in

three hours, deliver reproducible UPLC-MS glycan profiles of 48 mAb samples in four hours, and quantify both abundant and trace-level *N*-glycans. Importantly, this method may be adapted for samples prepared at any concentration in any buffer, regardless of RFMS-compatibility, and has potential use for other glycoproteins.

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720007854, February 2023