

Robust Automated High-Throughput N-Glycan Analysis Using the GlycoWorks *RapiFluor*-MS N-Glycan Kit for Automation

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APPLICATION BENEFITS

- Robust and fully automated solution enabling fast high-throughput N-glycan analysis.
- Simplification and standardization of a complex workflow through automation, minimizing potential human error, as well as increasing reproducibility and method transferability.
- Validated, comparable, precision performance relative to manual preparation with GlycoWorks *RapiFluor*-MS in the absence of significant bias and with the benefit of increased speed and significantly reduced manual labor.

WATERS SOLUTIONS

[GlycoWorks™ *RapiFluor*-MS™ N-Glycan Kit - Automation](#)

[GlycoWorks HILIC \$\mu\$ Elution™ Plate](#)

[RapiFluor-MS Glycan Performance Test Standard](#)

[RapiGest™ SF Surfactant](#)

[Intact mAb Mass Check Standard](#)

[RapiFluor-MS Intact mAb Standard](#)

[\(...continued on Page 11\)](#)

KEYWORDS

GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation, HILIC Chromatography, UPLC, HILIC SPE, GlycoWorks, Deglycosylation, *RapiFluor*-MS labeling, Tecan, Andrew Alliance, Hamilton, script, ACQUITY FLR Detector, High-throughput, glycoprotein, NIST mAb 8167 Reference Material, RFMS

INTRODUCTION

This application note demonstrates the robustness of the Waters GlycoWorks *RapiFluor*-MS N-Glycan automated solution as a reliable standardized method for the fast, fully automated, high-throughput sample preparation of glycoproteins for N-glycan profile determination.

The GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation couples the efficiencies of the *RapiFluor*-MS label and associated chemistries in a specially designed automation kit, with a verified and fully automated Tecan script. The combination of these technologies provides an automated high-throughput N-glycan sample preparation method without compromise in analytical performance. The robustness of the automated method has been extensively characterized through a combination of risk analysis and rational design, and subsequent robustness testing during product development.

The discussion begins with a review of the inherent robustness of the adopted *RapiFluor*-MS labeling chemistry and HILIC sample clean-up and then evaluates any new factors potentially introduced by the application of high-throughput automation to the established GlycoWorks *RapiFluor*-MS method.



EXPERIMENTAL

Analytical method conditions
(unless otherwise stated)

Universal N-glycan profiling method

LC conditions

LC system: ACQUITY UPLC I-Class OR ACQUITY UPLC H-Class Bio with ACQUITY UPLC FLR Fluorescence Detector

Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm
([p/n: 186004742](#))

Column temp.: 60 °C

Sample temp.: 10 °C

Detection: Ex 265/Em 425 nm

Data rate: 10 Hz

Initial flow rate: 0.4 mL/min

Mobile phase A: 50 mM Ammonium formate solution, pH 4.4 ([p/n: 186007081](#)) along with LC-MS-grade water [Fisher p/n W6 or equivalent] is recommended.)

Mobile phase B: 100% acetonitrile (LC-MS-grade ACN is recommended, Fisher p/n A955 or equivalent)

Seal Wash and LC Autosampler Wash - 70 % LC-MS-grade ACN, 30% LC-MS-grade water (v/v)

Injection volume: 10 µL suggested (= 30 µL, DMF/ACN diluted SPE eluate, 2.1 mm I.D. columns)

Injector needle: A stainless steel needle is recommended when injecting samples containing DMF as co-solvent

Gradient:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	0.4	25	75	6
35	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55	0.4	25	75	6

Sample description

RapiFluor-MS-labeled N-glycans were prepared from glycoproteins, including the Intact mAb Mass Check Standard (murine IgG1) ([p/n: 186006552](#)) and the NIST 8167 mAb Reference Material (humanized IgG1κ).

For the purpose of evaluating consistent results across the entire 96-well plate, glycoprotein samples were pooled after reconstitution and then aliquoted out into the sample tubes on the Tecan. Samples were prepared on the Tecan automated pipetting platform using the *RapiFluor*-MS Tecan Script (GlycoWorks *RapiFluor*-MS N-Glycan Starter Kit - Automation ([p/n: 176004152](#))) according to the guidelines provided in the GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation Care and Use Manual ([715005359EN](#)).

For comparison purposes, some samples were prepared manually using the already available GlycoWorks *RapiFluor*-MS N-Glycan Kit – 96 Samples ([p/n: 176003606](#)) according to the guidelines provided in the GlycoWorks *RapiFluor*-MS N-Glycan Kit Care and Use Manual ([715004793EN](#)).

RapiFluor-MS Glycan Performance Test Standard ([p/n: 186007983](#)) and *RapiFluor*-MS Intact mAb Standard ([p/n: 186008843](#)) were reconstituted in 50 µL water for chromatographic benchmarking and system suitability.

RESULTS AND DISCUSSION

The GlycoWorks *RapiFluor*-MS N-Glycan automated method can prepare up to 96 samples for N-glycan analysis in under 3 hours (including sample concentration normalization) without any loss in analytical performance and without the need for user intervention during the script run. Results delivered by the automated sample preparation are equivalent to results achieved by the existing manual preparation method. High-throughput (n = 48, 96) precision of <3% CV (FLR peak area %) is typical for the main N-glycan peaks used for performance evaluation.

A Tecan script is provided with an automation-compatible GlycoWorks *RapiFluor*-MS N-Glycan Kit, however the kit quantities are also compatible with other high-throughput automation platforms such as Hamilton and Agilent Bravo as demonstrated through a combination of Waters testing and external collaborations. The Tecan script includes useful features such as sample barcode scanning and sample concentration normalization. For other automation platforms, a script parameters document is available with critical information such as liquid classes and line by line script commands with tips and tricks.

Product design incorporated a thorough risk analysis process to ensure that adequate controls were built-in (such as product labeling) to minimize error and enhance product robustness. Extensive performance verification, validation, and robustness testing have been performed using the GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation and script parameters using the Intact mAb Mass Check Standard and the NIST mAb 8167 Reference Material (Figure 1-3). However, the script may be easily modified by users wishing to alter deglycosylation conditions or SPE clean-up which may be required for other glycoproteins (Ref: GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation Care and Use Manual [\(715005359EN\)](#).) Testing was validated on multiple Tecan EVO Freedom 100 instruments in different laboratory sites to demonstrate the transferability of the method.

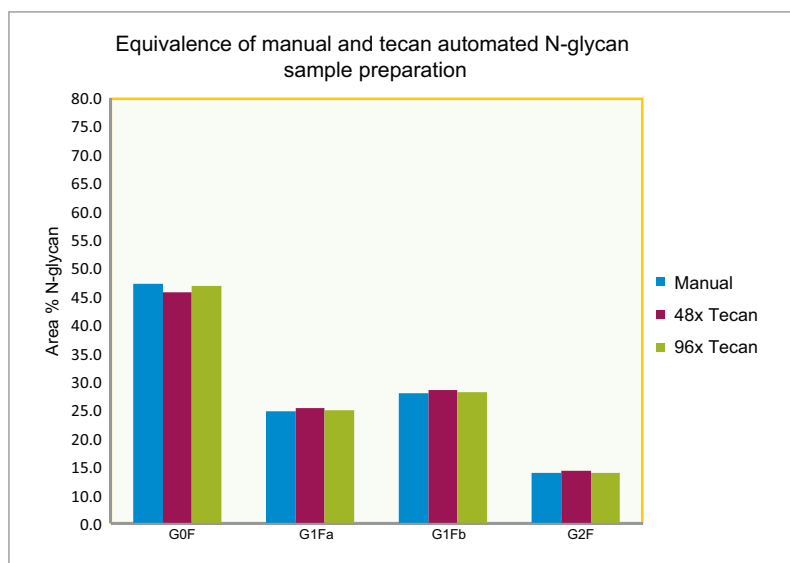


Figure 1. Performance equivalence of the automated GlycoWorks *RapiFluor*-MS sample preparation (reference data tables 1-2 Appendix 1).

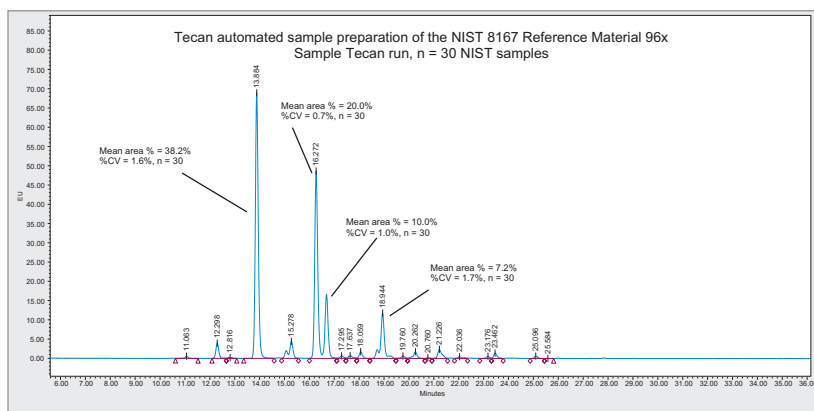


Figure 2. Precision performance of the automated GlycoWorks *RapiFluor*-MS sample preparation (NIST mAb 8167 Reference Material, humanized IgG1κ (Reference data tables Appendix 1 Table 3)).

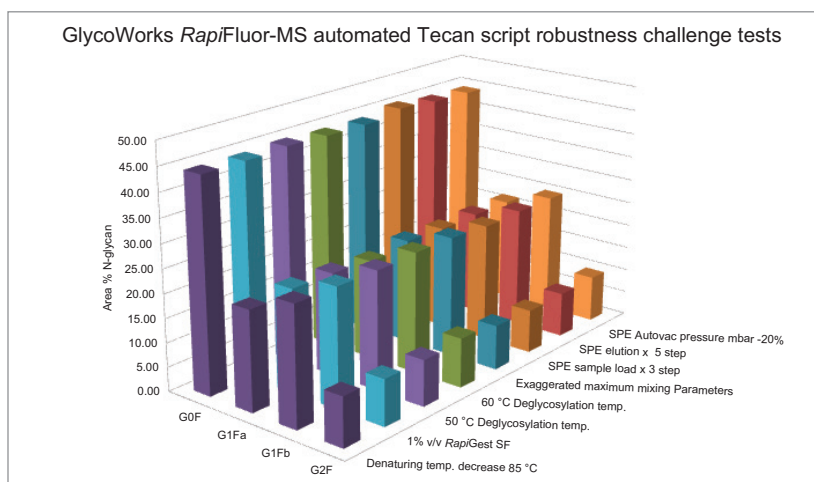


Figure 3. Summary comparison of robustness experiments for the GlycoWorks *RapiFluor*-MS N-Glycan Kit-Automation script (further details provided in discussion on next page).

METHOD DEVELOPMENT APPROACH

The foundation for the development of this robust automated N-glycan sample preparation method is attributed to the inherent robustness of the *RapiFluor*-MS chemistry and HILIC SPE protocol.^{1,2} An intermediate step towards the fully automated method was achieved by the creation of the automation-compatible-fixed volume pipetting protocol.³ This protocol uses 10 μ L volumes for the glycoprotein sample and reaction reagents at solution concentrations which ensure molar equivalence to the original protocol. The use of 10 μ L volumes allows for better precision on the liquid handler than the lower volumes specified in the manual protocol.

Subsequently, the development of the fully automated method (script) on the Tecan began with a high fidelity translation of these existing protocol parameters into the Tecan automation software (Freedom EVOware). This was accompanied by selection of suitable Tecan hardware and plastic consumable labware for executing the automated protocol. In parallel, the design and development of a compatible GlycoWorks *RapiFluor*-MS N-Glycan Kit for automation containing all of the labware and adequate quantities of reagents required to perform the automated sample preparation was undertaken. A significant script development effort was required to optimize the Tecan liquid handling parameters to achieve acceptable analytical performance compared to the familiar GlycoWorks *RapiFluor*-MS manual sample preparation. ([715004793EN](#))

In summary, the main factors which confer robustness to the new automated method are; inherent robustness of *RapiFluor*-MS labeling chemistry and SPE protocol, selection of compatible plastic consumables and hardware, set-up of the Tecan robotic and liquid handling arms (“teaching”), and optimization of programmable pipetting parameters for each liquid reagent (Tecan “liquid class” parameters).

The final script was subjected to extensive robustness testing using the automation kit to investigate whether the script is reliable under normal conditions of variation. For the purpose of this discussion the workflow may be seen as two stages; the reaction steps which result in labeling of released glycosylamines (denaturing, deglycosylation, labeling reactions), followed by HILIC SPE clean-up of the post reaction sample solutions.

The list of reaction parameters includes the following;

- Reaction chemistry:
 - Reagent and substrate volumes and concentrations
 - Reaction temperatures and reaction times
- Physical factors:
 - Selection of plastic consumables
 - Mixing speed and times, types of mixing (pipette or orbital plate mixer)
 - Programming robot for accurate dispensing into reaction wells and mass transfer of reaction solution between plates

The SPE stage parameters include;

- Conditioning, washing, and elution protocols (solvent, polarity, concentration, pH, volume)
- Elution dynamics: SPE vacuum pressure and time

ROBUST REACTION CHEMISTRY

Two existing Waters application notes by Lauber and colleagues provide a comprehensive description of the robustness testing for the *RapiFluor*-MS reaction chemistry parameters and the HILIC SPE protocol.^{1,2} Additionally, useful information on the genesis of the robust method chemistry is provided in the published article on the topic.⁴ Each procedural step in the GlycoWorks *RapiFluor*-MS N-glycan protocol has been optimized to be high yielding and to minimize the introduction of bias to an N-glycan profile.¹ Complete and robust deglycosylation of glycoprotein was demonstrated by denaturing at 90 °C for 3 minutes in the presence of *RapiGest* SF Surfactant, followed by 5 minutes at 50 °C in the presence of the PNGase F enzyme at reagent and substrate concentrations specified in the protocol. The released N-glycosylamines were shown to have a relatively long half-life of ca. 2 hours at 50 °C in case of delays beyond the specified 5 minute reaction time.¹ Similarly, extensive labeling reaction robustness testing is already reported in the existing application notes including; pH, temperature, ionic strength, time, buffer components, and reagent molar excess. An optimal molar excess of *RapiFluor*-MS label reagent has been incorporated into the protocol to ensure that the available nucleophilic protein amines or low concentrations of nucleophilic buffers (i.e. tris) do not interfere with the labeling of the released glycosylamines. The labeling reaction is also self-quenching, limiting the generation of unwanted side reactions.

Critical to the robustness of the new automated method, these previously optimized reaction conditions have remained unchanged thereby sustaining the favorable reaction kinetics of the existing chemistry. The adopted reagent reaction concentrations and molar ratios are unchanged as discussed below. Denaturing, deglycosylation, and labeling reaction temperatures and times have been programmed into the Tecan script. Therefore, the fundamental factors controlling the robustness of the method chemistry are inherent in the automated method as well. Internal well temperatures have been verified during testing to confirm adequate heat transfer for the selected plastic consumables (the PCR plate) and compatible adapter plate for the heat block. Robustness testing of the denaturing and deglycosylation temperatures (+/- 5 °C) was performed and shown not to affect N-glycan profile result for the Intact mAb Mass Check Standard (p/n: 186006552) (Figure 3); in any case the user may optimize these temperature set-points to achieve desired deglycosylation conditions. The main

caveats relating to these temperatures is to avoid excessively high temperatures during glycoprotein sample denaturing which might lead to “bumping” of reagent from the reaction well, and to avoid excessive deglycosylation temperatures which might lead to heat denaturing of the PNGase F enzyme.

The reagent volumes and concentrations specified in the GlycoWorks *RapiFluor*-MS N-Glycan Kit for the automation-friendly or [QC protocol](#) have been translated into the automated method. Delivered volumes may be carefully verified using calibrated handheld pipettes to confirm the working capability of the users' liquid handler. The standard 10 µL reaction reagent volumes adopted from this protocol promote increased precision on the liquid handler. As stated in the associated application note, the fundamental difference between the original flexible volume protocol and the adopted automation-friendly or fixed volume protocol is the effective concentration of *RapiGest* SF (p/n: 186002122) during the deglycosylation reaction, changing from 1.0% to 1.5%.³ During development, the automated method was tested for robustness at these two *RapiGest* SF concentrations and no significant difference in N-glycan profile was detected (Figure 3 & 4). However, the slightly higher *RapiGest* SF concentration may facilitate the deglycosylation of certain glycoproteins that are more resistant to denaturing. In any case, with this protocol difference tested, the reported robustness of the original variable volume protocol is expected to be unchanged by adopting the QC pipetting protocol and this is reflected in the final verification of performance equivalence of the automated method (Figure 1).

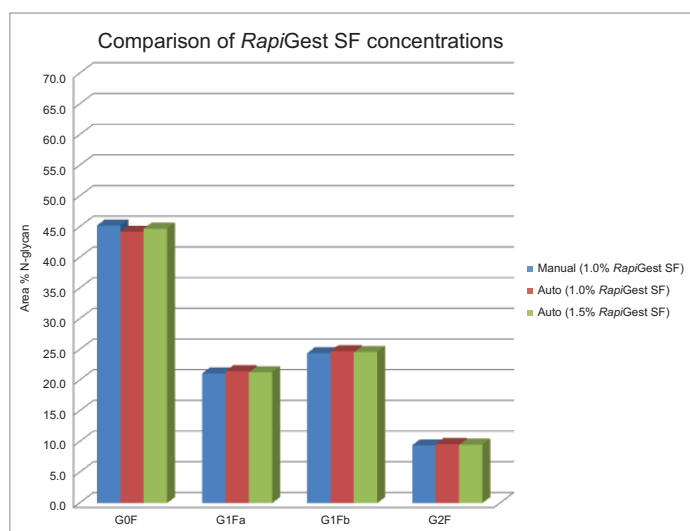


Figure 4. Demonstration of performance equivalence delivered by the updated *RapiGest* SF concentration (1.5% v/v) in the deglycosylation reaction solution.

ROBUSTNESS: PHYSICAL DESIGN FACTORS

a) Selection of plastic consumables

Concurrently, consideration was given to the selection of suitable plastic consumables for the automated method. For equivalence to the manual methodology, polypropylene was the material of construction selected to avoid introducing new sources of bias due to potential selective binding with alternative materials.

The design of the reaction plate warranted particular attention. The 200 μ L conical bottom 96-well plate was carefully selected to provide well dimensions compatible with the protocol reaction volumes and the Tecan instrument pipetting and robotic arm gripping capabilities.

The chosen reaction well design is suitable for containment of uniform and mixable reaction solutions neatly at the bottom of each well to facilitate optimal chemical transformation. The conical profile is compatible with a selected plate adapter for the INHECO heat block allowing efficient heat transfer to rapidly attain stable reaction solution temperatures.

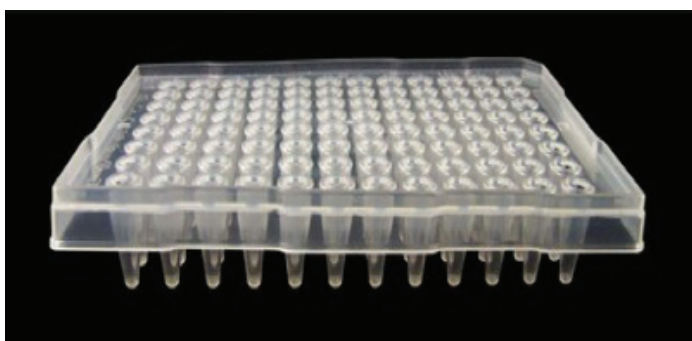


Figure 5. Selected 96-well PCR plate for automated GlycoWorks RapiFluor-MS N-glycan sample preparation.

These factors mimic the robust physical reaction conditions of the familiar manual method. The plate also has sufficient well depth to avoid losses due to reagent bumping at the high denaturing temperature. The conical well profile also facilitates quantitative transfer of the labeled reaction solution to the 1 mL round dilution plate pre-SPE loading. All of these factors were considered to sustain the robustness of the manual method in the automated method.

b) Mixing

The sufficient mixing of reaction solutions is a critical physical factor for ensuring reproducible chemical transformation. For the manual protocol, pipette mixing is achieved by repeated aspiration and dispensing of the reaction solution. In the automated method, an additional mixing tool is available with the Teleshake module. The Teleshake is an orbital plate mixer which can reproducibly provide thorough mixing of the individual contents of each well in the 96-well plate. This mode of mixing was selected for the mixing of the sample solution with RapiGest SF, PNGase F, and RapiFluor-MS label, as well as the final dilution of post SPE sample in 32:68 DMF/ acetonitrile. Initial Teleshake speeds were selected to provide adequate mixing when visually assessed without the risk of material losses at high speeds. Some refinements were made

to initial settings during yield optimization experiments without any impact on the measured glycan profiles. A final robustness test maximized all mixing parameters without regard to the overall script run time and there was no change in N-glycan profile for the resulting samples (Figure 6). Therefore, provided the solutions are visually mixed, a good degree of robustness is expected around variations in the actual mixing speed delivered from different instruments.

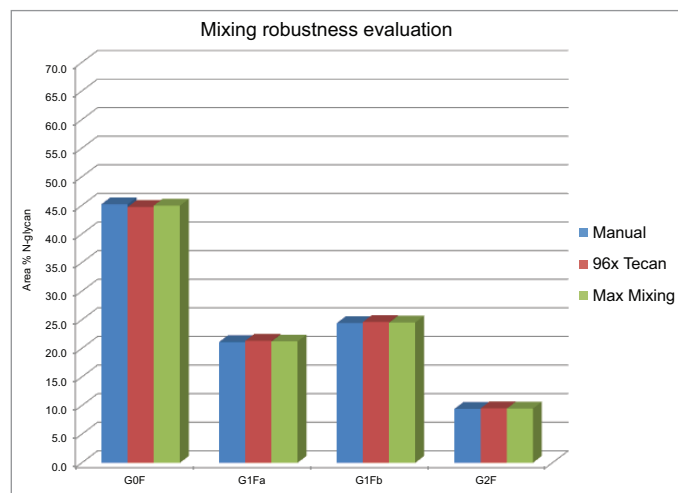


Figure 6. Evaluation of optimal script mixing conditions.

Note: A particular mixing protocol was adopted for the reaction solution for the labeling reaction in the automated method. Optimization experiments indicated more consistent N-glycan recoveries (60–70% yield G0F) across the entire 96-well plate when each set of 4 wells was mixed immediately after receiving the dispensed *RapiFluor*-MS label aliquot prior to moving on to the next set of 4 wells. The rationale for this choice was that the aqueous deglycosylation reaction medium and the *RapiFluor*-MS:DMF label solution are not readily miscible. Unless immediately mixed, it is likely that the labile *RapiFluor*-MS label hydrolyses to varying extent across the plate at the aqueous/organic interface prior to reaction with the released glycosylamines. Plate mixing is then allowed to continue during the 5 minute labeling reaction as the plate is already on the Teleshake module.

Another step in the protocol requiring specific pipette mixing is the dilution of the reaction solution pre-SPE loading with acetonitrile. This dilution must occur in a separate 1 mL round 96-well Sample Collection Plate ([p/n: 186002481](#)) (not positioned on the Teleshake) due to the volume restrictions of the 200 μ L reaction plate, and therefore must be performed using pipette mixing. It is important for optimal HILIC partitioning of the analyte that the sample solution is thoroughly mixed and loaded to the SPE plate in a uniform organic solvent polarity. The fixed pipette tips are washed thoroughly between sample mixing to eliminate cross contamination. Experiments using blank water samples to bracket glycoprotein samples showed no carryover of N-glycans.

c) Programming the robot for robust pipetting

One of the most critical factors for creating a robust automated method was the programming of the automated platform to ensure the accurate and precise pipetting of reagent volumes. To this end, the condition of the robotic instrument, worktable set-up, and accurate teaching of the liquid handling and robotic arms are considered prerequisites.

Even so, the very precise and accurate aspiration and dispensing action of the trained analyst who is familiar with the manual workflow must be conferred to the robot. Liquids have variable physical properties such as viscosity and surface tension which require subtle adjustments in manual aspiration and dispensing methods in order to achieve precise and accurate volume delivery. The trained analyst has the ability to gauge aspiration and dispense speeds and positions for the pipette and to detect possible sources of variation such as air bubbles. For the Tecan automated script, these parameters were addressed through careful

optimization of the multivariate Tecan Freedom EVOware liquid class parameters. Prior to this method development activity, the reproducibility of the automated sample preparation was unacceptable with unpredictable outlier results due to inconsistent volume delivery. The so called liquid class parameters control the accurate and precise aspiration and dispensing of liquids with different physical properties such as those provided in the kit (i.e., aqueous glycoprotein samples or the more viscous organic 32:68 DMF/acetonitrile). Over 30 individual parameters can be programmed including among others; aspirate/dispense speeds, dispense positions, delay times, conductivity for liquid detection, use of air gaps, break-off speeds, conditioning volumes, etc. Following extensive optimization and robustness testing, the optimal parameters have been empirically identified and integrated into the Tecan script. The optimal liquid class parameters enable high throughput precision and accuracy of N-glycan profile determination across an entire 96-well plate. Effectively, when the instrument hardware has been set-up correctly, the integrated liquid class parameters impart a very high degree of robustness and reproducibility to the automated method as demonstrated in the verification and validation test results across multiple Tecan instruments.

SPE ROBUSTNESS

The robustness of the HILIC SPE protocol for the clean-up of post labeling sample solutions has been reported previously in two Waters application notes.^{1,2} These application notes describe the optimization of the HILIC conditioning, washing and elution protocols (solvent, polarity, concentration, pH, volume) on the highly polar aminopropyl silica-based sorbent. Only one minor modification was made to the automated SPE protocol. The analyte elution from the SPE plate is achieved using five 18 μ L aliquots in the automated method instead of three 30 μ L aliquots in the manual protocol. This elution protocol was chosen to achieve some modest gains in analyte recovery but has no impact on the glycan area % profile and little impact on preparation time.

Other aspects of robustness were evaluated such as modifying vacuum set-points and times for SPE elution steps and changing the SPE sample loading protocol from a single step loading to a three step loading. None of these variations led to any significant gains in glycan recovery or had any impact on the observed glycan area % indicating excellent robustness and absence of bias mechanisms in the solid-phase extraction process (Figure 3). The bias free recovery of N-glycans from the SPE plate was found to have some tolerance to variation which might arise due to slight changes in vacuum pump performance between instrument service intervals.

The selected SPE elution conditions in the provided automation script were chosen to mimic elution flow characteristics observed for the manual sample preparation using the SPE vacuum manifold while avoiding conditions which might promote drying of the plate between elution steps. Drying of the plate wells could deplete the polar solvent layer at the HILIC stationary-phase surface and limit partitioning of the glycans during the low polarity sample loading conditions.

The absence of bias mechanisms in the SPE was demonstrated by comparing the N-glycan profile of pre- and post-SPE samples derived from pooled post-labeling reaction samples (Figure 6). The SPE protocol effectively eliminates interferences for MS and FLR detection without creating bias due to selectivity between released N-glycans.

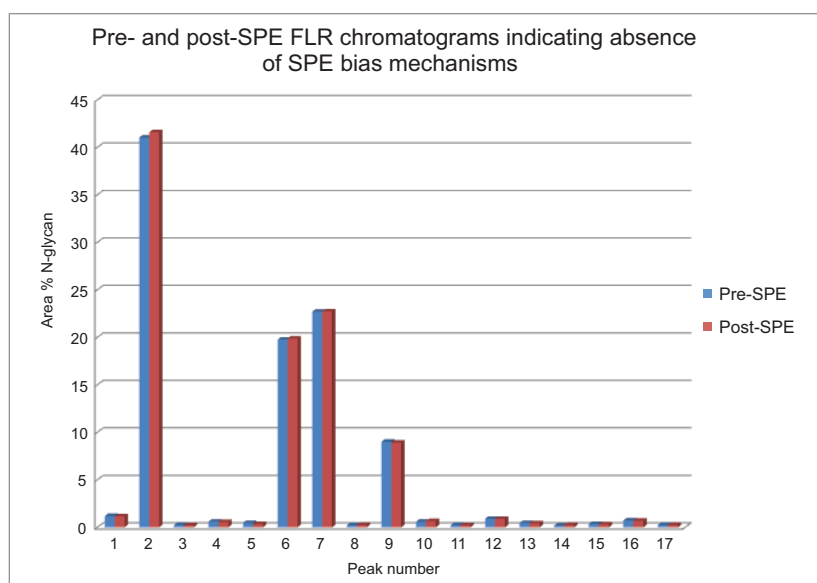


Figure 7. Absence of SPE bias mechanisms for pre/post-SPE samples.

ADDITIONAL ROBUSTNESS FEATURES AND TESTS

Sample concentration normalization

A further enhancement to this aspect of method robustness is provided with the automated method. Samples of varying glycoprotein concentration in the range of >1.5 to 20 mg/mL can be normalized (optionally) by the robot to a common starting concentration of 1.5 mg/mL which provides a consistent and optimal 15 µg of glycoprotein substrate to the reaction solution. Testing indicated very consistent instrument response for normalized samples of various concentrations in the specified range (Figure 8).

Reagent stability “On Board” the liquid handler worktable

A subtle difference between the manual and automated protocols is that all of the starting reagents are dispensed into open reagent troughs/reservoirs and placed on the liquid handling worktable at the start of the automation protocol. In contrast the manual workflow typically involves opening the reagents and pipetting at the required point in the workflow. Robustness tests were performed to confirm that the reagents have sufficient stability when presented in open reservoirs on the worktable for the duration of the automated workflow. Particular attention was given to the *RapiFluor*-MS in anhydrous DMF solution as the *RapiFluor*-MS is prone to hydrolysis. Testing confirmed that all of the reagents are stable on the worktable for the duration of the workflow and no evidence of adverse factors such as evaporation or hydrolysis were detected (Figure 9).

Stability of prepared N-glycan samples on-board the ACQUITY Sample Manager

Provision of the high-throughput sample preparation solution through automation required a confirmation of the stability of the prepared N-glycan solutions at 10 °C on-board the ACQUITY Sample Manager for the entire 96 sample data acquisition time. The universal intact mAb method has a runtime of 55 minutes therefore 96 samples requires an acquisition time of 88 hours (A shorter 10 minute runtime method is also available and is suitable for some applications). On-board stability testing verified that the prepared N-glycan samples are very stable in the ACQUITY Sample Manager at 10 °C for at least 88 hours. Samples reinjected after 88 hours had a mean % area % difference of ≤0.2% (maximum values: 0.9% difference T88 vs. T0) (Figure 10).

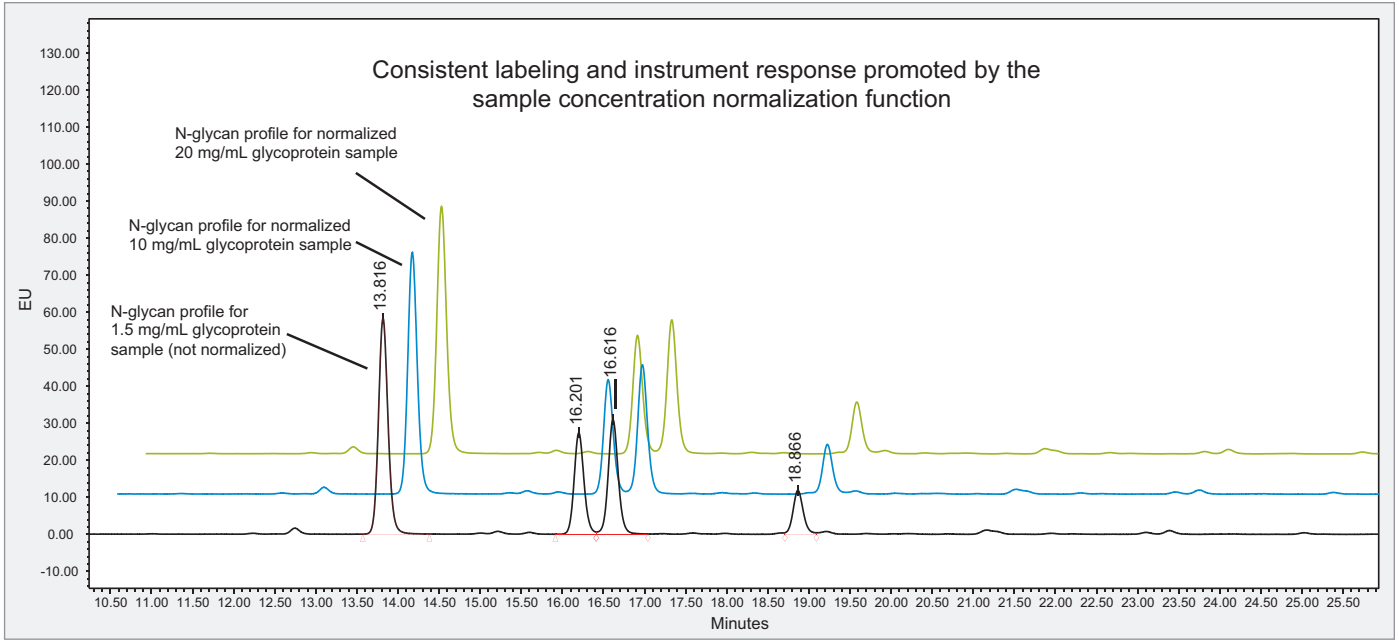


Figure 8. Uniform instrument response delivered through normalization of glycoprotein sample concentration to 1.5 mg/mL at the start of the sample preparation protocol.

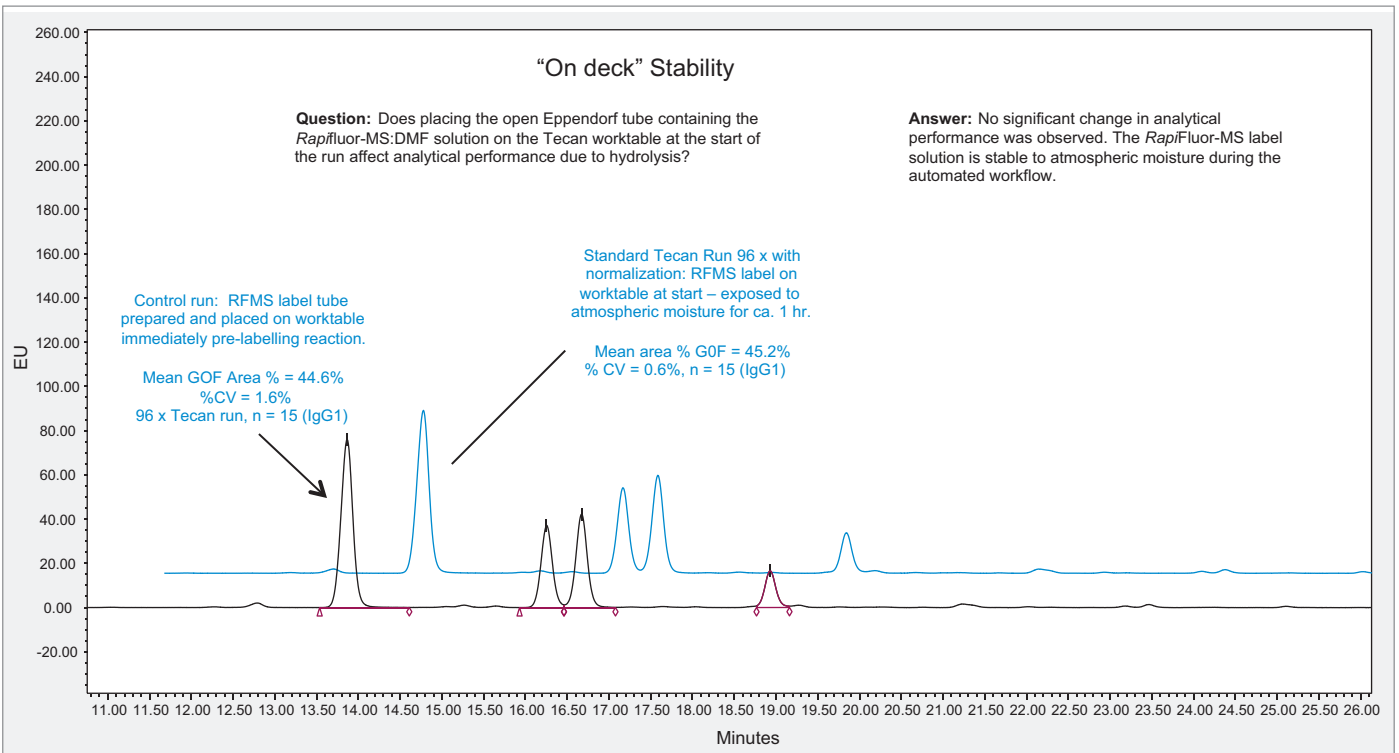


Figure 9. Confirmation of stability of reagents on the automation worktable.

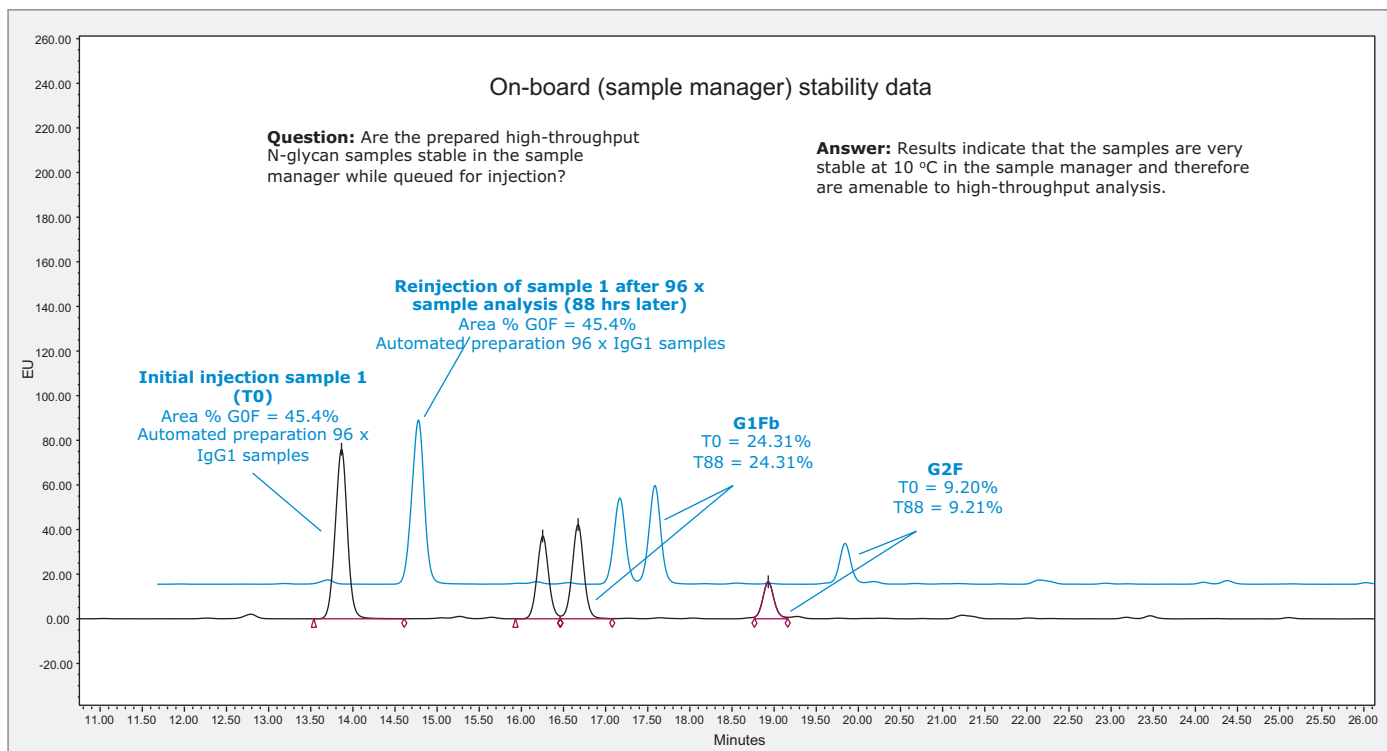


Figure 10. Confirmation of on-board stability of prepared N-glycan samples in the ACQUITY Sample Manager at 10 °C.

Intermediate precision

Although the main focus of this application note is on method robustness, other considerations such as intermediate precision also provide an additional indicator of method reliability. In this case, normal day to day variations in experimental inputs such as reagent lots, SPE sorbent lot, different glycoprotein substrate, liquid handling instrument, test instrument, column lot, analyst, etc., were incorporated in to product development testing. Provided the kit is used according to the provided instructions, none of these factors have any significant impact on method performance.

Reagent stability

Another important consideration for the reliability of the method is the stability of the reagents. The kit components for the GlycoWorks RapiFluor-MS product family are provided with specified storage conditions and undergo rigorous stability testing schemes. No deterioration in product performance has been detected under the specified storage and stability conditions provided with the kit.

CONCLUSIONS

The current technical and regulatory landscape poises N-glycan sample preparation as an excellent candidate for automated high-throughput sample preparation.⁵ A critical requirement is that this automation is provided with confidence in robustness and reproducibility. Robustness is defined by the regulatory agencies as a measure of the methods capacity to remain unaffected by small but deliberate changes in method parameters, providing an indication of reliability during normal usage.^{6,7,8}

The GlycoWorks *RapiFluor*-MS N-Glycan automated method has been designed with a high degree of reliability through a combination of rational design and risk analysis. The automated kit sustains the proven reliability and analytical performance of the existing manual GlycoWorks *RapiFluor*-MS N-Glycan Kit - by maintaining the favorable deglycosylation and labeling reaction kinetics and SPE clean-up performance.

The robustness of the analytical performance for the automated method has been demonstrated empirically by interrogating variations in selected method parameters which might be influenced specifically by the automation. The combination of robustness testing with intermediate precision, multisite performance validation, reagent stability studies and product risk analysis provide a high degree of assurance for overall product reliability following the detailed outline and exact workstation and script requirements used for this work. Any modification made by the user would need to be tested, however this application note provides guidance on how one could do this and what to look for that are critical.

FUTURE WORK

The GlycoWorks *RapiFluor*-MS N-Glycan Kit - Automation was verified using the Tecan script (provided with the kit), however the kit design is intended to be platform agnostic. Through internal testing on the Andrew Alliance and external collaborations on Hamilton and other liquid handling instruments, the reagent kit quantities have shown to be compatible across liquid handling platforms. The scalability and flexibility of the GlycoWorks *RapiFluor*-MS product family from manual sample preparation to semi-automated (Andrew Alliance) and fully-automated (Tecan) has been tested and is the subject of a future application note.

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ADDITIONAL WATERS SOLUTIONS

[Waters Ammonium Formate Solution – Glycan Analysis](#)

[ACQUITY™ UPLC™ Glycan BEH Amide Column](#)

[ACQUITY UPLC I-Class System](#)

(or [ACQUITY UPLC H-Class Bio System](#))

[ACQUITY UPLC FLR Detector](#)

[ACQUITY Sample Manager](#)

[96-well Sample Collection Plate](#)

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Appendix 1

The critical performance characteristic of the method is the reproducible determination of the area % N-glycan profile of glycoprotein samples across an entire 96-well plate. The baseline performance reference for the automated sample preparation is the area % data for the manual preparation of glycoproteins using the existing GlycoWorks RapiFluor-MS N-Glycan manual kit.

For the purpose of method comparison, a target of $\leq 5\%$ CV for area % results of the G0F, G1Fa, G1Fb, G2F glycan peaks was applied and all results were required to be within $\pm 6\sigma$ of the mean to verify the absence of outlier results.

The performance verification data in Table 1 and 3 below demonstrates acceptable performance equivalence of the automated method and the familiar manual GlycoWorks RapiFluor-MS N-Glycan sample preparation method.

Table 1. Summary: Area % comparison (Intact mAb Mass Check Standard).

	Mean N-glycan area %			
	G0F	G1Fa	G1Fb	G2F
Automated 48x Tecan run (n = 48)	43.6	21.6	25.0	9.8
Automated 96x Tecan run 2 (n = 96)	44.7	21.3	24.6	9.5
Grand mean area %				
Automated Tecan runs	44.1	21.4	24.8	9.7
Mean area % manual (n = 15)	45.2	21.1	24.4	9.4
% Difference mean automated - mean manual	-2.5	1.4	1.6	-3.1

Table 2. Summary precision comparison (Intact mAb Mass Check Standard).

N-glycan	N-glycan % CV			
	G0F	G1Fa	G1Fb	G2F
Automated 48x Tecan run (n = 48)	1.7	0.9	1.2	2.5
Automated 96x Tecan run (n = 96)	1.0	0.6	0.8	1.4
Mean % CV Automated Tecan runs	1.4	0.8	1.0	2.0
% CV manual (n = 15)	0.2	0.1	0.3	0.7

Table 3. Summary NIST precision data (NIST 8167 mAb).

	Results	N-glycan			
		G0F	G1Fa	G1Fb	G2F
96x Run 4 n = 30 NIST 8167 mAb Reference Material samples (Different N-glycan profile to Intact mAb)	Mean	38.2	28.0	10.0	7.2
	SD	0.6	0.2	0.1	0.1
	% CV	1.6	0.7	1.0	1.7
Precision 96x run 1 n = 95 intact mAb samples	Mean	44.1	21.4	24.8	9.7
	SD	0.6	0.2	0.2	0.2
	% CV	1.4	0.9	1.0	2.4