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Quality Control and Automation Friendly GlycoWorks *Rapi*Fluor-MS N-Glycan Sample Preparation

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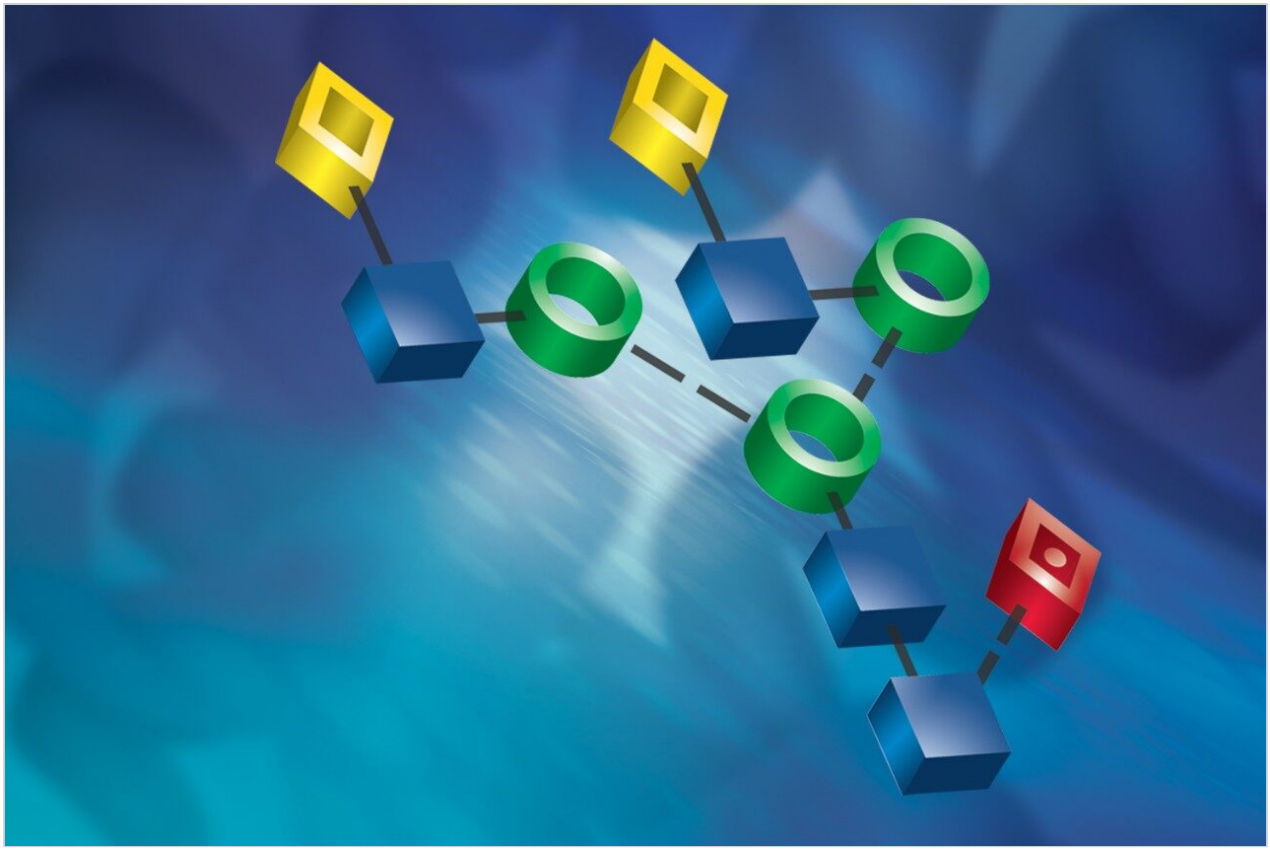
Waters Corporation



Abstract

The following work demonstrates that RFMS labeled glycan samples prepared using this alternative sample preparation scheme are comparable to those produced by the previously published flexible volume procedure. By virtue of its simplification and use of larger volumes, this protocol should be an excellent fit for adoption of RFMS into quality control (QC) environments and automated platforms.

Benefits

*Rapi*Fluor-MS glycan labeling procedure with larger volume, simplified liquid transfer to improve ease of use and automatability.

Introduction

Waters RapiFluor-MS (RFMS) is a novel labeling reagent that provides a fast, efficient, and reproducible sample preparation workflow and unsurpassed fluorescent and MS sensitivity for released N-glycan profiling.[1,2] This initial methodology was designed to accommodate the lowest possible glycoprotein sample concentration and, as result, calls for several low volume (1.2 to 7 µL) liquid transfers. Looking to minimize the impact of pipetting volume inaccuracies, we have redesigned this sample preparation to make pipetting volumes larger (≥10 µL) and thereby reduce the variation in the absolute quantities of analytes and reagents that get delivered during the denaturation, PNGase F deglycosylation, and RFMS labeling steps of the procedure.

The following work demonstrates that RFMS labeled glycan samples prepared using this alternative sample preparation scheme are comparable to those produced by the previously published flexible volume procedure. By virtue of its simplification and use of larger volumes, this protocol should be an excellent fit for adoption of RFMS into quality control (QC) environments and automated platforms.

Experimental

Method conditions

LC system:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC FLR Detector with analytical flow cell
Wavelength:	265 nm excitation, 425 nm emission
Column:	ACQUITY UPLC Glycan BEH Amide, 130 Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)
Column temp.:	60 °C
Sample temp.:	10 °C
Injection volume:	10.0 µL
Mobile phase A:	50 mM ammonium formate (pH 4.4) LC-MS grade water, from a 100 x concentrate (p/n 186007081)
Mobile phase B:	LC-MS grade acetonitrile
Sample vials:	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL (p/n 186002640)
Data management:	MassLynx 4.1 Software

Gradient:

Time	Flow rate(mL/min.)	%A	%B	Curve
0.0	0.4	25	75	6

Time	Flow rate(mL/min.)	%A	%B	Curve
35.0	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	0.4	25	75	6

MS conditions for *Rapi*Fluor-MS released N-glycans

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~ 40,000)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr

MS conditions for *Rapi*Fluor-MS released N-glycans	
Calibration:	NaI, 1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray:	300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds
Data management:	MassLynx 4.1 Software

Results and Discussion

Comparison of GlycoWorks RapiFluor-MS protocols

The GlycoWorks *Rapi*Fluor-MS sample preparation procedure (Table 1) was developed to allow for maximum flexibility with respect to the concentration of the sample being prepared for released N-glycan analysis.[3] By altering the addition of water, this method is capable of preparing samples with concentrations as low as 0.66 mg/mL. While this procedure was designed with significant molar excesses of the critical reagents, such as denaturant, enzyme, and the RFMS label, to produce reproducible results,[4] a potential drawback of this procedure is that several of the aliquoted volumes are well below 10 µL. As such, the methodology is not as amenable for adoption into certain QC laboratories, depending on their internally imposed method requirements, or for use in specific robotic platforms. Pipetted volume accuracy and precision increases with volume and, based on the International Organization for Standardization (ISO) requirements for mechanical pipette accuracy and precision, it is at 10 µL or more that the lowest permissible systematic and random errors are obtained (Figure 1, Adapted from Reference 5). It should be noted that these maximum permissible errors are doubled for the use of multi-channel pipettes. For this reason, some laboratories prefer to avoid procedures with pipetted volumes lower than 10 µL.

Component	Flexible volume standard tube (1 mL tube)	Flexible volume PCR tube (200 µL tube)
2.0 mg/mL sample	7.5 µL	7.5 µL
5% *Rapi*Gest[1]	6.0 µL	3.0 µL
Water	15.3 µL	3.3 µL
PNGaseF	1.2 µL	1.2 µL
Total volume of released N-glycan sample	30 µL	15 µL
RFMS[2]	12.0 µL	6.0 µL
Total volume of the labeled N-glycan sample	42 µL aliquot	21 µL aliquot
ACN dilution	358 µL	179 µL
Total volume of HILIC SPE Load	400 µL	200 µL

Table 1. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit flexible-volume protocols for 1 mL and 200 µL tubes.

[1]RapiGest reconstitution: 10 mg with 200 µL buffer or 3 mg with 60 µL buffer.

[2]RFMS reconstitution: 23 mg in 335 µL DMF and 9 mg in 131 µL DMF (68.7 µg/µL) for Standard Protocol or 23 mg in 168 µL DMF and 9 mg in 66 µL DMF (136.4 µg/µL) for PCR Protocol.

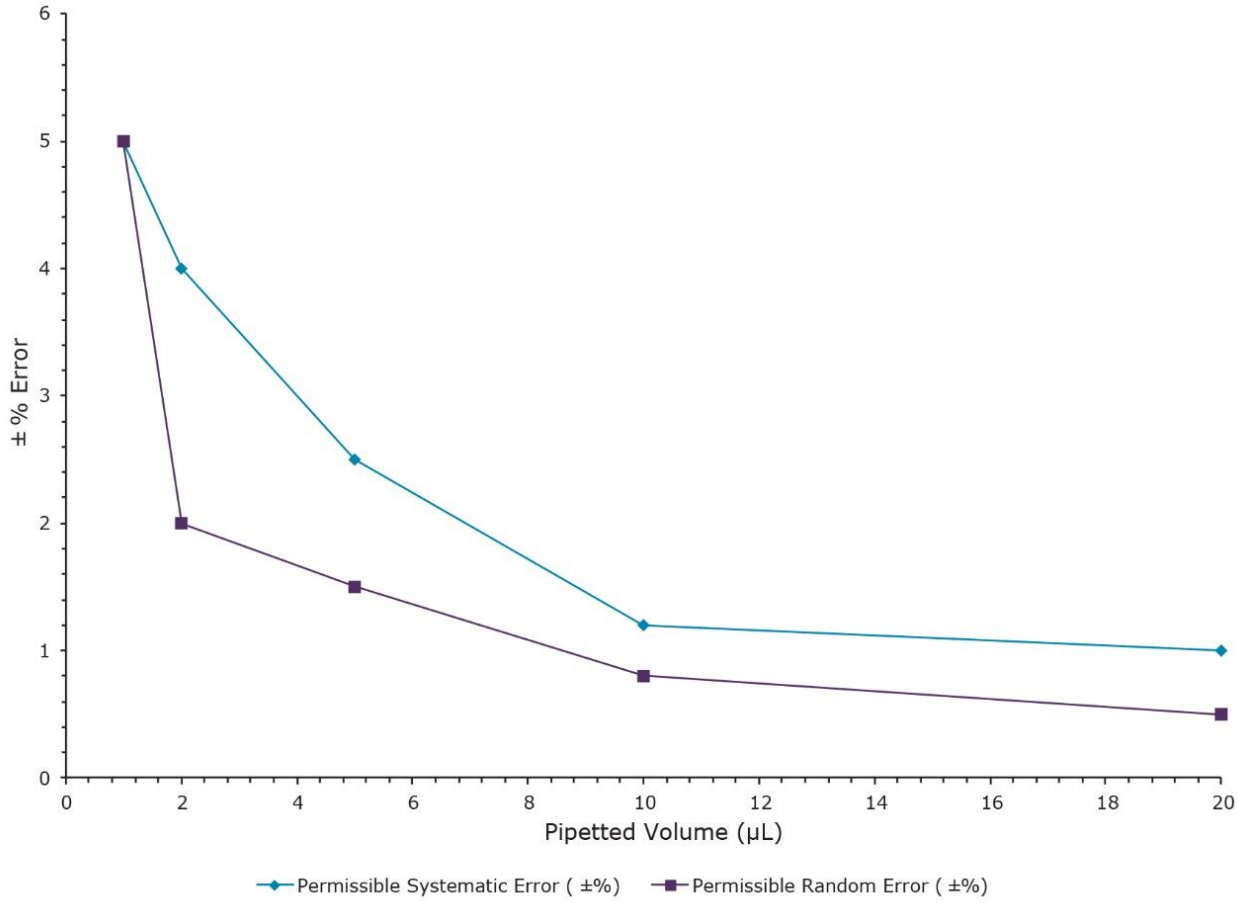


Figure 1. Trends in the maximum permissible pipette volume errors based on ISO 8655.

To provide a protocol with transfer volumes of 10 µL or more, the standard RFMS sample preparation procedure was reconsidered. The primary changes made to the deglycosylation procedure were that the addition of water was removed, and more dilute solutions of *Rapi*Gest SF surfactant and PNGase F were used. The final recommended conditions are presented in Table 2. This revised procedure is designed to give optimal results using a 10 µL sample of glycoprotein at a concentration of 1.5 mg/mL (15 µg of glycoprotein), however, samples that are more or less concentrated can still potentially produce quality results. It should be noted that at significant deviations from the optimal sample quantity, i.e. <5 µg and >30 µg, the labeling reaction can produce undesirable side reactions or low yields, so it is recommended to assess these situations on a case-by-case basis.

Component	Automated /QC standard tube (1 mL tube)	Automated/QC PCR tube (200 µL tube)
1.5 mg/mL sample	10 µL	10 µL
3% *Rapi*Gest[1]	10 µL	10 µL
Water	0 µL	0 µL
PNGaseF (diluted)[2]	10 µL	10 µL
Total volume of released N-glycan sample	30 µL	30 µL
RFMS[3]	10 µL	10 µL
Total volume of the labeled N-glycan sample	40 µL aliquot	Divide into 2 x 20 µL aliquots
ACN dilution	360 µL	2 x 180 µL
Total volume of HILIC SPE Load	400 µL	200 + 200 = 400 µL

Table 2. Aliquoted volumes for GlycoWorks RapiFluor-MS Kit automation and QC volume protocols for 1 mL and 200 µL tubes.

[1]RapiGest reconstitution: 10 mg with 200 µL buffer + 135 µL water or 3 mg with 60 µL buffer + 40 µL water.

[2]PNGase F dilution (contents of vial 30 µL + 220 µL water).

[3]RFMS reconstitution: 23 mg in 280 µL DMF or 9 mg in 110 µL DMF (82.5 µg/µL).

The only fundamental difference in this revised procedure is that the concentration of *Rapi*Gest SF is slightly higher (1.5%) during the denaturation step versus the previous procedure (1.0%). This increase is not

predicted to cause any deleterious effects and may provide some benefit for certain glycoprotein samples that are particularly resistant to denaturation. In the following step, PNGase F deglycosylation, concentrations of the principal components (glycoprotein, *Rapi*Gest SF, and PNGase F) are equivalent to the standard, flexible volume procedure.

In modifying the labeling step, the aliquoted amount of the RFMS label solution was decreased from 12 to 10 µL to be consistent with the other lowest pipetted volumes of the procedure. To account for this volume change, the concentration of the RFMS reagent was increased proportionally such that the final ratio of RFMS to glycoprotein remains equivalent. In addition to the protocol using the 1 mL reaction tubes provided in the kit, this revised procedure, like the previous procedure, has also been adapted for use with a 200 µL thermocycler tube. If using a thermocycler with this new QC and automation friendly protocol, it is necessary to divide the final released and labeled glycan sample into two aliquots, or to transfer sample to a larger tube, prior to dilution and SPE clean-up.

Comparing RapiFluor-MS labeled N-glycan profiles

To compare the standard, flexible volume procedure with its newly designed, QC and automation-friendly analog, analyses of the Waters Intact mAb Mass Check Standard were performed. Samples from this murine monoclonal antibody were prepared and analyzed following the two different protocols along with 1 mL sample tubes and single-channel pipettes. A comparison of representative chromatograms for each of these sample preparations are presented in Figure 2. The labeled peaks were integrated and the quantitative results are presented in Figure 3. The glycan species observed in this profile were assigned using online ESI-MS detection with a Xevo G2-XS QTof Mass Spectrometer (Table 3). As can be clearly seen, the two procedures produce both qualitative and quantitative results that are comparable and reproducible for peaks with relative abundances as low as 0.06%.

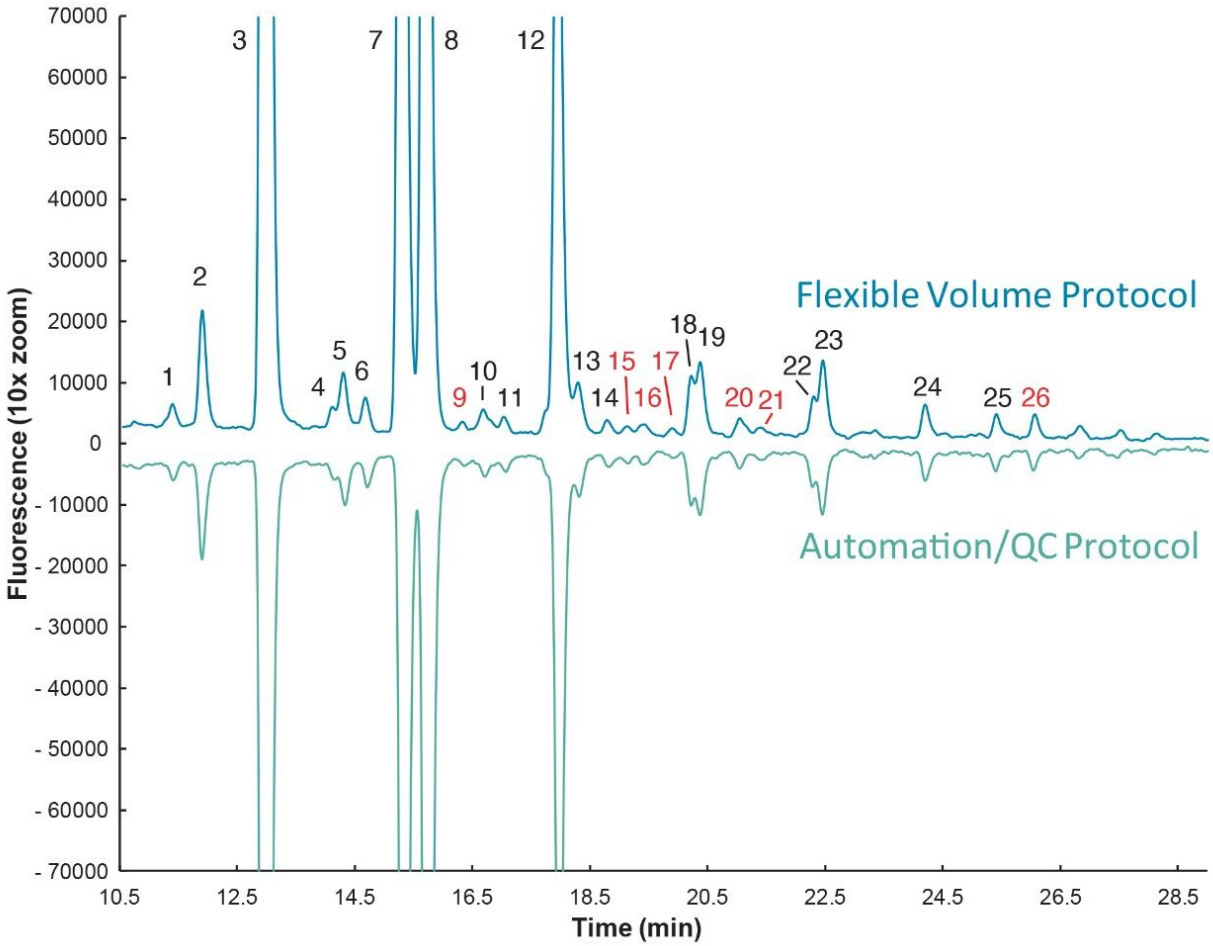


Figure 2. Chromatographic comparison of RFMS labeled glycans prepared using GlycoWorks RapiFluor-MS Kit flexible volume protocol and proposed automation and QC volume protocol. Putatative peak identifications shown in Table 3 are based on mass (Waters Xevo G2-S QTof) .

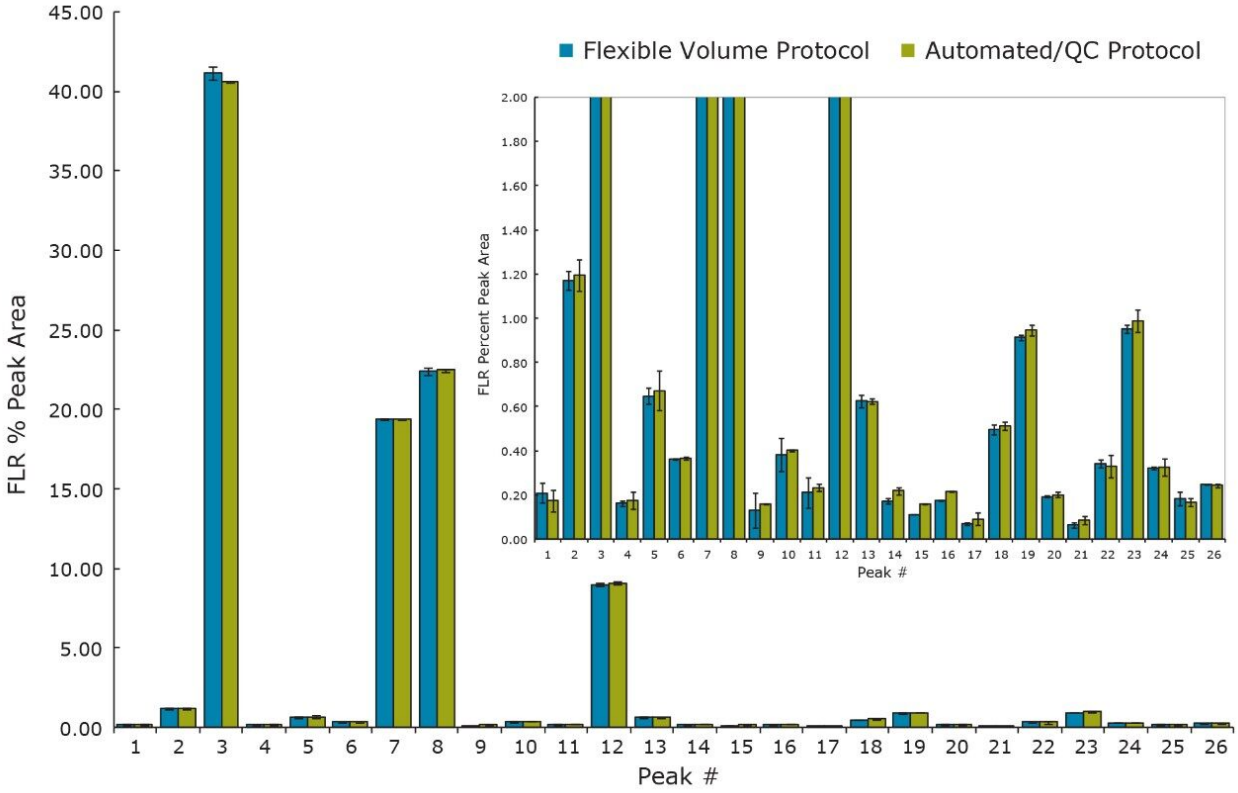


Figure 3. Quantitative comparison of RFMS labeled glycans prepared using GlycoWorks RapiFluor-MS Kit flexible volume protocol and proposed automation and QC volume protocol. Inset shows zoomed view of results

Peak #	Peak ID
1	FA1
2	A2
3	FA2
4	M5
5	FA1G1 A2G1
6	A2G1 (iso)
7	FA2G1
8	FA2G1 (iso)
10	FA2G1B
11	FA2G1B (iso)
12	FA2G2
13	FA2G1Ga1
14	FA2BG2
18	FA2G2Ga1
19	FA2G2Ga1 (iso)
22	FA2G2Sg1
23	FA2G2Ga2
24	Fa2G2GaSg1
25	Fa2G2GaSg1 (iso)
9, 15, 16, 17, 20, 21, 26	unidentified

Table 3. Figure 1 peak identifications based on mass (Xevo G2-S QTof).

Conclusion

The GlycoWorks *Rapi*Fluor-MS N-glycan sample preparation procedure has been successfully adapted to be more amenable to automation and QC use by adjusting pipetted volumes to ≥10 µL. This supplemental procedure requires a glycoprotein sample concentration of 1.5 mg/mL to obtain optimal results. As an added

benefit, the dispensed aliquots of the sample and principal reagents are equivalent in volume (10 µL), thereby providing greater assurance that the relative amounts of these components will be equivalent regardless of the systematic accuracy of the pipetting device that is used, which should result in greater intra-laboratory and inter-laboratory reproducibility.

References

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4. MA Lauber, MF Morris, DW Brousmiche, SM Koza. Robustness of *Rapi*Fluor-MS N-Glycan Sample Preparations and Glycan BEH Amide HILIC Chromatographic Separations, Application Note [serial on the Internet]. 2015; (720005370en).
5. ISO 8655-2:2002, pg. 6.

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