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Note d'application

A Modified GlycoWorks Procedure With Enhanced Detection Sensitivity

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Abstract

Here, we present a modified GlycoWorks™ RapiFluor-MS™ procedure that enhances FLR and MS sensitivity for the detection of low-abundance released N-glycans without sacrificing data quality or ease-of-use. This updated procedure was shown to outperform an NHS-carbamate modified procainamide (InstantPC™) based method in both increased detection sensitivity and reduced N-glycan over-labelling.

Benefits

- A modified GlycoWorks procedure that yields a 3-fold increase in FLR sensitivity and a 2-fold increase in MS sensitivity without sacrificing ease-of-use
- 1.5-times greater FLR sensitivity and 2.5-times greater MS sensitivity than the Agilent™ AdvanceBio™ Gly-X™ N-Glycan Prep kit with IPC
- 1.5–2-times less N-glycan over-labelling than the Agilent AdvanceBio Gly-X N-Glycan Prep kit with IPC

Introduction

N-linked glycosylation is a critical quality attribute (CQA) that is closely monitored during biotherapeutic drug development. The N-glycan profile of a biotherapeutic drug can affect clearance rate, effector function and immunogenicity.[1,2] For instance, high mannose glycans have been reported to reduce serum half-life of therapeutic antibodies.[3] Therefore, rapid and robust methods for N-glycan analysis are necessary for efficient development and approval of biotherapeutic drugs. Moreover, these methods must be sensitive enough to detect changes in low abundance glycoforms, such as high-mannose 5 (Man5).

The Waters™ GlycoWorks RapiFluor-MS (RFMS) N-Glycan labelling kit provides a rapid and facile method to release and label N-glycans for sensitive fluorescence and MS detection. The procedure involves enzymatically releasing N-glycans with rapid PNGase F, labelling the released N-glycans with RFMS, and cleaning samples up with HILIC-SPE. The entire process takes 1–2 hours, depending on how many samples are prepared, and can be adapted to a wide range of samples.[4–7]

In this application note, the FLR and MS detection sensitivity of RFMS-labelled N-glycans is increased 3- and 2-fold, respectively, by increasing the starting sample amount. The N-glycan profile and HILIC-SPE recovery for samples prepared using this enhanced-sensitivity protocol are reported and compared to the Agilent AdvanceBio Gly-X N-Glycan Prep and Cleanup kits. Finally, over-labelling of released N-glycans is assessed. The GlycoWorks kit yields 1.5–2-times less over-labelled N-glycans than Agilent's AdvanceBio Gly-X N-Glycan Prep kit with InstantPC (IPC).

Experimental

GlycoWorks Sample Preparation

Released and RFMS-labelled N-glycans were prepared using the GlycoWorks RapiFluor-MS N-Glycan Eco Kit (p/n: 176005289 <https://www.waters.com/nextgen/global/shop/application-kits/176005289-glycoworks-rapi-fluor-ms-n-glycan-eco-starter-kit---24-samples.html>). Reagents and samples were prepared following the QC/Automation-Friendly Protocol published in Application Note 720005506. Briefly, 10 µL of sample (4 mg/mL for 40 µg or 1.5 mg/mL for 15 µg) was mixed with 10 µL RapiGest™ SF Surfactant (3%) and heated at 90 °C for three minutes. After cooling at room temperature for three minutes, 10 µL of diluted PNGase F was added and samples were heated at 50 °C for five minutes. Samples were cooled at room temperature for three minutes, treated with 10 µL RFMS (82.5 µg/µL), mixed, and placed at room temperature for five minutes. Samples were cleaned up via HILIC-SPE following the GlycoWorks Care and Use Manual (715005359 <

https://www.waters.com/waters/support.htm?lid=134956186&lcid=134956185&type=USRM>) and analyzed directly without dilution.

Gly-X Sample Preparation

Released and IPC-labelled N-glycans were prepared using Agilent's AdvanceBio Gly-X N-Glycan Prep kit with IPC (p/n: GX96-IPC) following the procedure outlined in the user manual (5994-1231EN < https://www.agilent.com/cs/library/usermanuals/public/5994-1231EN.pdf>). All reagents were prepared as outlined in the user manual. Briefly, 20 µL of sample (2 mg/mL for 40 µg or 0.75 mg/mL for 15 µg) was mixed with 2 µL Gly-X Denaturant and heated at 90 °C for three minutes. After cooling at room temperature for two minutes, 2 µL of N-Glycanase Working Solution was added and samples were heated at 50 °C for five minutes. 5 µL of IPC Dye Solution was immediately added and samples were heated at 50 °C for one minute. Samples were cleaned up via HILIC-SPE using Agilent's AdvanceBio Gly-X InstantPC Cleanup Module (p/n: GX96-102) following the procedure outlined in the user manual and were analyzed directly without dilution.

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class (FLR) or ACQUITY Premier UPLC (MS)
Column:	ACQUITY Premier Glycan BEH™ Amide, 130 Å, 1.7 µm, 2.1 x 100 mm (p/n: 186009523)
Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	1 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	0.40	25	75	Initial
23.33	0.40	46	54	6
24.33	0.40	100	0	6
26.33	0.40	100	0	6
28.73	0.40	25	75	6
31.73	0.40	25	75	6
36.67	0.40	25	75	6

ACQUITY UPLC FLR detector Settings

λ_{ex} (RFMS):	265
λ_{em} (RFMS):	425
λ_{ex} (IPC):	285
λ_{em} (IPC):	345
Sample rate:	2 Hz

Xevo G2-XS QToF detector Settings

Mass range:	50–2000 *m/z*
Ionization mode:	ESI+
Analyzer mode:	Sensitivity
Experiment type:	MS^E

Sample rate:	1 Hz
Capillary voltage:	3.00 kV
Cone voltage:	80 V
Source temperature:	120 °C
Desolvation temperature:	300 °C
Cone gas flow:	50 L/h
Desolvation gas flow:	800 L/h
Collision energy:	6 V
High energy ramp:	15–40 V

Results and Discussion

Enhanced Sensitivity of RFMS-Labelled N-Glycans

A modified GlycoWorks procedure with an increased starting sample amount was used to release and label N-glycans from a mixture of a monoclonal antibody (NISTmAb) and bovine fetuin. This glycoprotein mixture was chosen to cover a broad range of N-glycans ranging from low antennarity neutral forms that are more abundant on the mAb to high antennarity and highly sialylated forms present on fetuin. Figure 1 shows the HILIC-FLR chromatography of RFMS-labelled N-glycans prepared from the traditional (15 µg of each glycoprotein) and modified (40 µg of each glycoprotein) GlycoWorks procedures. Both chromatograms display the expected N-glycan profile of a NISTmAb/fetuin mixture. The sample prepared using the modified procedure yields ~3-times higher signal in the HILIC-FLR chromatogram relative to the sample prepared using the traditional GlycoWorks procedure. A similar signal increase is exhibited in HILIC-MS chromatography (see signal-to-noise data below).

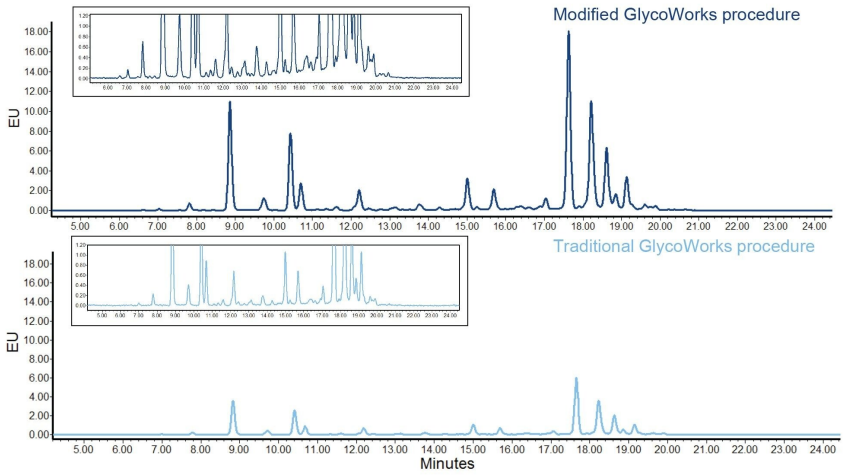

Figure 1. HILIC-FLR chromatograms of released and labelled NISTmAb/fetuin glycans prepared using the traditional and modified GlycoWorks procedures. The modified GlycoWorks procedure yields ~3-times higher signal with comparable glycan profile.

The relative FLR peak abundances of selected NISTmAb and fetuin N-glycans released from 40 and 15 µg sample with the GlycoWorks kit are shown in Figure 2. For both NISTmAb and fetuin, the N-glycan distribution does not change when the starting sample amount is increased from 15 to 40 µg. These results demonstrate the efficacy of the kit across a wide range of sample concentrations and confirm that enhanced detection sensitivity can be achieved without deleterious effects on the release and label reactions. The relative abundances are shown before and after HILIC-SPE cleanup to demonstrate the non-selective nature of the cleanup step regardless of sample concentration.

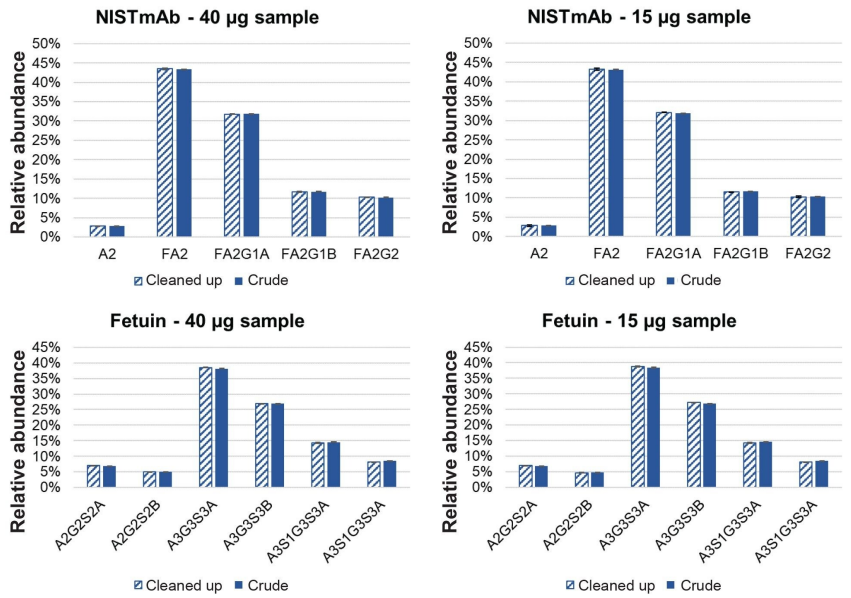

Figure 2. Comparison of the relative N-glycan profiles before and after sample cleanup prepared using the traditional (15 µg) and modified (40 µg) GlycoWorks procedures. Error bars represent one standard deviation (n=4). The N-glycan distribution does not change when the starting sample amount is increased from 15 to 40 µg.

Comparison to Agilent's AdvanceBio Kit

Agilent's AdvanceBio Gly-X N-Glycan Prep kit with IPC was also used to release and label N-glycans from the same NISTmAb/fetuin mixture at two concentrations (40 µg and 15 µg of each glycoprotein) to compare to the traditional and modified GlycoWorks procedures. The GlycoWorks and AdvanceBio kits deliver comparable N-glycan profiles, regardless of starting sample amount (see MS chromatography in Figure 3).

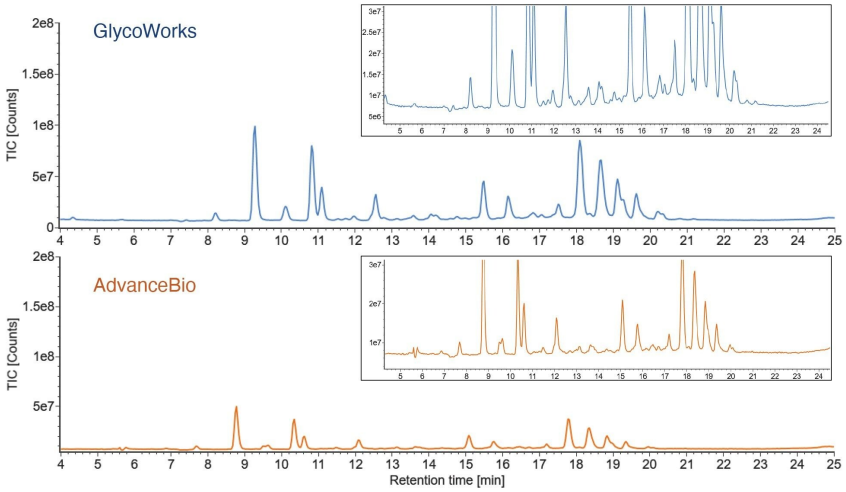

Figure 3. HILIC-MS chromatograms of released and labelled NISTmAb/fetuin glycans prepared using the GlycoWorks and AdvanceBio kits. Both chromatograms display released and labelled N-glycans prepared from 40 µg of each glycoprotein in the mixture and deliver comparable N-glycan profiles. The GlycoWorks kit yields ~2-fold stronger MS signal.

HILIC-SPE recovery for both kits was assessed by comparing the FLR peak area of the FA2 N-glycan before and after sample cleanup (Figure 4). Both kits yield comparable SPE recoveries with 15 µg of each glycoprotein, but the GlycoWorks cleanup kit yields 1.5-times higher recovery than the AdvanceBio kit with 40 µg of each glycoprotein. The SPE recovery of the GlycoWorks kit is improved with increased sample amount. This is expected because the relative level of sample loss during cleanup is reduced when the total sample concentration is increased. The SPE recovery of the AdvanceBio kit decreases with increased sample amount, likely because of the limited capacity of the SPE procedure (recommended 40 µg glycoprotein maximum).

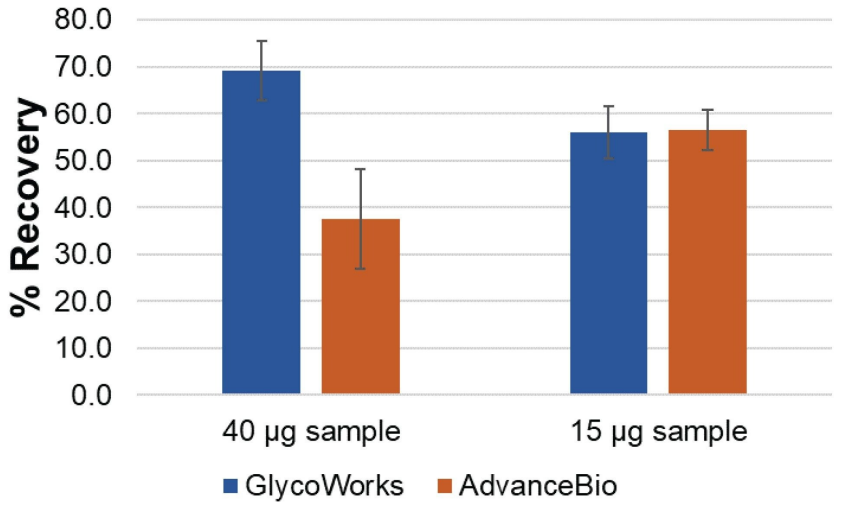

Figure 4. HILIC-SPE recovery for the GlycoWorks and AdvanceBio kits for 15 µg and 40 µg of each glycoprotein in the mixture. Error bars represent one standard deviation (n=4). Both kits yield comparable SPE recoveries with 15 µg of each glycoprotein, but the GlycoWorks cleanup kit yields 1.5-times higher recovery than the AdvanceBio kit with 40 µg of each glycoprotein.

Signal-to-noise ratios (SNRs) should be considered when choosing a sample preparation kit for released N-glycan analysis to ensure detection of changes in low-abundance glycoforms. SNRs for FLR and MS detection of released and labelled N-glycans prepared using the GlycoWorks and AdvanceBio kits are shown in Figure 5. The GlycoWorks kit yields more sensitive MS detection than the AdvanceBio kit regardless of starting sample amount. With FLR detection, the GlycoWorks kit yields more sensitive detection than the AdvanceBio kit when starting with 40 µg of each glycoprotein, but less sensitive detection when starting with 15 µg of each glycoprotein. The GlycoWorks kit likely outperforms the AdvanceBio kit in FLR sensitivity at higher sample amounts because of its higher SPE recovery.

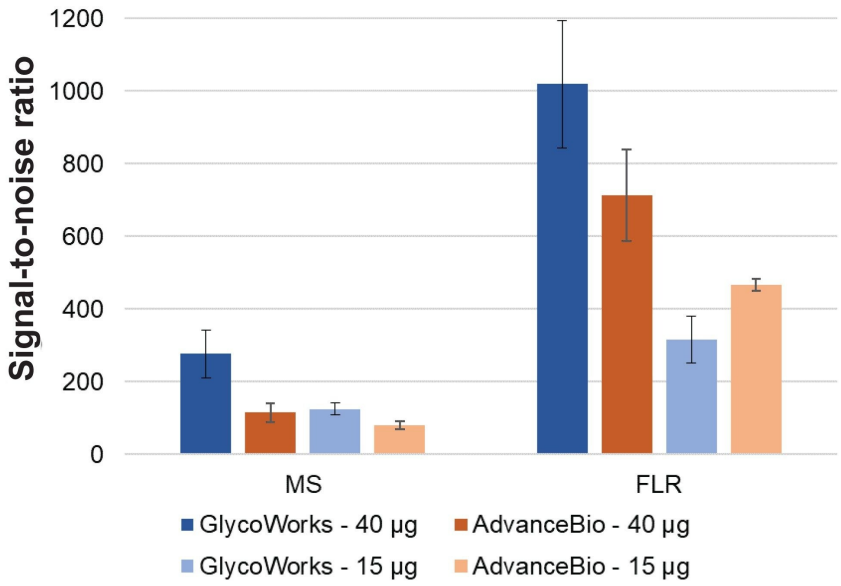

Figure 5. Signal-to noise ratios for FLR and MS detection of released and labelled N-glycans prepared using the GlycoWorks and AdvanceBio kits. Error bars represent one standard deviation (n=4). When starting with 40 µg of sample, the GlycoWorks kit yields 2.5-times higher SNR than the AdvanceBio kit for MS detection and 1.5-times higher SNR for FLR detection. When starting with 15 µg of sample, the GlycoWorks kit yields 1.5-times higher SNR for MS detection but 1.5-times lower SNR for FLR detection.

Over-labelling of Released N-Glycans

The GlycoWorks and AdvanceBio kits rely on the chemical crosslinking of free glycosylamines with N-hydroxysuccinimide (NHS) esters, similar to the cross-linking of lysine residues in proteins with modified NHS esters. However, cross-linking of proteins with NHS esters can result in over-labelling through reactivity with nucleophilic sites other than lysine (***e.g.***, N-termini, tyrosines, serines, and threonines).[8] Similarly, released and labelled N-glycans prepared with the GlycoWorks and AdvanceBio kits should be scrutinized for over-labelling with more than one RFMS or IPC molecule. Over-labelling of released N-glycans will cause retention time shifts that may result in inaccurate identification and quantification of low-abundance N-glycans.

Over-labelling with RFMS and IPC was measured by integrating the extracted ion chromatogram (EIC) of doubly- and singly-labelled FA2 and A3G3S3 N-glycans. Figure 6 shows the % of doubly-labelled species relative to the sum of doubly- and singly-labelled species for samples prepared using the GlycoWorks and AdvanceBio kits. For both kits, the A3G3S3 N-glycan exhibits more over-labelling than the FA2 N-glycan, and there is a slight increase in over-labelling when increasing the starting sample amount from 15 µg to 40 µg. Notably, samples prepared using the GlycoWorks kit exhibit 1.5-2-times less over-labelling than the AdvanceBio kit. These results highlight a significant advantage of the GlycoWorks kit, mitigating the risk of inaccurate identification and quantification of low-abundance N-glycans resulting from over-labelling.

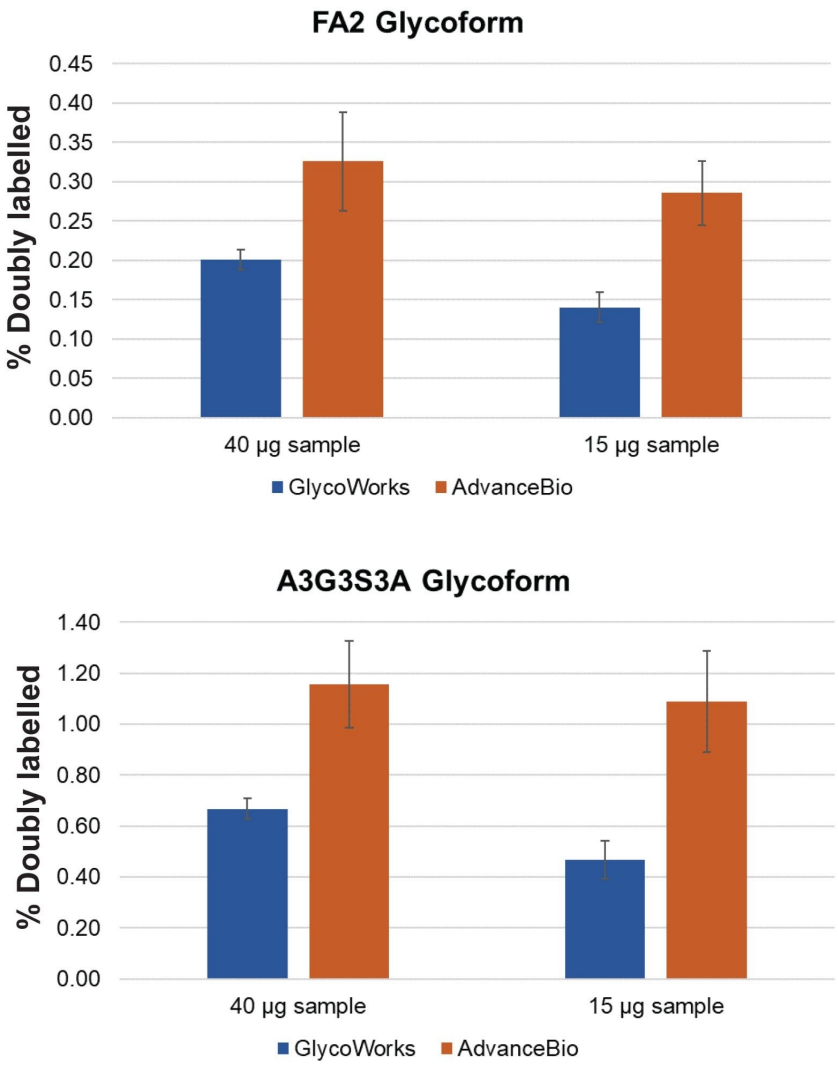

Figure 6. Over-labelling of the FA2 and A3G3S3 N-glycans for samples prepared using the GlycoWorks and AdvanceBio kits. Error bars represent one standard deviation (n=4). Samples prepared using the GlycoWorks kit exhibit 1.5–2-times less over-labelling than the AdvanceBio kit.

Conclusion

Rapid, robust, and sensitive methods for N-glycan analysis are necessary for efficient development and approval of biotherapeutic drugs. A modified GlycoWorks procedure was established that enhances detection sensitivity without deleterious effects on the relative quantification of the released and labelled N-glycans. The new procedure yields a 3-fold increase in FLR SNR and a 2-fold increase in MS SNR by increasing the starting sample amount. At these higher sample concentrations, the GlycoWorks kit exhibits increased SPE recovery, increased FLR sensitivity (1.5-fold higher) and increased MS sensitivity (2.5-fold higher) compared to Agilent's AdvanceBio Gly-X N-Glycan Prep kit with IPC. Moreover, samples prepared using the GlycoWorks kit yield 1.5–2-times less over-labelling than those prepared with the AdvanceBio kit.

References

1. Zhang, P., Woen, S., Wang, T., Liau, B., Zhao, S., Chen, C., Yang, Y., Song, Z., Wormald, M. R., Yu, C., Rudd, P. M. Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs. Drug Discovery Today. 2016, 21 (5), 740–765.

2. Reusch, D., Tejada, M.L. Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology. 2015, 25 (12), 1325–1334.

3. Goetze A. M., Liu Y. D., Zhang Z., Shah B., Lee E., Bondarenko P. V., Flynn GC. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. Glycobiology. 2011, 21 (7), 949–959.

4. Lauber, M. A.; Yu, Y.-Q.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Magnelli, P.; Guthrie, E.; Taron, C. H.; Fountain, K. J. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Labeling Reagent That Facilitates Sensitive Fluorescence and ESI-MS Detection. Anal. Chem. 2015, 87 (10), 5401–5409.

5. Koza, S. M.; McCall, S. A.; Lauber, M. A.; Chambers, E. E. Quality Control and Automation Friendly GlycoWorks RapiFluor-MS N-Glycan Sample Preparation (720005506). Waters Corporation May 2020.

6. Zhang, X.; Reed, C. E.; Birdsall, R. E.; Yu, Y. Q.; Chen, W. High-Throughput Analysis of Fluorescently Labeled N-Glycans Derived from Biotherapeutics Using an Automated LC-MS-Based Solution. SLAS Technology. 2020, 24 (4), 380–387.

7. Hanna, C.M.; Koza, S. M., Yu, Y. Q. Automated RapiFluor-MS™ Labeled N-Glycan Sample Preparation for

Protein A Purified Monoclonal Antibodies (720007854). Waters Corporation February 2023.

8. Kalkhof, S., Sinz, A. Chances and pitfalls of chemical cross-linking with amine-reactive N-hydroxysuccinimide esters. Anal Bioanal Chem 392, 305–312 (2008).

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720008222, February 2024