Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Absolute Quantitation of *Rapi*Fluor-MS Labeled N-Glycans by Calibration of Fluorescence Response

Qi Wang and Matthew A. Lauber
Waters Corporation, Milford, MA, USA

GOAL

To demonstrate the absolute quantitation of *Rapi*Fluor-MS® labeled N-glycans using the *Rapi*Fluor-MS Quantitative Glycan Standard.

BACKGROUND

Glycosylation plays an important role in the structure and function of proteins, and altered glycosylation is implicated in numerous diseases, such as cancer,[1,2] autoimmune diseases,[3] and neurodegenerative disorders.[4] Furthermore, glycosylation is frequently determined to be a critical quality attribute for therapeutic glycoproteins.[5,6] Just like characterization, quantitation of protein glycosylation is needed for various reasons, including the discovery of glycan biomarkers for diagnosis, prognosis, or risk prediction,[7] and drug metabolism and pharmacokinetics (DMPK) studies.[8] Quantitative measurements are also essential to assessing the proficiency of an analytical method, where they can be helpful in estimating yields, facilitating validation studies, and confirming the success of transferring a method to a different laboratory. Recently, a novel analytical approach was introduced for the preparation and analysis of N-glycans.

Together, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and *Rapi*Fluor-MS Quantitative Glycan Standard provide a powerful new approach to quantitatively analyze N-glycans for proficiency testing, and to facilitate glycan-centric biomarker, as well as DMPK, studies.

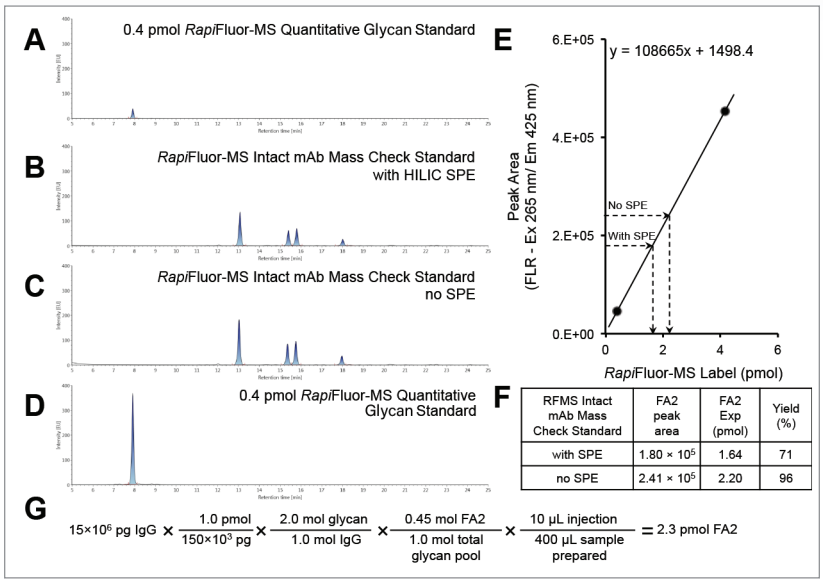

Figure 1. Quantitation of RapiFluor-MS labeled N-glycans. HILIC-fluorescence chromatograms of 0.4 pmol (A) and 4.0 pmol (D) of RapiFluor-MS Quantitative Glycan Standard and RapiFluor-MS labeled N-glycans obtained from Intact mAb Mass Check Standard with (B) and without HILIC SPE cleanup (C) using the GlycoWorks® RapiFluor-MS N-Glycan Kit. Glycans were profiled with an ACQUITY UPLC® H-Class Bio System and a fluorescence detector using an ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 × 150 mm. A two-point external calibration curve was constructed with the RapiFluor-MS Quantitative Glycan Standard (E) to enable quantitation of the RapiFluor-MS labeled FA2 glycan obtained from the Intact mAb Mass Check Standard (F). (G) Calculation for the theoretical yield of the FA2 glycan obtained from Intact mAb Mass Check Standard. Analyses were performed in duplicate.

The strategy of this approach is based on combining the rapid release and labeling of N-glycans via the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit with high-resolution hydrophilic interaction chromatography (HILIC) and sensitive fluorescence (FLR)-mass spectrometric (MS) analyses.[9] In this work, we expand upon the capabilities of this approach and show how the *Rapi*Fluor-MS Quantitative Glycan Standard can be used to calibrate fluorescence response and thereby enable the absolute quantitation of *Rapi*Fluor-MS labeled N-glycans.

THE SOLUTION

The *Rapi*Fluor-MS Quantitative Glycan Standard (p/n: 186008791) was purposefully designed to help analysts calibrate fluorescence responses observed during HILIC-FLR-MS separations of *Rapi*Fluor-MS labeled glycans. It is a synthetic peptide uniquely constructed of hydrophilic residues. Most importantly, it is derivatized by one mole equivalent of the *Rapi*Fluor-MS label and rigorously tested to ensure applicability to quantitative measurements. With a HILIC glucose unit (GU) value of approximately 4, it can be used as either an internal or external calibrant for N-glycan quantitation without interference issues from sample matrix void peaks or *Rapi*Fluor-MS labeled N-glycans, as demonstrated in Figure 1A–D. Since fluorescence response is linearly proportional to the amount of *Rapi*Fluor-MS label, fluorescence peak areas can be used to quantify specific *Rapi*Fluor-MS labeled glycans. For example, a linear regression can be graphed to relate observed fluorescence peak areas to different injected amounts of the *Rapi*Fluor-MS Quantitative Glycan Standard. Analyses have shown that a 4-point calibration curve consisting of 0.4, 0.8, 2.4, and 4.0 pmol of the standard provides an R^2 value of 1.0000. A quick 2-point (0.4 and 4.0 pmol) calibration curve for *Rapi*Fluor-MS labeled species can therefore be used to perform reliable absolute quantitation (Figure 1E).

Among the different reasons to perform a quantitative analysis, one is to check the proficiency of a GlycoWorks *Rapi*Fluor-MS

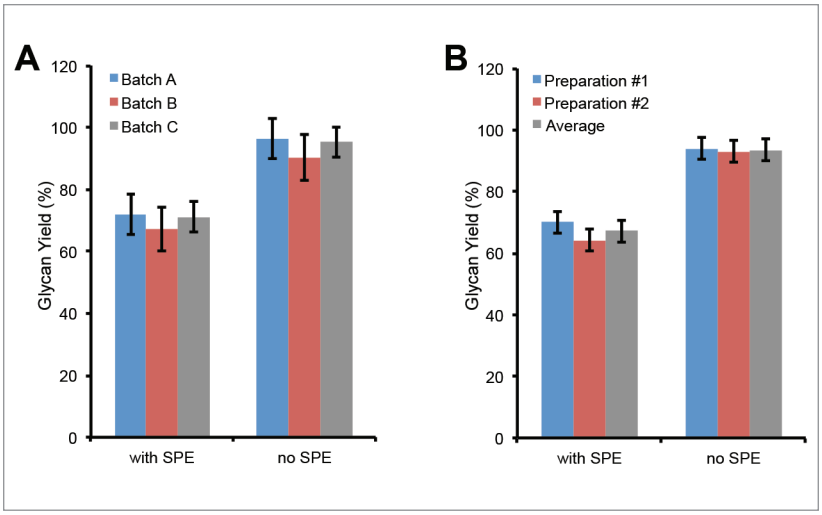

Figure 2. Yields of RapiFluor-MS labeled glycans obtained from Intact mAb Mass Check Standard. RapiFluor-MS labeled glycans obtained from the Intact mAb Mass Check Standard with and without HILIC SPE cleanup using the GlycoWorks RapiFluor-MS N-Glycan Kit were quantified with three different batches of the RapiFluor-MS Quantitative Glycan Standard (A). Glycan quantitation was performed on two GlycoWorks RapiFluor-MS N-Glycan Kit preparations of the Intact mAb Mass Check Standard (B).

N-Glycan Kit (p/n: 176003713) sample preparation. In such a case, a control standard, like the Intact mAb Mass Check Standard (p/n: 186006552), can be used to study proficiency and yields. Here, we have focused on the amounts of FA2 glycan obtained from the Intact mAb Mass Check Standard with and without HILIC solid phase extraction (SPE) cleanup, which we have calculated, from their measured fluorescence peak areas, to be 1.64 and 2.20 pmol (Figure 1F). Since 45% of the glycans released from the Intact mAb Mass Check Standard are known to correspond to the FA2 glycan, the yield of glycans released and labeled from the Intact mAb Mass Check Standard using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit with and without HILIC SPE cleanup can be calculated to be 71% and 96%, respectively (Figure 1F-G). As previously reported, this result confirms that a *Rapi*Fluor-MS N-glycan sample preparation is effectively lossless, save for the SPE step.[9] The value of the SPE step should not, however, be discounted as its losses have been confirmed to be unbiased, and its use can improve the lifetime of the downstream HILIC column.

The above glycan yields were obtained with one vial of the *Rapi*Fluor-MS Quantitative Glycan Standard. With three different batches of *Rapi*Fluor-MS Quantitative Glycan Standard, the glycan yields of the same preparation of *Rapi*Fluor-MS labeled N-glycans ranged from 67% to 72% with HILIC SPE cleanup and 90% to 96% without SPE (Figure 2A). The displayed error bars correspond to the relative standard deviations of glycan yields obtained from three vials of *Rapi*Fluor-MS Quantitative Glycan Standard within the same batch. In sum, these findings demonstrate the noteworthy vial-to-vial and batch-to-batch reproducibility of the *Rapi*Fluor-MS Quantitative Glycan Standard. The average glycan yields obtained from the three batches of *Rapi*Fluor-MS Quantitative Glycan Standard were 70% with SPE and 94%

without SPE (Figure 2B, preparation #1). In the same fashion, glycan quantitation was also performed on a second N-glycan preparation, and the yields were found to be 64% with SPE and 93% without SPE (Figure 2B, preparation #2). These data clearly demonstrate the high reproducibility of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit for glycan release and labeling. As they are, these results also provide evidence in support of the proficiency with which the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit sample preparations were performed.

SUMMARY

We have demonstrated the absolute quantitation of N-glycans through combining HILIC-FLR analyses with the use of the *Rapi*Fluor-MS Quantitative Glycan Standard. In this work, we defined an external calibration curve from the fluorescence responses obtained with two different injected amounts of the *Rapi*Fluor-MS Quantitative Glycan Standard. It was thereby possible to quantify the amounts of specific *Rapi*Fluor-MS labeled N-glycans. As applied to proficiency testing, use of the *Rapi*Fluor-MS Quantitative Glycan Standard made it possible to determine and compare the yields of two *Rapi*Fluor-MS N-glycan sample preparations. In turn, it was demonstrated that release, labeling, and SPE clean-up of glycans with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit is achieved with very high reproducibility. Together, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and *Rapi*Fluor-MS Quantitative Glycan Standard provide a powerful new approach to quantitatively analyze N-glycans. Here, its utility for proficiency testing was highlighted, but it should also be noted that the *Rapi*Fluor-MS Quantitative Glycan Standard could be used to facilitate glycan-centric biomarker and DMPK studies.

References

1. Mechref, Y. et al. Defining putative glycan cancer biomarkers by MS. *Bioanalysis* 2012, 4 (20), 2457–2469.
2. Ruhaak, L. R., Miyamoto, S. and Lebrilla, C. B. Developments in the identification of glycan biomarkers for the detection of cancer. *Molecular & Cellular Proteomics* 2013, 12 (4), 846–855.
3. Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A. and Dwek, R. A. Glycosylation and the immune system. *Science* 2001, 291 (5512), 2370–2376.
4. Lefebvre, T. et al. Does O-GlcNAc play a role in neurodegenerative diseases? *Expert Review of Proteomics* 2005, 2 (2), 265–275.
5. Dalziel, M., Crispin, M., Scanlan, C. N., Zitzmann, N. and Dwek, R. A. Emerging principles for the therapeutic exploitation of glycosylation. *Science* 2014, 343 (6166), 1235681.
6. Beck, A., Wagner-Rousset, E., Ayoub, D., Van Dorsselaer, A. and Sanglier-Cianférani, S. Characterization of therapeutic antibodies and related products. *Analytical Chemistry* 2012, 85 (2), 715–736.
7. Etxebarria, J. & Reichardt, N.-C. Methods for the absolute quantification of N-glycan biomarkers. *Biochimica et Biophysica Acta (BBA)-General Subjects* 2016, 1860 (8), 1676–1687.
8. Cong, Y., Zhang, Z., Zhang, S., Hu, L. and Gu, J. Quantitative MS analysis of therapeutic mAbs and their glycosylation for pharmacokinetics study. *PROTEOMICS-Clinical Applications* 2015.
9. Lauber, M. A. et al. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection. *Analytical Chemistry* 2015, 87 (10), 5401–5409.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com