RAPID PREPARATION OF RELEASED N-GLYCANS FOR HILIC ANALYSIS USING A NOVEL FLUORESCENCE AND MS-ACTIVE LABELING REAGENT

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INTRODUCTION

Conventional approaches to the preparation of *N*-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity.[1] In the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans can be readily detected by fluorescence but are difficult to detect by electrospray ionization mass spectrometry (ESI-MS). Variations of conventional approaches for *N*-glycan sample preparation have been explored, but have not yet presented a solution that combines the desired attributes of simplicity, high MS sensitivity, and high throughput. One example is rapid tagging procedures that yield labeled glycans in a matter of minutes. Cook and co-workers have presented the use of a rapid tagging analog of aminobenzamide (AB).[2] In a rapid reaction, the precursor glycosylamines of reducing, aldehyde terminated glycans are modified via a urea linked aminobenzamide. Although this rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced ionization efficiencies needed in modern *N*-glycan MS analyses.

To address the above shortcomings, we have developed a sample preparation solution that enables unprecedented FLR and MS sensitivity for released *N*-glycan detection while also improving the throughput of *N*-glycan sample preparation. A novel labeling reagent has been synthesized that rapidly reacts with glycosylamines following their release from glycoproteins. Within a 5 minute reaction, *N*-glycans are labeled with *Rapi*Fluor-MS, a reagent comprised of an *N*-hydroxysuccinimide (NHS) carbamate rapid tagging group, an efficient quinoline fluorophore, and a tertiary amine for enhancing ionization. To further accelerate the preparation of *N*-glycans, rapid tagging has been directly integrated with a Rapid PNGase F deglycosylation procedure involving RapiGest SF surfactant and a HILIC µElution SPE clean-up step that provides highly quantitative recovery of the released and labeled glycans with the added benefit of not requiring a solvent dry-down step prior to the LC-FLR-MS analysis of samples.

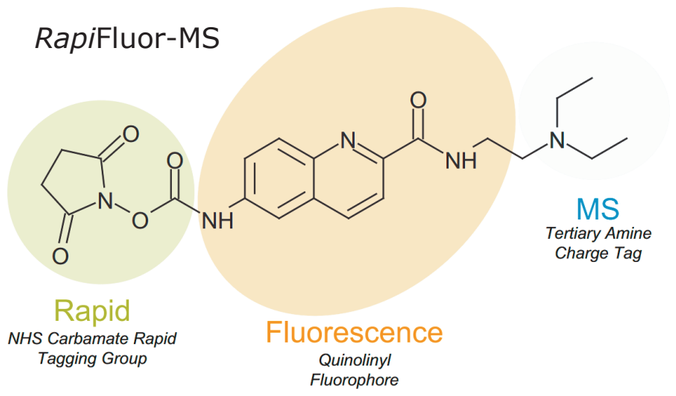


Figure 1. *Rapi*Fluor-MS Molecular Structure.

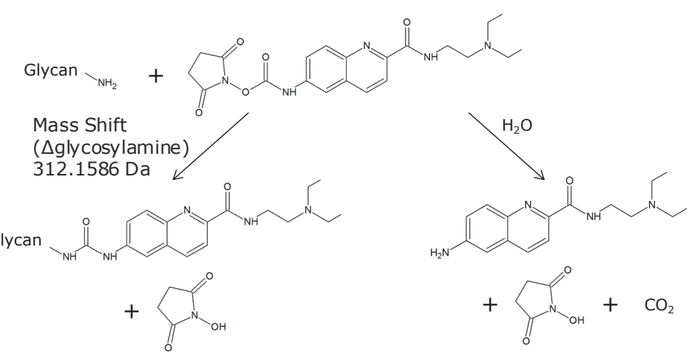


Figure 2. Reaction Schematic for *Rapi*Fluor-MS Derivatization of an *N*-Glycosylamine.

METHODS

SAMPLE DESCRIPTION:
N-glycans were prepared using a GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit (p/n 176003606) and the manufacturer suggested protocols provided in the Care and Use Manual (715004793EN). To compare the response factors of Instant AB™ and *Rapi*Fluor-MS labeled glycans, labeling reactions were performed with equivalent molar excesses of reagent, and crude reaction mixtures were directly analyzed by HILIC-FLR-MS in order to avoid potential biases from SPE clean-up procedures. To compare the response factors of 2-AB labeled versus *Rapi*Fluor-MS labeled glycans, equivalent quantities of labeled *N*-glycans from pooled human IgG were analyzed by HILIC-FLR-MS. Column loads were calibrated using external quantitative standards.

METHOD CONDITIONS (unless otherwise noted):

LC Conditions:

LC system:	ACQUITY UPLC H-Class Bio System
Sample Temp.:	5 °C
Analytical Column Temp.:	60 °C
Flow Rate:	0.4 mL/min
Fluorescence Detection:	Ex 265 / Em 425 nm (RapiFluor-MS) Ex 278 / Em 344 nm (Instant AB™) Ex 330 / Em 420 nm (2-AB) 5 Hz scan rate Gain =1
Column:	ACQUITY UPLC Glycan BEH Amide 130 Å 1.7 µm, 2.1 x 50 mm (p/n 186004740)

Gradient used with 2.1 x 50 mm columns:
Mobile Phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)
Mobile Phase B: ACN (LC-MS grade)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
11.7	0.4	46	54	6
12.2	0.2	100	0	6
13.2	0.2	100	0	6
14.4	0.2	25	75	6
15.9	0.4	25	75	6
18.3	0.4	25	75	6

For more experimental details and additional results, see Waters Application Note "Rapid Preparation of Released *N*-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent "

MS Conditions

MS system:	Synapt G2-S HDMS
Analyzer mode:	ESI+, TOF MS, Resolution Mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Acquisition:	500–2500 m/z, 1 Hz scan rate
Data management:	MassLynx Software (V4.1)

RESULTS AND DISCUSSION

Rational Design of a New *N*-Glycan Labeling Reagent

A new labeling reagent for facilitating *N*-glycan analysis has been synthesized based on rational design considerations (**Figure 1**) that would afford rapid labeling kinetics, high fluorescence quantum yields, and significantly enhanced MS detectability. Within a 5 minute reaction, *N*-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (**Figure 2**). In addition to rapid tagging capabilities, the new labeling reagent also supports high sensitivity for both MS and flourescence detection.

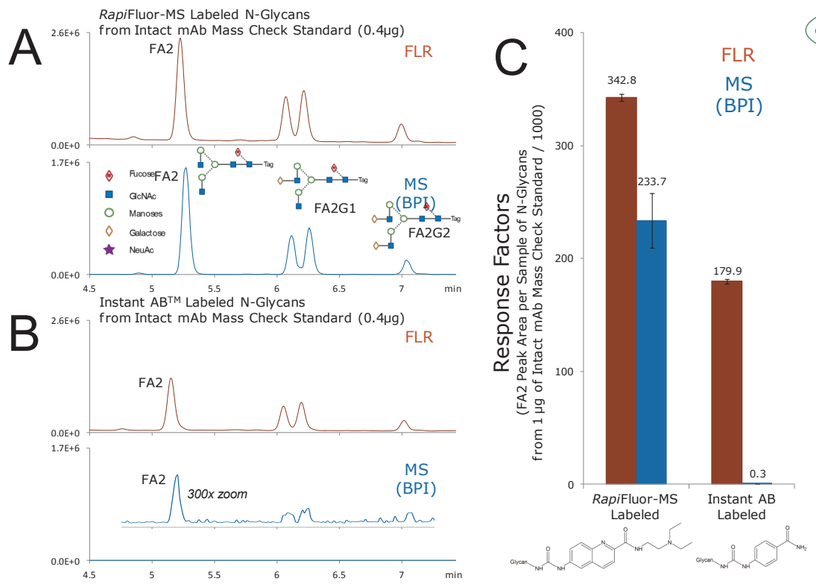


Figure 3. HILIC-FLR-MS of (A) RapiFluor-MS and (B) Instant AB™ Labeled *N*-Glycans from Intact mAb Mass Check Standard. Fluorescence (FLR) chromatograms are shown in orange and base peak intensity (BPI) MS chromatograms are shown in blue. (C) Response factors for RapiFluor-MS and Instant AB labeled glycans (measured as the FA2 peak area per sample of *N*-glycans resulting from 1 µg of Intact mAb Mass Check Standard).

***Rapi*Fluor-MS Enables High Sensitivity Fluorescence and MS Detection**

The response factors of *Rapi*Fluor-MS labeled glycans have been benchmarked against those observed for glycans labeled with alternative reagents. The most closely related, commercially available alternative to *Rapi*Fluor-MS is an NHS carbamate analog of aminobenzamide, known as Instant AB™.[2] **Figures 3A** and **3B** present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of *N*-glycans released from a murine monoclonal antibody (Intact mAb Mass Check Standard, p/n 186006552) and labeled with *Rapi*Fluor-MS and Instant AB, respectively. Based on the observed chromatographic peak areas, response factors for fluorescence and MS detection were determined for the most abundant glycan in the IgG profile, the fucosylated, biantennary FA2 glycan (**Figure 3C**) and indicate that *Rapi*Fluor-MS labeled glycans produce 2 times higher fluorescence signal and, more astoundingly, 780 times greater MS signal than *N*-glycans labeled with Instant AB. In a similar fashion, *Rapi*Fluor-MS labeling has also been compared to conventional 2-AB labeling. To summarize our observations, we have plotted the response factors of Instant AB and 2-AB as percentages versus the response factors of *Rapi*Fluor-MS (**Figure 4**).

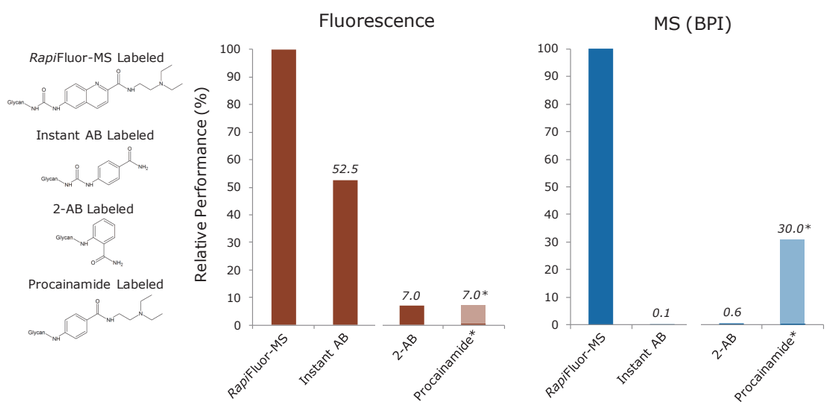


Figure 4. Relative performance of glycan labels. Response factors normalized to the fluorescence and MS response factors of *Rapi*Fluor-MS labeled *N*-glycans. (*) Comparative result extrapolated from a published comparison of N-glycans (Klapoetke et al. 2010).[3]

Rapid Deglycosylation with a Novel Formulation of Rapid PNGase F and RapiGest SF Surfactant

*Rapi*Fluor-MS labeling revolutionizes *N*-glycan sample preparation and can be readily adopted in the laboratory with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit. This complete solution from Waters and New England BioLabs was purposefully designed to simplify and accelerate -glycan sample preparation. The optimized *N*-glycan sample preparation workflows require a minimum of three steps, including deglycosylation (to release glycans from a glycoprotein), labeling (to impart a detectable chemical entity to glycans), and a clean-up step (to eliminate potential interferences corresponding to deglycosylation and labeling byproducts from the sample) (**Figure 5**). Conventional approaches to *N*-glycan sample preparation can be very time consuming due to not only lengthy labeling procedures but also lengthy deglycosylation steps that range from 1 to 16 hours.

The GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit includes a novel formulation of Rapid PNGase F and RapiGest SF surfactant that can be used to completely deglycosylate glycoproteins in an approximately 10 minute procedure. RapiGest SF, an anionic surfactant, is used to ensure that *N*-glycans are accessible to Rapid PNGase F and that the glycoproteins remain soluble upon heat denaturation. Most importantly, RapiGest is an enzyme-friendly reagent and can therefore be used at high concentrations without hindering the activity of Rapid PNGase F. In the developed method, a glycoprotein is subjected to a high concentration of RapiGest (1% w/v) and heated to 80°C for 2 minutes. Subsequently and without any additional sample handling, Rapid PNGase F is added to the solution and the mixture is incubated at an elevated (50°C) temperature for 5 minutes to achieve complete, unbiased deglycosylation for most glycoproteins. Using SDS-PAGE based gel shift assays, we have confirmed this fast deglycosylation process to be effective for a diverse set of glycoproteins (**Figure 6**).

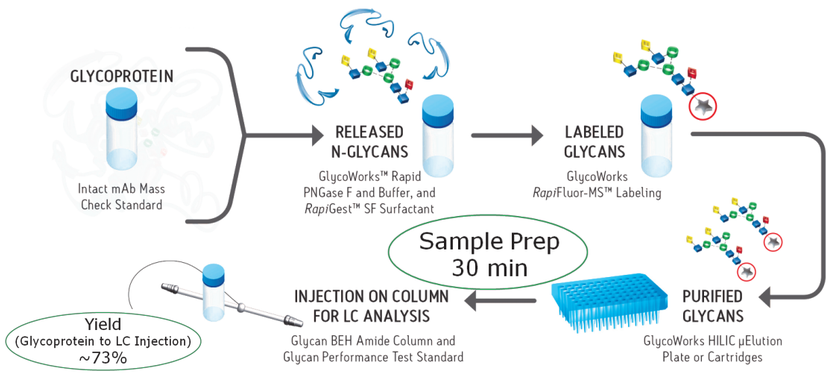
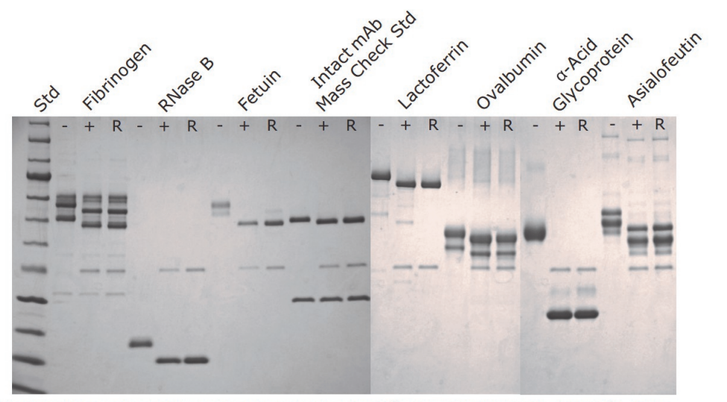


Figure 5. Workflow for the rapid preparation of *N*-glycans using the *Rapi*Fluor-MS *N*-Glycan Kit.



(-) Neg Control / Conventional / 2 Steps without PNGase F: SDS + DTT, 95°C, 2 min / NP-40 + Reaction Buffer, 37°C, 30 min
(+) Pos Control / Conventional / 2 Steps with PNGase F: SDS + DTT, 95°C, 2 min / NP-40 + Reaction Buffer + PNGase F, 37°C, 30 min
(R) 2 Step Rapid Deglycosylation / Reaction Buffer + RapiGest 80°C, 2 min / + PNGase F, 50°C, 5 min

Figure 6. Gel electrophoresis assay for deglycosylation of glycoproteins.

Robust, Quantitative HILIC SPE

The final step in an *N*-glycan sample preparation is SPE designed to selectively extract *Rapi*Fluor-MS labeled N-glycans from labeling reaction byproducts, which can otherwise interfere with analysis of the labeled glycans by HILIC column chromatography (**Figure 7A**). A highly polar, aminopropyl silica-based sorbent was selected for this application. Glycans were adsorbed to the sorbent via a HILIC mechanism, then the sample was washed to remove sample matrix. An acidic wash solvent was employed in this step to introduce electrostatic repulsion between the aminopropyl HILIC sorbent and labeling reaction byproducts and to enhance the solubility of the matrix components. After washing, *Rapi*Fluor-MS labeled *N*-glycans were eluted from the aminopropyl sorbent using an eluent comprised of a pH 7 solution of 200 mM ammonium acetate in 5% acetonitrile. The eluted *Rapi*Fluor-MS labeled glycans were then diluted with a mixture of organic solvents (ACN and DMF) and directly analyzed by HILIC column chromatography, as shown in **Figure 7B**.

Figure 7C shows the relative abundances for four glycans (FA2, FA2G2S1, A3G3S3, and A3S1G3S3) as determined after one pass and two passes of the SPE process, respectively. These results demonstrate that this SPE technique provides a mechanism to immediately analyze a sample of RFMS labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of *N*-glycans.

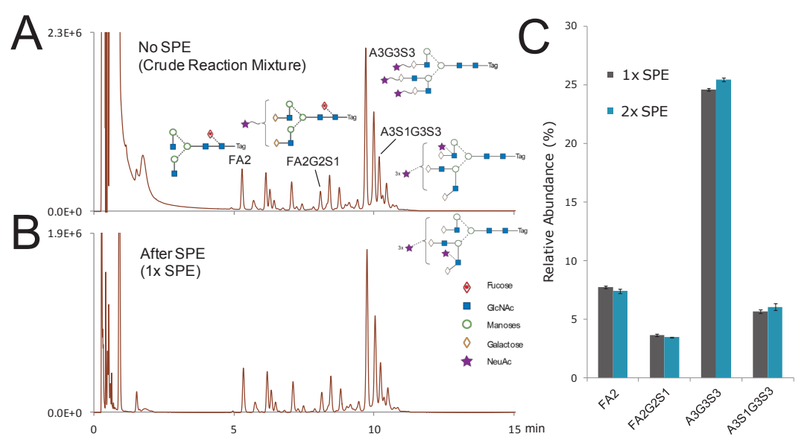


Figure 7. Extraction of *Rapi*Fluor-MS labeled *N*-glycans by SPE with a GlycoWorks HILIC µElution plate. (A) Crude reaction mixture comprised of *Rapi*Fluor-MS labeled glycans from pooled human IgG and bovine fetuin separated on a 2.1 x 50 mm ACQUITY UPLC BEH Amide 130Å 1.7µm column and detected via fluorescence (labeled *N*-glycans from 0.4 µg glycoprotein, 10 µL injection of ACN/DMF diluted sample). (B) The mixture after extraction by HILIC SPE. (C) Relative abundances determined for this set of *Rapi*Fluor-MS labeled glycans after one versus two passes of GlycoWorks HILIC SPE.

CONCLUSION

- **Preparation of labeled *N*-glycans (from glycoprotein to analysis ready sample) in 30 minutes**
- **Unprecedented sensitivity for labeled *N*-glycans (2 and nearly 800 fold increases to fluorescence and MS signal compared to Instant AB)**
- **Complete deglycosylation to produce unbiased results**
- **Simple, streamlined protocol provided with the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit**
- **Accurate profiling based on robust SPE for neutral to tetrasialylated *N*-glycans**

References

1. Mechref, Y.; Hu, Y.; Desantos-Garcia, J. L.; Hussein, A.; Tang, H., Quantitative glycomics strategies. *Mol Cell Proteomics* 2013, *12* (4), 874-84.
2. Cook, K. S.; Bullock, K.; Sullivan, T., Development and qualification of an antibody rapid deglycosylation method. *Biologicals* 2012, *40* (2), 109-17.
3. Klapoetke, S.; Zhang, J.; Becht, S.; Gu, X.; Ding, X., The evaluation of a novel approach for the profiling and identification of *N*-linked glycan with a procainamide tag by HPLC with fluorescent and mass spectrometric detection. *J Pharm Biomed Anal* 2010, *53* (3), 315-24.

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