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Robustness of *Rapi*Fluor-MS N-Glycan Sample Preparations and Glycan BEH Amide HILIC Chromatographic Separations

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APPLICATION BENEFITS

- High yield sample preparation with quantitative recovery to ensure accurate and repeatable profiling of N-glycans
- Comparability to historical 2-AB based released glycan analysis approaches
- RapiFluor-MS Glycan Performance Test Standard for method familiarization, troubleshooting, and benchmarking
- Robust Glycan BEH Amide HILIC separations supported by GU calibration with the novel *Rapi*Fluor-MS Dextran Calibration Ladder

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS™ N-Glycan Kit

GlycoWorks HILIC µElution Plate

*Rapi*Fluor-MS Glycan Performance Test Standard

*Rapi*Fluor-MS Dextran Calibration Ladder

ACQUITY UPLC® Glycan BEH Amide, 130Å Column

XBridge® Glycan BEH Amide, 130Å Column

ACQUITY UPLC H-Class Bio System

ACQUITY® QDa® Mass Detector

Xevo® G2-XS QTof MS

SYNAPT® G2-S*i* HDMS

KEY WORDS

GlycoWorks, *Rapi*Fluor-MS, RapiGest™ SF, Rapid Tagging, PNGase F, Deglycosylation, ACQUITY UPLC H-Class Bio System, BEH Amide 130Å, Glycans, Glycoproteins, Glycosylation, HILIC, Fluorescence

INTRODUCTION

N-glycosylation of proteins is routinely characterized and monitored because of its significance to the detection of disease states[1-3] and the manufacturing of biopharmaceuticals.[4-5] Glycosylation profiles are most often assessed by means of released glycan analyses, wherein samples are often prepared by techniques that are notoriously time-consuming or lead to compromises in MS sensitivity.[6-7] With the development of the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, we have addressed these shortcomings by enabling unprecedented sensitivity for glycan detection while also improving the throughput of N-glycan sample preparation.[8] Using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, glycoproteins are deglycosylated in 10 minutes to produce N-glycosylamines that are then rapidly reacted with the novel *Rapi*Fluor-MS labeling reagent (Figure 1). In a final step, the resulting labeled glycans are extracted from reaction byproducts by means of an SPE method that facilitates immediate analysis of samples. As a result, an analyst can now complete an N-glycan sample preparation, from glycoprotein to ready-to-analyze sample, in just 30 minutes and be poised to perform high sensitivity N-glycan profiling using hydrophilic interaction chromatography (HILIC) and mass spectrometric (MS) or fluorescence (FLR) detection.

Equally important as the efficiency and sensitivity gains afforded by this new sample preparation approach is its robustness and its ability to produce results consistent with historical N-glycan profiling. Within this application note, we will discuss these attributes of the *Rapi*Fluor-MS based sample preparation and the corresponding HILIC-based LC analyses.

EXPERIMENTAL

Method conditions

(unless otherwise noted)

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Fluorescence detection:	Ex 265/Em 425 nm (*Rapi*Fluor-MS)
	Ex 330 / Em 420 nm (2-AB)
	(2 Hz scan rate [150 mm column], Gain =1)
	(5 Hz scan rate [50 mm column], Gain=1)
Injection volume:	≤1 µL (aqueous diluents with 2.1 mm I.D. columns)
	≤30 µL (DMF/ACN diluted samples with 2.1 mm I.D. columns)
Columns:	ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186004740)
	XBridge Glycan BEH Amide XP, 130Å, 2.5 µm, 2.1 x 150 mm (p/n 186007265)
	Agilent AdvanceBio Glycan Mapping Rapid Resolution HD, 1.8 µm, 2.1 x 150 mm
	Thermo Scientific Accucore™ 150 Amide HILIC, 2.6 µm, 2.1 x 150 mm
Sample collection/vials:	Sample Collection Module (p/n 186007988)
	Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640)

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: Acetonitrile (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

Gradient used with 2.1 x 150 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: Acetonitrile (LC-MS grade)

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

Intact mass analysis was performed by LC-MS with a Xevo G2-QTof

MS conditions

MS system:	Xevo G2 QTof
Ionization mode:	ESI+
Analyzer mode:	TOF MS, resolution mode (~20 K)
Capillary voltage:	3.0 kV
Cone voltage:	45 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Desolvation gas flow:	800 L/Hr
Calibration:	NaI, 1 µg/µL from 500–5000 *m/z*
Acquisition:	500–5000 *m/z*, 1 Hz scan rate
Data management:	UNIFI® 1.7/MassLynx® Software (v4.1)

Sample description

*Rapi*Fluor-MS labeled N-glycans were prepared from glycoproteins, including Intact mAb Mass Check Standard (p/n: 186006552), using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n: 176003606) according to the guidelines provided in its Care and Use Manual (715004793).

2-AB labeled N-glycans were prepared using a Prozyme GlykoPrep® Rapid N-Glycan Preparation with 2-AB Kit according to the manufacturer's recommended protocol. In addition, 2-AB labeled N-glycans were also prepared using an approach combining the use of a Prozyme GlykoPrep Digestion Module, an in-house optimized 2-AB labeling protocol, and a GlykoPrep Cleanup Module (Prozyme, Hayward, CA).

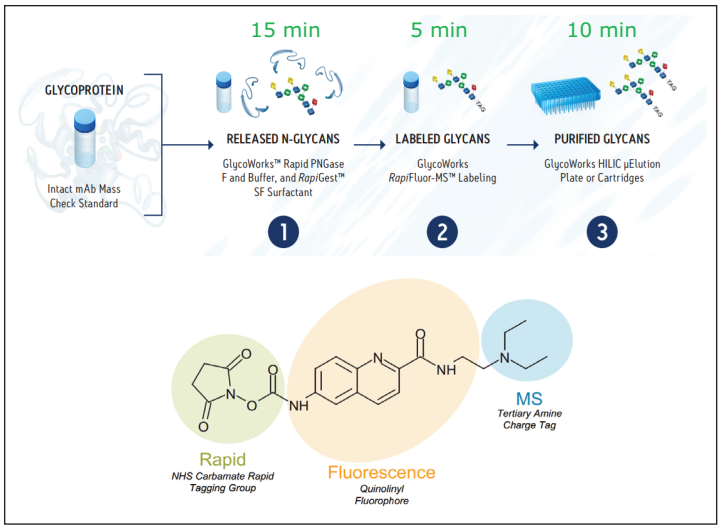


Figure 1. GlycoWorks RapiFluor-MS N-Glycan Kit sample preparation workflow and the chemical structure of the RapiFluor-MS Reagent.

*Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983) was reconstituted in 50 µL of water and injected as a 1 µL volume for chromatographic benchmarking and lifetime testing experiments. *Rapi*Fluor-MS Dextran Calibration Ladder (p/n: 186007982) was reconstituted in 100 µL of water and injected as a 1 µL volume for retention time calibrations.

Percent yields for the sample preparation workflows were determined by means of quantitative analyses. Column loads were calibrated using external quantitative standards of 2-AB labeled triacetyl chitotriose and *Rapi*Fluor-MS derivatized propylamine obtained in high purity (confirmed by HPLC and 1H NMR).

To determine percent yields, the measured quantities of FA2 glycan from Intact mAb Mass Check Standard (p/n: 186006552) were compared to theoretical yields calculated for the preparation. For example, the theoretical yield for the FA2 glycan resulting from the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit was calculated as follows:

$$1.5 \times 10^7 \text{ pg IgG} \times \frac{1 \text{ pmol}}{150{,}000 \text{ pg}} \times \frac{2 \text{ pmol glycan}}{1 \text{ pmol IgG}} \times \frac{0.45 \text{ pmol FA2}}{1 \text{ pmol total glycan pool}} \times \frac{10 \text{ µL injection}}{400 \text{ µL sample prepared}} = 2.3 \text{ pmol}$$

**This calculation is based on the assumption that the sample of Intact mAb Mass Check Standard was 15 µg, that the mAb has a molecular weight of 150 kDa, that there are only 2 N-glycans per one mAb, that the N-glycan profile of the mAb contains the FA2 glycan at a relative abundance of 45%, and that only 2.5% of the sample was analyzed.*

RESULTS AND DISCUSSION

Robust sample preparation: Deglycosylation

Each procedural step in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit has been optimized to be high yielding and to minimize the introduction of bias to an N-glycan profile. Previous work based on SDS PAGE gel shift assays has demonstrated that the rapid deglycosylation procedure developed for this kit produces complete deglycosylation of a diverse set of glycoproteins.[8] This completeness of deglycosylation is also supported by intact mass analysis using LC-MS, where the deglycosylation of a monoclonal antibody (mAb) can be readily tracked. Figure 2 presents deconvoluted ESI mass spectra for Intact mAb Mass Check Standard, a murine IgG1 mAb. The top spectrum shows the mAb before it had been subjected to rapid deglycosylation (Figure 2A). The bottom spectrum meanwhile presents the mAb after it was processed according to the approach specified in the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit, wherein glycoproteins are subjected to 1% (w/v) RapiGest SF Surfactant-assisted heat denaturation followed by incubation with Rapid PNGase F at 50 °C for 5 minutes (Figure 2B). The masses observed in these spectra confirm that these samples differ in terms of glycan occupancy. The control sample contains the mAb in its doubly glycosylated, native form (one glycan on each heavy chain). In contrast, the sample subjected to the proposed 2-step rapid deglycosylation procedure is homogenous with an observed molecular weight that is in agreement with the predicted molecular weight of the fully deglycosylated mAb (145.3 kDa). And although high temperatures are employed in this method for the purpose of heat denaturation, no detrimental effects on an N-glycan profile have been observed. To this point, notice that there are no differences in an N-glycan profile prepared from pooled human IgG when using an excessive 20 minute heat denaturation at 90 °C versus the rapid 3 minute procedure (Figure 3).

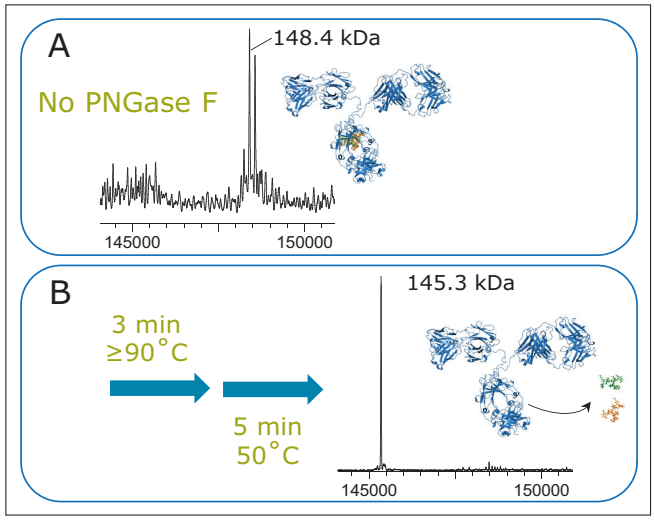


Figure 2. Intact mass analysis of Intact mAb Mass Check Standard (A) before and (B) after rapid deglycosylation with the GlycoWorks RapiFluor-MS N-Glycan Kit.

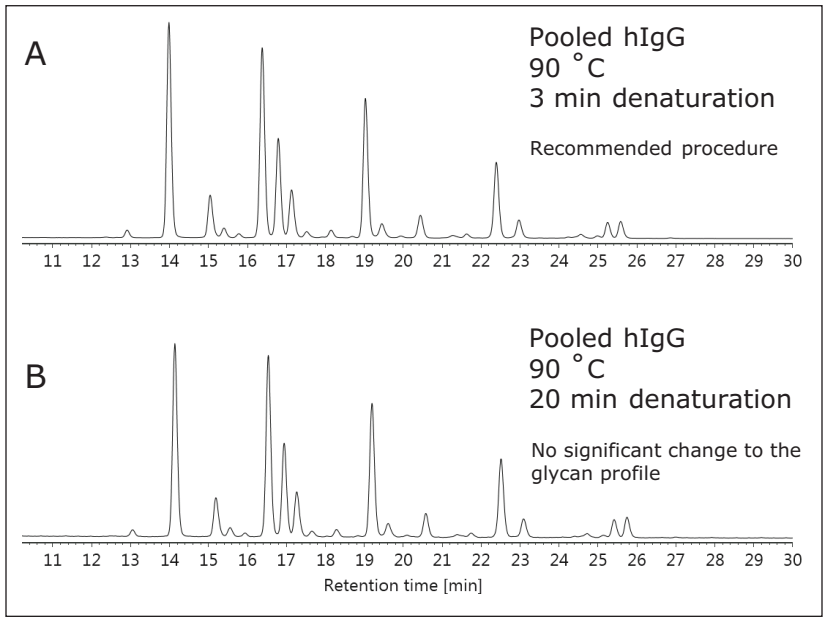
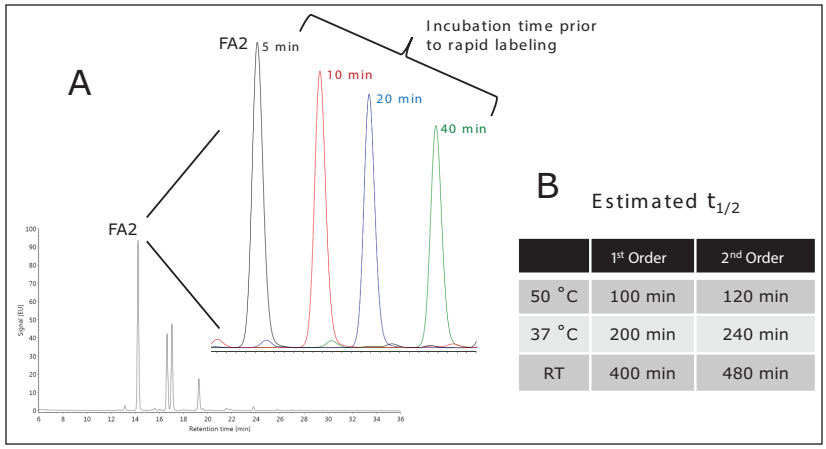


Figure 3. Testing the effects of subjecting human IgG and its N-glycans to heat denaturation. (A) The RapiFluor-MS N-glycan profile as observed using the recommended 3-minute heat denaturation versus (B) the RapiFluor-MS N-glycan profile as observed using a 20-minute heat denaturation.

Robust sample preparation: Rapid labeling

The efficiency of the sample preparation carries over from deglycosylation to *Rapi*Fluor-MS labeling. A primary concern in this step is the relative stability of the PNGase F released N-glycosylamines, which are required for *Rapi*Fluor-MS labeling, in the pH 7.9 GlycoWorks Rapid Buffer. A time-course study involving varying delays between deglycosylation and *Rapi*Fluor-MS labeling steps has shown that the N-glycosylamines have a relatively long half-life of approximately 2 hours at 50 °C (Figure 4). That is, with our 5 minute deglycosylation step, there should be little concern over sample loss (< 3% loss) due to hydrolysis of the glycosylamine. In addition, sample losses from the labeling reaction are minimal. Many experimental parameters were explored during the development of the rapid labeling reaction specified in the *Rapi*Fluor-MS N-Glycan Kit, including pH, temperature, ionic strength, time, buffer components, and reagent molar excess. Figure 5 shows an example of optimizing the reagent molar excess as needed to maximize labeling yield. Fluorescence chromatograms for labeled, released N-glycans from Intact mAb Mass Check Standard are stacked on the left (Figure 5A). Note that with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit proteins are purposely not depleted from the sample after deglycosylation to save time and to give better control over the labeling. The *Rapi*Fluor-MS Reagent is therefore used in a molar excess over all of the nucleophiles from the glycoprotein, which for an IgG corresponds to approximately seventy five protein amines and two N-glycosylamines. Each of the corresponding samples was obtained from labeling a fixed glycoprotein concentration of 0.36 mg/mL with *Rapi*Fluor-MS Reagent at concentrations varying from 18 to 108 mM. As shown in Figure 5B, plotting of the fluorescence peak areas for the resulting N-glycan profile indicates that labeling is maximized near a *Rapi*Fluor-MS Reagent concentration of 36 mM, the conditions designed into the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Moreover, molar excess conditions both higher and lower than the 36 mM reagent condition produced comparable fluorescence profiles, underscoring the robustness of *Rapi*Fluor-MS labeling.



	1st Order	2nd Order
50 °C	100 min	120 min
37 °C	200 min	240 min
RT	400 min	480 min

Figure 4. Estimating the half-life of N-glycosylamine hydrolysis through a time-course on deglycosylation incubation. (A) Fluorescence traces for RapiFluor-MS labeled FA2 from Intact mAb Mass Check Standard observed after implementing varying incubation times for deglycosylation (50°C incubations). (B) Approximation of the N-glycosylamine half-life assuming 1st or 2nd order reaction kinetics.

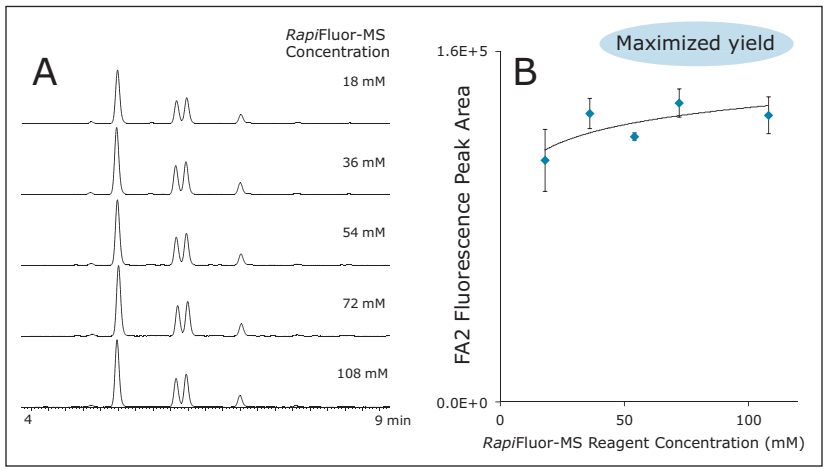


Figure 5. Optimization of labeling reagent molar excess for the GlycoWorks RapiFluor-MS N-Glycan Kit. (A) Fluorescence chromatograms for labeled glycans obtained by titration of 0.36 mg/mL deglycosylated Intact mAb Mass Check Standard with varying concentrations of RapiFluor-MS Reagent. Separations were performed with labeled glycans from 0.4 µg of glycoprotein and an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 50 mm Column. (B) Fluorescence peak area as a function of RapiFluor-MS Reagent concentration.

Robust sample preparation: µElution HILIC SPE

The last step in the sample preparation involves extraction of the *Rapi*Fluor-MS labeled glycans from reaction byproducts using HILIC SPE. This technique has been routinely used for preparations of 2-AB labeled N-glycans and has now been optimized for *Rapi*Fluor-MS labeled species.[8-9] Previous studies have shown that *Rapi*Fluor-MS labeled glycans are obtained through this SPE processing at relatively high yields of approximately 74%.[8] Nearly all of the observed sample losses in this step are non-specific. Figure 6 plots fluorescence peak areas for preparations of N-glycans from Intact mAb Mass Checked Standard, in which the final SPE elution volume was either 30, 90 or 180 µL. This plot shows that SPE recovery is a function of elution volume and that highest recoveries are achieved when employing large elution volumes. To facilitate direct analyses, however, a compromise is made such that a 90 µL elution volume is used in order to obtain a relatively concentrated glycan eluate. Regardless of the elution volume and absolute yield of glycans from the SPE sorbent, the most important characteristic of this clean-up is that the observed sample losses have been determined to be non-specific with no significant bias being introduced to a glycan profile for a wide range of glycans with diverse chemical properties, including small, neutral glycans up to large, tetrasialylated glycans (see Reference 8 for more details about GlycoWorks HILIC SPE).

Yield of *Rapi*Fluor-MS labeled N-glycans

In another measurement of robustness, it is worth looking at the yield of N-glycans through the entire workflow. This was evaluated in order to measure the collective efficiency of combining fast deglycosylation, rapid labeling, and HILIC SPE extraction of *Rapi*Fluor-MS labeled glycans (Figure 7). *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were prepared, analyzed by HILIC-FLR, and quantified by means of an external calibration. Based on a calculated theoretical yield (see experimental) and duplicate analyses, it was determined that the percent yield through the entire *Rapi*Fluor-MS N-Glycan Kit sample preparation was approximately 73%. To provide perspective, we evaluated the yield of 2-AB labeled N-glycans from an alternative sample preparation workflow involving the use of a GlykoPrep Rapid N-Glycan Preparation with 2-AB Kit.

Quantitative analyses showed that 2-AB labeled N-glycans are prepared using this kit with a relatively low yield of approximately 35%, though it has been found that the yield of this kit can be dramatically improved by optimization and lengthening of the labeling step. Comparatively speaking, though, these results show that not only does the *Rapi*Fluor-MS approach quicken a historically time-consuming sample preparation, it also exhibits reasonably high yields.

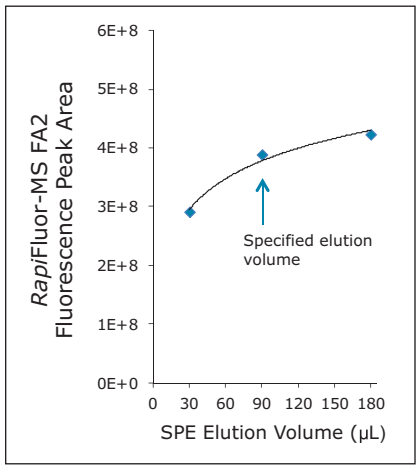


Figure 6. Fluorescence peak area as a function of SPE elution volume. The specified elution volume in the GlycoWorks RapiFluor-MS N-Glycan Kit is 90 µL.

Step	Yield	Testing to confirm minimal bias
Deglycosylation	Complete	▪ Intact mass analysis/subunit LC-MS ▪ Gel shift assays
Labeling	>95%	▪ Released glycan profile vs. subunit derived glycan information
SPE	~74%	▪ Recovery measurements ▪ Glycan profile before vs. after SPE
GlycoWorks RapiFluor-MS N-Glycan Kit	~73% Yield	
GlykoPrep® Rapid N-Glycan Preparation with 2-AB	~35% Yield	

Figure 7. Percent yield for the preparation of RapiFluor-MS labeled N-glycans with the GlycoWorks RapiFluor-MS N-Glycan Kit. Testing that has been performed to confirm minimal sample loss and quantitative recovery is listed for each procedural step. The percent yield that has been measured for the preparation of 2-AB labeled N-glycans with a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit is also provided. These results may not be representative of all applications.

Minimal impact to glycan profiling with reagent batch variation

Lastly, sample preparations with the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit have proven to be robust with respect to reagent manufacturing. A robustness study was performed to test the impact of changing the batches of each reagent that plays a critical role in the preparation of *Rapi*Fluor-MS labeled N-glycans, namely *Rapi*Gest SF, GlycoWorks Rapid Buffer, GlycoWorks Rapid PNGase F, *Rapi*Fluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE Plate, and the SPE Elution Buffer. Three sets of these reagents, each varying by batch, were tested in their application to profiling the N-glycans from Intact mAb Mass Check Standard. Average relative abundances observed for the glycan species in this standard with the three different reaction sets are presented in Figure 8. Relative abundances of N-glycans were observed to be largely comparable across the different preparations with an average RSD for the labeled N-glycan species being 2.3%.

Comparability to 2-AB N-glycan profiling

Another critical aspect to the *Rapi*Fluor-MS N-glycan sample preparation is that it can be used in place of legacy 2-AB methods without requiring significant adaptations to existing analytical techniques. With the speed of the sample preparation and the enhanced method sensitivity afforded by the *Rapi*Fluor-MS tag,[8] the task of analyzing N-glycosylation is in fact made significantly easier.

Just like 2-AB labeled glycans, *Rapi*Fluor-MS labeled glycans are ideally suited for HILIC separations with an amide bonded stationary phase, such as that found in the Waters Glycan BEH Amide Columns. Figure 9 shows example separations for 2-AB and *Rapi*Fluor-MS labeled glycans obtained from Intact mAb Mass Check Standard. The 2-AB labeled glycans, in this case, were prepared using the previously mentioned GlykoPrep Kit and an approximately 3.5 hour protocol, whereas the *Rapi*Fluor-MS labeled glycans were prepared in less than 30 minutes using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit.

Percent Abundance (%)

Species	Reaction set 1	2	3	Avg	Std Dev	RSD
A2	1.23	1.25	1.21	1.23	0.021	1.70
FA2	44.54	43.95	43.29	43.93	0.622	1.42
FA2G1	19.81	20.01	19.95	19.92	0.101	0.51
FA2G1'	22.54	22.86	22.91	22.77	0.199	0.87
FA2G2	8.93	9.02	9.42	9.12	0.264	2.90
FA2G2Sg1/FA2G2Ga2	0.92	0.82	0.94	0.89	0.060	6.71

For each reaction set: unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer.

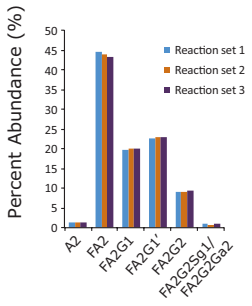


Figure 8. Characterization of batch-to-batch variation in the RapiFluor-MS sample preparation. Percent abundances were measured for the preparation of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard using three different sets of materials. Each reaction set was represented by unique batches of RapiGest SF, Rapid Buffer, Rapid PNGase F, RapiFluor-MS Reagent, DMF Reagent Solvent, GlycoWorks µElution SPE plate, SPE Elution Buffer. Testing was performed in triplicate. FA2G1' denotes the structural isomer of FA2G1.

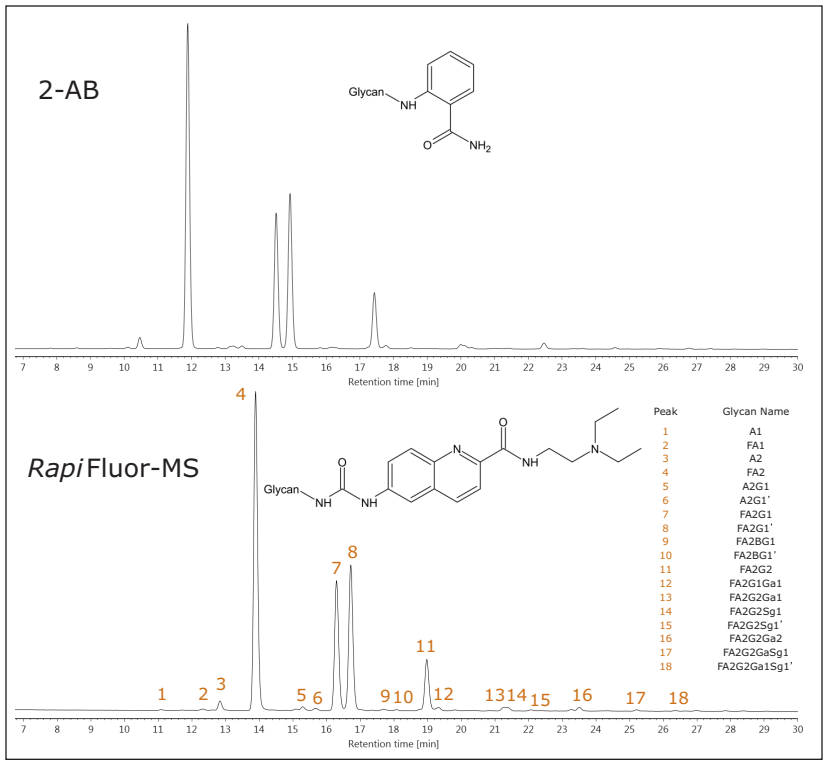


Figure 9. Similarity between 2-AB and RapiFluor-MS N-glycan HILIC profiles for a typical mAb. Fluorescence chromatograms for labeled glycans from Intact mAb Mass Check Standard using an ACQUITY UPLC BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications for the RapiFluor-MS labeled N-glycans are provided. 2-AB labeled N-glycans were prepared using a GlykoPrep Rapid N-Glycan Preparation with 2-AB kit. (') denotes a structural isomer.

So that chromatograms exhibiting equivalent signal-to-noise could be compared, the *Rapi*Fluor-MS sample was analyzed in this study at a significantly lower mass load than the 2-AB labeled sample. Despite being prepared by different approaches, it can be seen that the labeled N-glycans are resolved by the HILIC separation into very similar profiles. For a typical mAb profile, *Rapi*Fluor-MS and 2-AB labeling both yield HILIC glycan separations with similar selectivity. However, as a result of its additional hydrogen bonding donors/acceptors, the *Rapi*Fluor-MS label introduces a slight shift of the mAb N-glycan profile to higher retention times. This change in the absolute retention window of an N-glycan profile is predictable and can therefore be easily accounted for when transitioning from 2-AB to *Rapi*Fluor-MS based methods.

Consistency in results observed for the *Rapi*Fluor-MS-based approach compared to historical 2-AB techniques was also evaluated. N-glycan profiling of the same monoclonal IgG1 reference sample has been studied between these different methodologies. Figure 10 displays N-glycan information obtained for this mAb sample throughout 160 different profiling experiments involving 2-AB labeling and HPLC chromatography. Likewise, Figure 10 provides data from 12 recent experiments using *Rapi*Fluor-MS labeling and UPLC® chromatography. Comparable relative abundances are observed for this sample in a direct comparison (Figure 10, center panel) and a control chart demonstrates the ability to transition between these two methods (Figure 10, right panel). This consistency in N-glycan profiling makes it possible to replace time-consuming 2-AB/HPLC methods with *Rapi*Fluor-MS/UPLC techniques.

N-Glycan	2-AB with HPLC (n=160)	*Rapi*Fluor-MS with UPLC (n=12)
	Avg (%)	Avg (%)
A1	0.33	0.28
FA1	0.23	0.42
A2	5.98	5.76
FA2	45.44	45.88
Man5	1.20	1.08
A2G1	1.91	1.86
A2G1'	0.95	0.79
FA2G1	27.36	26.29
FA2G1'	9.66	9.23
A2G2	0.29	0.32
FA2G2	6.00	5.58
FA2G2S1	0.31	0.51

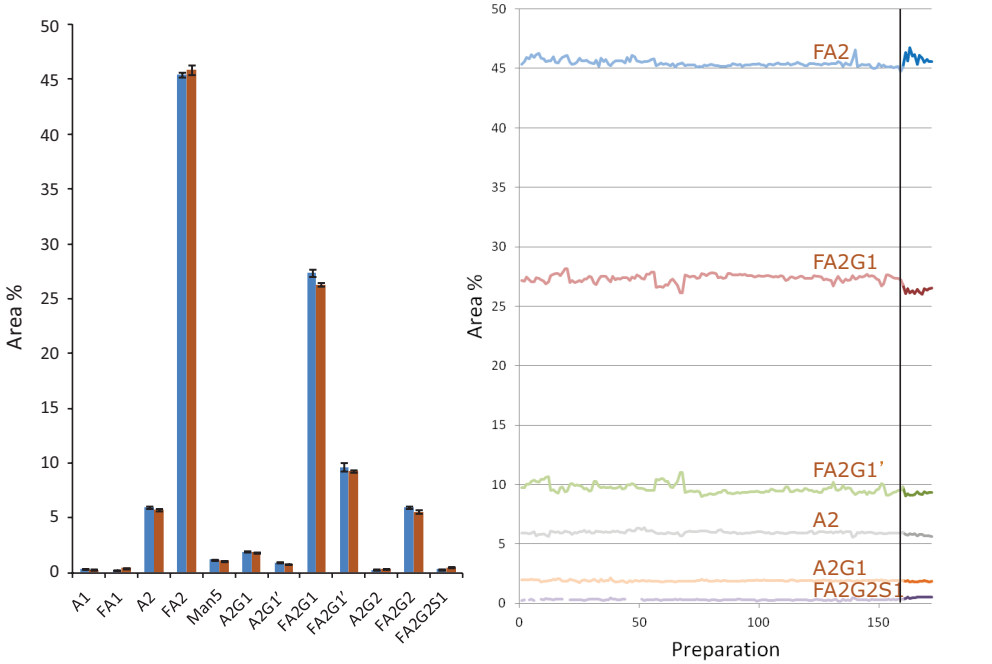
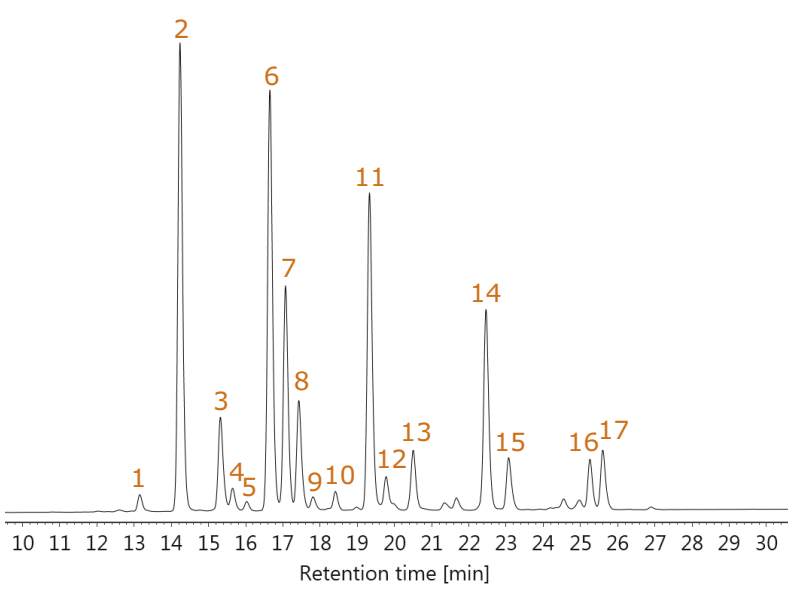


Figure 10. Consistency between UPLC-based RapiFluor-MS N-glycan profiling and HPLC-based 2-AB N-glycan profiling of a humanized monoclonal IgG1. Comparison of relative abundances for N-glycans detected using a method combining the GlycoWorks RapiFluor-MS N-Glycan Kit with a UPLC separation (n=12) versus a historical 2-AB sample preparation combined with an HPLC separation (n=160). Trending data for the N-glycans from the human monoclonal IgG1 (light colored lines = 2-AB/HPLC, dark colored lines = RapiFluor-MS/UPLC). FA2G1' and A2G1' denote the structural isomers of FA2G1 and A2G1, respectively.

Robustness of *Rapi*Fluor-MS N-glycan separations with glycan BEH amide columns

The robustness and resolving power of the HILIC column chromatography is critically important to successfully implementing this methodology. To this end, a test standard called *Rapi*Fluor-MS Glycan Performance Test Standard is available for method familiarization, system suitability, troubleshooting, and benchmarking studies. This standard contains a complex mixture of *Rapi*Fluor-MS N-glycans from human IgG that has been isolated from pooled human serum. Its composition of approximately 20 different major constituents makes it useful for evaluating the resolving power of a separation and the sensitivity of detection methods (Figure 11).



	Glycan	*Rapi*Fluor-MS Labeled Glycan Composition	M_i (Da)	M_{avg} (Da)
1	A2	C67H105O37N9	1627.6611	1628.5887
2	FA2	C73H115O41N9	1773.7190	1774.7299
3	FA2B	C81H128O46N10	1976.7984	1977.9224
4	A2G1	C73H115O42N9	1789.7140	1790.7293
5	A2G1'	C73H115O42N9	1789.7140	1790.7293
6	FA2G1	C79H125O46N9	1935.7719	1936.8705
7	FA2G1'	C79H125O46N9	1935.7719	1936.8705
8	FA2BG1	C87H138O51N10	2138.8512	2140.0630
9	FA2BG1'	C87H138O51N10	2138.8512	2140.0630
10	A2G2	C79H125O47N9	1951.7668	1952.8699
11	FA2G2	C85H135O51N9	2097.8247	2099.0111
12	FA2BG2	C93H148O56N10	2300.9041	2302.2036
13	FA2G1S1	C90H142O54N10	2226.8673	2228.1251
14	FA2G2S1	C96H152O59N10	2388.9201	2390.2657
15	FA2BG2S1	C104H165O64N11	2591.9995	2593.4582
16	FA2G2S2	C107H169O67N11	2680.0150	2681.5203
17	FA2BG2S2	C115H182O72N12	2883.0949	2884.7128

Figure 11. RapiFluor-MS Glycan Performance Test Standard. An example fluorescence chromatogram obtained from an 8 pmole load of the standard and a separation with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. Peak identifications are provided. FA2G1', A2G1', and FA2BG1' denote the structural isomers of FA2G1, A2G1, and FA2BG1 respectively.

In line with its intended purpose, we have used the *Rapi*Fluor-MS Glycan Performance Test Standard to benchmark the chromatographic performance of four different columns containing amide bonded stationary phases designed for glycan separations. Two of the columns were UPLC-based and contained sub-2-µm particles while the remaining two were intended for use on HPLC instrumentation and contained 2.5 µm and 2.6 µm particles. Figure 12 shows representative chromatograms obtained with each of these columns run under equivalent conditions and linear velocities. Four glycan species spread across these separations were monitored to measure retention windows, average peak widths, and peak capacities. Notice that whether performing a separation with a phase intended for UPLC or HPLC chromatography, Glycan BEH Amide Columns provide exemplary resolving power and comparable selectivities thereby enabling the seamless transfer of this glycan separation between HPLC and UPLC platforms.[10]

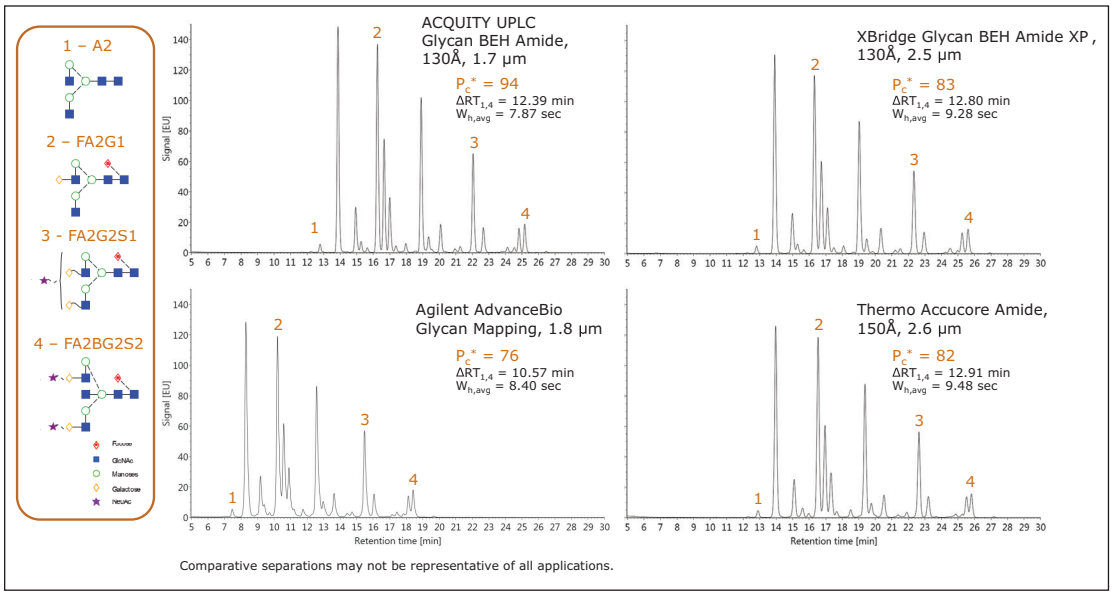


Figure 12. Chromatographic benchmarking of HILIC columns containing amide bonded stationary phases designed for glycan separations. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained from an 8 pmole load of the standard and separations with 2.1 x 150 mm columns. All separations were performed at the same linear velocity on an ACQUITY UPLC H-Class Bio System. Four glycan species spread across the separations were monitored to measure retention windows, average peak widths, and peak capacities.

Separations of *Rapi*Fluor-MS labeled glycans with glycan BEH amide columns have also proven to be very robust. In demonstration of this, a single Glycan BEH Amide, 130Å, 1.7 µm Column was subjected to lifetime testing and 300 sequential runs. At every 20th run, *Rapi*Fluor-MS Glycan Performance Test Standard was separated in order to track any changes in the retentivity and selectivity of the column.

Chromatograms corresponding to the 1st and 300th runs are provided in Figures 13A and 13B, respectively. Quite clearly, near identical separations were obtained at the onset as well as at the end of this approximately 2-week constant use scenario, with no significant shifts in retention times of the labeled N-glycans having been observed throughout the testing (Figure 13C).

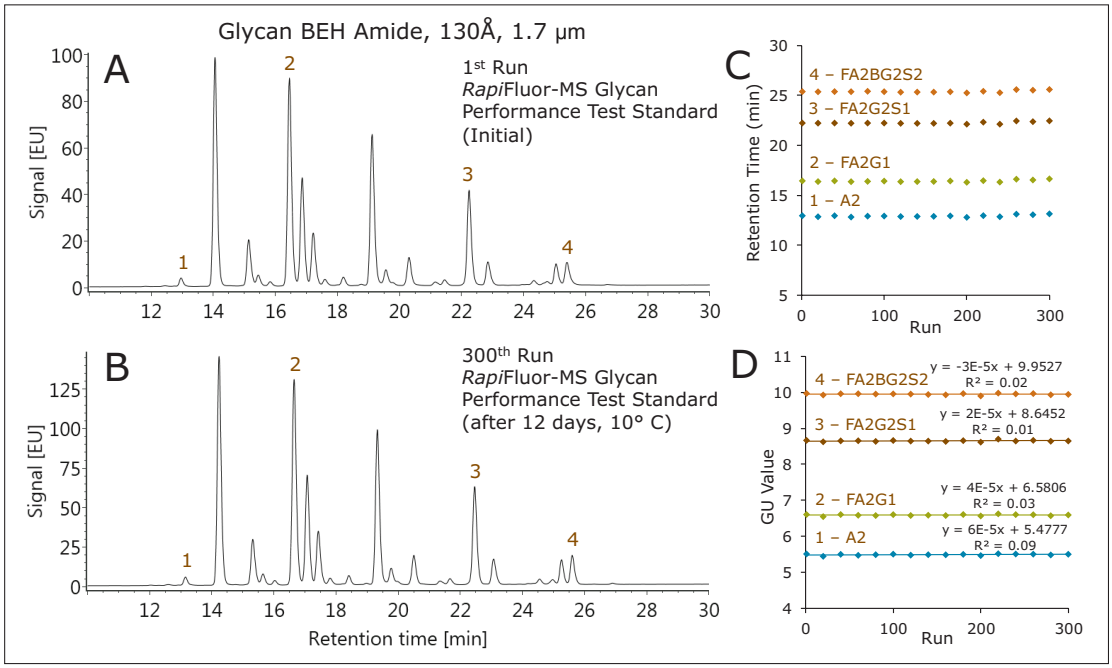


Figure 13. Robustness testing of an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm 2.1 x 150 mm Column for separations of RapiFluor-MS labeled N-glycans. Fluorescence chromatograms of the RapiFluor-MS Glycan Performance Test Standard were obtained at every 20th run from an 8 pmole load of the standard. Four glycan species spread across the separations were monitored to track the retentivity of the stationary phase and column. Fluorescence chromatograms are shown for the (A) 1st run and the (B) 300th run with the column. (C) Retention times as a function of run. (D) Glucose unit (GU) values as a function of run.

In this testing, LC calibrations were performed after every separation of the glycan mixture through application of a dextran ladder and assignment of glucose unit (GU) values. Separations with glycan BEH amide columns can be used in conjunction with glucose unit (GU) values as a means to calibrate HILIC-based glycan separations. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[11] To assign GU values, a dextran ladder (comprised of glucose multimers of increasing length) is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values.

The development of a dextran calibration ladder suitable for use with *Rapi*Fluor-MS labeled glycans was essential yet technically challenging. Given that dextran is a reducing sugar without a strong nucleophile, it cannot, unlike N-glycosylamines, be readily labeled with *Rapi*Fluor-MS Reagent. Because of the distinctive urea linkage imparted to N-glycans upon their derivatization with rapid tagging reagents, *Rapi*Fluor-MS labeled N-glycans have very unique fluorescence maxima at approximately 265 nm (excitation) and 425 nm (emission) (Figure 14A). In a novel labeling approach, we have prepared a *Rapi*Fluor-MS Dextran Calibration Ladder by first reductively aminating dextran with ethanolamine and then labeling it with *Rapi*Fluor-MS.

The resulting urea-linked dextran derivatives exhibit identical fluorescence properties to those of *Rapi*Fluor-MS labeled N-glycans. Furthermore, the obtained dextran is tuned for desired HILIC retention because of the hydroxyl group being incorporated through ethanolamine. A representative fluorescence chromatogram for this novel dextran ladder is provided in Figure 14B, and an example cubic spline fit of the retention data is shown in Figure 14C.

The impact of implementing GU value calibration is exemplified in Figure 13D, where the retention time data throughout the Glycan BEH Amide lifetime testing are reported in GU values. In comparing the retention time data shown in Figure 13C to the GU data in Figure 13D, one can see that the subtle fluctuations in retention times across the 2-week lifetime testing are compensated for by the GU calibration. In fact, RSDs for the GU value data are reduced by a factor of 2 compared to the RSDs in the retention time data.

Also, linear regression analysis of the GU value data shows that there is essentially no drifting in the HILIC retention data once calibrated using a dextran ladder. This analysis therefore clearly demonstrates the value of GU calibration with respect to improving the quality of reported data.

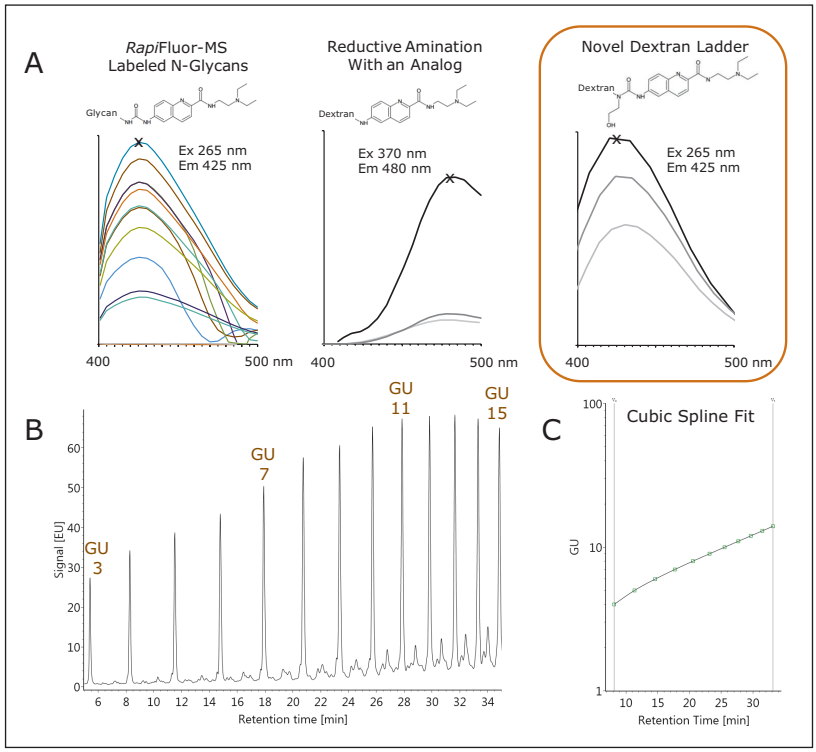


Figure 14. Assignment of Glucose Unit (GU) values with RapiFluor-MS labeling. (A) Chemical structures and fluorescence spectra of RapiFluor-MS labeled N-glycans versus dextrans derivatized with RapiFluor-MS-like labels. The novel dextran ladder that has been commercialized as the RapiFluor-MS Dextran Calibration Ladder is highlighted. (B) An example fluorescence chromatogram for the RapiFluor-MS Dextran Calibration obtained for a 0.5 µg mass load with an ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 x 150 mm Column. (C) Calibration curve resulting from cubic spline fitting.

CONCLUSIONS

In this application note, we have demonstrated the robustness of *Rapi*Fluor-MS N-glycan preparations and Glycan BEH Amide HILIC Column chromatography. The *Rapi*Fluor-MS N-Glycan Kit enables analysts to perform a high yielding sample preparation with quantitative recovery that ensures accurate and repeatable profiling of N-glycans that is highly comparable to HPLC, 2-AB based methodologies. Moreover, it has been demonstrated that Glycan BEH Amide Columns afford exemplary resolving power and ruggedness for separations of *Rapi*Fluor-MS labeled N-glycans. Additionally, this separation can be readily transferred between UPLC and HPLC platforms. To further ensure success with these new methodologies, two standards have been commercialized, and their use to facilitate *Rapi*Fluor-MS analyses has been demonstrated. The *Rapi*Fluor-MS Glycan Performance Test Standard has been used for benchmarking studies, while the novel *Rapi*Fluor-MS Dextran Calibration Ladder has been employed to enhance the reproducibility of chromatographic retention time data. In summary, the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit and supporting standards and columns can significantly reduce the burdens associated with N-glycan profiling while providing accurate, reproducible, and sensitive analyses.

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