

The background of the slide features a soft-focus photograph of a tropical beach. A palm tree is visible on the right side, and a person is walking along the shoreline. The overall color palette is light and airy, with shades of blue, green, and white.

The Road to Glycan Analysis Without Compromise

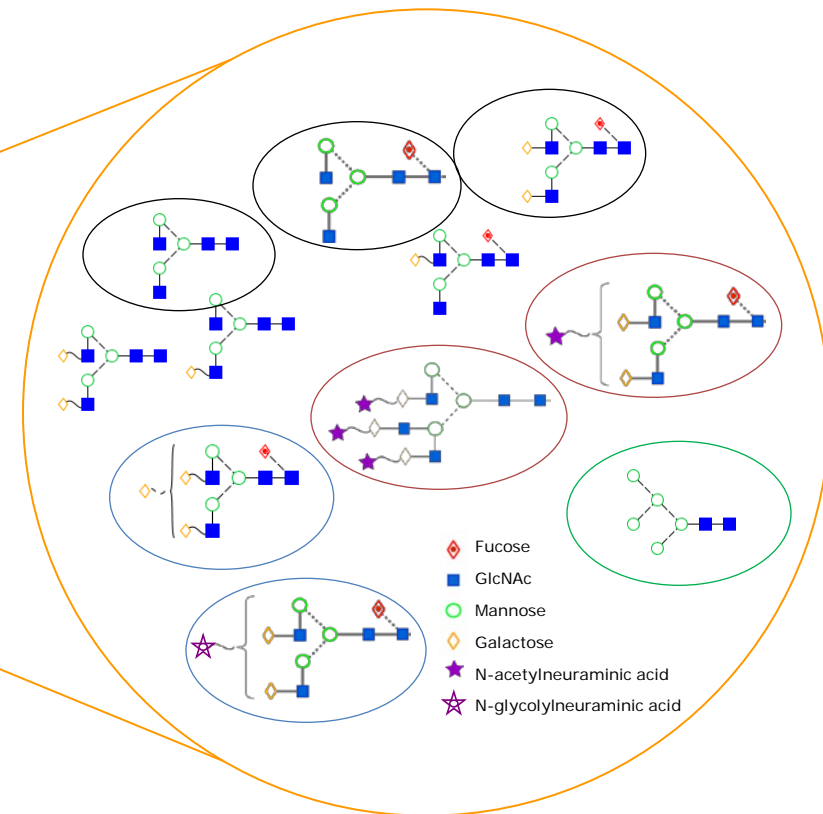
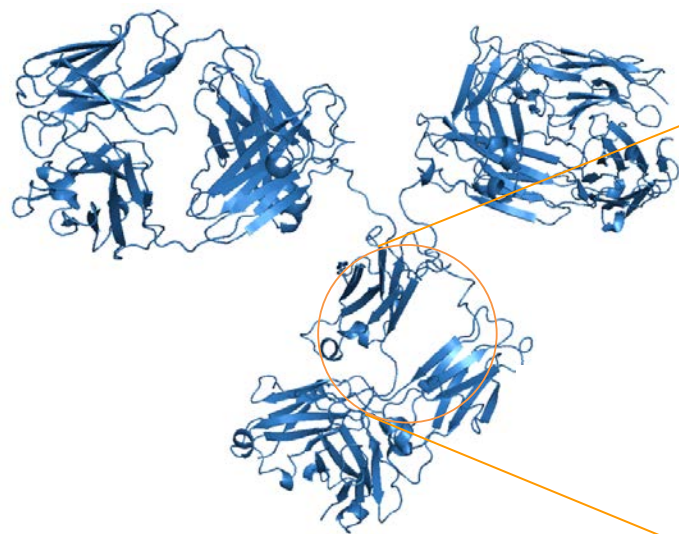
WCBP 2015

Waters Technical Seminar

Jan 27, 2015 Washington, DC

Today's Agenda

- Overview and Introduction
- ***RapiFluor-MS N-Glycan Labeling***: A breakthrough technology for released glycan LC and MS analysis
Matthew A. Lauber (Consumables Business Unit, Waters)
- ***RapiFluor-MS Technology & Glycan Characterization***
Ying Qing Yu (Biopharmaceutical Sciences, Waters)
- **Impact of *RapiFluor-MS* Technology on Released Glycan Profile Monitoring**
Sean M. McCarthy (Biopharmaceutical Sciences, Waters)
- Scientific Panel – Questions & Discussion



Effector Functions (ADCC/CDC)
(fucosylation/galactosylation)

Low Half Life
(high mannose)

Anti-Inflammatory
(sialylation)

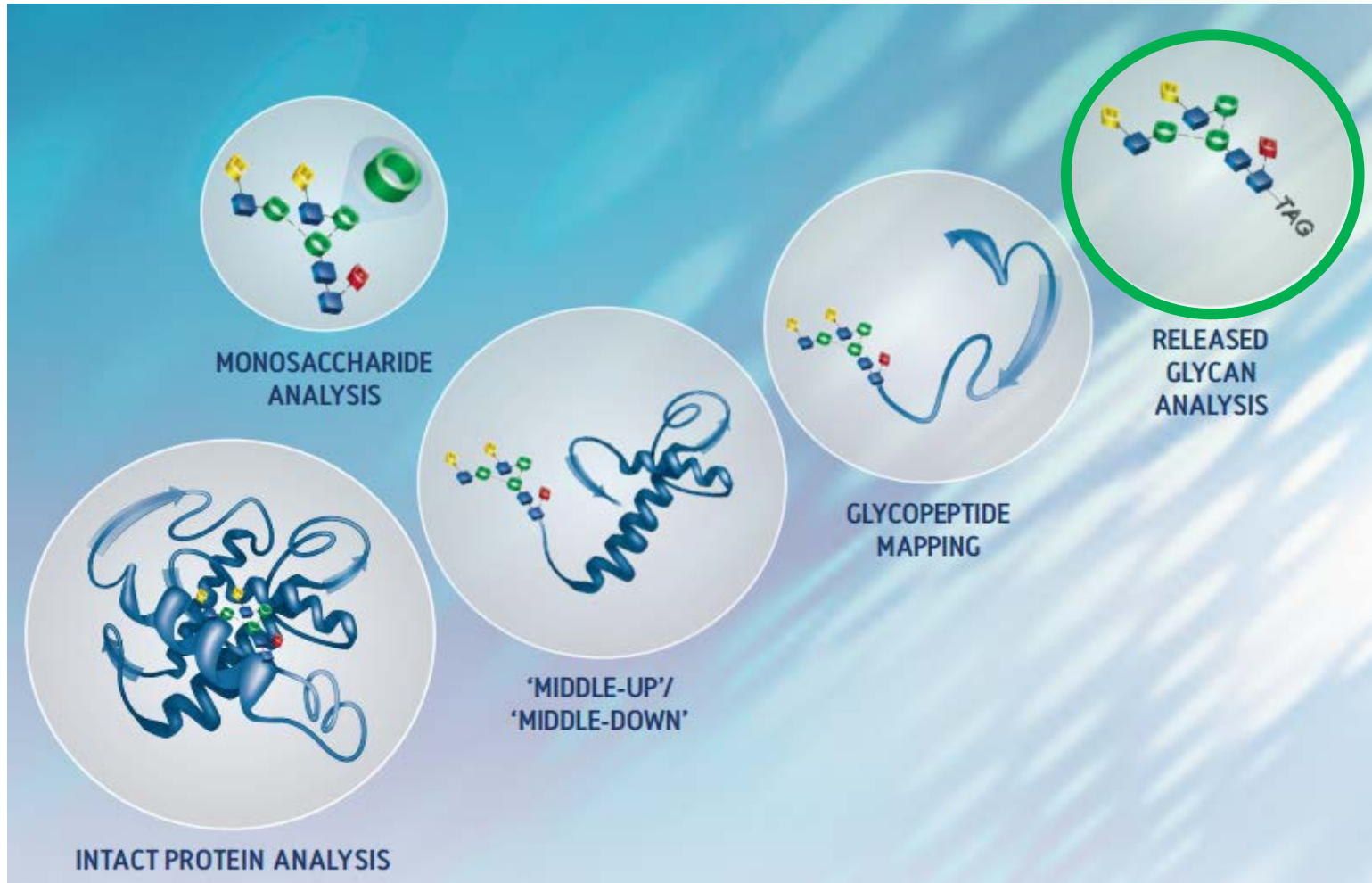
Immunogenic
(α Gal / N-glycolylneuraminic acid)

Overall profile sensitive to
manufacturing conditions

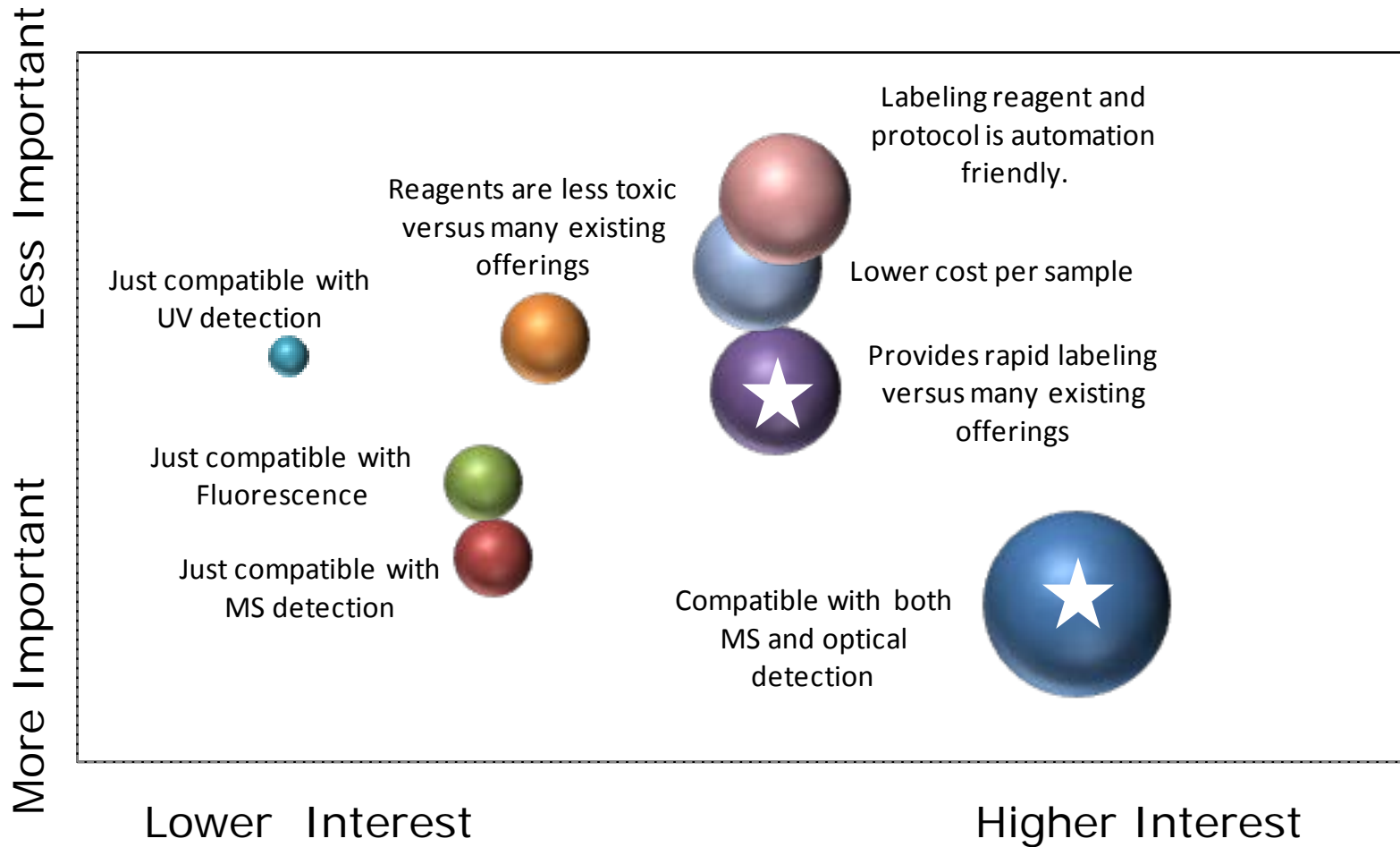
- N-glycosylation is a quality attribute of biotherapeutics
- Glycosylation profiles are characterized and routinely monitored

Glycoprotein Characterization

Multiple Strategies – Complementary Information



Customer voice brought us focus



RELEASED GLYCAN ANALYSIS

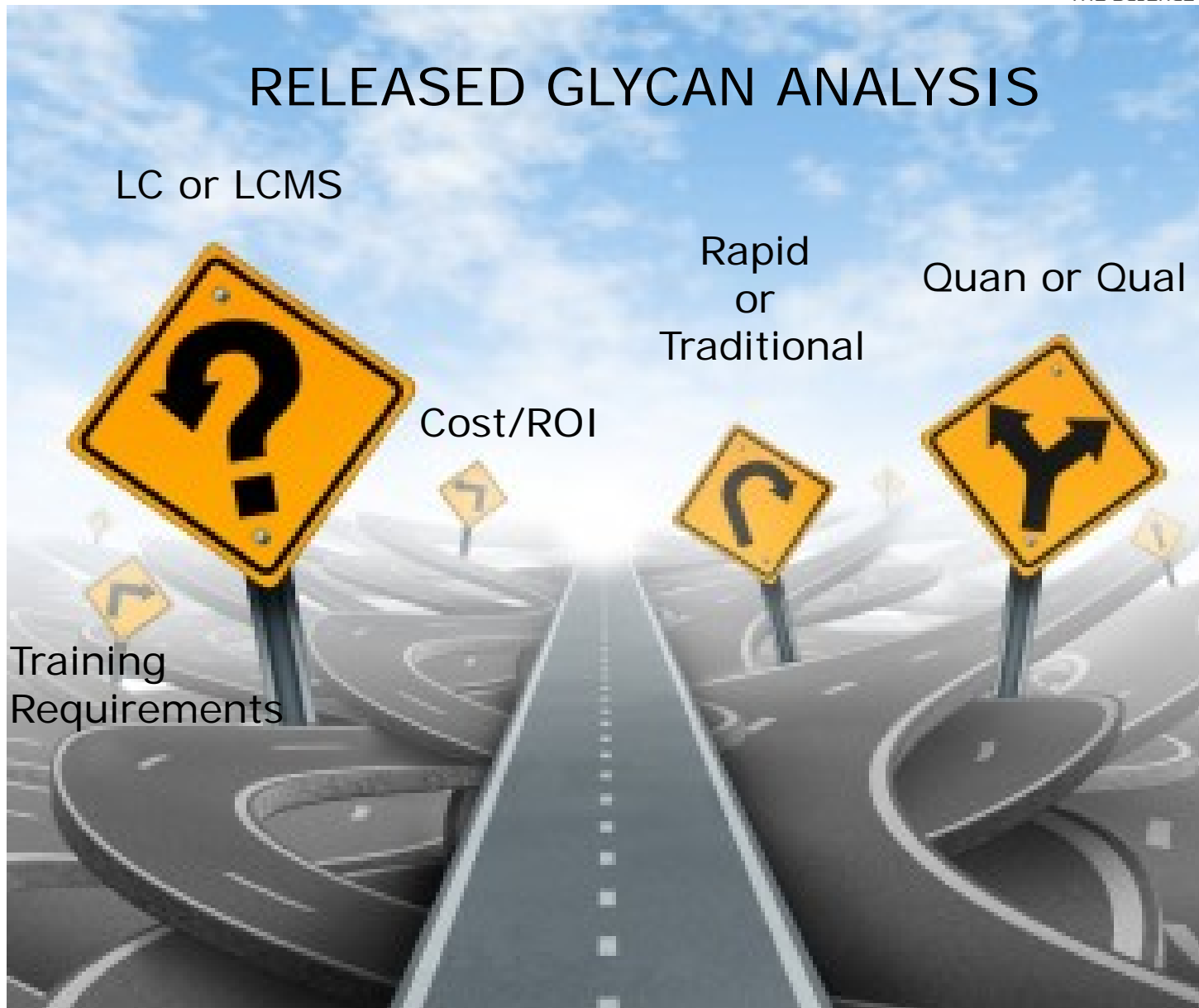
LC or LCMS

Rapid
or
Traditional

Quan or Qual

Cost/ROI

Training
Requirements



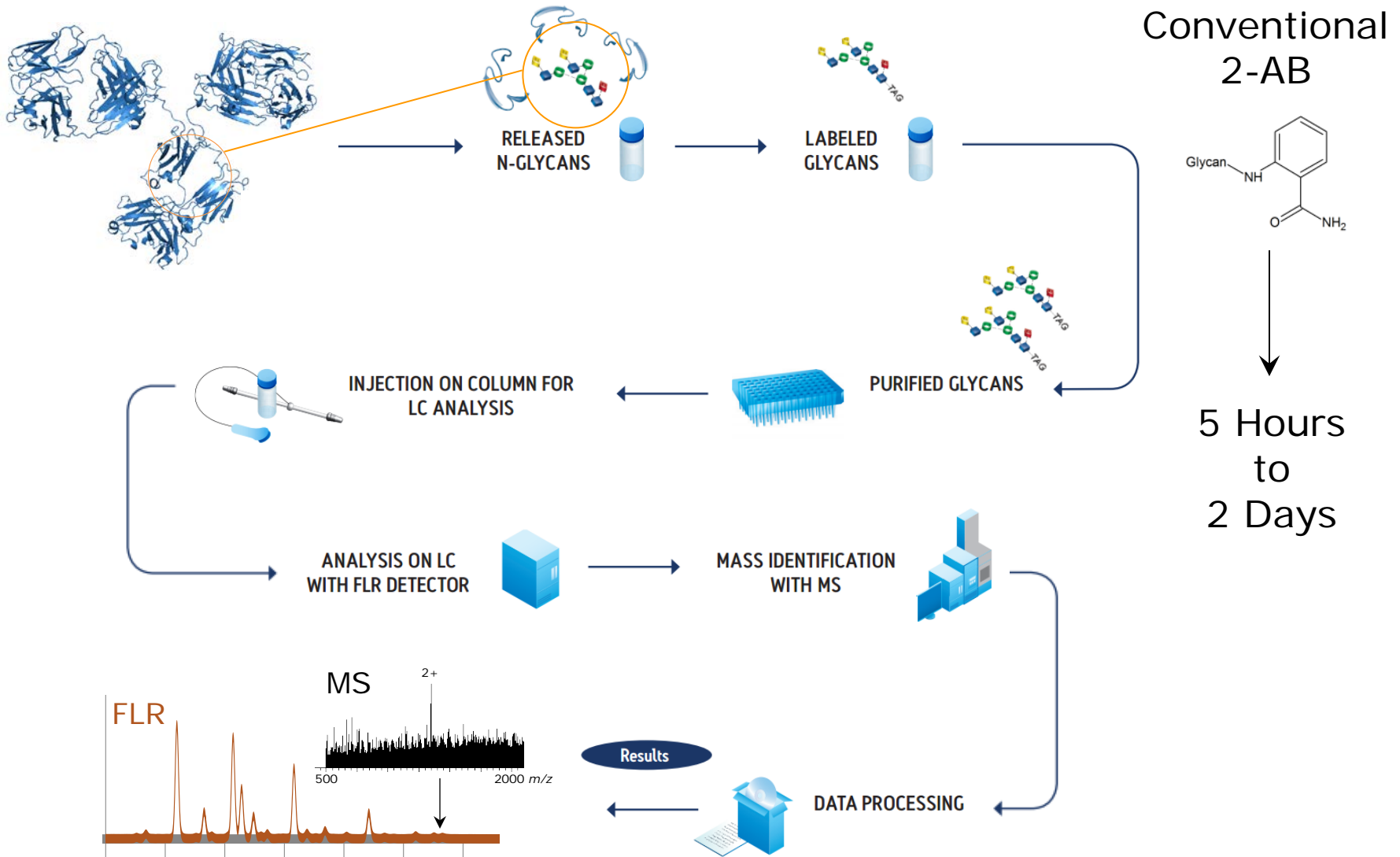


*Rapi*Fluor-MS N-Glycan Labeling:
A breakthrough technology for
released glycan LC and MS analysis

Released Glycan Analysis

HILIC Profiling

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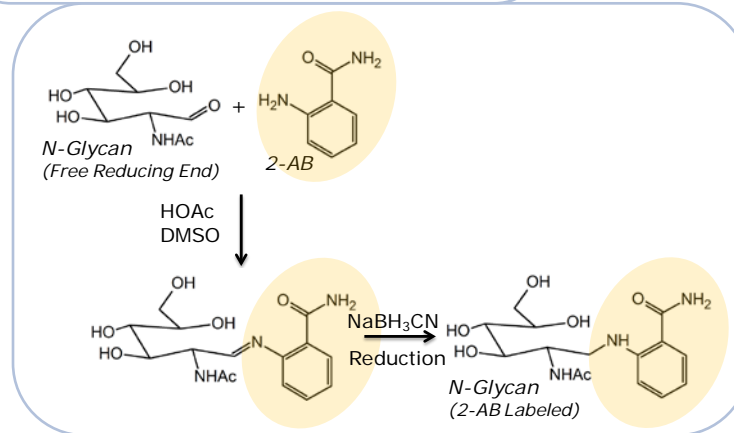
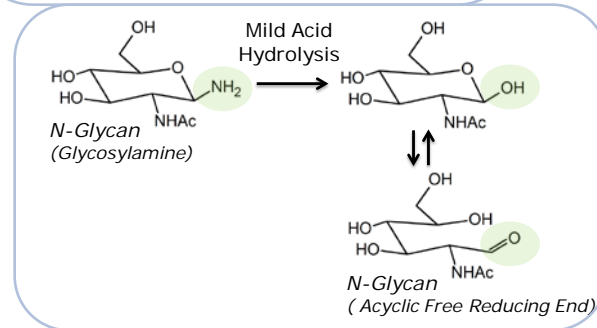
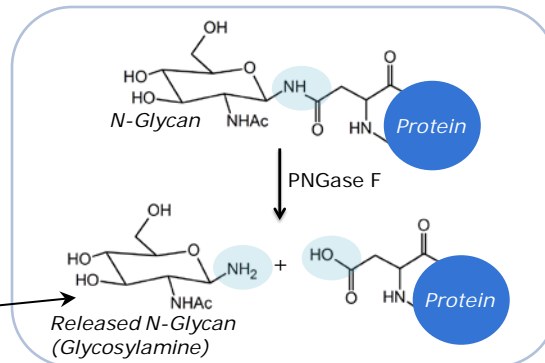


Out with Conventions

Reductive Amination is Laborious

Rapid Tagging

Glycosylamine labeling circumvents these issues



Reductive Amination (*conventional*)

- Anhydrous sample
- Numerous chemical conversions
- Laborious
- Heterogenous reaction products

RapiFluor-MS™ Reagent

Built Upon Our Expertise

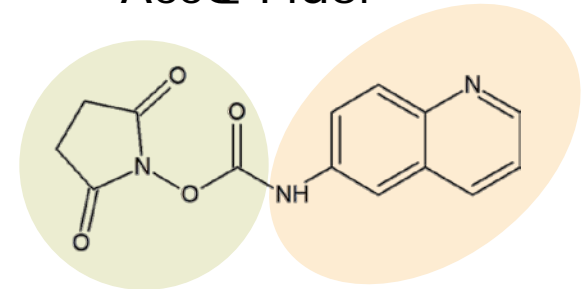
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From Waters' expertise in rapid, fluorescence labeling of amino acids

Enhanced chemical properties for glycan analysis:

- Rapid Tagging
- Efficient Fluorescence
- Enhanced Ionization Efficiency

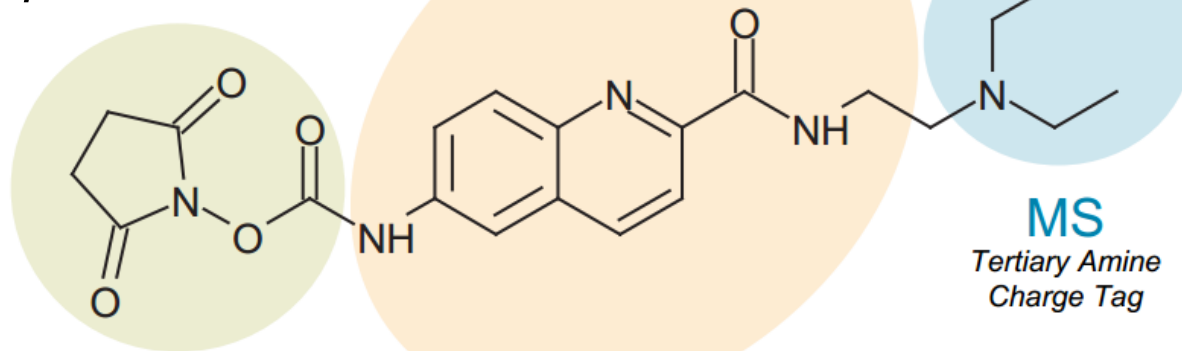
AccQ·Fluor™



Rapid

Fluorescence

RapiFluor-MS



Rapid

NHS Carbamate Rapid
Tagging Group

Fluorescence

Quinolinyne
Fluorophore

MS

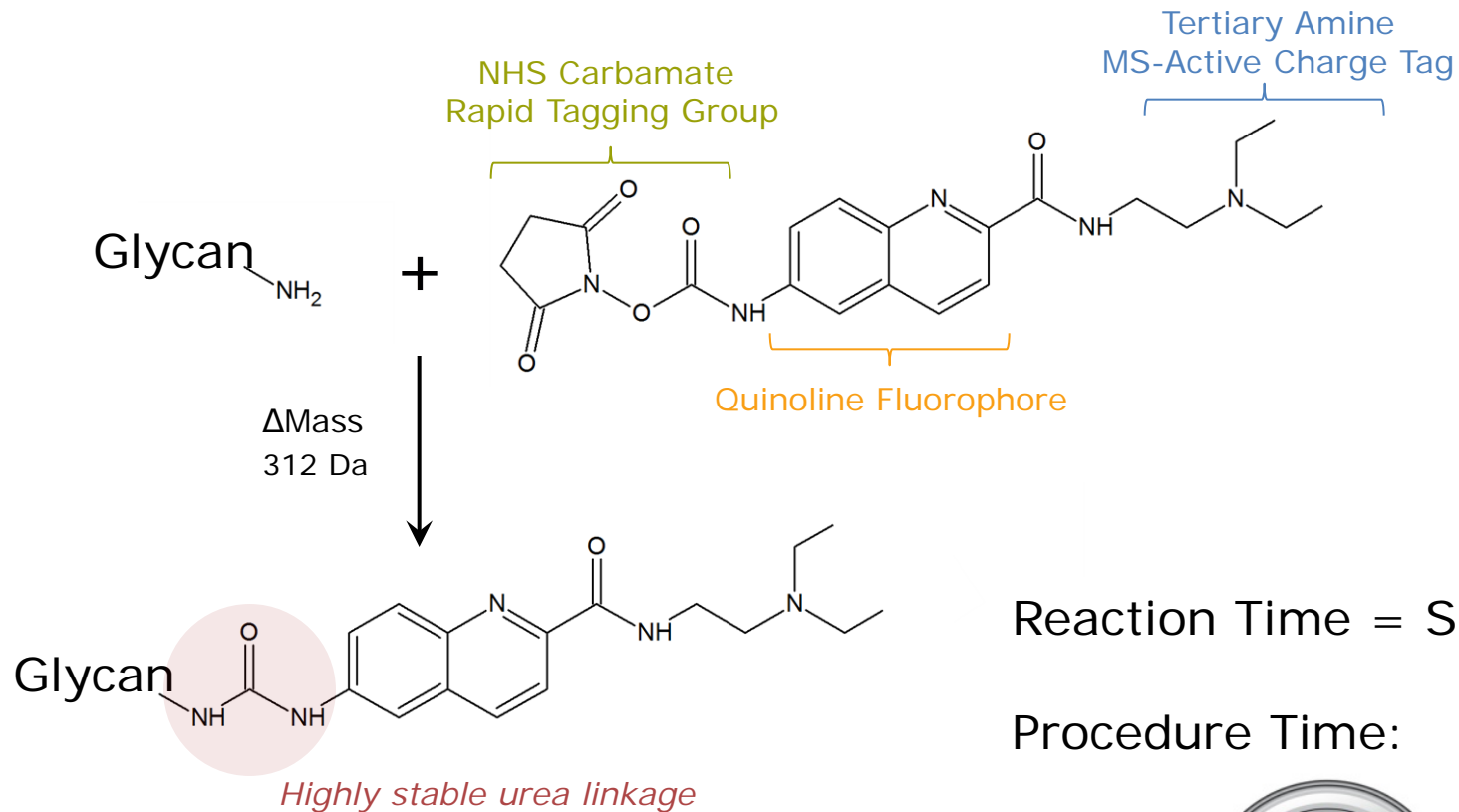
Tertiary Amine
Charge Tag

Patent Pending

RapiFluor-MS Reagent

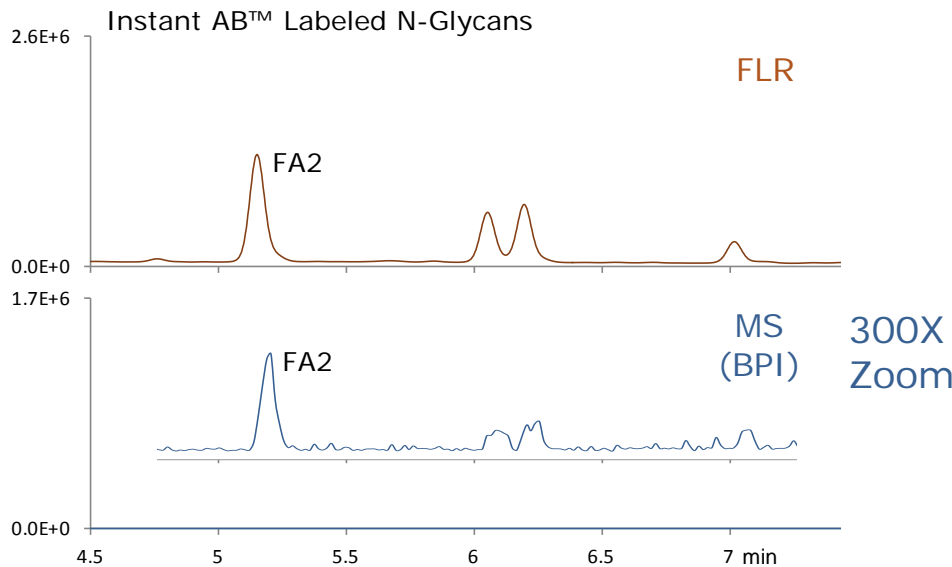
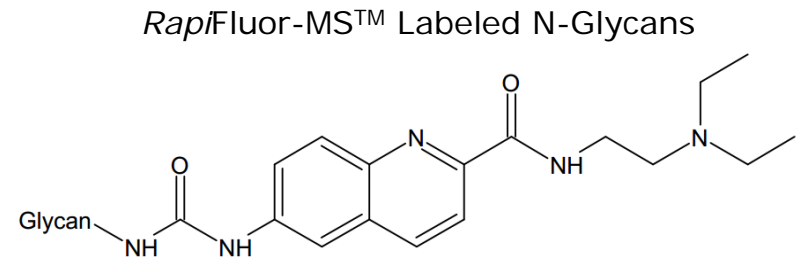
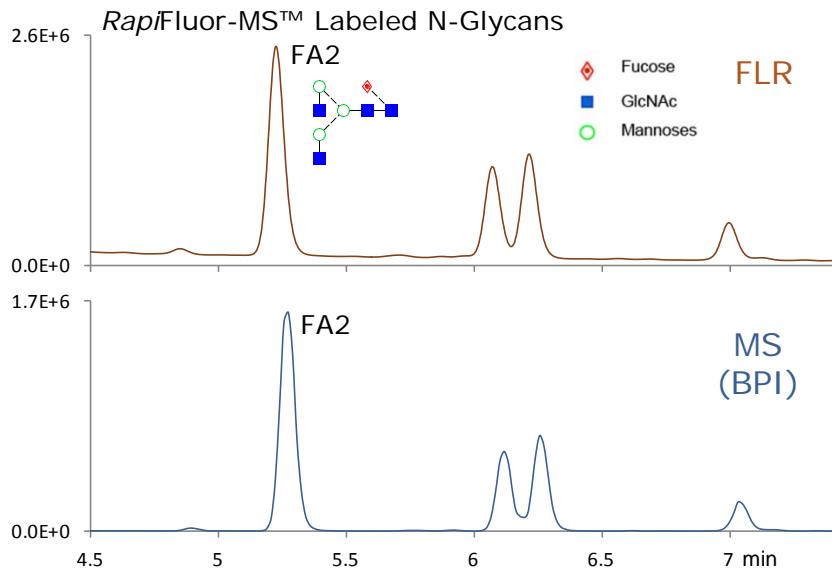
Rapid Reaction Kinetics

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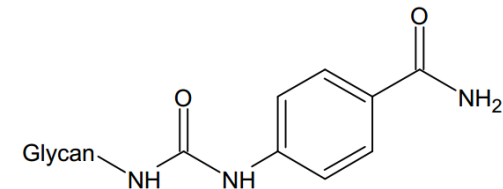


RapiFluor-MS Reagent

Sensitivity Comparison – Instant AB

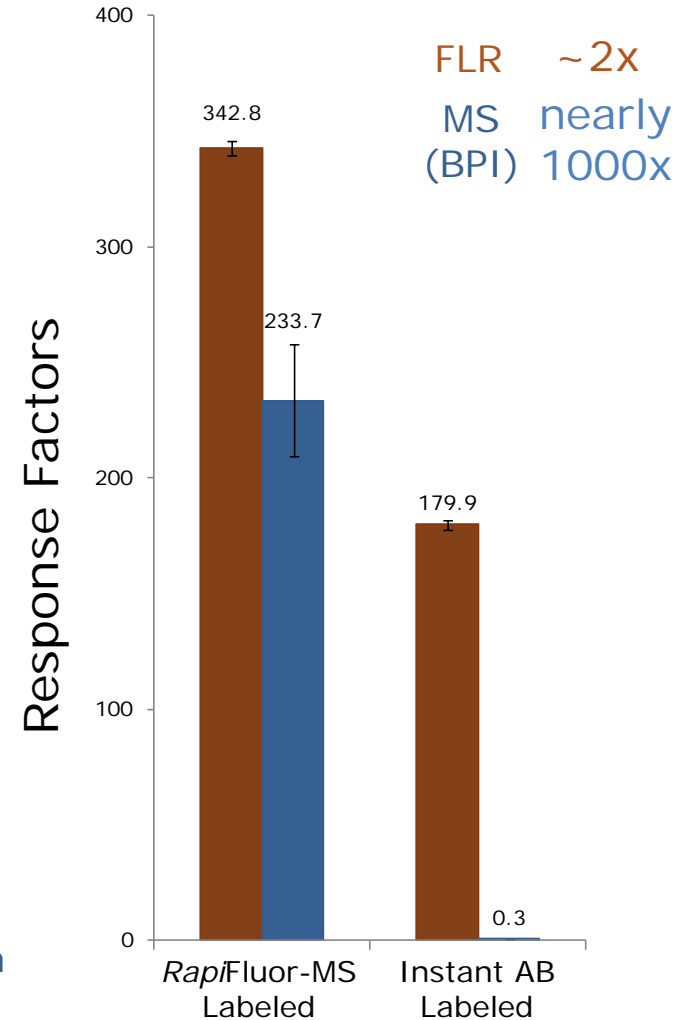
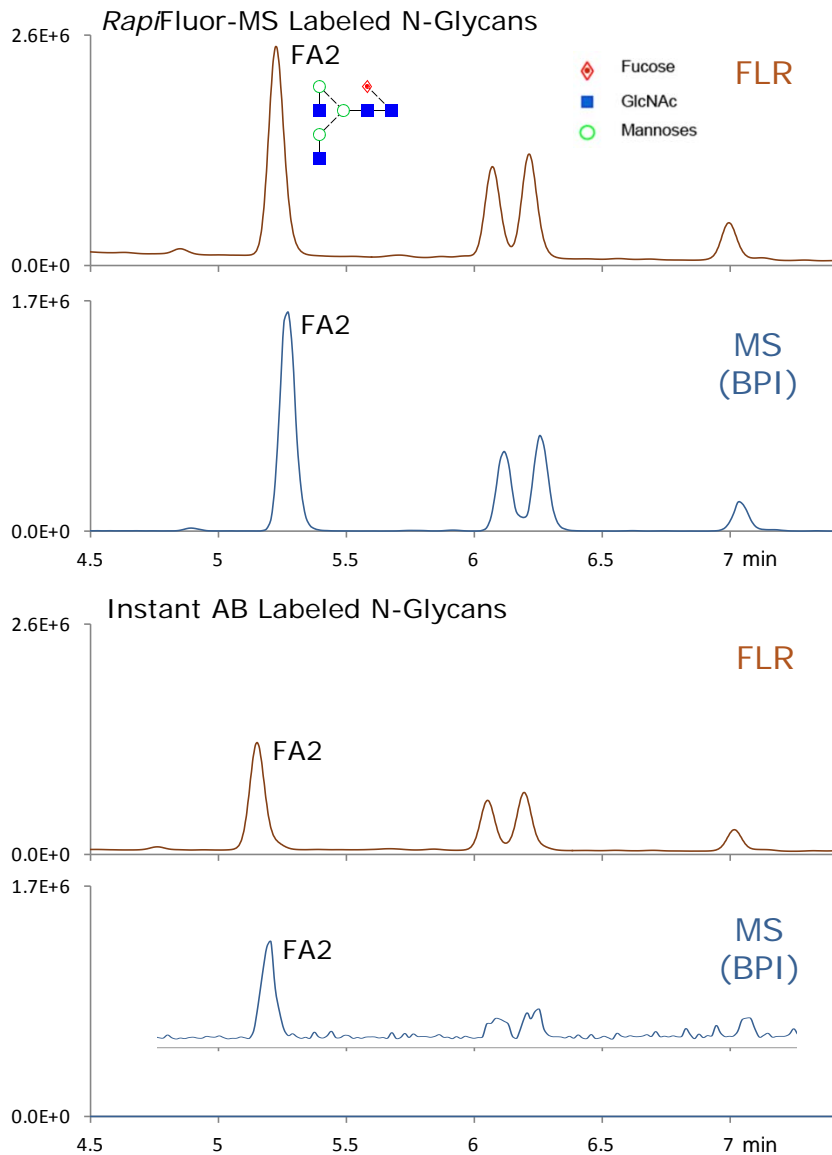


Instant AB™ Labeled N-Glycans



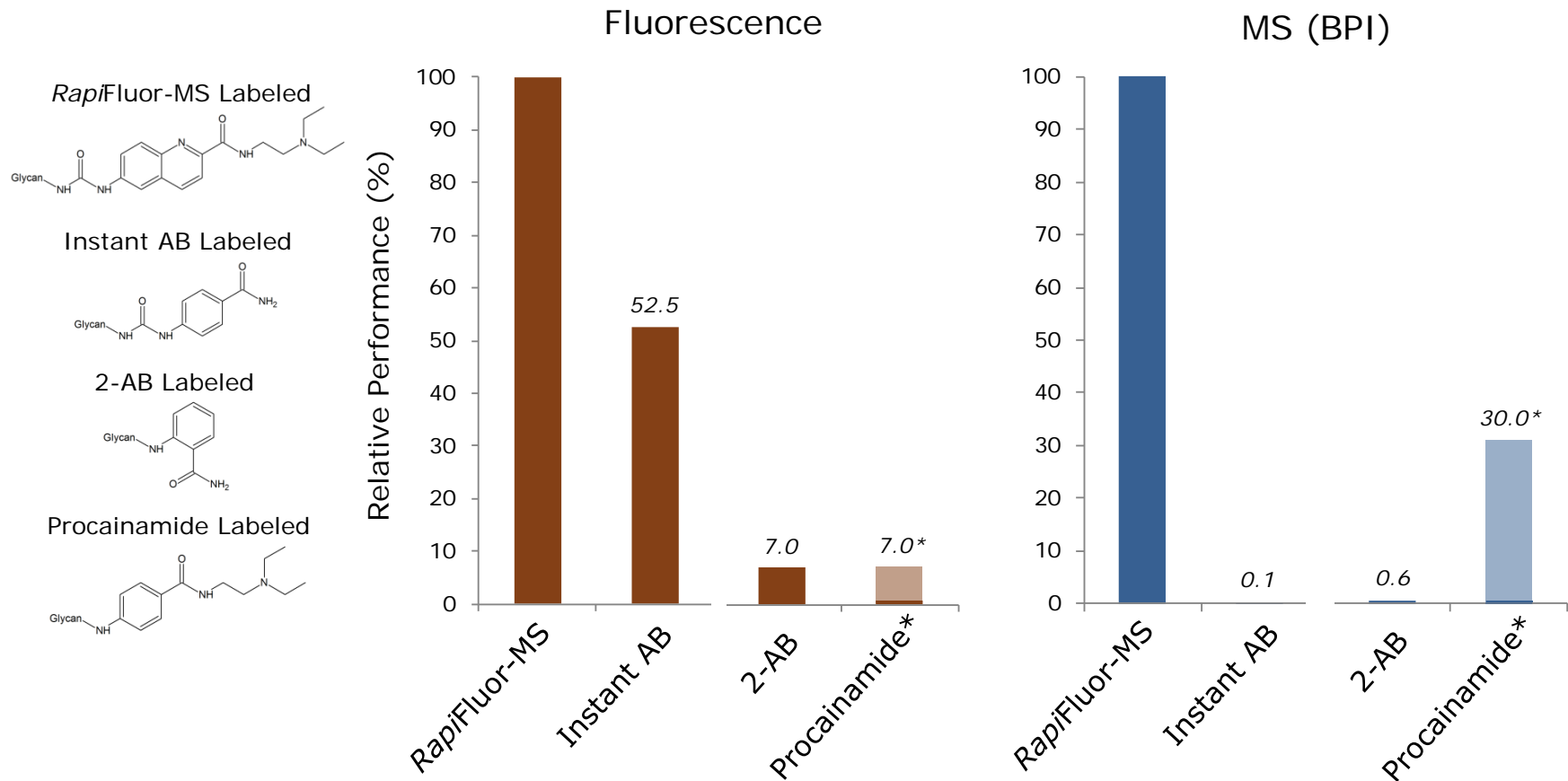
RapiFluor-MS Reagent

Sensitivity Comparison – Instant AB



RapiFluor-MS Reagent

Sensitivity Comparison



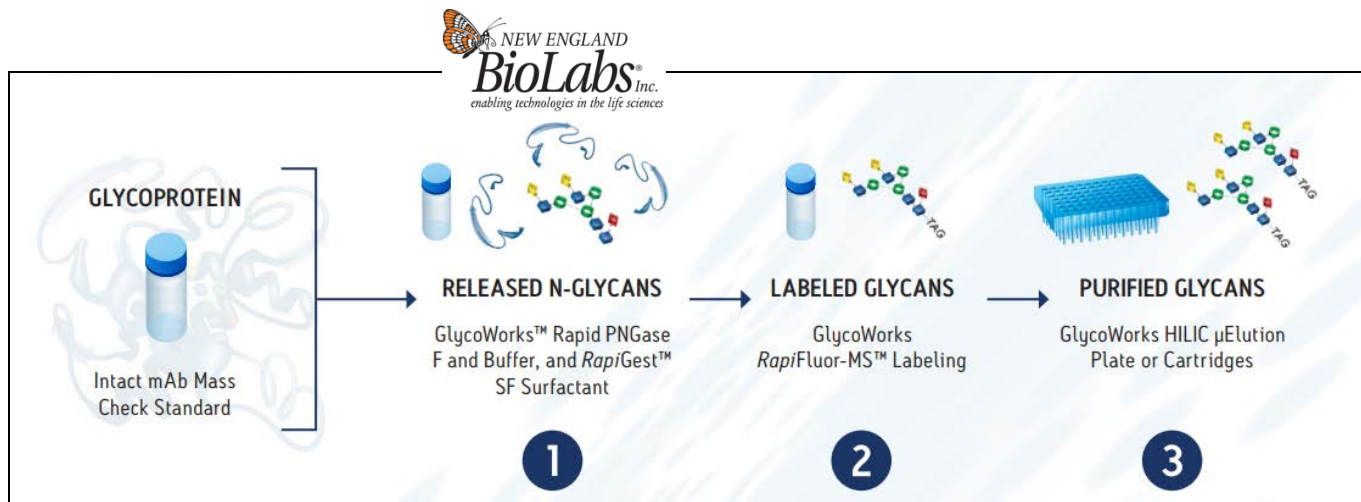
(*) Comparative result extrapolated from a published comparison of N-glycans, wherein it was found that procainamide provided comparable fluorescence and up to 50 fold greater ESI-MS sensitivity when compared to 2-AB(Klapoetke et al. 2010).

Simplified Workflow

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Conventional

5 Hours
to
2 Days



Direct Analysis
(Organic Solvent Dilution)

10 min

5 min

10 min

Total
Sample Prep Time

30 min

GlycoWorks™
RapiFluor-MS™ N-Glycan Kit



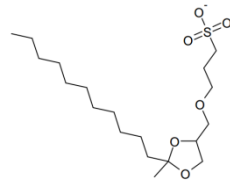
Patent Pending

Rapid Deglycosylation

RapiGest™ SF Assisted

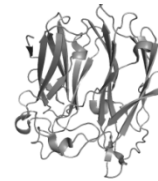
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NEW ENGLAND
BioLabs Inc.
enabling technologies in the life sciences



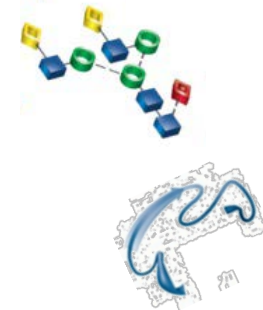
1% RapiGest SF
Surfactant
GlycoWorks
Rapid Buffer

2 min
≥80°C



GlycoWorks
Rapid PNGase F
Enzymatic
Deglycosylation

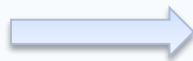
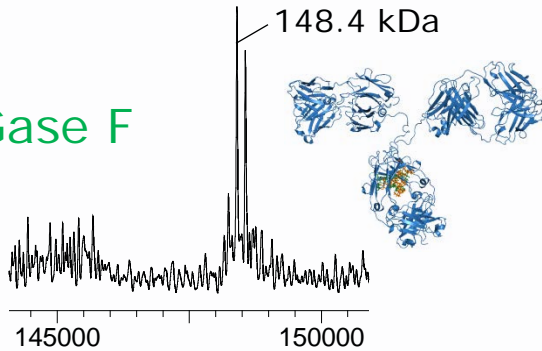
5 min
50°C



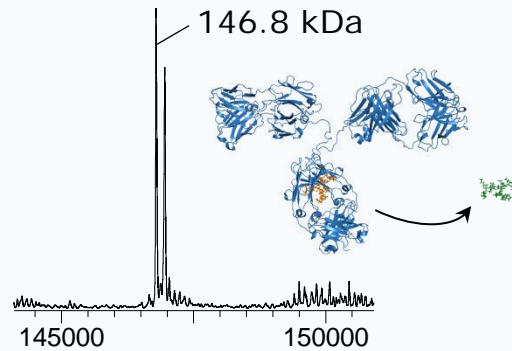
Rapid Deglycosylation

RapiGest™ SF Assisted

No PNGase F
(control)



5 min
50°C

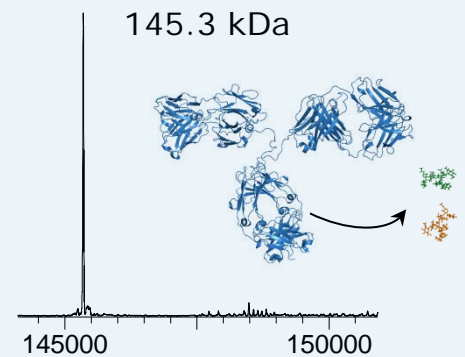


2 Step

2 min
Heat Denaturation

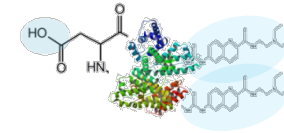
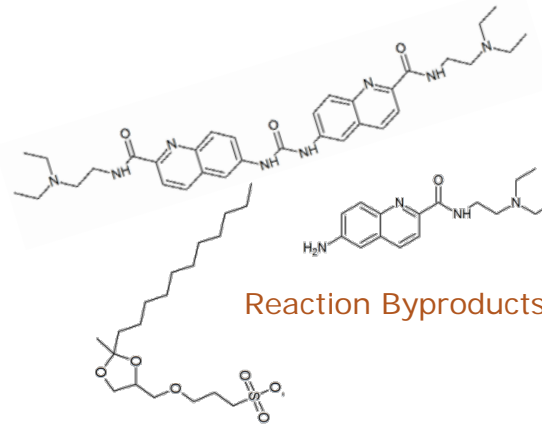
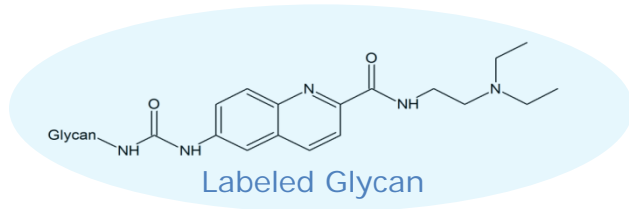


5 min
50°C

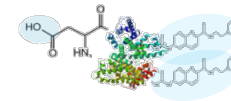
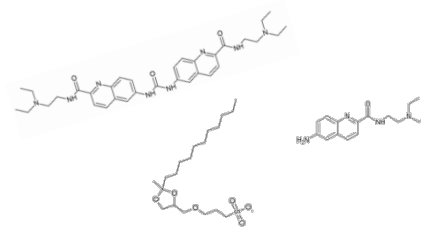
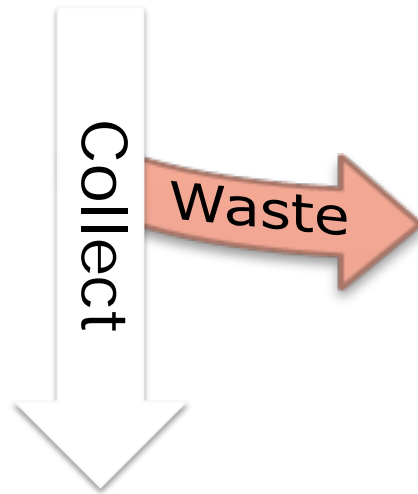


Robust HILIC SPE

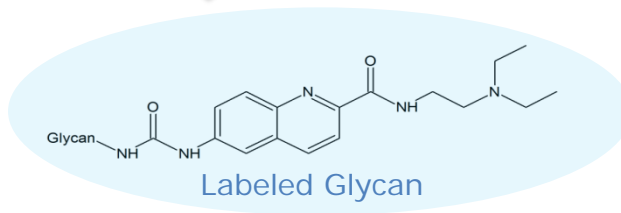
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GlycoWorks
HILIC SPE



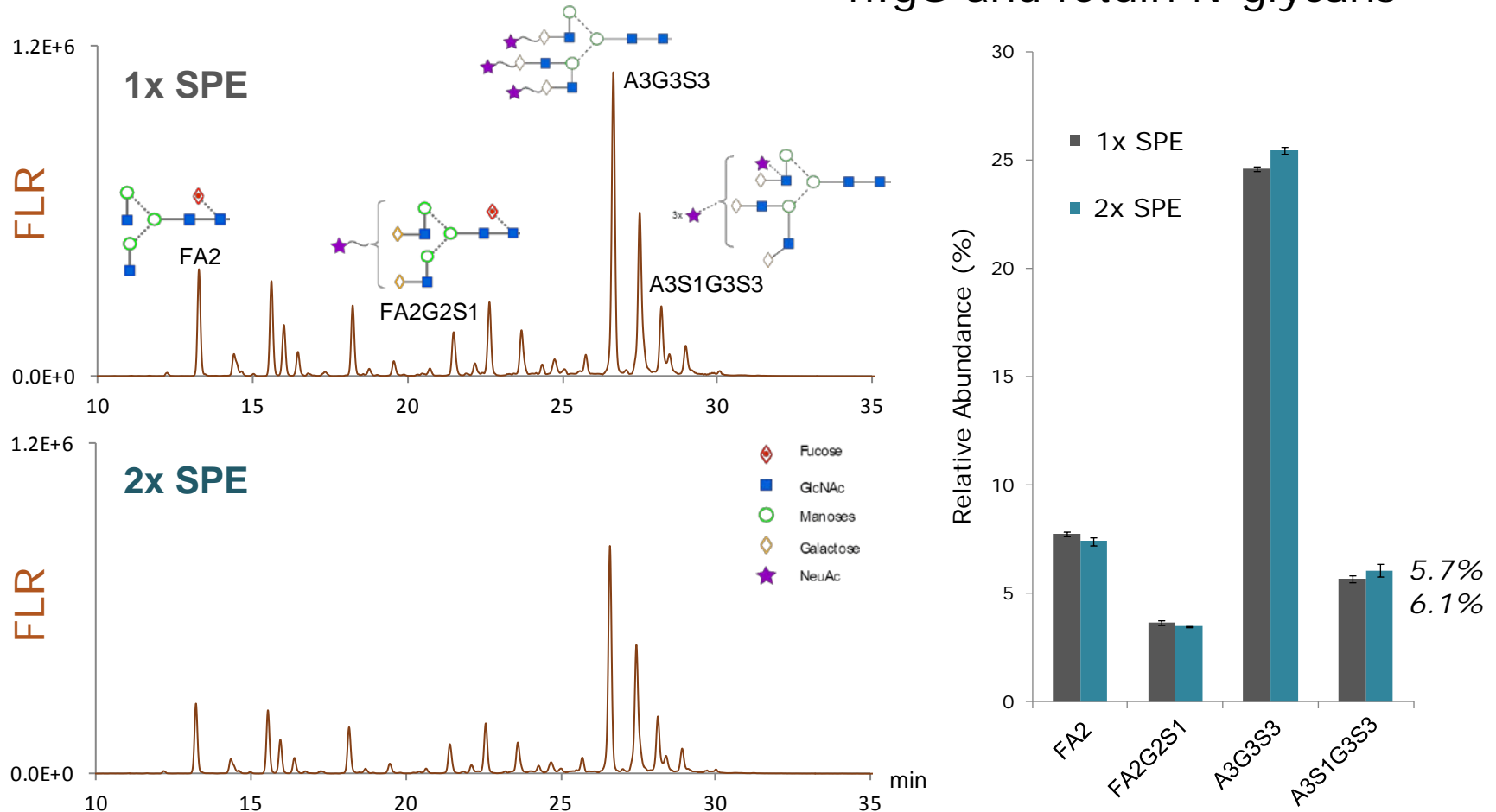
Byproducts



Robustness

Quantitative Extraction

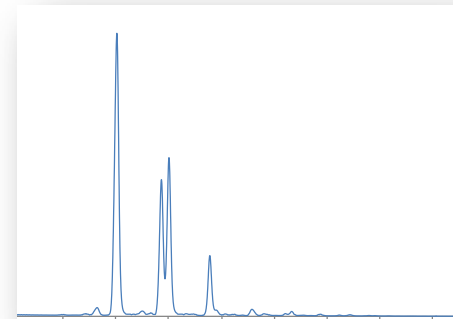
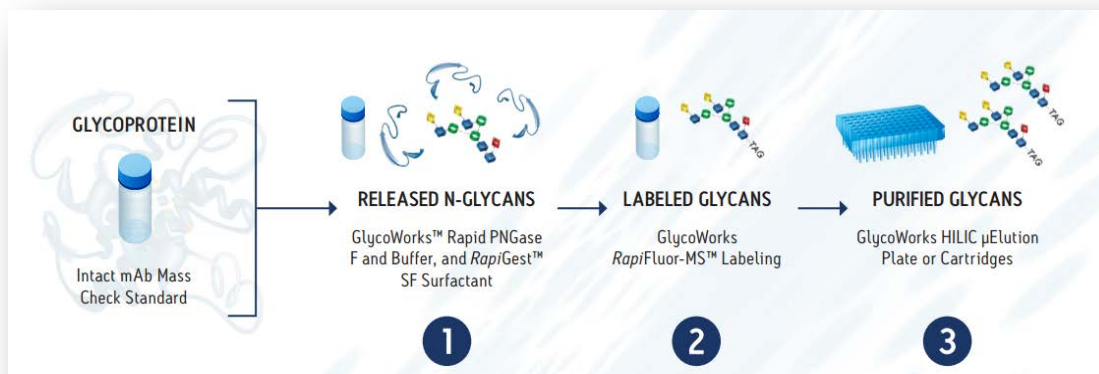
hIgG and fetuin N-glycans



- GlycoWorks HILIC SPE of *RapiFluor*-MS N-glycans is quantitative
- No significant deviation in the glycan profile upon SPE processing

Robustness

High Yield and Minimal Bias



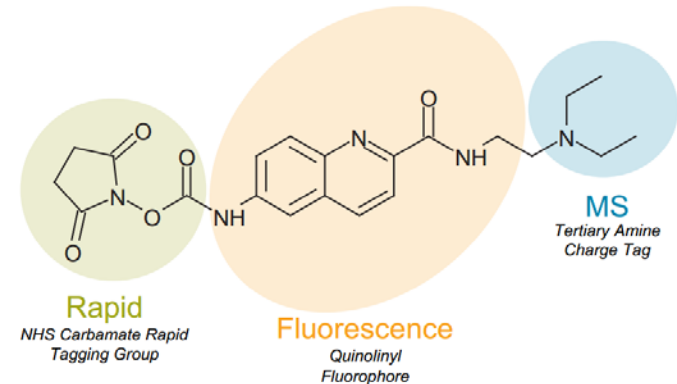
Step	Yield	Testing to confirm minimal bias
Deglycosylation	Complete	<ul style="list-style-type: none"> Intact mass analysis Gel shift assays Subunit LC-MS
Labeling	>95%	<ul style="list-style-type: none"> Released glycan profile vs subunit derived glycan information
SPE	~74%	<ul style="list-style-type: none"> Recovery measurements Glycan profile before vs after SPE
Entire Workflow (experimentally determined)	~73% Yield	

Summary

GlycoWorks™ RapiFluor-MS™ N-Glycan Kit

- Simple, streamlined protocol
- Fast and complete deglycosylation
- Rapid and efficient labeling
- Unbiased and robust SPE for neutral to tetrasialylated *N*-glycans
- Unprecedented FLR and MS sensitivity

RapiFluor-MS



Glycoprotein



Analysis-Ready *N*-glycans

30 min

GlycoWorks RapiFluor-MS N-Glycan Kit

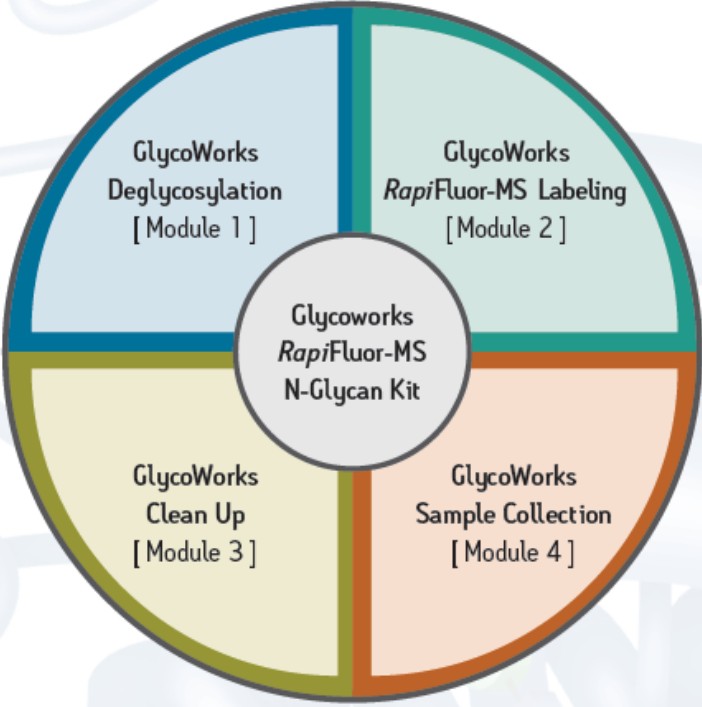


GlycoWorks™ RapiFluor-MS™ Kit

Smart Workflow with No Compromise

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4 x 24 Format= 96 samples



Available
February 2015



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

Matthew A. Lauber,¹ Darryl W. Brousic,¹ Zhengmao Hua,¹ Stephan M. Kozz,¹ Ellen Guthrie,² Paula Magnelli,² Christopher H. Taron,¹ Kenneth J. Fountain¹
¹Waters Corporation, Milford, MA
²New England Biolabs, Ipswich, MA

INTRODUCTION

Conventional approaches to the preparation of N-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromise in sensitivity. 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 detection have been explored, but have not yet presented a solution that combines the desired attributes of sensitivity, high mass resolution, and high throughput. One example is rapid tagging procedures that yield labeled glycans in a matter of minutes. Such methods have presented the use of a rapid tagging reagent of aminobenzamide (AB).¹ In a rapid reaction, the precursor glycosidases of releasing, sialidase terminal glycosidases are modified with a sialine aminobenzamide. Although this rapid tagging reagent accelerates the labeling procedure, it does not provide the enhanced sensitivity efficiency needed in modern N-glycan MS analyses.

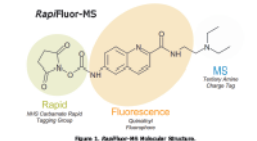
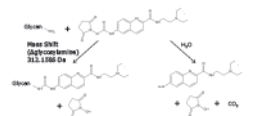


Figure 1. RapFluor-MS Molecular Structure.



METHODS

SAMPLE DESCRIPTION: Glycans were prepared using a glycosylating RapFluor-MS N-Glycan Kit (Cat # 116599) and the manufacturer suggested protocols provided in the Care and Use Manual (116599-01). To compare the response factors of Instant ABSM and RapFluor-MS labeled glycans, labeling reactions were performed with equivalent molar amounts of reagent, and equal reaction conditions were checked relative to HILIC-MS. To compare the response factors of 2-AB labeled versus RapFluor-MS labeled glycans, equivalent quantities of 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.

METHODS CONTINUED (Values are arbitrary units):
LC-MS/MS:
LC system: ACQUITY UPLC H-Class Bio System
Mobile Phase: A: 0.1% TFA
Flow Rate: 0.4 µL/min
Fluorescence Detection: 315 nm (Excitation) / 315.1588 nm (Emission)
MS system: ACQUITY UPLC Q-ToF MS System
Scan Rate: 1000 scans/min
Scan Range: 100-1000 m/z
Scan Resolution: 10,000

Statistical Analysis: Student's t-test was used to compare the response factors of Instant ABSM and RapFluor-MS labeled glycans. The results are shown in Figure 4. The response factors of Instant ABSM and RapFluor-MS labeled glycans were compared to the response factors of 2-AB labeled glycans. The results are shown in Figure 5. The response factors of Instant ABSM and RapFluor-MS labeled glycans were compared to the response factors of 2-AB labeled glycans. The results are shown in Figure 6.

MS Conditions:
MS system: Synapt G2-S HDMS
Analyser mode: ESI+, TOF MS, Resolution Mode (>100 K)
Capillary voltage: 2.5 kV
Cone voltage: 120 V
Source temperature: 120 °C
Desolvation gas flow: 600 L/h
Sulfuric acid concentration: 100-200 µM, 1.0 µmol/min
Data management: MassLynx Software (V4.1)

RESULTS AND DISCUSSION

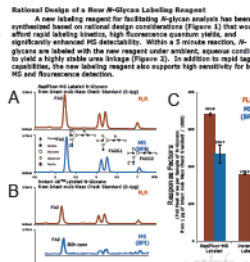


Figure 3. HILIC-MS analysis of 2-AB labeled and Instant ABSM labeled N-glycans. The response factors of Instant ABSM labeled N-glycans were compared to the response factors of 2-AB labeled glycans. The results are shown in Figure 4. The response factors of Instant ABSM and RapFluor-MS labeled glycans were compared to the response factors of 2-AB labeled glycans. The results are shown in Figure 5. The response factors of Instant ABSM and RapFluor-MS labeled glycans were compared to the response factors of 2-AB labeled glycans. The results are shown in Figure 6.

RapFluor-MS Enables High Sensitivity Fluorescence and MS Detection: The response factors of RapFluor-MS labeled glycans have been characterized against those measured for glycans labeled with alternative reagents. The most closely related, commercially available alternative to RapFluor-MS is Instant ABSM. Figures 3A and 3B present HILIC-MS chromatograms and mass peak intensity (MS) chromatograms for equivalent quantities of glycans released from a mixture of commercial antibodies (DMS) with Instant ABSM and RapFluor-MS, respectively. Based on the observed chromatographic peak areas, response factors were determined for the most abundant glycan in the MS profile, the fucosylated, biantennary F2C glycan. Figure 4C indicates that RapFluor-MS labeled glycans produce 2.988 higher fluorescence signal and, more importantly, 760 times greater MS signal than glycans labeled with Instant AB. In a similar fashion, RapFluor-MS labeling has also been compared to conventional 2-AB labeling. To corroborate our observations, we have plotted the response factors of Instant AB and 2-AB as percentages versus the response factor of RapFluor-MS (Figure 5).

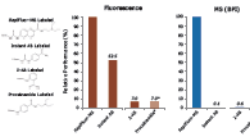


Figure 4. MS performance of glycan labels. Response factors normalized to the fluorescence and MS response factors of RapFluor-MS labeled glycans. (%) comparative response factors of a released concentration of glycans (DMS) of 0.1000 µg.

Rapid Deglycosylation with a Novel Formulation of Rapid PNGase F and RapPhase F Surface: RapFluor-MS labeling necessitates N-glycan sample preparation and can be readily adopted in the laboratory with the GlycoWorks RapFluor-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 workflow requires 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 reagents competing 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 RapFluor-MS N-Glycan Kit includes a novel formulation of Rapid PNGase F and RapPhase F surface that can be used to completely deglycosylate glycoproteins in an approximately 10 minute procedure. RapPhase F is an acidic surfactant used to ensure that glycoproteins are accessible to Rapid PNGase F and that the glycoproteins remain stable upon reagent denaturation. Most importantly, Rapid PNGase F and RapPhase F can also 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 Rapid PNGase F 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 10 minutes to achieve complete, unbiased deglycosylation for most glycoproteins. Using MS/MS based gel electrophoresis, we have confirmed this fast deglycosylation process to be effective for a diverse set of glycoproteins (Figure 6).

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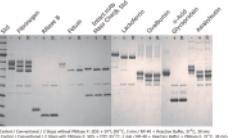
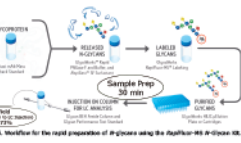


Figure 6. MS/MS chromatogram for deglycosylation of glycoproteins. The MS/MS profile of a glycoprotein before and after deglycosylation, demonstrating the release of individual glycan fragments.

Robust, Quantitative HILIC SPE The final step in an N-glycan sample preparation is SPE designed to selectively extract RapFluor-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, anion-exchange silica-based column was selected for this application. Glycans were adsorbed to the column as a HILIC mechanism. After the sample was washed to remove sample matrix, an acidic wash solvent was employed in the flow to introduce electrostatic repulsion between the anion-exchange HILIC column and labeling reaction byproducts and to enhance the elution of the matrix components. After resulting, RapFluor-MS labeled N-glycans were eluted from the anion-exchange column using an almost constant flow of 7 volumes of 200 mM ammonium acetate in 5% acetonitrile. The eluted RapFluor-MS labeled glycans were then diluted with a mixture of organic solvents (ACN and DMSO) and directly analyzed by HILIC column chromatography, as shown in Figure 7B.

Figure 7C shows the relative abundances for four glycans (F2C, F2D2G2L, AD2G2L, and AS2G2L2S) as determined after one pass and two passes of the SPE process, respectively. These results demonstrate that the SPE technique provides a mechanism to immutably analyze a sample of PNGe labeled glycans and does so without significant compromise to the accuracy of the relative abundances determined for a wide range of N-glycans.

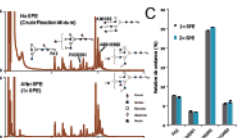


Figure 7. HILIC-MS analysis of N-glycans. Panel A shows the HILIC-MS chromatogram of N-glycans. Panel B shows the HILIC-MS chromatogram of N-glycans after SPE. Panel C is a bar chart comparing the relative abundances of four glycans before and after SPE.

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 RapFluor-MS N-Glycan Kit
- Accurate profiling based on robust SPE for neutral to tetraantennary N-glycans

References
1. Hildner, M., et al. (2008) J. Biol. Chem. 283, 11111-11116.
2. Hildner, M., et al. (2008) J. Biol. Chem. 283, 11117-11122.
3. Hildner, M., et al. (2008) J. Biol. Chem. 283, 11123-11128.
4. Hildner, M., et al. (2008) J. Biol. Chem. 283, 11129-11134.
5. Hildner, M., et al. (2008) J. Biol. Chem. 283, 11135-11140.

WCBP 2015 Poster P-216-W

Rapid Preparation of N-Glycans Using a Novel Fluorescence and MS Active Labeling Reagent

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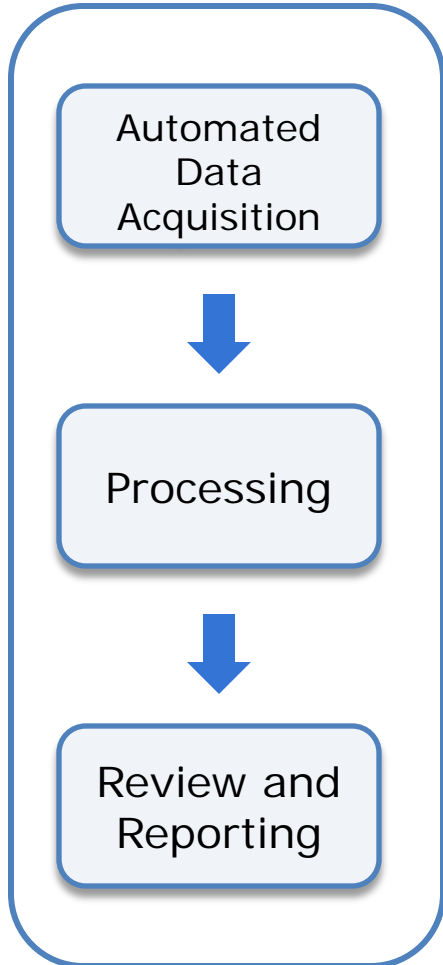
RapiFluor-MS:
Enhanced Workflows
for Glycan Characterization

Glycan Characterization

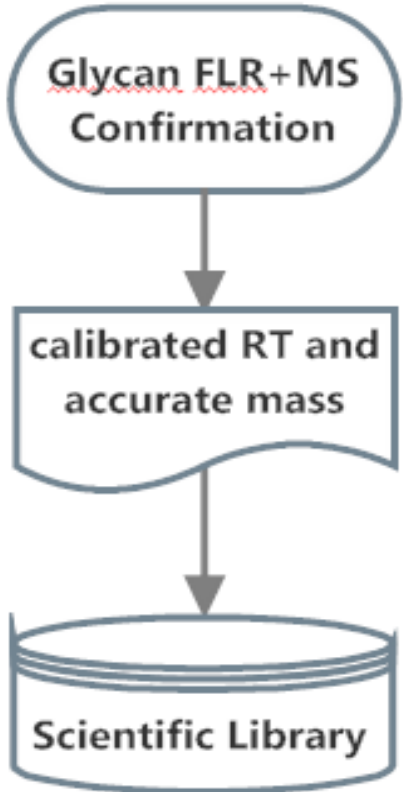
Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

- ACQUITY UPLC® H-Class Bio System
- ACQUITY UPLC Column Manager
- ACQUITY UPLC FLR Detector
- Xevo® G2-XS QToF MS
- UNIFI® Glycan Application Solution or MassLynx® Informatics
- GlycoWorks™ *RapiFluor-MS*™ N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column

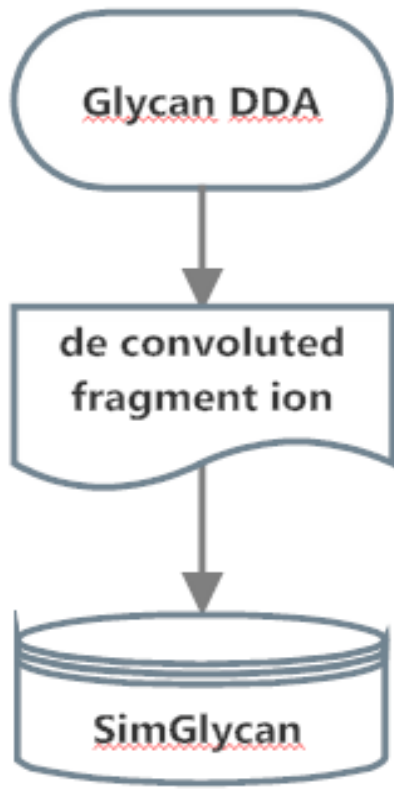




HILIC FLR GU + Accurate Mass



HILIC FLR GU + DDA MS/MS

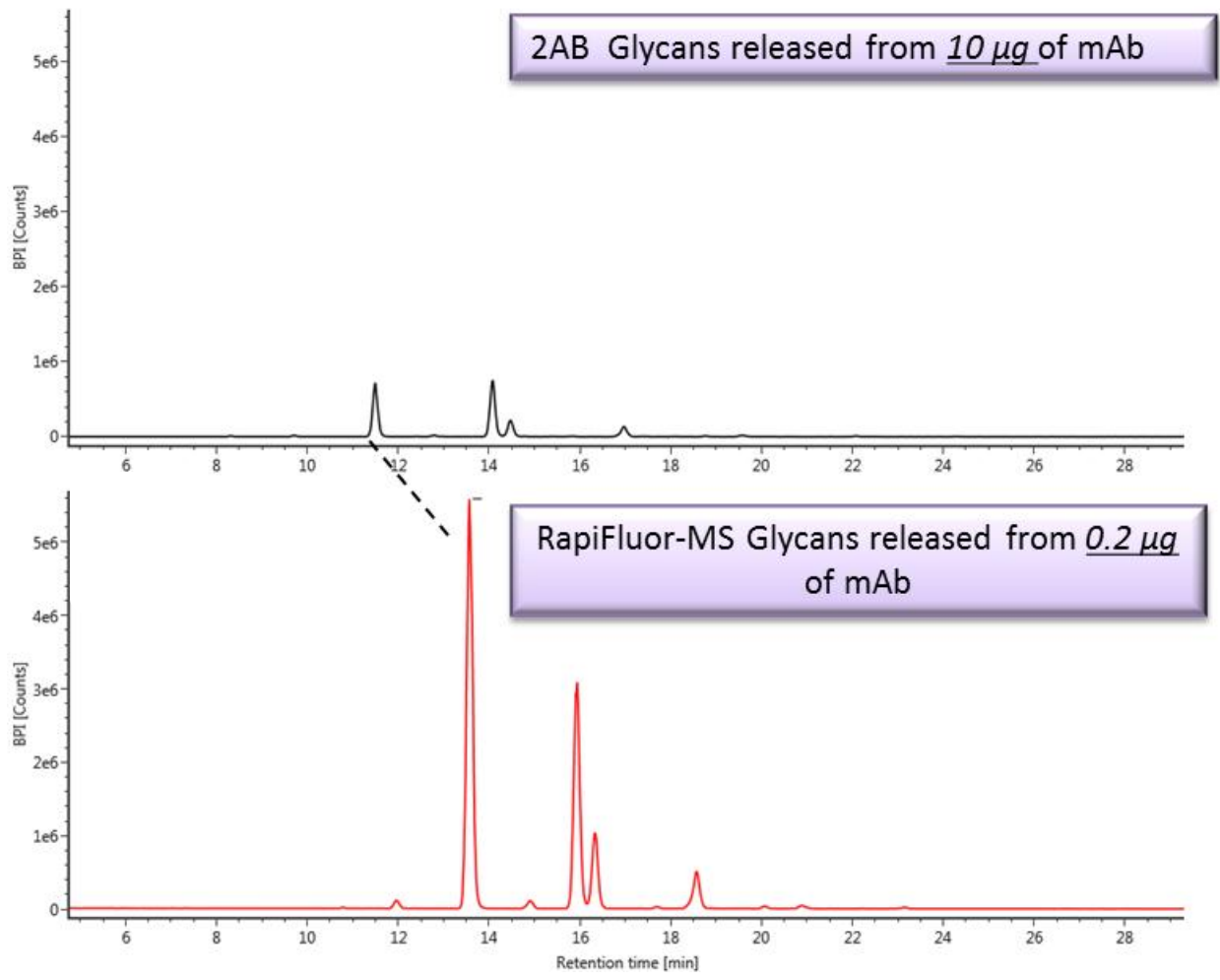


Workflows support conventional glycan labels and new *RapiFluor-MS*TM label technology

RapiFluor-MS™ Labeling for Characterization

Greater than 100x MS response over 2AB labeling

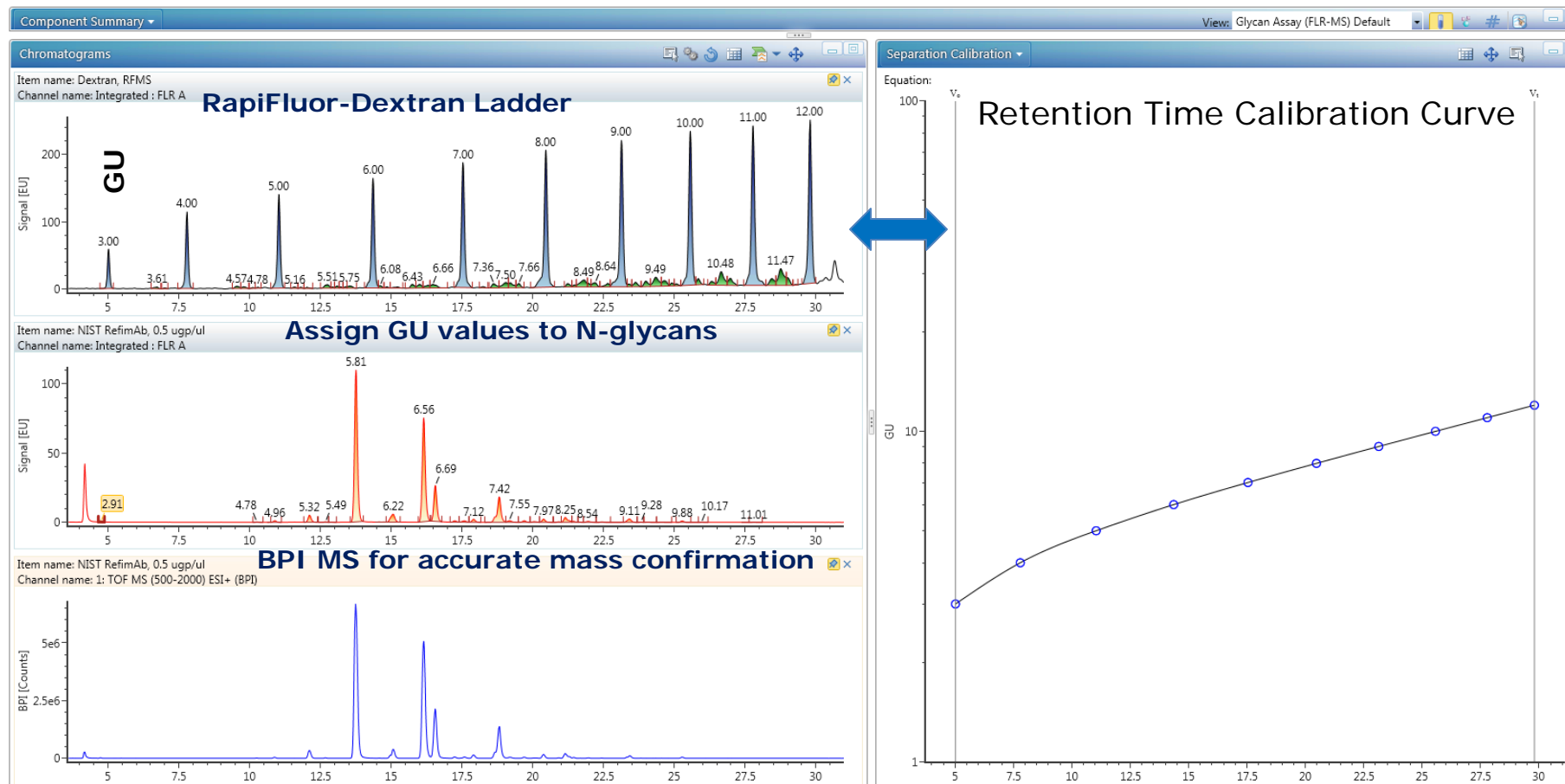
BPI MS



Sample: NIST RM 8670 mAb lot #3F1b

HILIC FLR GU + Accurate Mass

Method Robustness and Transferability, Confident Assignments



RapiFluor-MS Labeled Dextran and System Performance Standard (hIgG) are now available to support this GU workflow

UNIFI[®] Scientific Library for Automated GU or GU+Mass Glycan Confirmation



F(6)A2 [2AB-Glycan] Reagent: RFMS

Property	Value
Item type	Glycan
Item description	
IUPAC name	
Formula	C ₅₆ H ₉₄ N ₄ O ₄₀
Hill formula	C ₅₆ H ₉₄ N ₄ O ₄₀
Average molar mass	1463.3484
Monoisotopic mass	1462.5444
Item tag	Infiximab, Human IgG, Mouse IgG, Human Serum, Herceptin
InChI	

Residues: 8
 Hexose: 3
 N-acetyl hexose: 4
 Sialic acid: 0
 Mannose: 3
 Fucose: 1
 Mass [RFMS]: 1773.7190 g/mol

mass

FLR label

Properties ▾

Synonyms		Identifiers		Physical properties	
Synonym	Synonym type	Identifier	Value	Property	Value
F(6)A2		NIBRT GlycoBase	43	GU value	5.87
*		*		GU value standard deviation	0.071

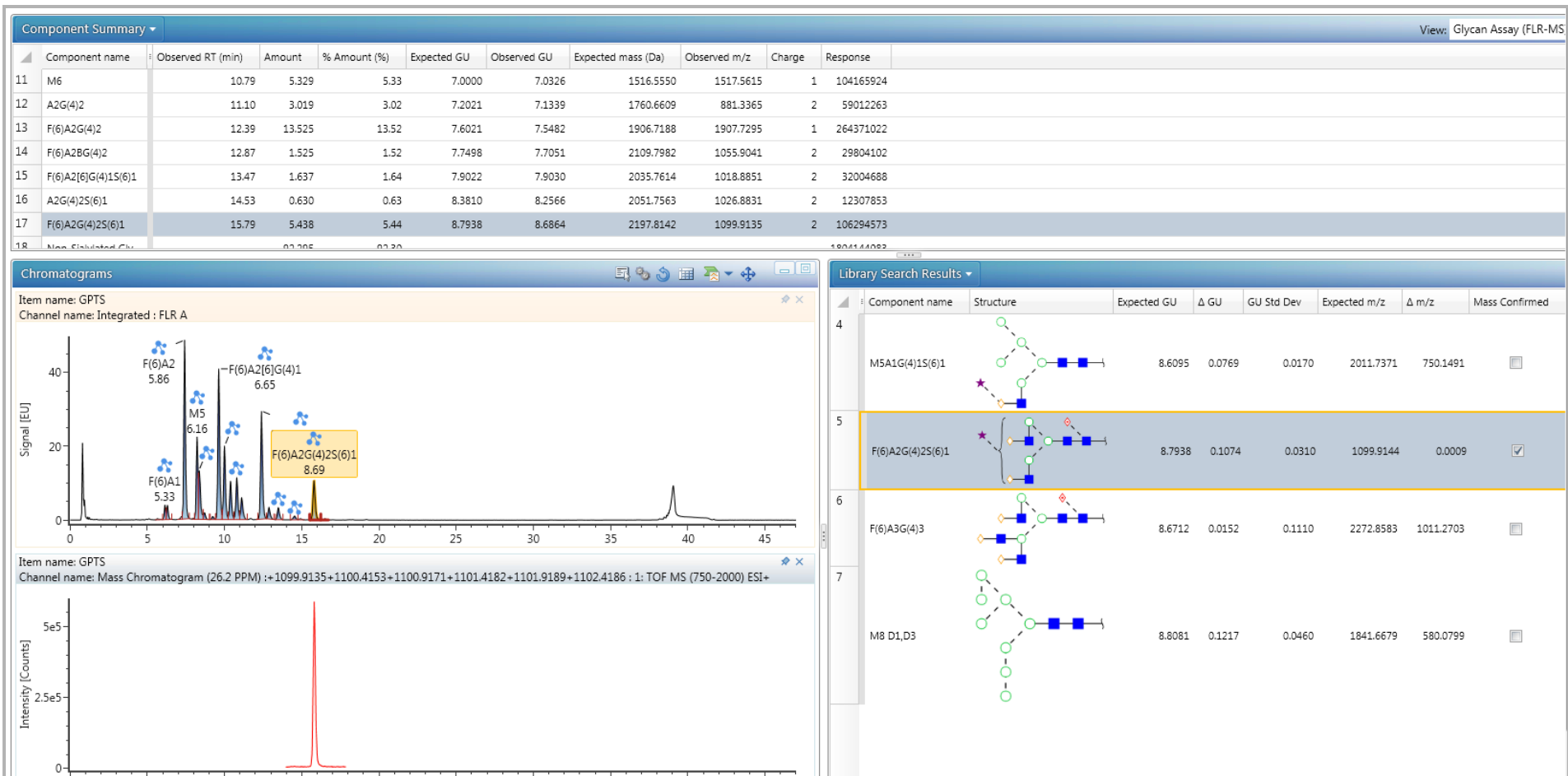
Experimental GU value

Waters Glycan GU Library:

- Experimentally derived GU Retention (> 10 injections/protein)
- Data from proteins representing spectrum of glycan diversity
- All entries confirmed with exoglycosidase digestion

UNIFI® Scientific Library for Confident Glycan Assignments

Waters
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Powerful UNIFI® Reporting Architecture Simplifies Communication of Results

Example

Summary Table

Summarized by: % Amount (%)

Item name	A1	M3B	M4	F1R3A1	A2	M4A1	F1R3A2	MS	A2G411	F1R3A1G411
1 9CM	1.29	0.15	3.17	1.99	0.57	47.11	4.58	0.41		
2 9CM	1.28	0.13	3.16	2.01	0.57	47.11	4.56	0.41		
3 9CM	1.28	0.14	3.15	2.00	0.57	47.11	4.55	0.41		
4 RLM	1.08	0.11	0.28	2.68	1.90	0.58	44.09	4.46	0.48	
5 RLM	1.08	0.11	0.26	2.66	1.88	0.57	44.09	4.45	0.47	
6 RLM	1.08	0.11	0.28	2.67	1.91	0.57	44.10	4.44	0.46	
7 OBB	1.07	0.13	0.31	3.38	1.53	0.62	48.19	4.59	0.28	2.21
8 OBB	1.08	0.14	0.36	3.38	1.53	0.61	48.18	4.55	0.29	2.15
9 OBB	1.08	0.14	0.34	3.36	1.54	0.61	48.18	4.57	0.28	2.19
Mean	1.147	0.124	0.292	3.069	1.810	0.586	46.477	4.528	0.402	2.279
% RSD	8.89	14.64	31.45	10.29	11.78	5.51	5.96	1.50	22.08	8.44
Std dev	0.103	0.018	0.089	0.316	0.213	0.021	1.841	0.099	0.089	0.078

Sample List

Analysis Injection List

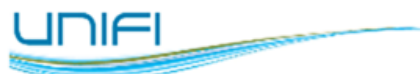
	Sample name	Sample type	Replicate number	Sample position	Injection volume (µL)	Processing options
1	blank	Blank		1:1:A,1	1.00	
2	B005_2	Reference		1:1:B,3	2.00	
3	Dextran 6	Standard		1:1:A,2	1.00	Separation standard
4	Dextran 4	Standard		1:1:A,2	1.00	Separation standard
5	Dextran 7	Standard		1:1:A,2	1.00	Separation standard
6	Dextran 5	Standard		1:1:A,2	1.00	Separation standard
7	9CM	Unknown		1:1:B,4	2.00	
8	9CM	Unknown		2:1:B,4	2.00	
9	RLM	Unknown		1:1:B,5	2.00	

Analysis Method

Analysis Method Report

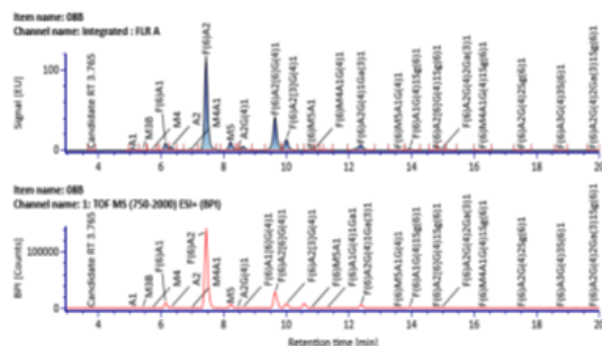
Purpose

Item name: Infilmat_2nd_data_innovator_targeted	Quantify enabled: No
Description:	Compare enabled: No
Instrument system type:	Discover enabled: No
Analysis type: Glycan Assay (FLR with MS Confirmation)	Matrix Factor Enabled: No
Analysis type description: Discovers and Quantifies Glycans using peaks in FLR chromatograms, identifies them via Library Search and Confirms identification using MS data	Separation enabled: No
Screen mass: Accurate mass	Analyte class: Glycan
Screen enabled: Yes	Analyte class description: Glycan Analyte



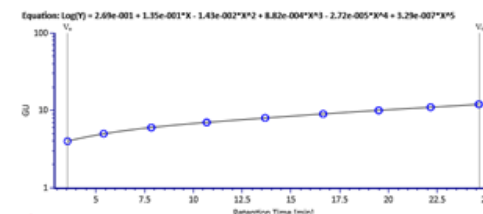
Report

Processed UPLC/FLR/MS Chromatogram and Result Table



Component name	Observed RT (min)	Observed GU	% Amount (%)
A1	5.09	4.84	1.07
M3B	5.41	4.99	0.13
M4	5.71	5.13	0.33
F1R3A1	6.55	5.33	3.38

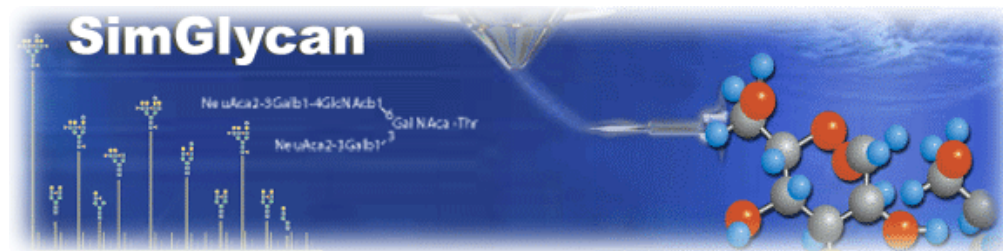
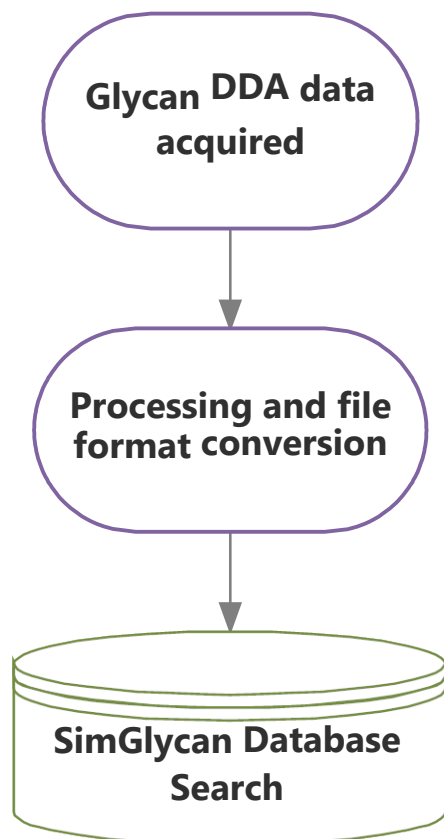
RT Calibration Curve



Library Search Result

Library name	Component name	Structure	Expected GU	A GU	Expected m/z	z m/z
Waters Glycan Library	F1R3A2G411		7.000	0.0420	174.8800	0.0133
Waters Glycan Library	M4 G2		7.000	0.0220	137.5621	0.0442
Waters Glycan Library	A2G411G411		7.000	0.0980	176.6662	0.22180

UNIFI® Glycan DDA Workflow



Optional UNIFI[®] Export to SimGlycan for MS/MS Database Search

A2G2S1

Scan35@1122.43_1 (20141031_YEA439_yqy_Human IgG_DDA_01)

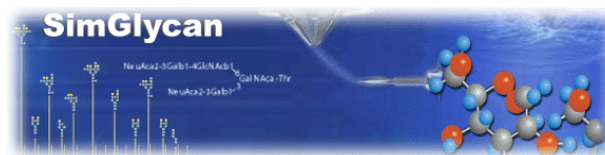
MS Profile Search Results Annotated Peaklist

Rank	Glycan ID	Glycan Sequence	Proximity score
1	G04162	NeuAc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	78.2395
1	G00837	NeuAc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	78.2395
2	G00393	NeuAc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	78.2191
2	G02190	NeuAc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	78.2191
3	G00205	NeuAc(a2-3)Gal(b1-4)GlcNAc(b1-2)Ma...	78.1882
3	G12014	NeuAc(a2-3)Gal(b1-4)GlcNAc(b1-2)Ma...	78.1882
4	G03954	NeuAc(a2-)Glc(b1-4)GlcNAc(b1-2)Man(...	78.1781
5	G00203	NeuAc(a2-3)Gal(b1-4)GlcNAc(b1-2)Ma...	78.1676
6	G04207	NeuAc(a2-3)Gal(b1-3)GlcNAc(b1-2)Ma...	78.1161
7	G04314	NeuAc(a2-)Gal(b1-4)GlcNAc(b1-2)Man(...	78.07
8	SG28781	NeuAc(a2-6)GalNAc(b1-4)GlcNAc(b1-2)...	78.0311
9	G04319	NeuGc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	76.7915
10	G04226	NeuGc(a2-6)Gal(b1-4)GlcNAc(b1-2)Ma...	75.7974

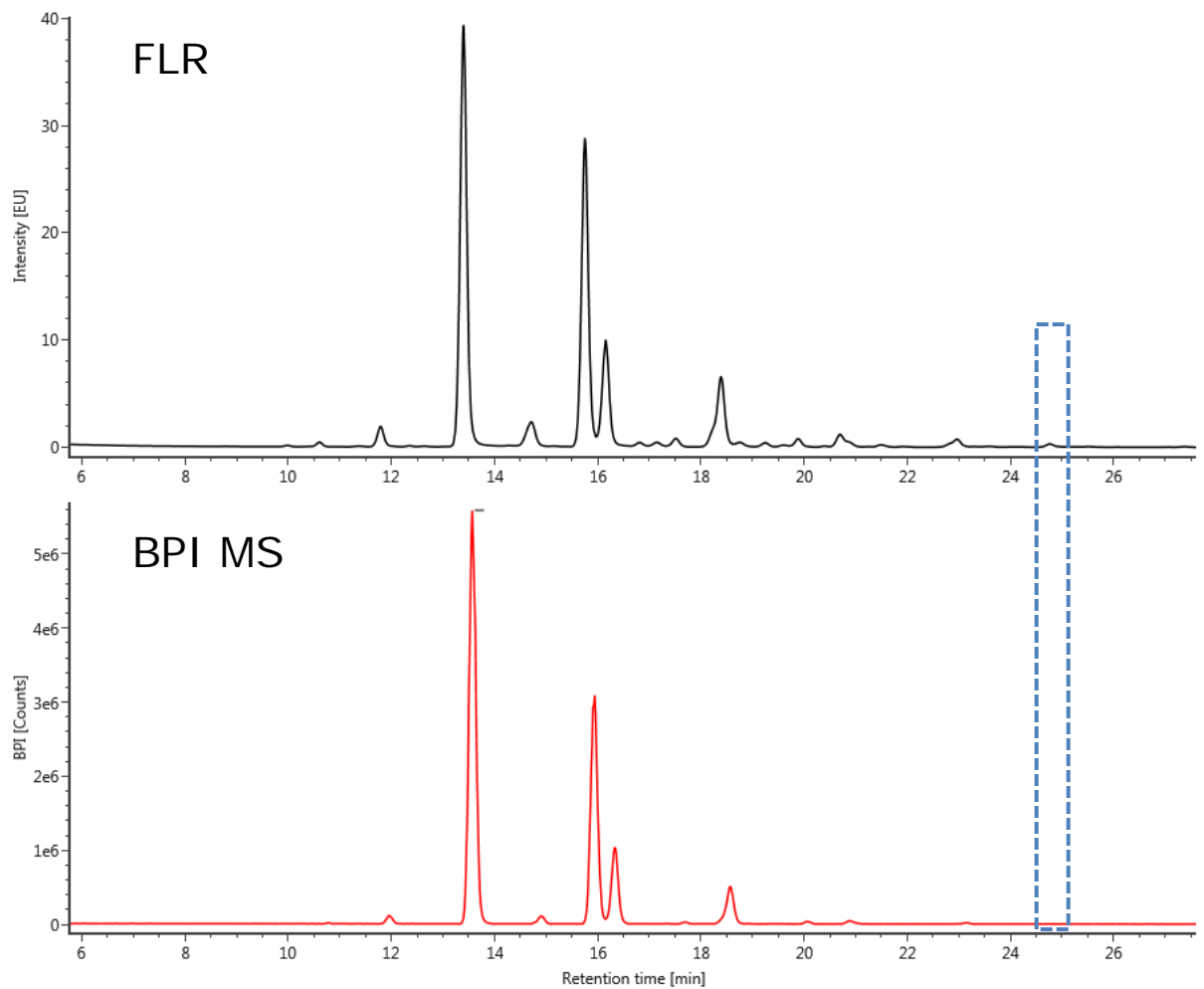
Glycan Structure (G00837)

Sequence:
NeuAc (a2-6) Gal (b1-4) GlcNAc (b1-2) Man (a1-6) [Gal (b1-4) GlcNAc (b1-2) Man (a1-3)] Man (b1-4) GlcNAc (b1-4) GlcNAc

Carbohydrate Name



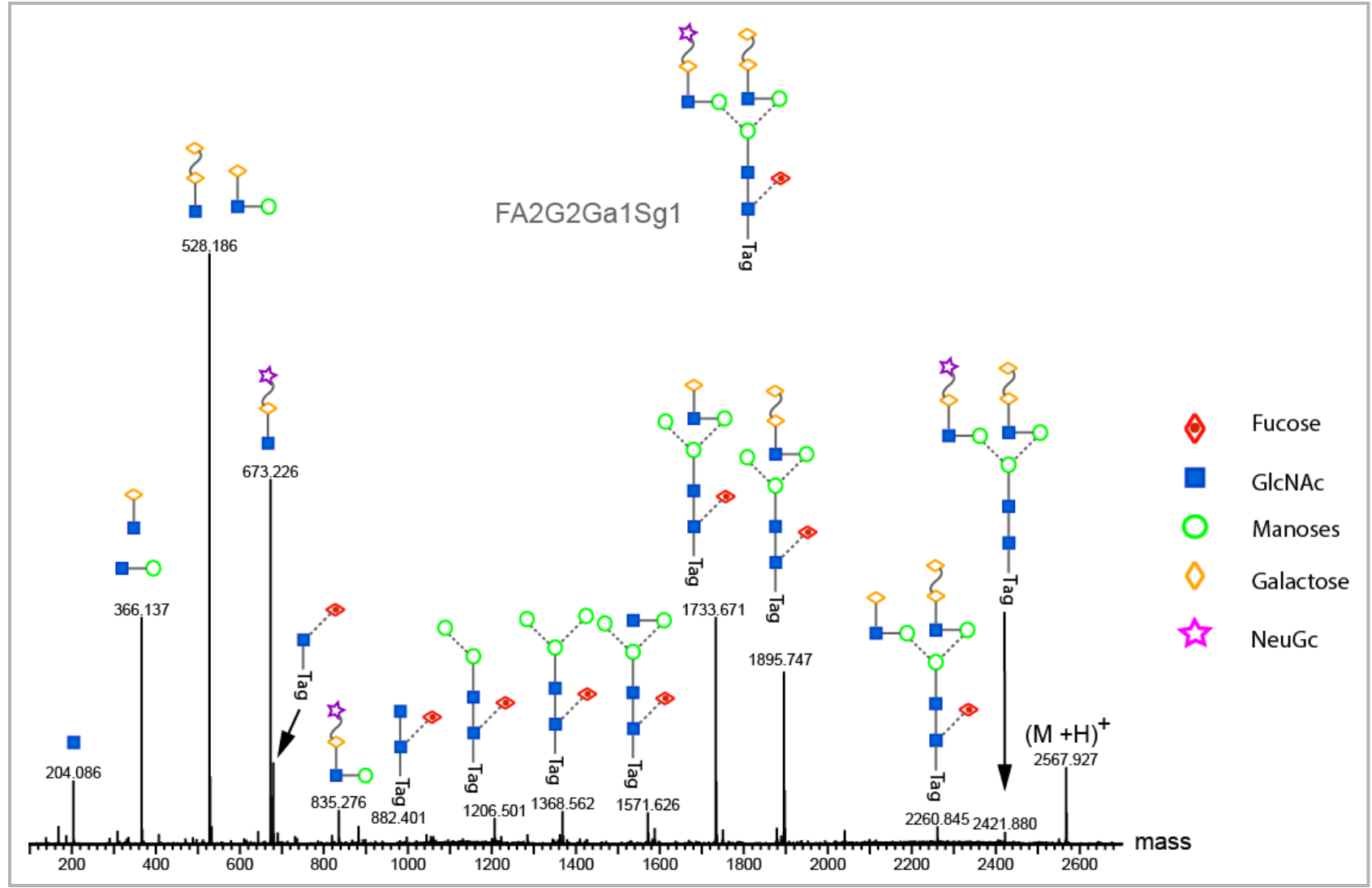
MS performance extends to fragmentation data



↓ MSMS

Enhanced MSMS with *RapiFluor*-MS Labeling

MS performance extends to MS/MS fragmentation data



Developing a Scientific Library for UPLC/FLR/MS Analysis of Released N-glycans Labeled with a Novel Tagging Reagent



Mark Hilliard¹, Naoibh McLoughlin¹, Pauline M Rudd¹, Ying Qinn Yu¹
¹NIBRT, Fosters Avenue, Mount Merrion, Blackrock, Dublin 4, Ireland, ²Waters Corporation, Milford, MA.



INTRODUCTION

A new glycan fluorescent label, RapIFluor-MS™, is used to label N-linked glycans. This innovative label improves FLR and MS signals for glycan characterization and profiling analysis.

Waters and NIBRT are co-developing a new scientific library for RapIFluor-MS labeled N-glycans that identifies glycans based on HILIC-UPLC retention time (in Glucose Unit, GU) and accurate mass information (Ref.1).

- Each glycan contained in the scientific library is fully characterized structurally using a combination of exoglycosidase array and MS analysis. The "unknown" glycan is confirmed by matching its retention time (in GU value) and its accurate mass with the experimental data composites inside the scientific library. Glycan assignment is based on the best matched GU value and exact mass.

- NIST mab reference standard (candidate NIST RM 8670 mab lot #3F1b) is being used as a proof of concept sample to kick start this new scientific library development.

RESULTS

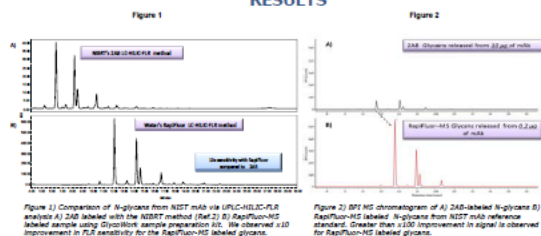


Figure 1) Comparison of N-glycans from NIST mab via UPLC-HILIC-FLR analysis. A) ZAB labeled with the NIST method (Ref.2) B) RapIFluor-MS labeled sample using Dioprotein sample preparation kit. We observed a 10 improvement in FLR sensitivity for the RapIFluor-MS labeled glycans.

Figure 2) BPI MS chromatogram of A) ZAB-labeled N-glycans B) RapIFluor-MS labeled N-glycans from NIST mab reference standard. Greater than 100 improvement in signal is observed for RapIFluor-MS labeled glycans.

METHODS

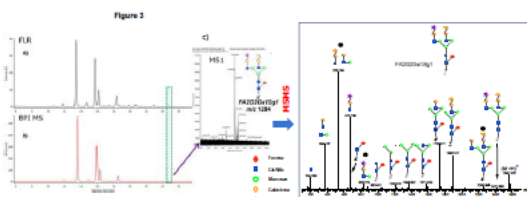
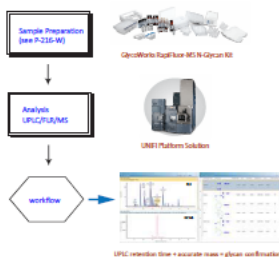


Figure 3) Characterization of low abundant RapIFluor-MS labeled glycan: UPLC-HILIC-FLR analysis of N-glycans from NIST mab. A) FLR chromatogram and B) BPI MS chromatogram. C) Spectrum insert shows good S/N for a low abundant glycan, F43220438.

Figure 4) Collision induced fragmentation of the ion (from Fig. 3c) shows that the fragmentation mechanism of RapIFluor-MS labeled glycan is very similar to those of ZAB-labeled glycans in that glycosidic bond cleavage is observed as the predominant fragmentation pathway. Structurally informative fragments (with asterisks) are observed for this low abundant ion (< 0.1% relative abundance). This fragmentation also suggests that the glycan contains alpha and beta-linked N-acetylglucosamine residues.



Figure 5) Exoglycosidase digestion array of RapIFluor-MS labeled NIST mab. The exoglycosidase array is used to fully characterize the glycans to provide information on the composition, linkage and branching of the glycans present. The experimental retention time (in GU) from the exoglycosidase array and the accurate mass for each glycan are the key glycan attributes to be included in the scientific library.



Figure 6) Generation of the scientific library using RapIFluor-MS labeling chemistry. The key glycan features such as structure, MW (with or without the label), and the experimental GU value associated with each glycan are shown in the screen capture above. The GU value and the mass or LC/MS/MS are used to assign and confirm the structure of an unknown glycan.

CONCLUSIONS

-RapIFluor-MS™ labeling chemistry enhances both FLR and MS signals for N-Glycan analysis: 10x for FLR, and 100x for MS, compared to the ZAB label.

-Waters and NIBRT are developing a new scientific library specifically for RapIFluor-MS™ labeled glycans. This new library will be used for automated glycan assignment based on the HILIC-UPLC retention time (in GU) and accurate mass measurement. We will work on generating GU values for RapIFluor tagged N-glycans from variety of therapeutic proteins.

References:
1. Waters Application Note: 720004856en, T.Q. Yu, "Novel Glycan Characterization and Profiling: Combining the Power of Accurate Mass, Reference Standard, and UPLC Software to Confine Glycan Assignments".
2. Hsieh, L.; Karki, C. M.; Davis, R. A.; Rudd, P. H. Methods Mol. Biol. 2006, 947, 123-43.

WCBP 2015 Poster P-115-T

Developing a Scientific Library for UPLC/FLR/MS Analysis of Released N-glycans Labeled with a Novel Labeling Reagent

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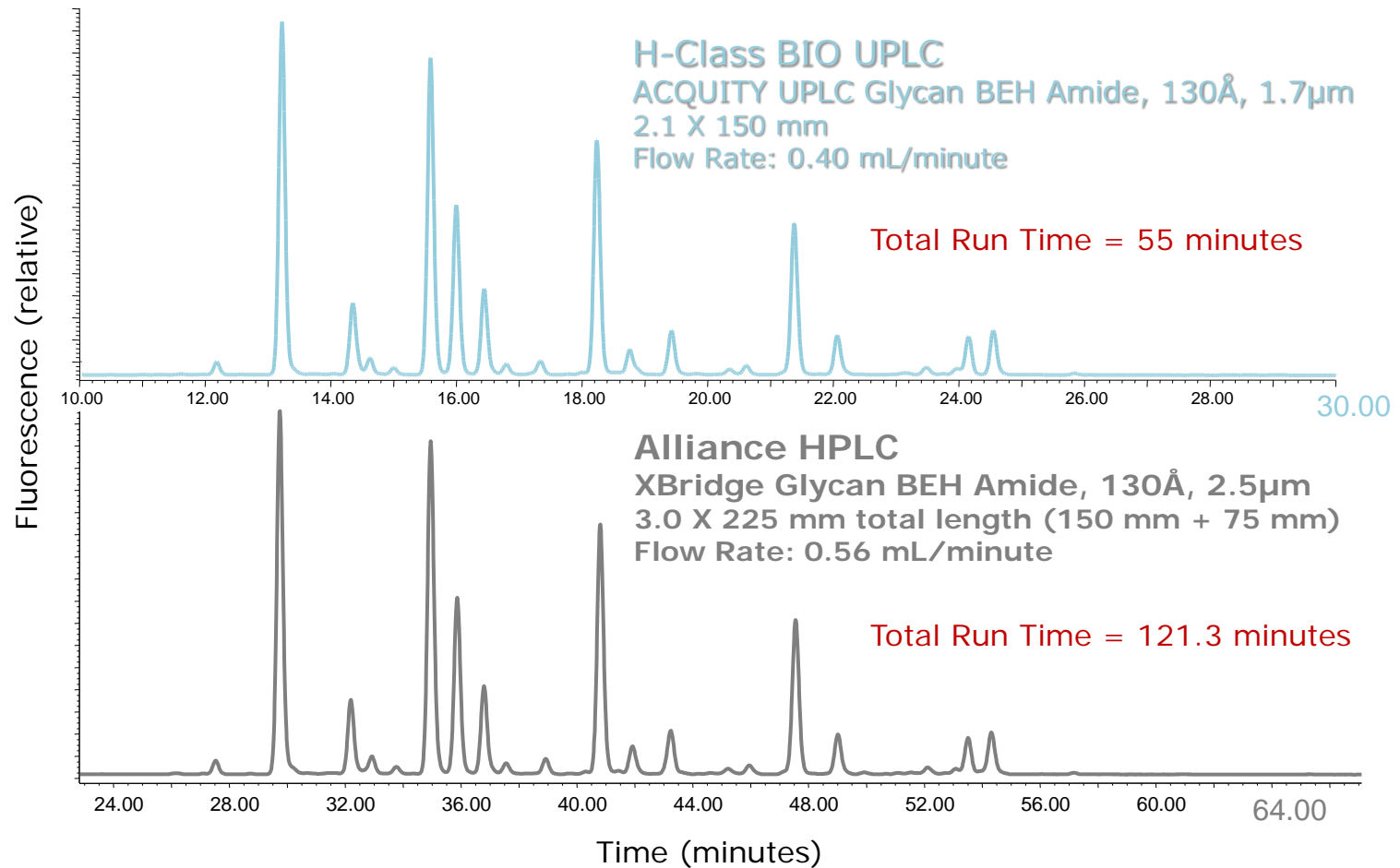


RapiFluor-MS:
Enhanced Workflows
for Glycan Monitoring

Transferability between UPLC® and HPLC Waters

RapiFluor-MS™ Labeled Glycans

THE SCIENCE OF WHAT'S POSSIBLE.®



- Comparable sensitivity and resolution – 3x sample load / 2x increase in time
- Transfer between labs with different LC equipment capabilities

Glycan Monitoring

Waters
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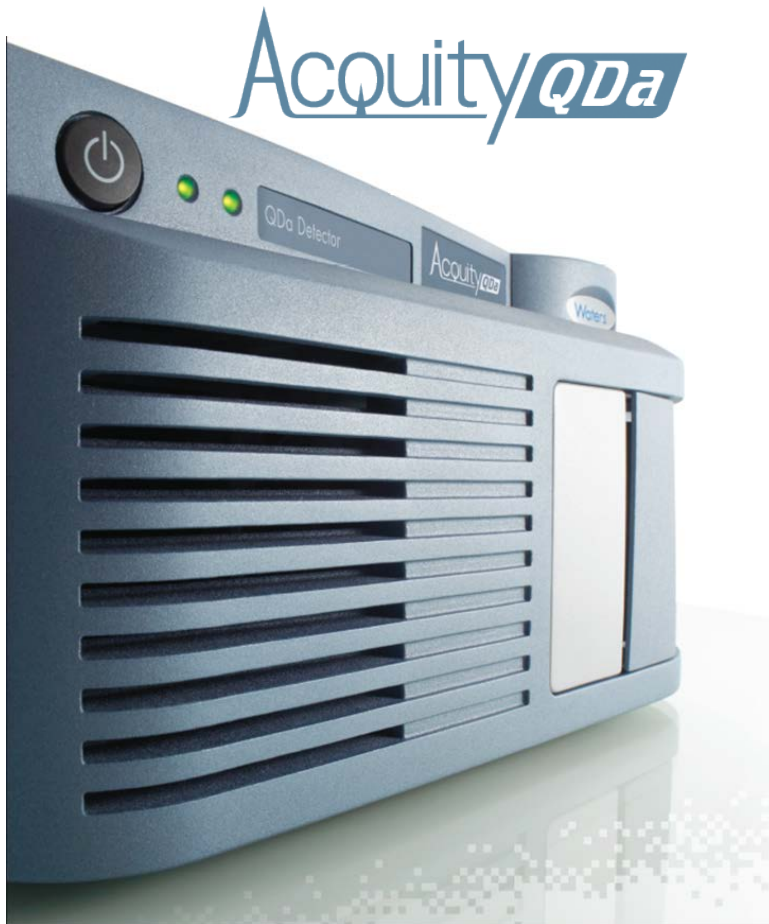
- ACQUITY UPLC® H-Class Bio System
- ACQUITY UPLC Column Manager
- ACQUITY UPLC FLR Detector
- ACQUITY® QDa® Mass Detector
- Empower® or MassLynx® Informatics
- GlycoWorks™ *RapiFluor-MS*™ N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column



ACQUITY® QDa® Mass Detector

A breakthrough product with mass appeal

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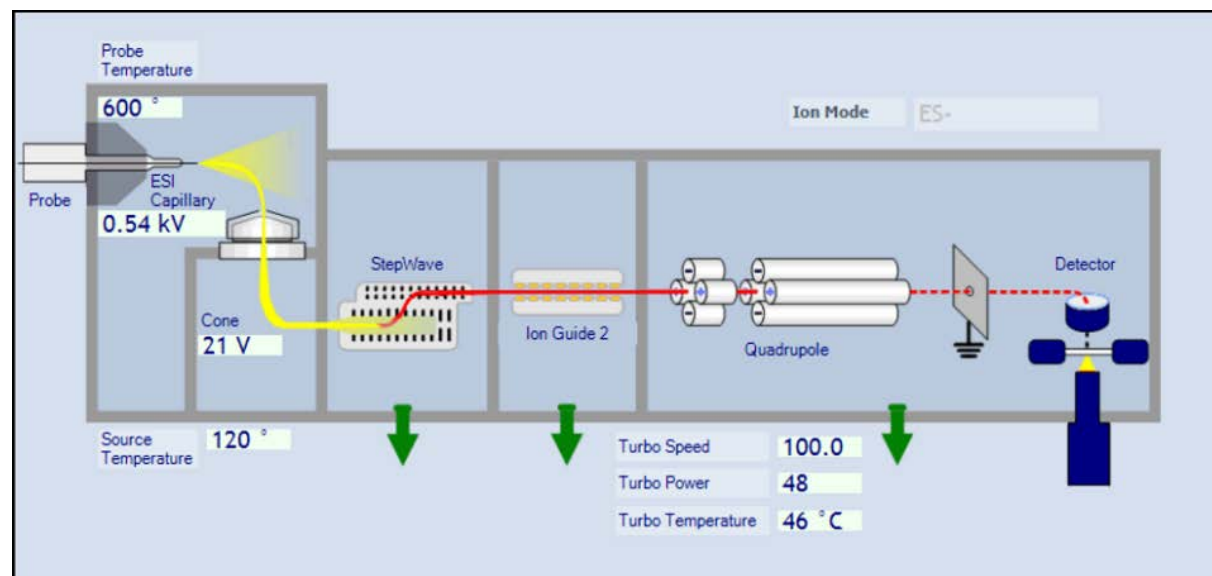


- Revolutionary innovative design focused on ease of use for analysts
- Empowering analytical chemists everywhere with orthogonal mass detection – added information with every sample
- Compact, robust and affordable: Built for constant use with a wide variety of chromatographic conditions
- Seamlessly integrates with Empower based HPLC & UPLC®

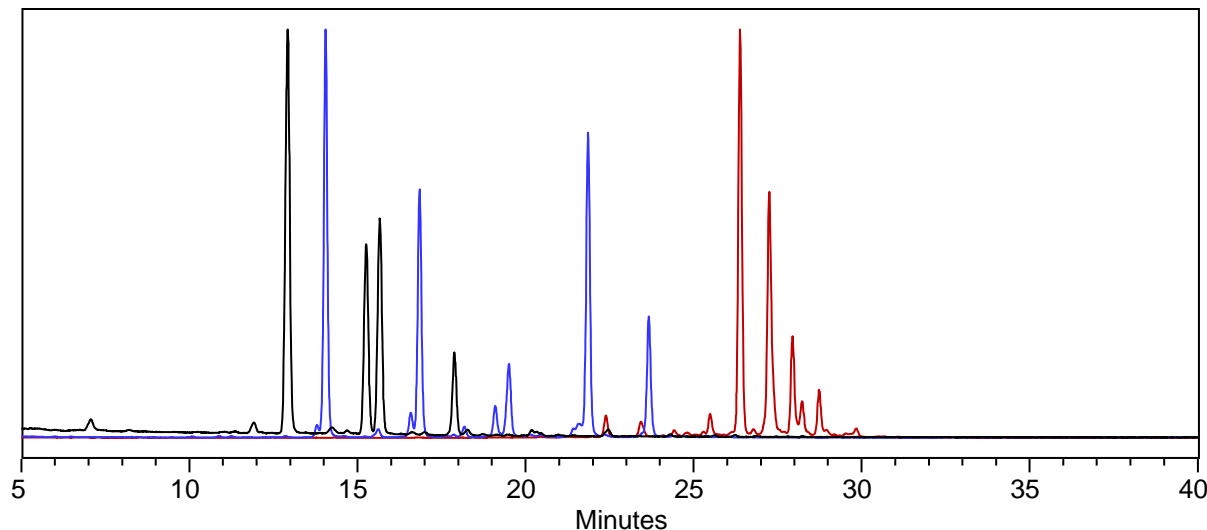
Automated Start Up Provides Robust, Reproducible Performance

- **Automated resolution and calibration** occurs with each start-up ensuring mass information is accurate and precise
- **ESI interface optimized** for UPLC[®] performance to ensure chromatographic resolution, sensitivity and throughput is preserved
- **Disposable** sample aperture and capillary for easy maintenance

Graphic QDa[®] monitor display enables easy viewing and adjustment of system parameters



Routine N-Glycan Detection with Comparable FLR and MS response



Detection across a broad range of glycoforms:

IgG

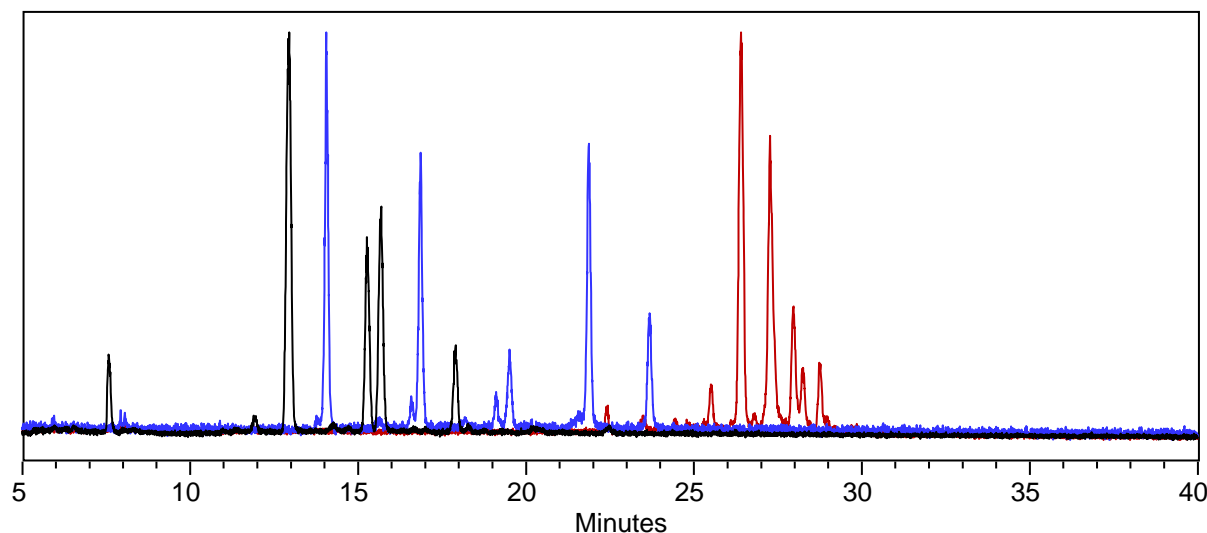
Simple bi-antennery structures

RNase B

High mannose structures

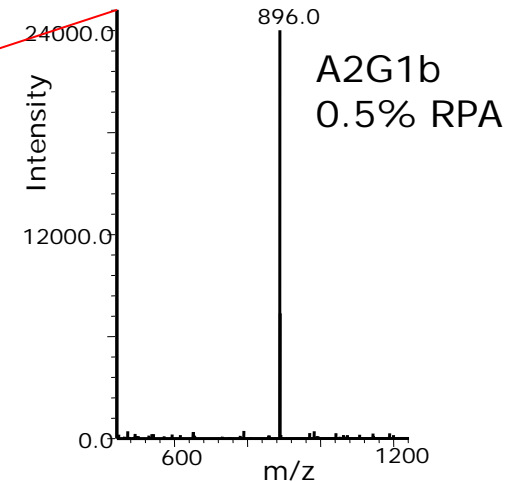
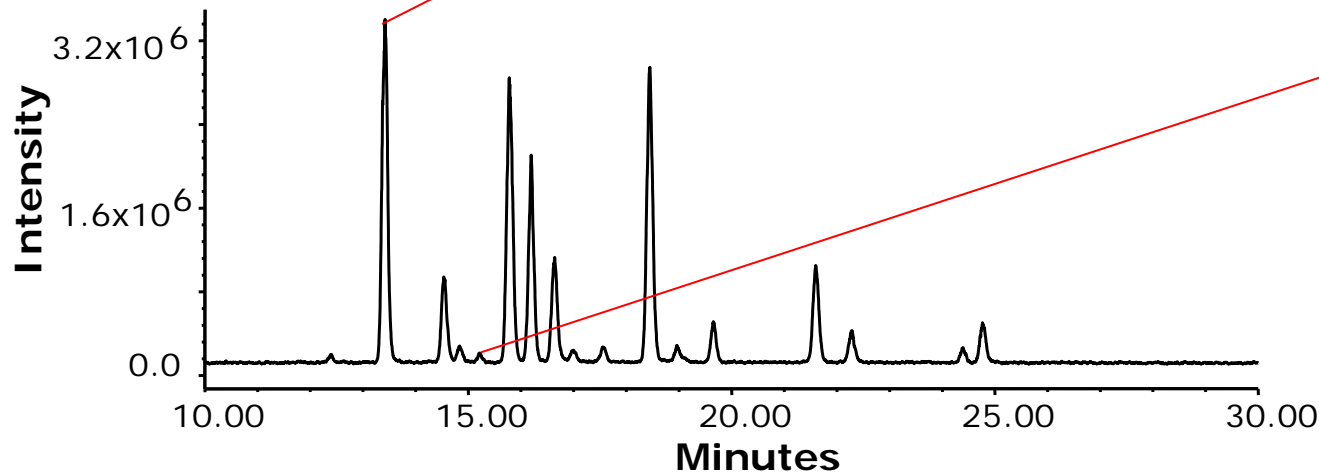
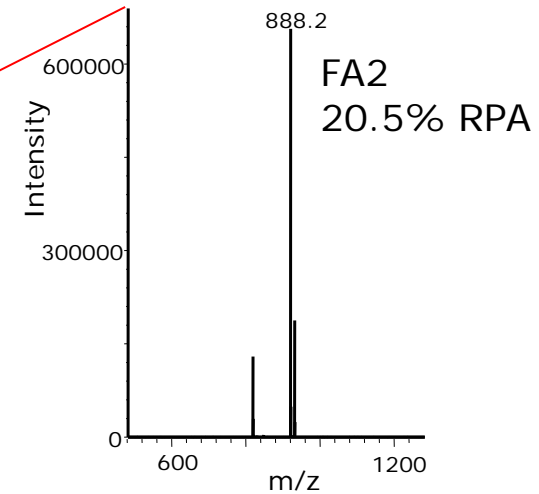
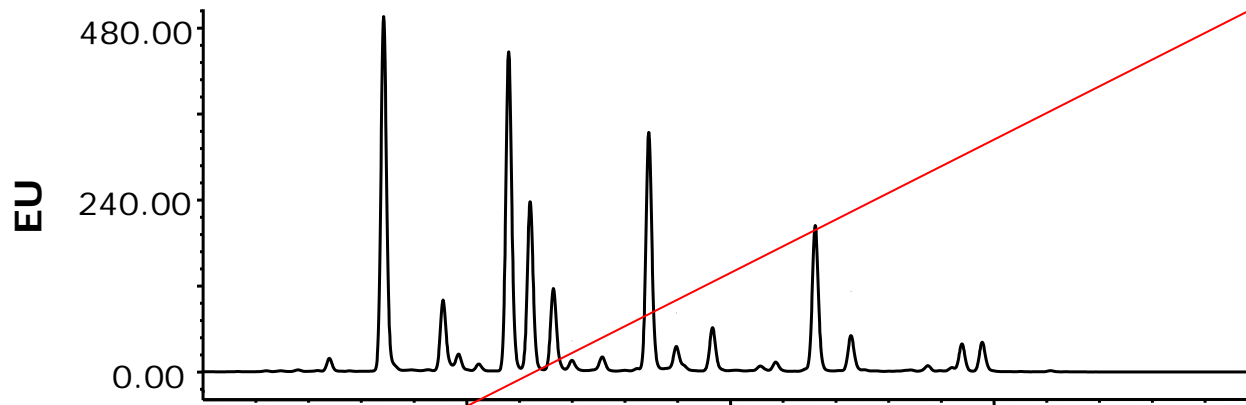
Fetuin

Large, complex structures



IgG Glycan Profile and Structure Confirmation Using ACQUITY® QDa®

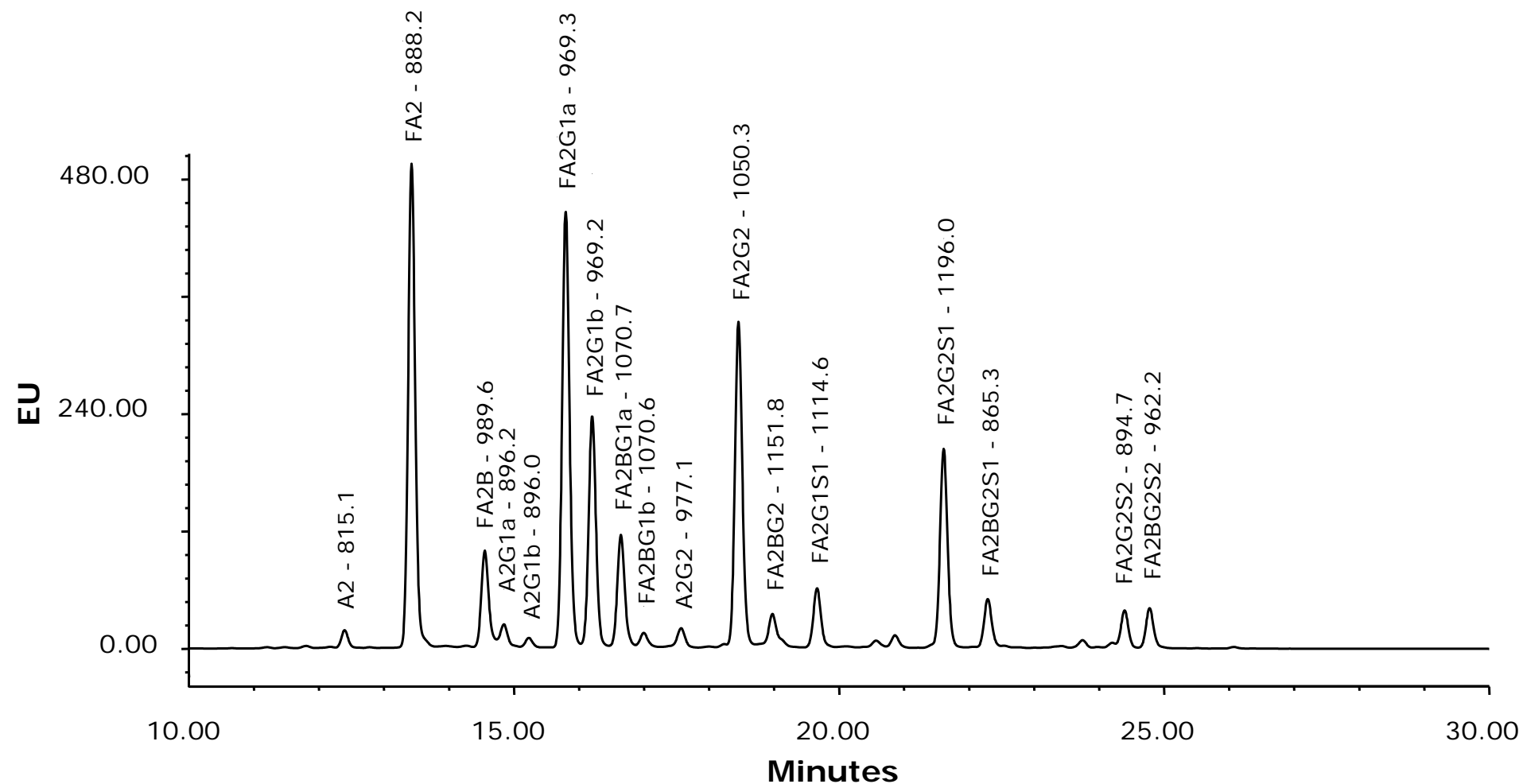
Waters
THE SCIENCE OF WHAT'S POSSIBLE.®



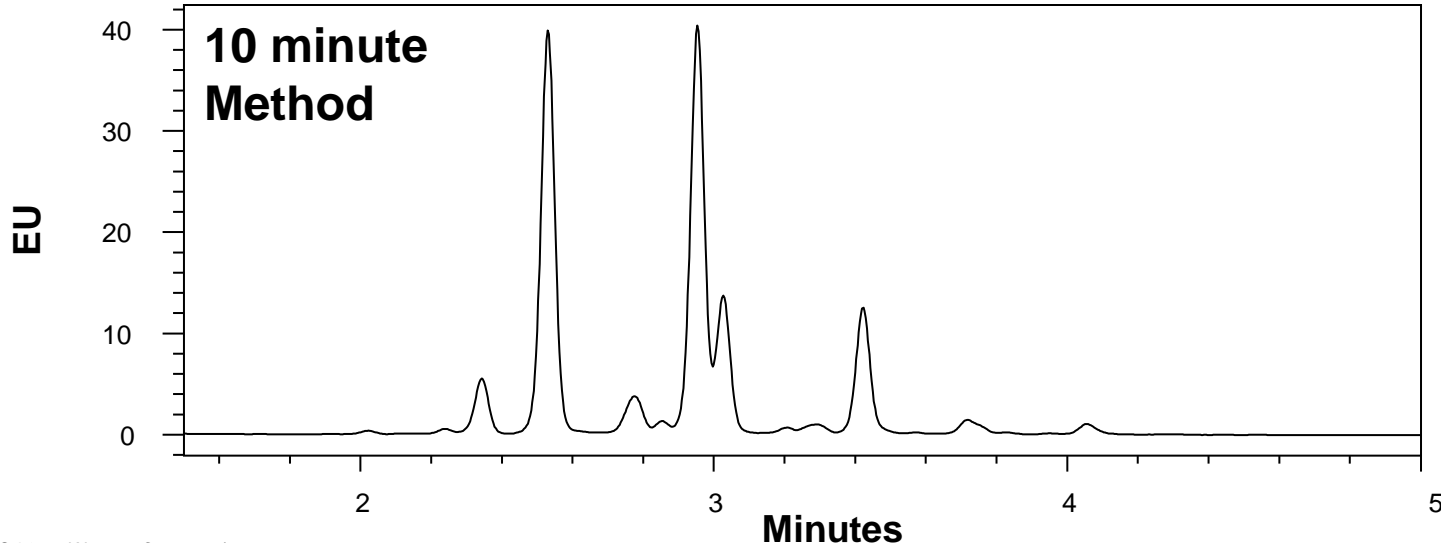
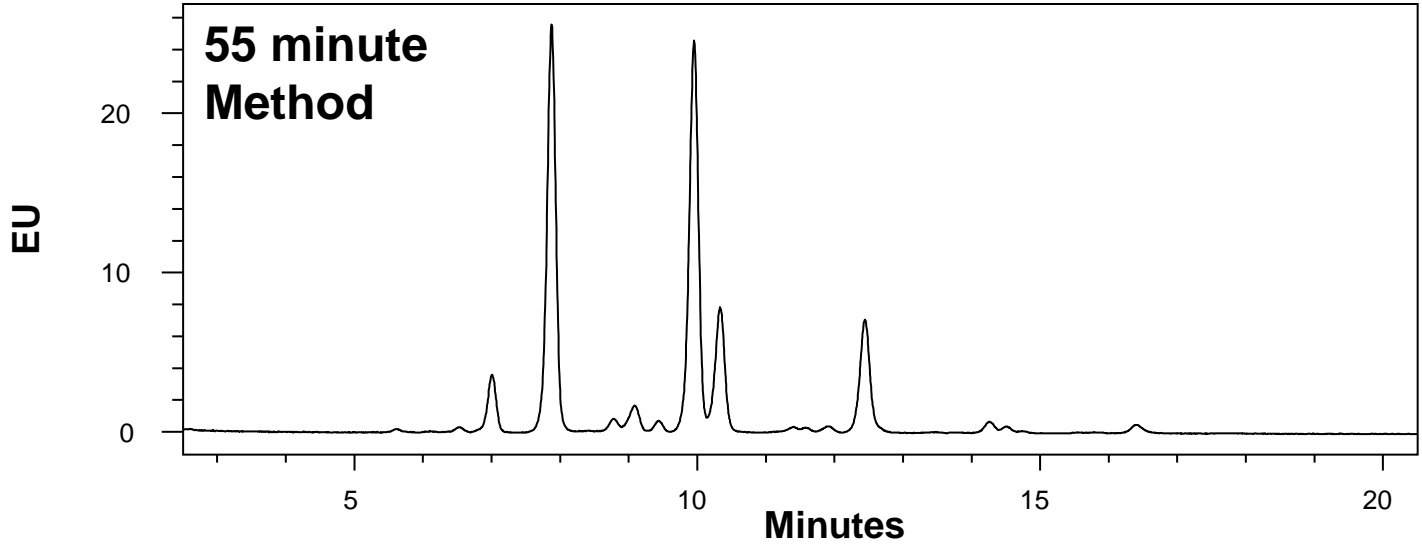
Key take away: Large dynamic range – can clearly see most abundant and least abundant glycoforms.

Intuitive GMP compliant Reporting

Empower® integration enables annotation of peaks with names and m/z



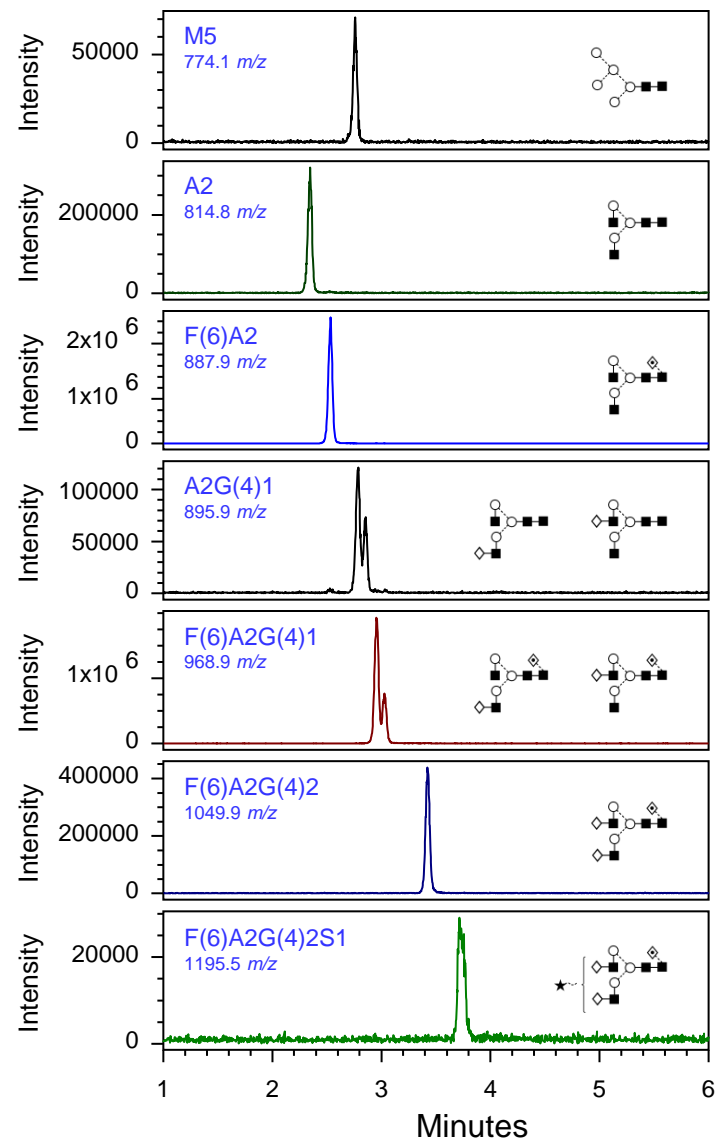
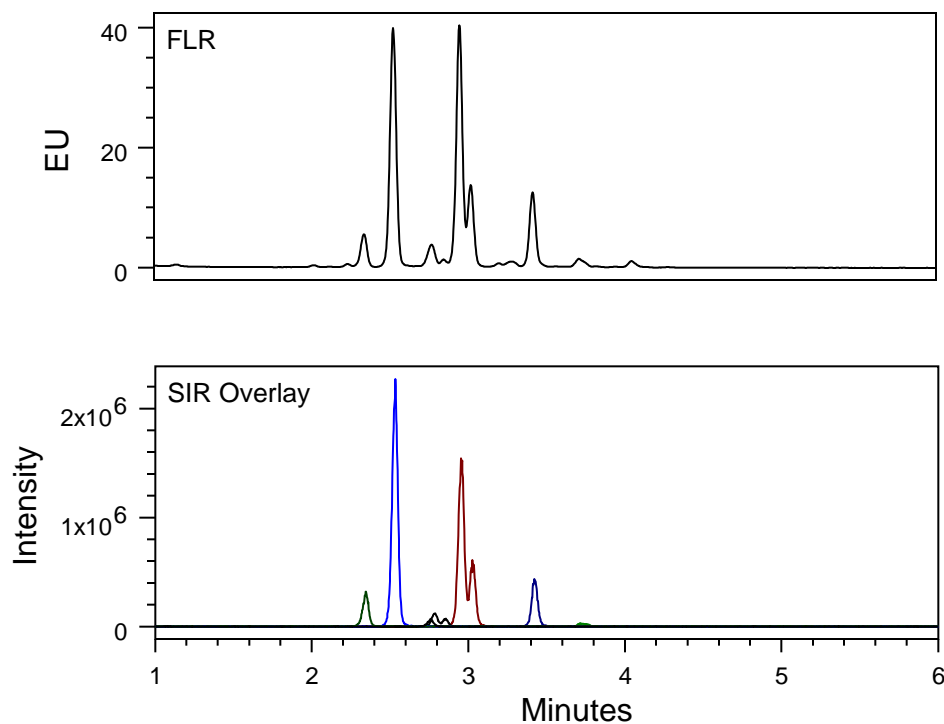
Developing a Rapid Method for Glycan Analysis



Rapid Screening Process for Development Samples

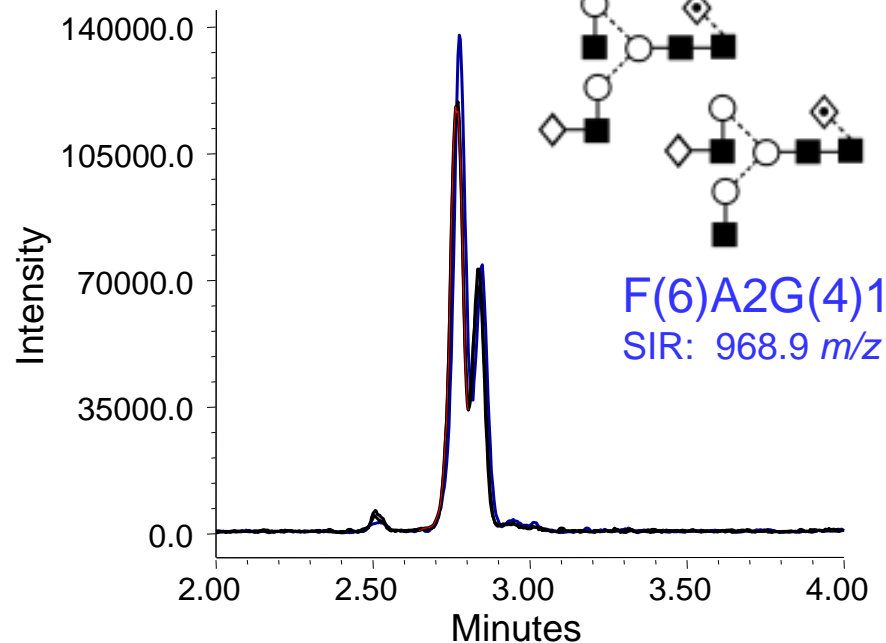
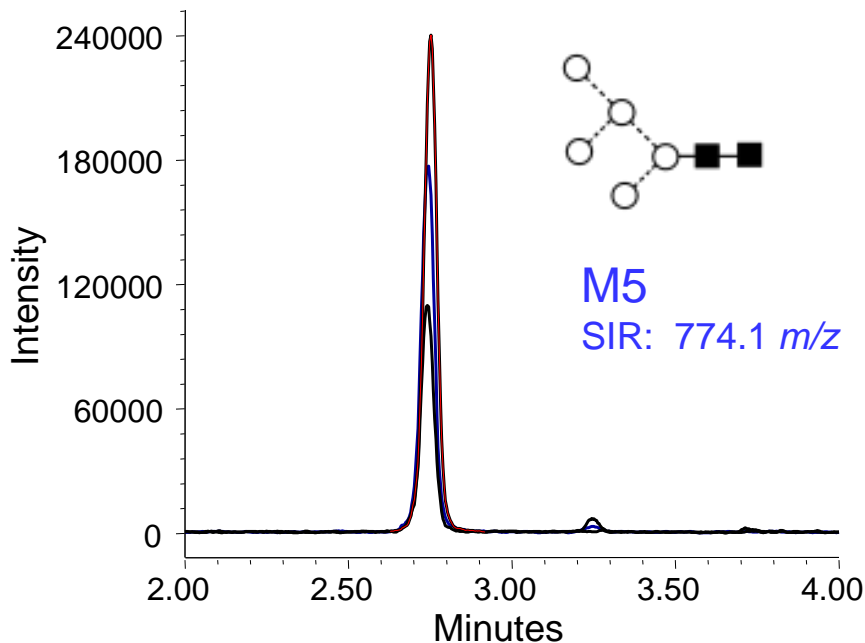
Trastuzumab N-Glycan Analysis

RapiFluor-MS™ labeled glycans: 10 minute method



Monitoring Glycan Ratios

Keeping Tabs on Mannose 5



Man5: F(6)A2G(4)1 Ratio

	Mannose 5 spiked in		
	Low	Medium	High
Inj 1	0.61	0.96	1.20
Inj 2	0.57	0.90	1.11
Inj 3	0.55	0.86	1.18
Mean	0.58	0.91	1.16
StDev	0.03	0.05	0.04
% RSD	5.44	5.47	3.85

Released N-Glycan UPLC Analysis Workflows

SAMPLE PREP

GlycoWorks™ Kits
RapiFluor-MS™
N-Glycan Kit



Deglycosylation, Labeling
and Clean-up in 30 min

Unmatched sensitivity
for FLR and MS detection

SEPARATION

ACQUITY UPLC®
Glycan BEH
Amide Column



DETECTION & INFORMATICS



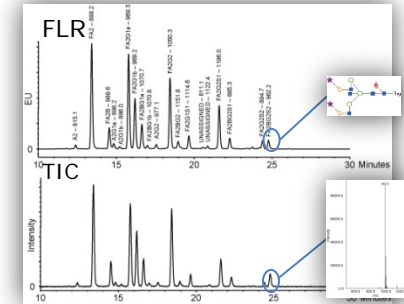
ACQUITY® FLR/QDa
and Empower® 3
Software

Glycan
Monitoring

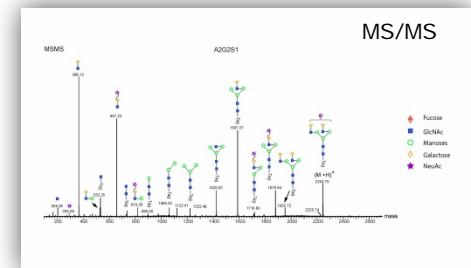
Glycan
Characterization



FLR/Xevo® G2-XS QToF MS
and UNIFI® Scientific
Information System



FLR Quantification
GU Retention
MS Confirmation



FLR Quantification
GU Retention
Accurate Mass
Confirmation
MS/MS Fragmentation

ROUTINE MONITORING OF N-GLYCANS USING A NOVEL LABELING REAGENT WITH FLUORESCENCE AND MASS DETECTION

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Ben H. Cragg, Nathan A. Laidler, Clayton H. Fox, and Sam W. McCarty
Waters Corporation, 380 Main St, Milford, MA 01850, USA

Introduction

The great variety of glycoproteins in complex biological systems is critical to the function of a protein and its ability to interact with other molecules. Using various techniques, the generation of detailed glycan profiles is important for clinical, biotechnological, and pharmaceutical research. The increasing number of glycoproteins in complex systems, such as those used in drug development, has led to a need for more sensitive and specific methods to monitor glycan profiles and the need to establish what is most appropriate.

To address these challenges, we have developed a novel labeling reagent with significantly improved fluorescence and mass spectrometry detection. The new reagent enables researchers to obtain detailed glycan profiles from complex biological systems in addition to routine glycan analysis. The novel labeling reagent leads to more precise fluorescence detection and the new profile will feature more accurate mass spectrometry data. These improvements allow us to monitor glycan profiles and modify glycan profiles with less time and more accuracy.

We present the use of the novel labeling reagent with fluorescence and mass spectrometry for monitoring glycan profiles in complex biological systems. The novel labeling reagent allows for improved fluorescence and mass spectrometry data for glycan profiles and the ability to monitor glycan profiles in complex biological systems. The novel labeling reagent allows for improved fluorescence and mass spectrometry data for glycan profiles and the ability to monitor glycan profiles in complex biological systems.

RESULTS

FLR and QDs for Routine Identification of N-Glycans

Fluorescence labeling of glycans is made possible by the use of a novel labeling reagent with improved fluorescence and mass spectrometry detection. The new reagent enables researchers to obtain detailed glycan profiles from complex biological systems in addition to routine glycan analysis. The novel labeling reagent leads to more precise fluorescence detection and the new profile will feature more accurate mass spectrometry data. These improvements allow us to monitor glycan profiles and modify glycan profiles with less time and more accuracy.

UPLC-FLR-QDs Workflow Simplifies High-Throughput Glycan Monitoring

Analysis of glycan profiles in a single glycan profile allows for improved fluorescence and mass spectrometry detection. The new reagent enables researchers to obtain detailed glycan profiles from complex biological systems in addition to routine glycan analysis. The novel labeling reagent leads to more precise fluorescence detection and the new profile will feature more accurate mass spectrometry data. These improvements allow us to monitor glycan profiles and modify glycan profiles with less time and more accuracy.

Summary

- High-resolution glycan analysis is made possible by the use of a novel labeling reagent with improved fluorescence and mass spectrometry detection.
- The new reagent enables researchers to obtain detailed glycan profiles from complex biological systems in addition to routine glycan analysis.
- The novel labeling reagent leads to more precise fluorescence detection and the new profile will feature more accurate mass spectrometry data.
- These improvements allow us to monitor glycan profiles and modify glycan profiles with less time and more accuracy.

Methods

Sample Preparation

High-Resolution Glycan Analysis

Sample	Time (min)	Intensity
1	10	100
2	20	200
3	30	300
4	40	400
5	50	500
6	60	600
7	70	700
8	80	800
9	90	900
10	100	1000
11	110	1100
12	120	1200

FastFlow-MS™ Glycans Detected by the QDs

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Routine Monitoring of N-Glycans Using a Novel Labeling Reagent with Fluorescence and Mass Detection

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RapiFluor-MS N-Glycan Labeling:
A breakthrough technology for
released glycan LC and MS analysis