The Road to Glycan Analysis Without Compromise

WCBP 2015

Waters Technical Seminar

Jan 27, 2015 Washington, DC

Today's Agenda

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

  Matthew A. Lauber (Consumables Business Unit, Waters)
- ***Rapi*Fluor-MS Technology & Glycan Characterization**

  Ying Qing Yu (Biopharmaceutical Sciences, Waters)
- **Impact of *Rapi*Fluor-MS Technology on Released Glycan Profile Monitoring**

  Sean M. McCarthy (Biopharmaceutical Sciences, Waters)
- Scientific Panel – Questions & Discussion

Glycosylation of Biotherapeutics

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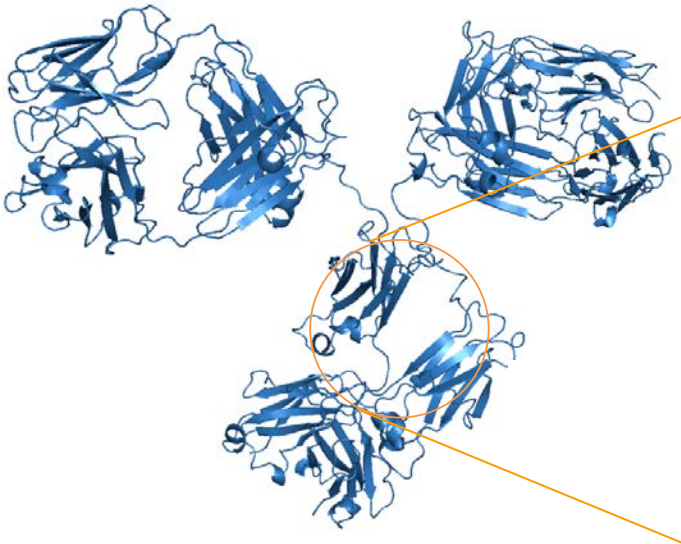

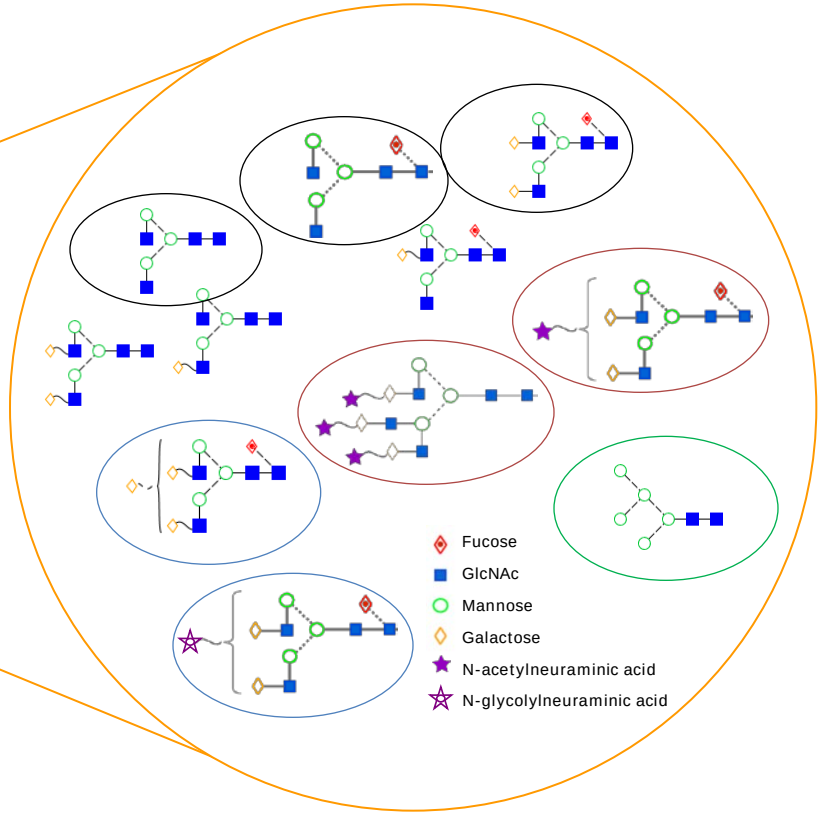

Effector Functions (ADCC/CDC)
(fucosylation/galactosylation)

Low Half Life
(high mannose)

Anti-Inflammatory
(sialylation)

Immunogenic
(αGal / N-glycolylneuraminic acid)

Overall profile sensitive to
manufacturing conditions

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

Anal Chem **2013**, *85* (2), 715-36.

Glycoprotein Characterization

Multiple Strategies – Complementary Information

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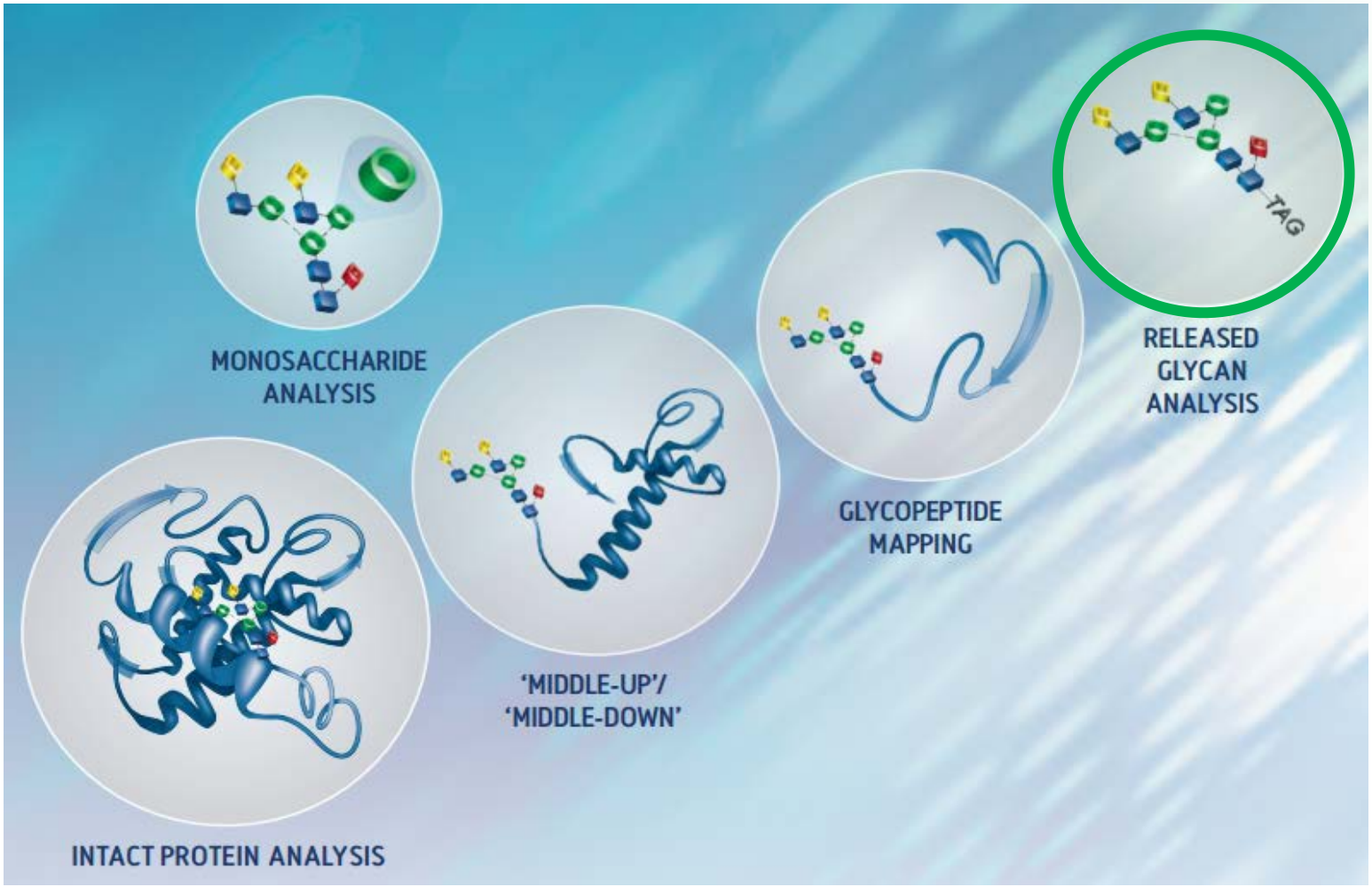

Customer voice brought us focus

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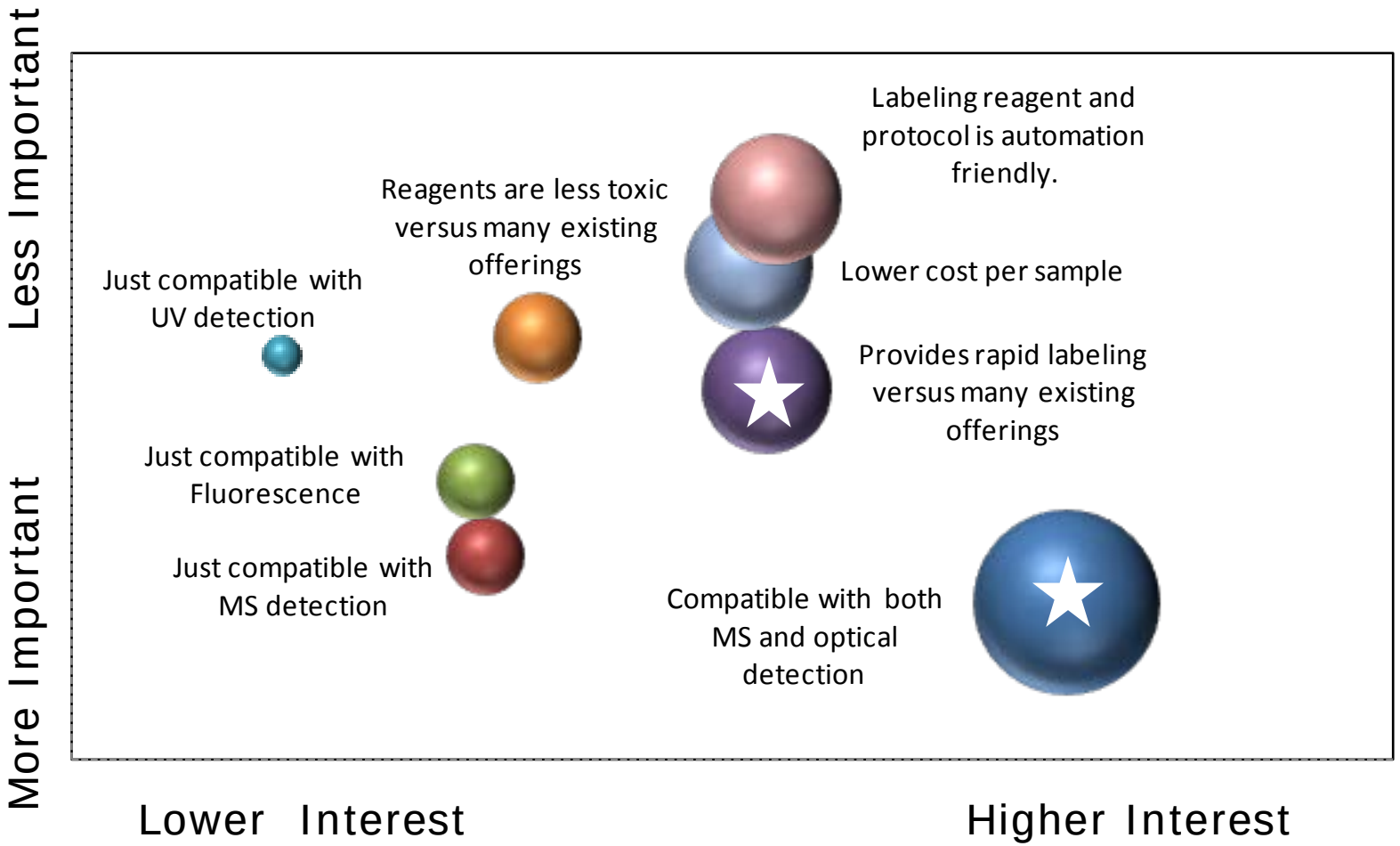

The Road to Glycan Analysis Without Compromise

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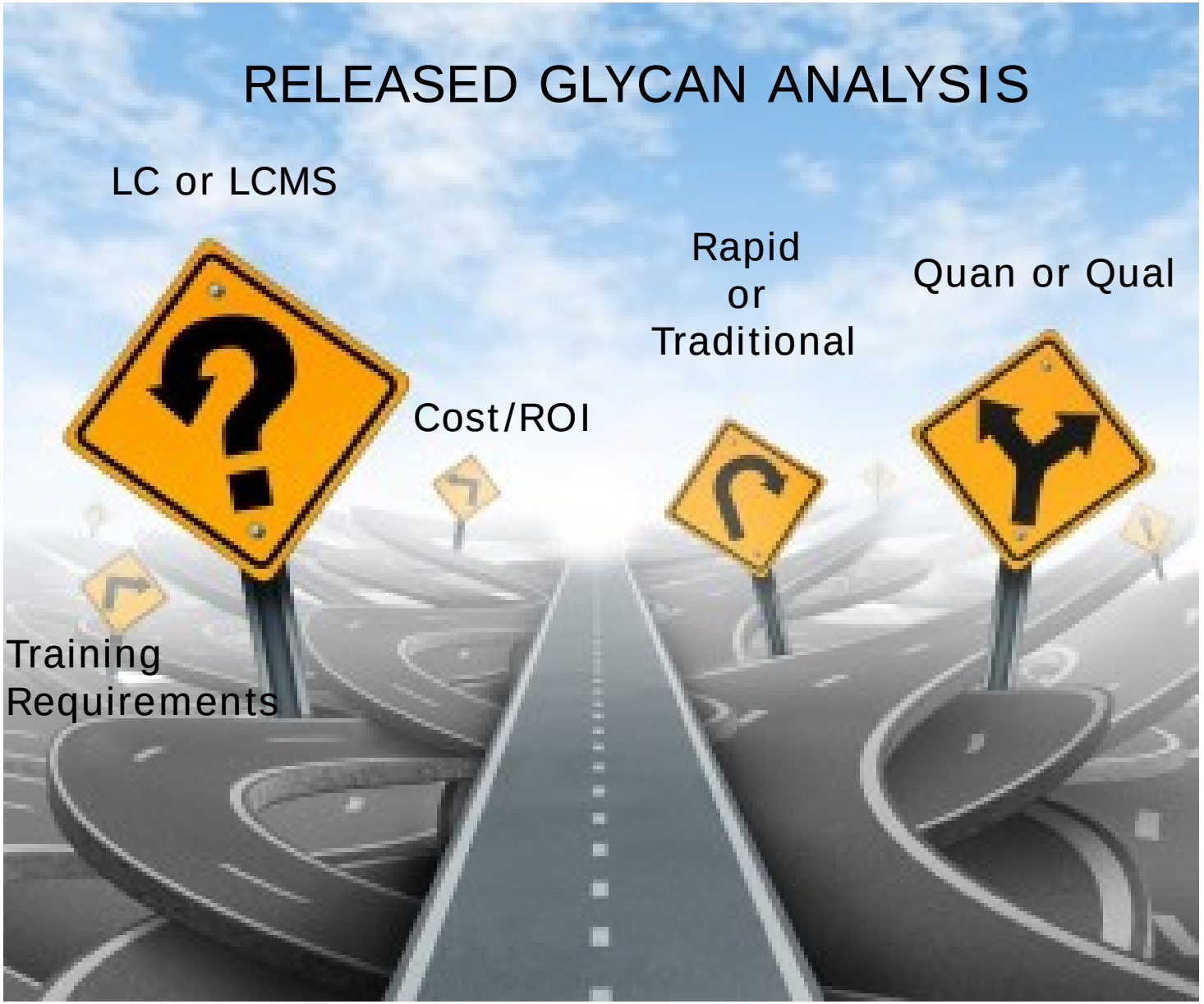

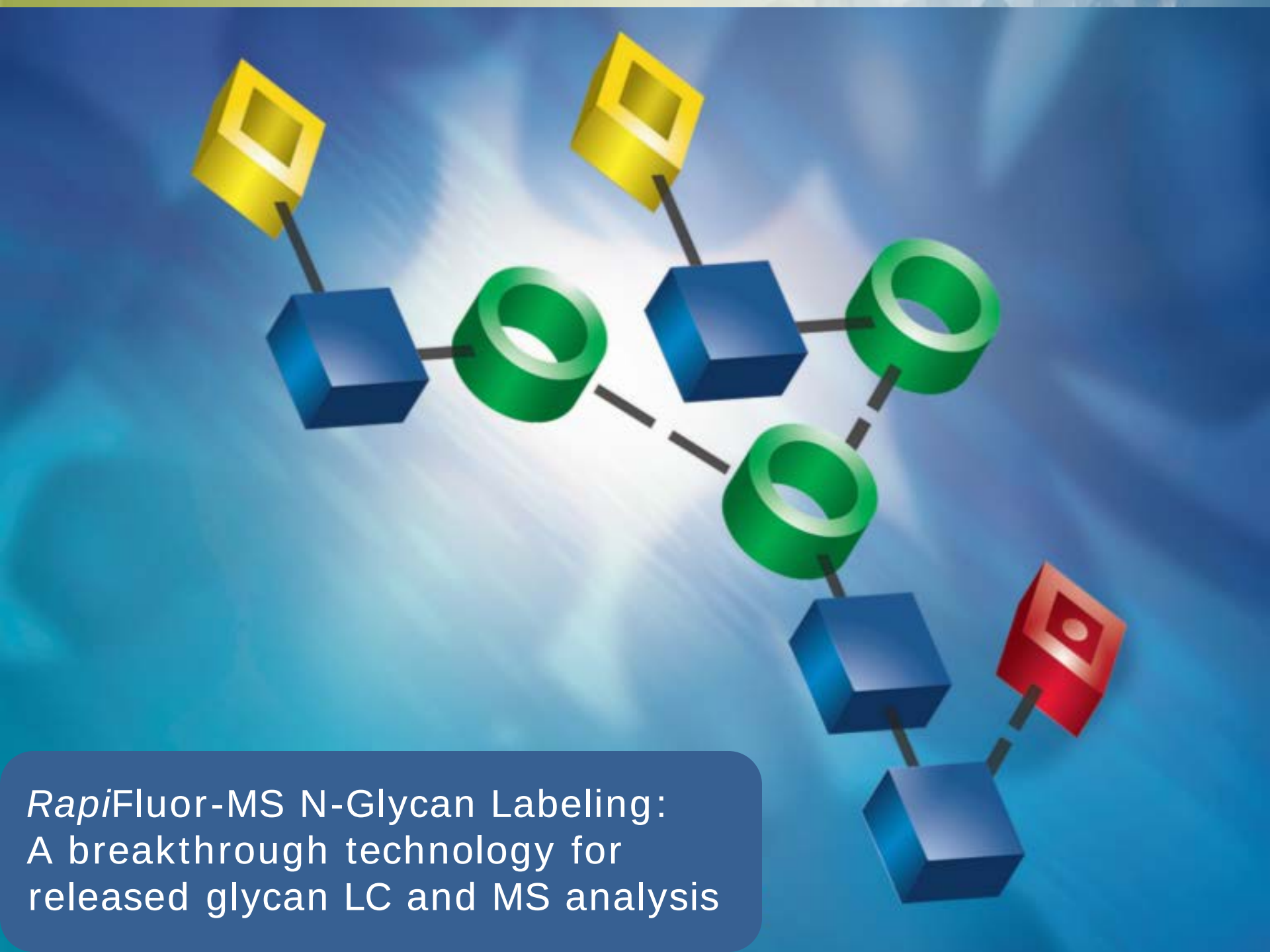

*Rapi*Fluor-MS N-Glycan Labeling: A breakthrough technology for released glycan LC and MS analysis

Released Glycan Analysis

HILIC Profiling

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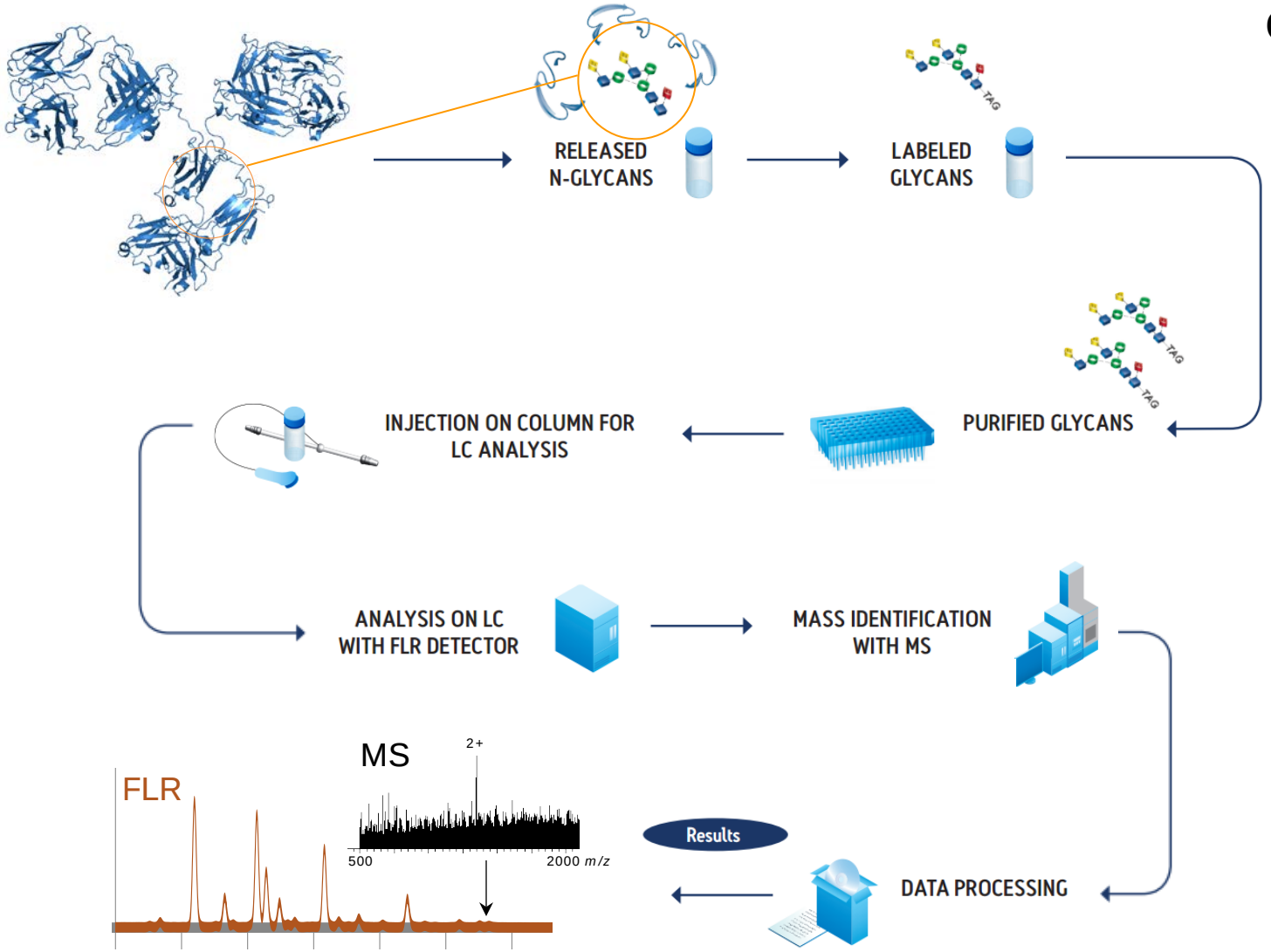
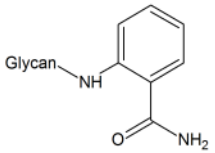


Conventional 2-AB



5 Hours to 2 Days

Out with Conventions

Reductive Amination is Laborious

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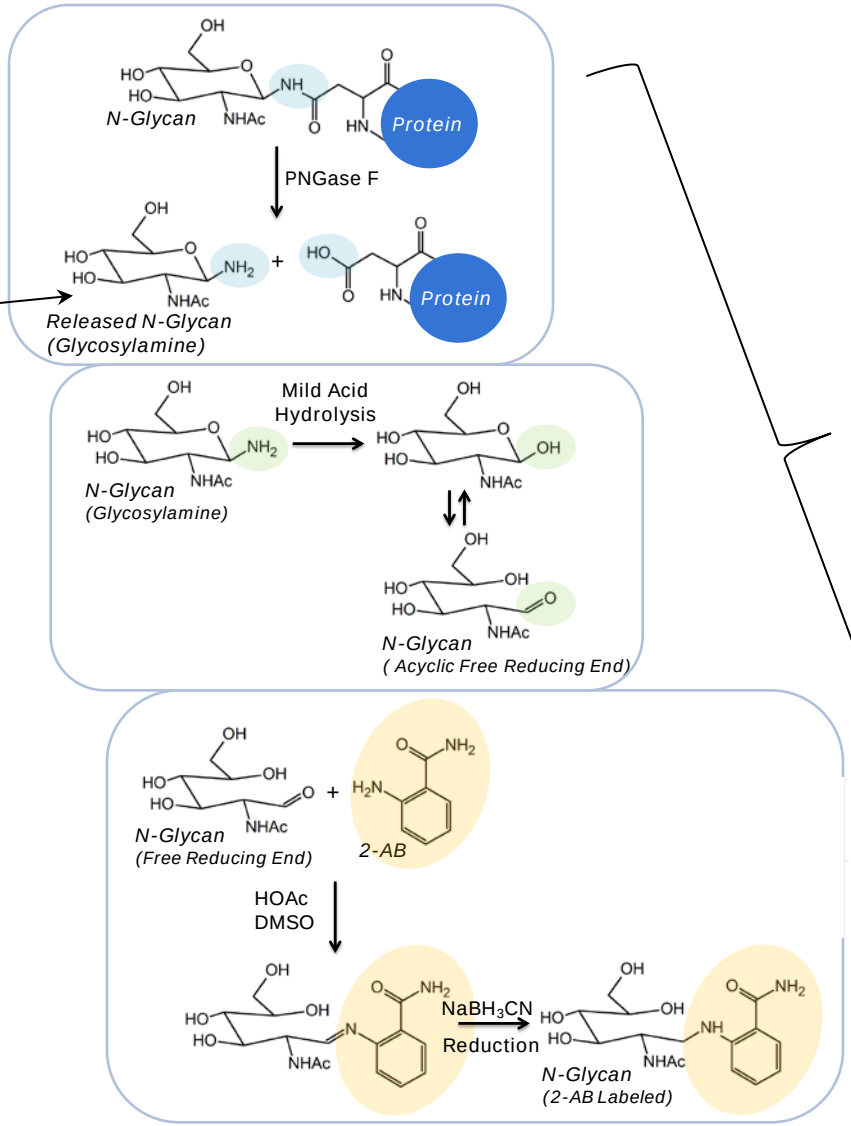
Rapid Tagging

Glycosylamine labeling circumvents these issues



Reductive Amination (*conventional*)

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

*Rapi*Fluor-MS™ Reagent

Built Upon Our Expertise

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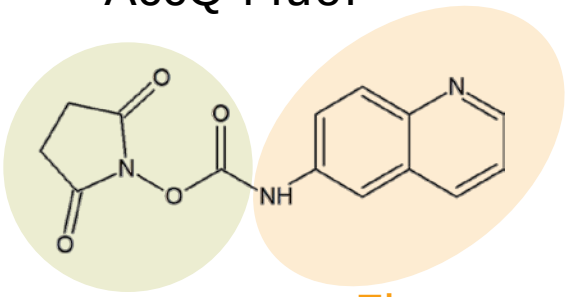
From Waters' expertise in rapid, fluorescence labeling of amino acids

Enhanced chemical properties for glycan analysis:

- Rapid Tagging
- Efficient Fluorescence
- Enhanced Ionization Efficiency

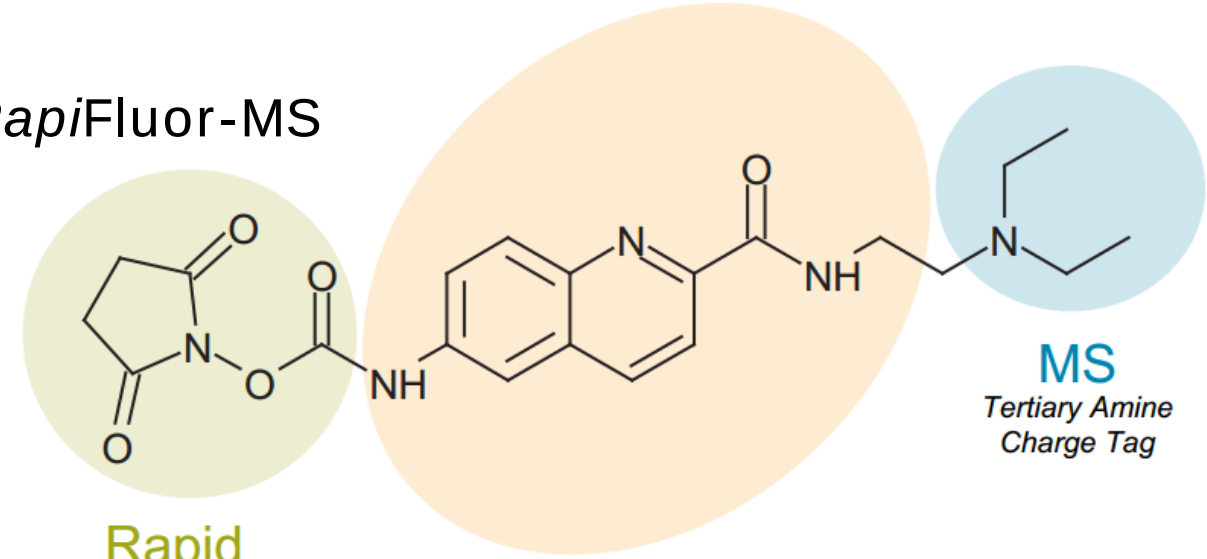
AccQ·Fluor™



Rapid

Fluorescence

*Rapi*Fluor-MS



Rapid
NHS Carbamate Rapid Tagging Group

Fluorescence
Quinolinyl Fluorophore

MS
Tertiary Amine Charge Tag

Patent Pending

*Rapi*Fluor-MS Reagent

Rapid Reaction Kinetics

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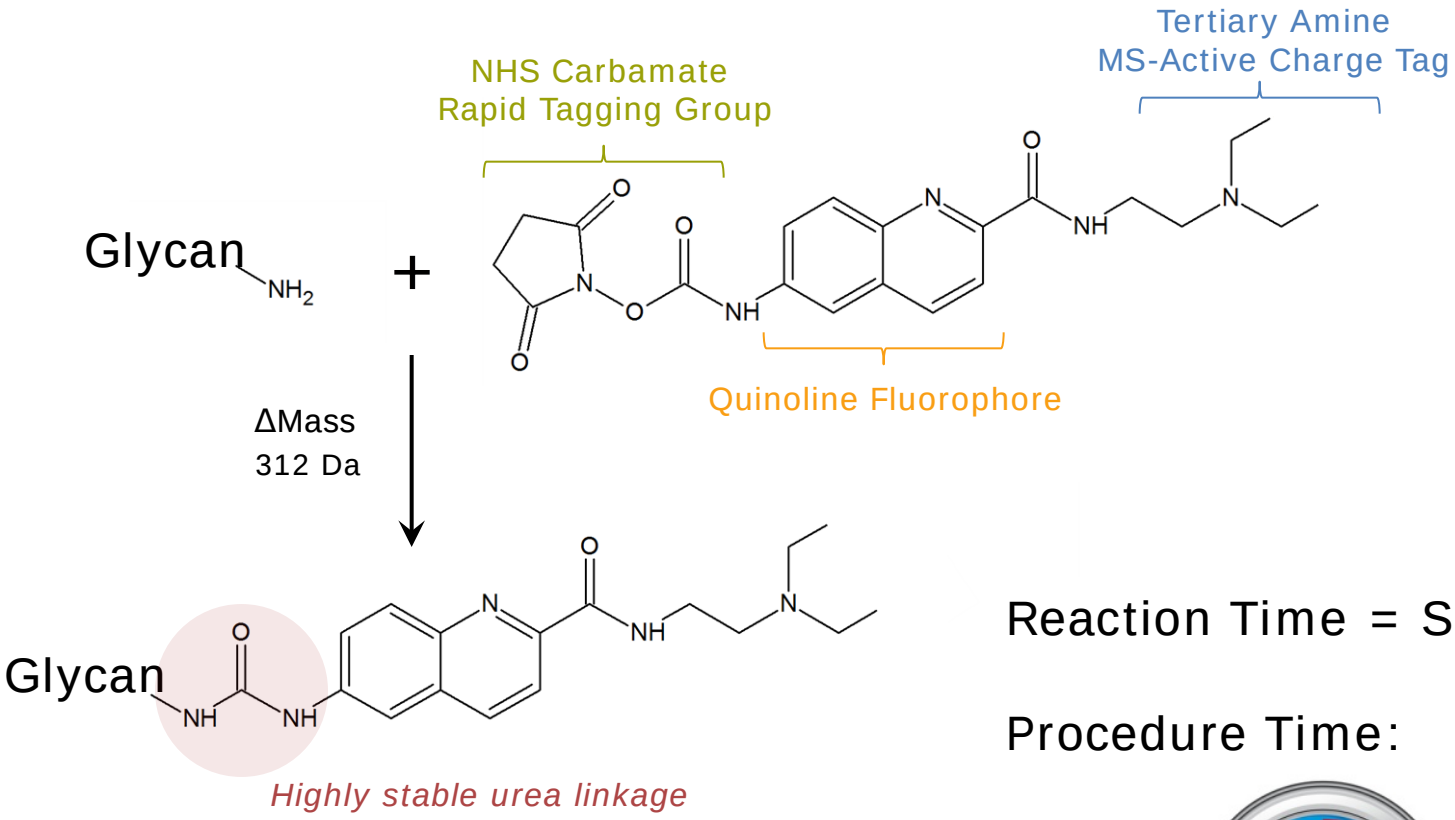

Reaction Time = Seconds

Procedure Time:

*Rapi*Fluor-MS Reagent

Sensitivity Comparison – Instant AB

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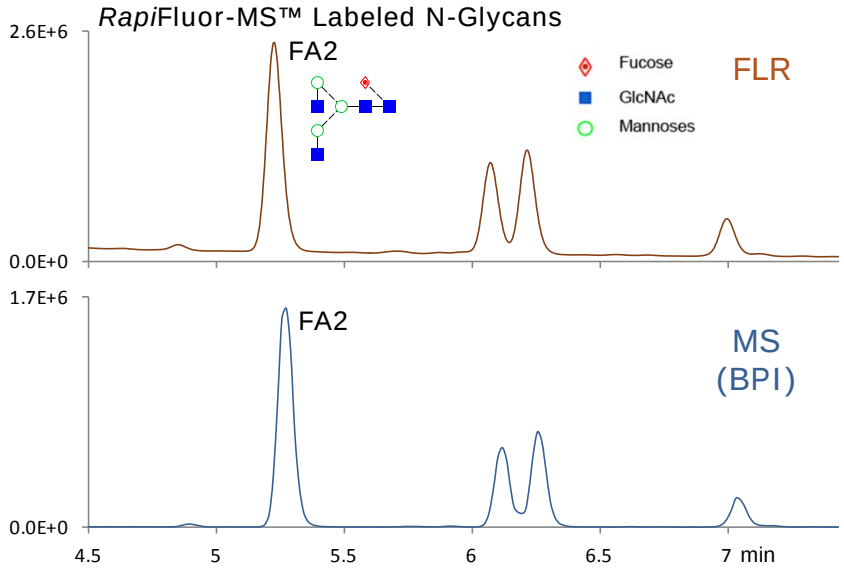
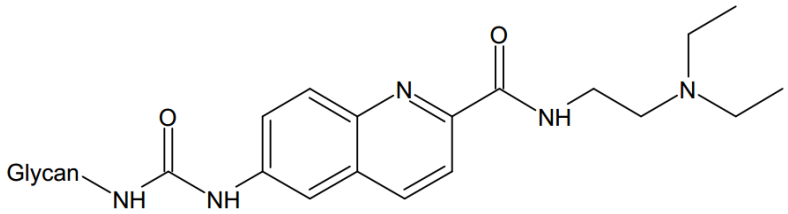
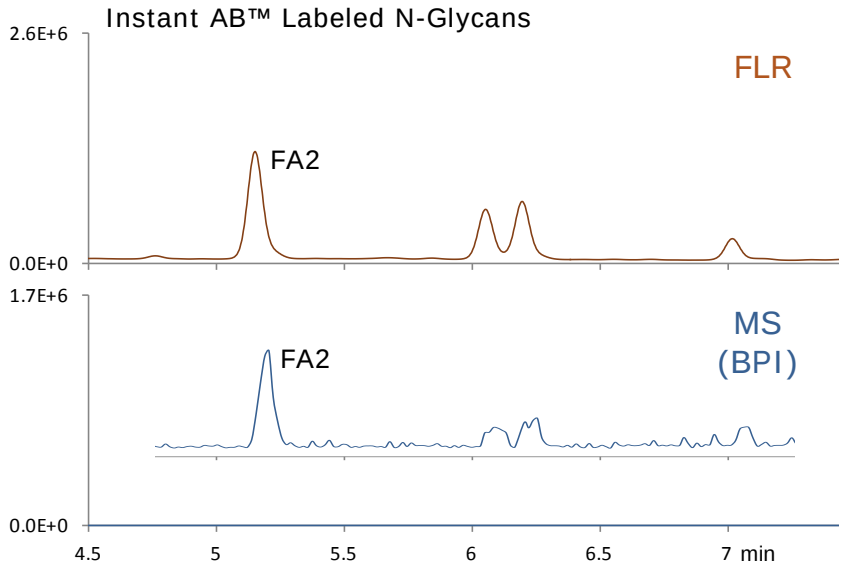


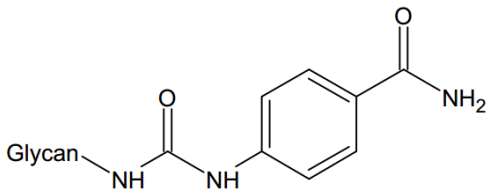
*Rapi*Fluor-MS™ Labeled N-Glycans





300X Zoom

Instant AB™ Labeled N-Glycans



Instant AB is a trademark of Prozyme Inc.

*Rapi*Fluor-MS Reagent

Sensitivity Comparison – Instant AB

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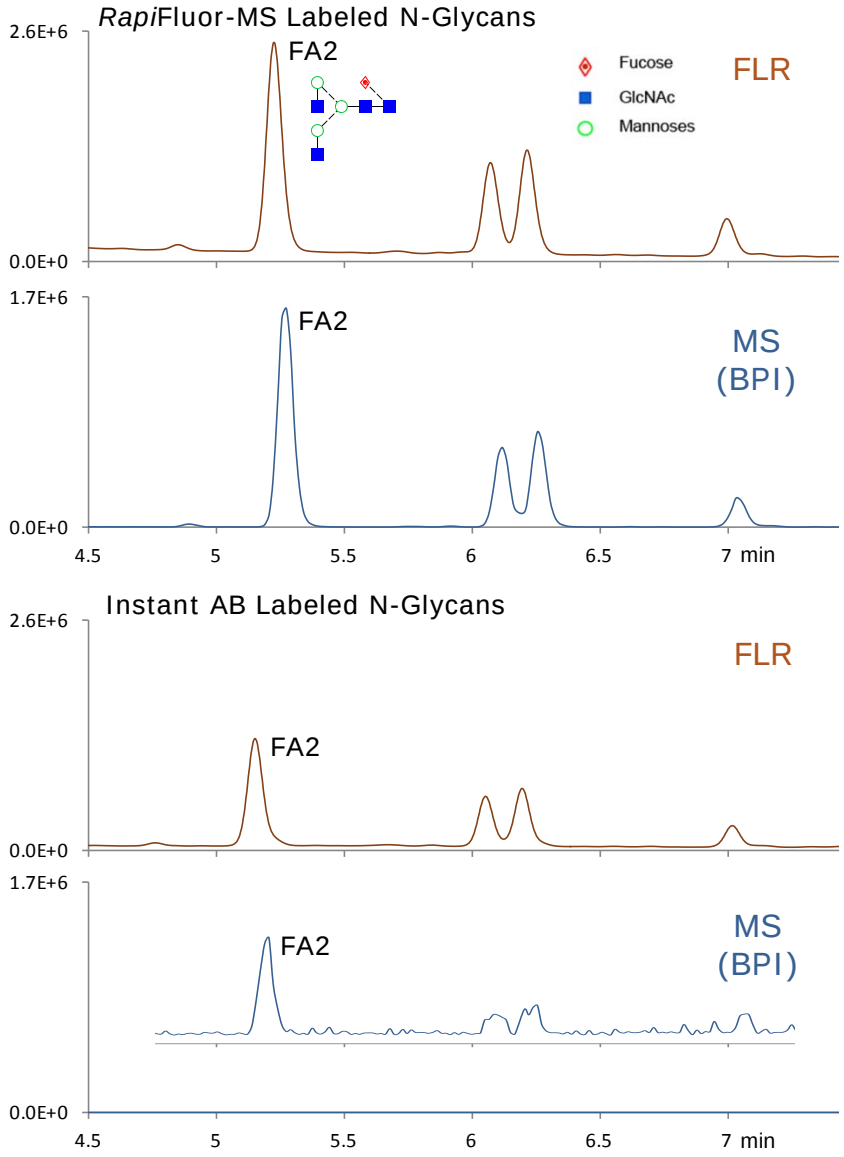

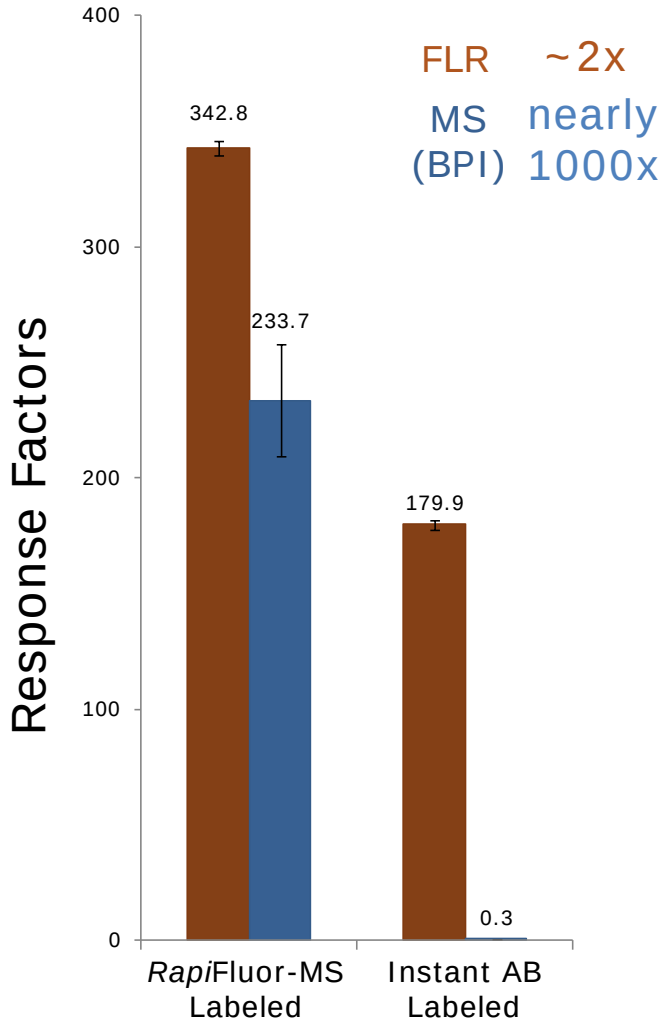

*Rapi*Fluor-MS Reagent

Sensitivity Comparison

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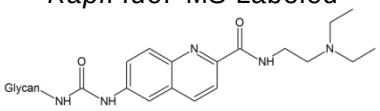
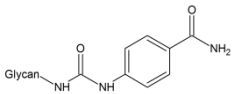
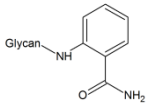
*Rapi*Fluor-MS Labeled



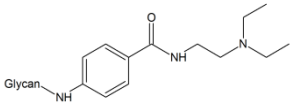
Instant AB Labeled

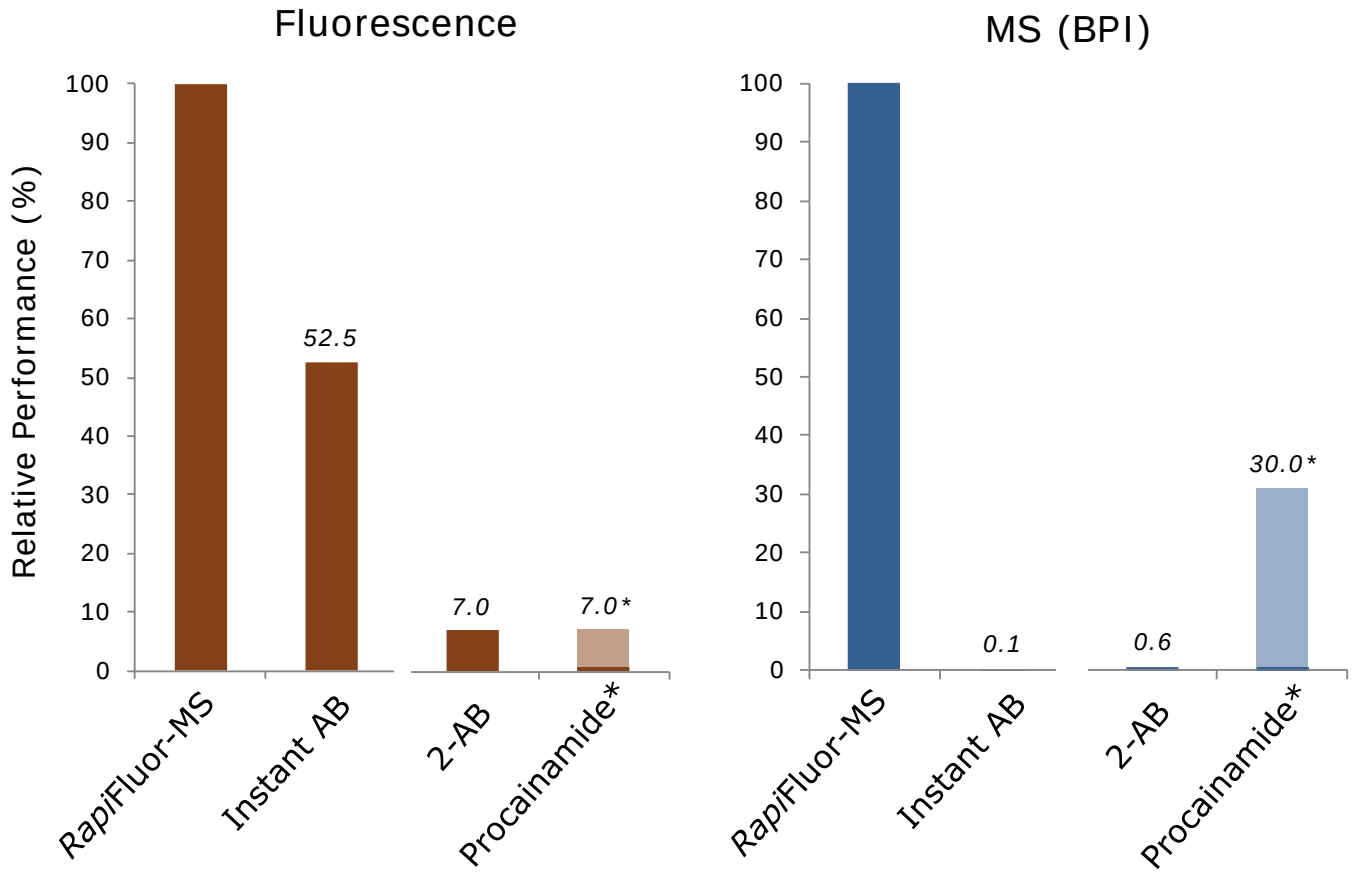


2-AB Labeled



Procainamide Labeled





(*) 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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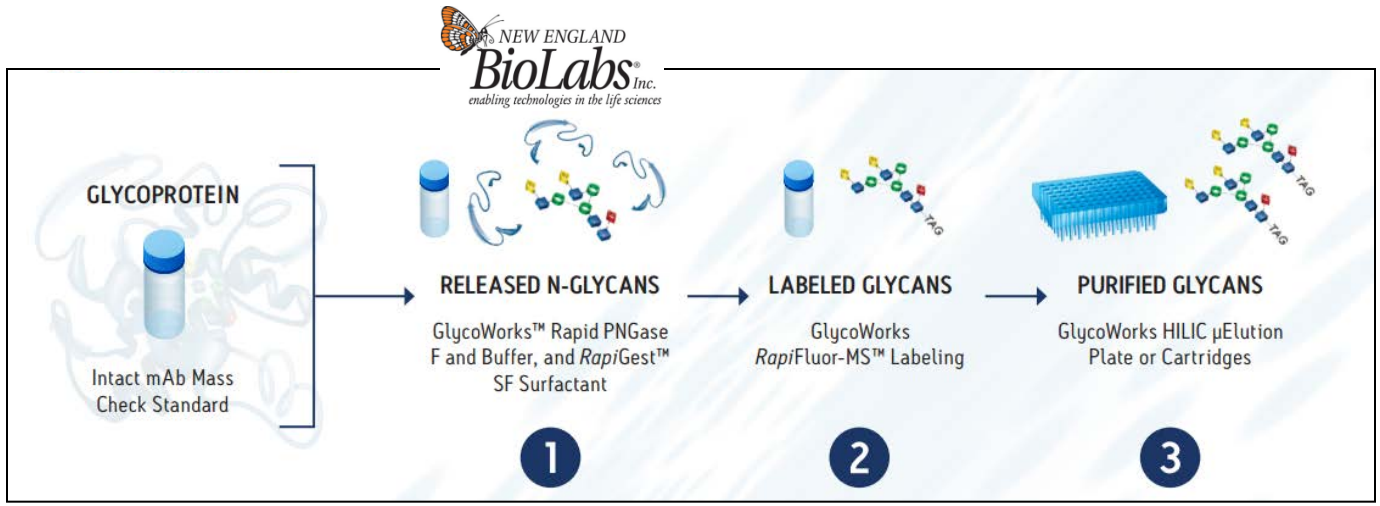
Conventional → 5 Hours to 2 Days



Direct Analysis
(Organic Solvent Dilution)

10 min *5 min* *10 min*

Total
Sample Prep Time

30 min

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

Patent Pending

Rapid Deglycosylation

RapiGest™ SF Assisted

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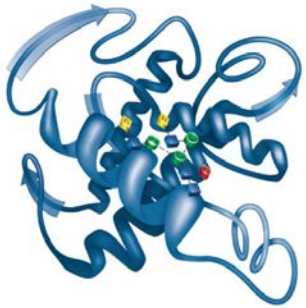
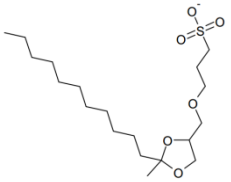
NEW ENGLAND BioLabs Inc.
enabling technologies in the life sciences

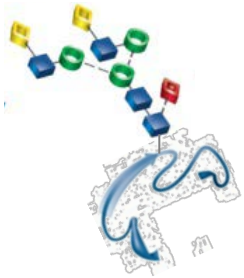


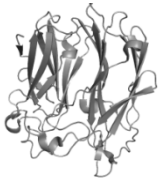


1% RapiGest SF Surfactant

GlycoWorks Rapid Buffer

2 min
≥80°C





GlycoWorks Rapid PNGase F

Enzymatic Deglycosylation

5 min
50°C

Rapid Deglycosylation

RapiGest™ SF Assisted

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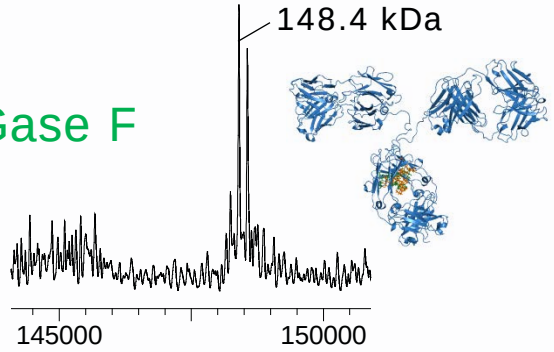
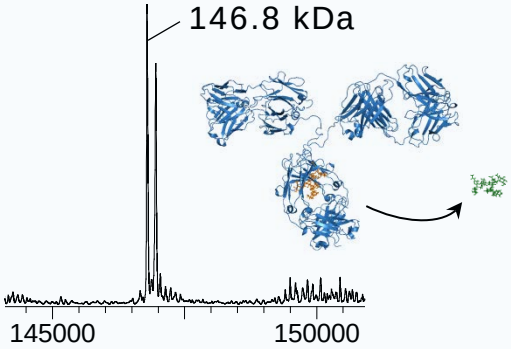
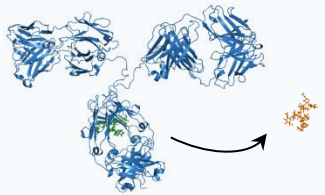
No PNGase F
(control)



5 min
50°C

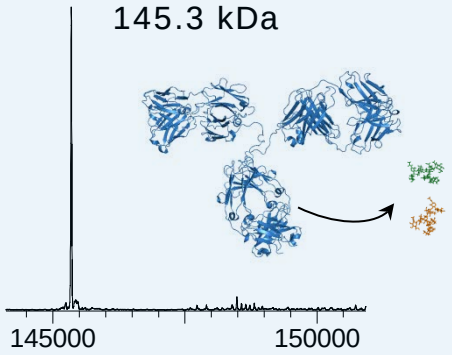




2 Step

2 min
Heat Denaturation

5 min
50°C



Robust HILIC SPE

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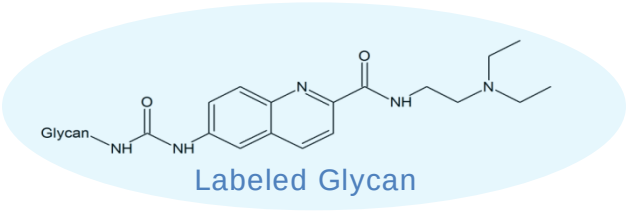
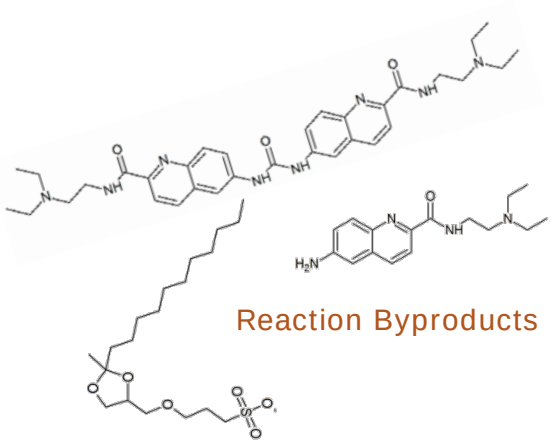
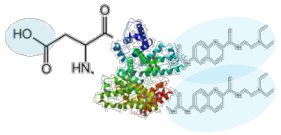


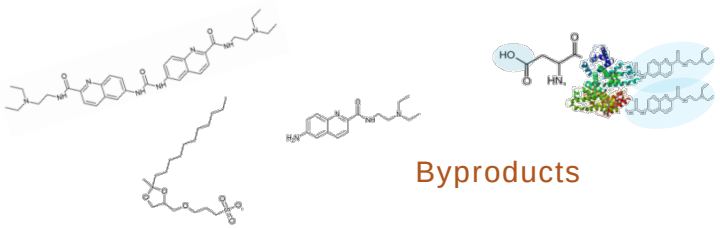


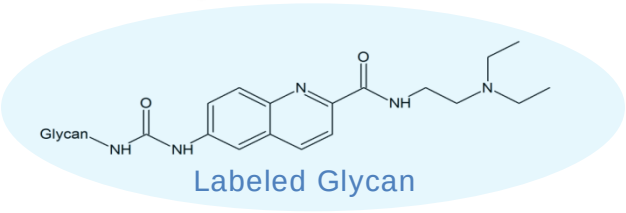


GlycoWorks
HILIC SPE

Collect

Waste





Robustness

Quantitative Extraction

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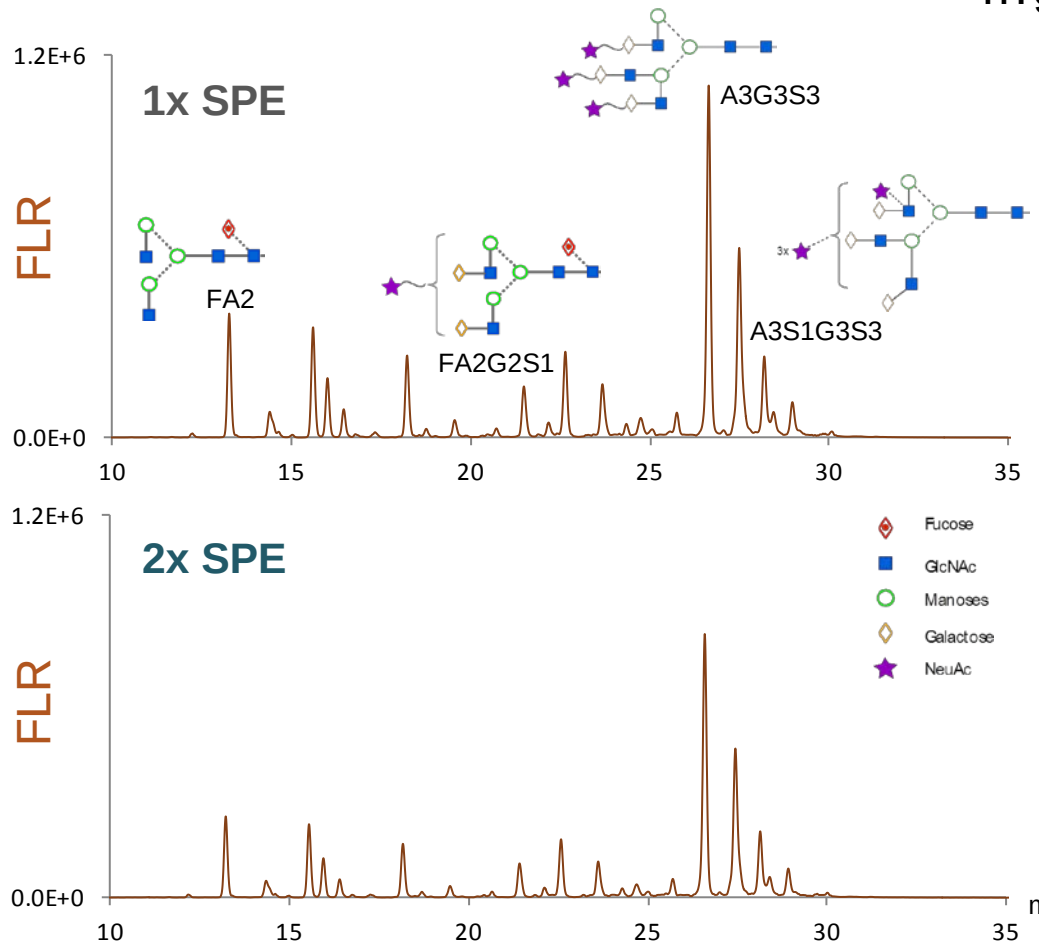
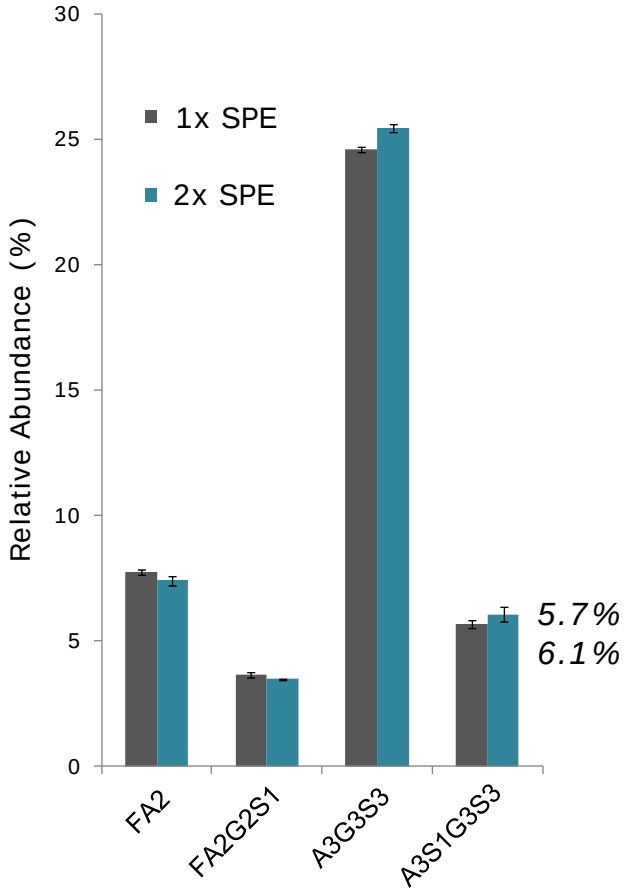
hIgG and fetuin N-glycans





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

Robustness

High Yield and Minimal Bias

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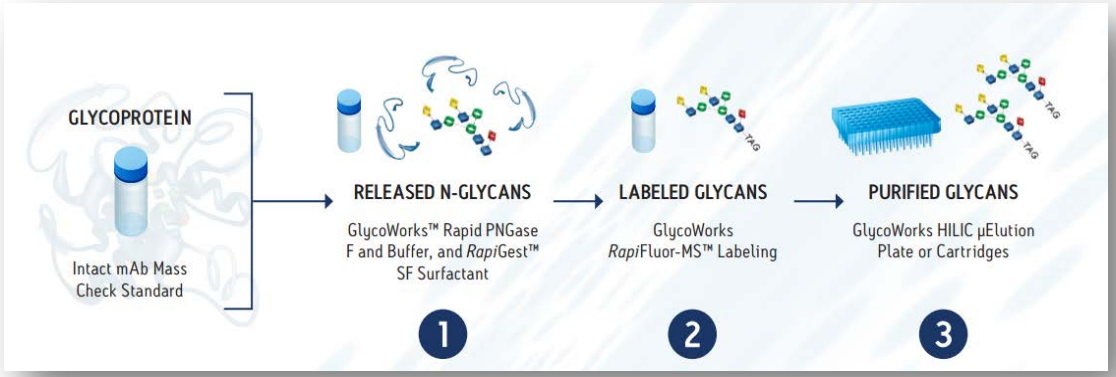

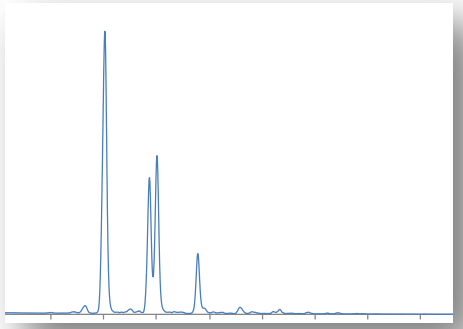

Step	Yield	Testing to confirm minimal bias
Deglycosylation	Complete	▪ Intact mass analysis ▪ Gel shift assays ▪ Subunit LC-MS
Labeling	>95%	▪ Released glycan profile *vs* subunit derived glycan information
SPE	~74%	▪ Recovery measurements ▪ Glycan profile before *vs* after SPE
Entire Workflow (experimentally determined)	~73% Yield	

Summary

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

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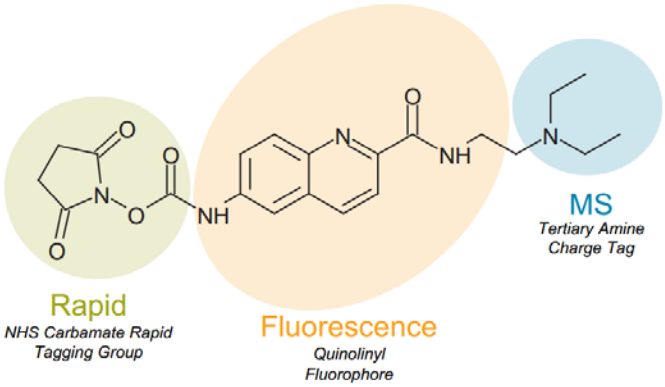
- Simple, streamlined protocol
- Fast and complete deglycosylation
- Rapid and efficient labeling
- Unbiased and robust SPE for neutral to tetrasialylated *N*-glycans
- Unprecedented FLR and MS sensitivity

*Rapi*Fluor-MS



Glycoprotein

Analysis-Ready *N*-glycans

30 min

GlycoWorks
*Rapi*Fluor-MS N-Glycan Kit

GlycoWorks™ *Rapi*Fluor-MS™ Kit

Smart Workflow with No Compromise

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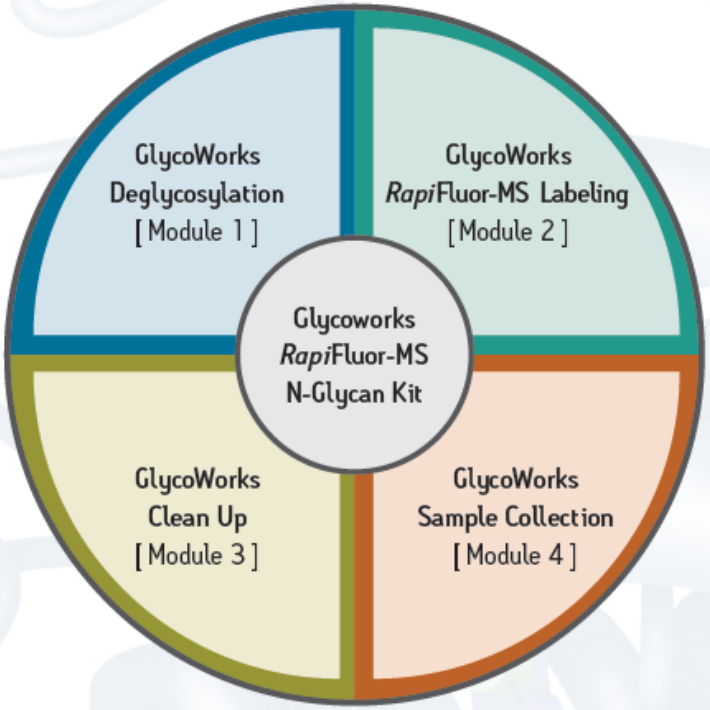
4 x 24 Format= 96 samples



Available
February 2015

Poster

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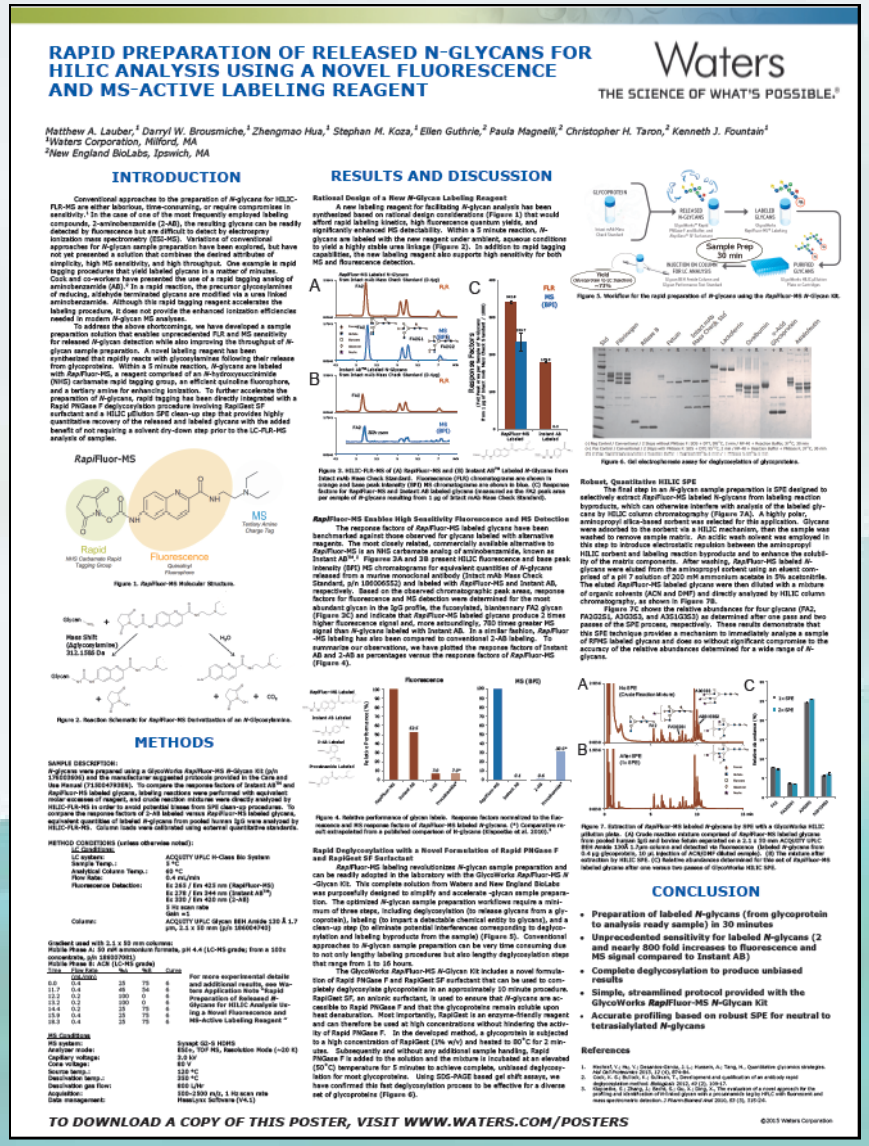

RAPID PREPARATION OF RELEASED N-GLYCANS FOR HILIC ANALYSIS USING A NOVEL FLUORESCENCE AND MS-ACTIVE LABELING REAGENT

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INTRODUCTION

RESULTS AND DISCUSSION

METHODS

CONCLUSION

References

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WCBP 2015 **Poster P-216-W**

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

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*Rapi*Fluor-MS: Enhanced Workflows for Glycan Characterization

Glycan Characterization

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- 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™ *Rapi*Fluor-MS™ N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column

UNIFI® Glycan Workflows

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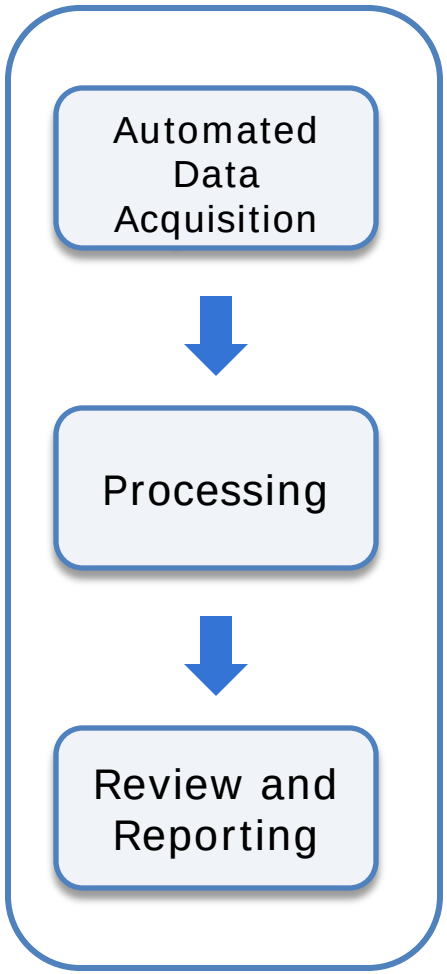
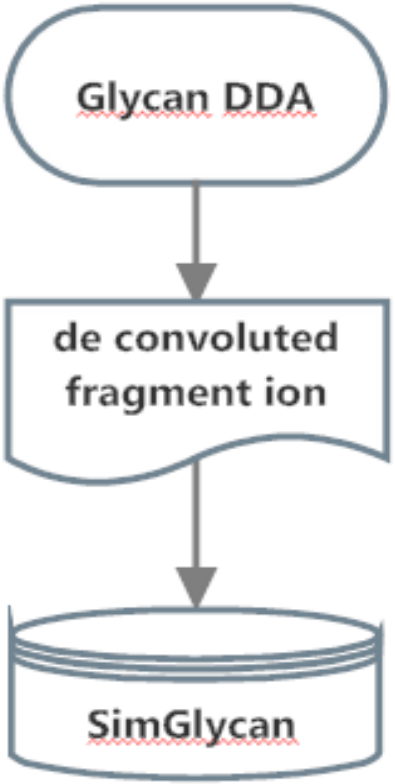


HILIC FLR GU +Accurate Mass

HILIC FLR GU + DDA MS/MS



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

*Rapi*Fluor-MS™ Labeling for Characterization

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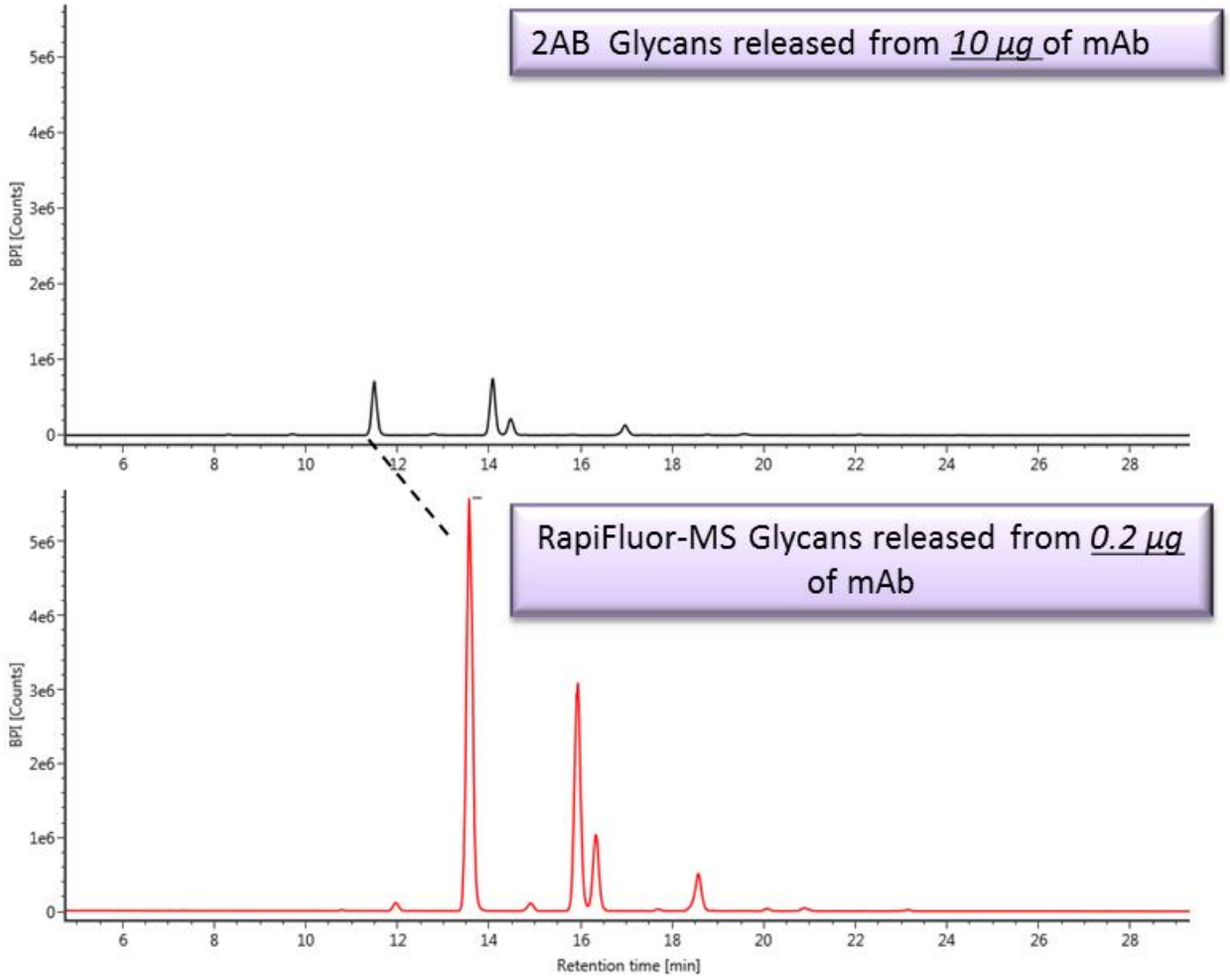
Greater than 100x MS response over 2AB labeling

BPI MS



Sample: NIST RM 8670 mAb lot #3F1b

HILIC FLR GU + Accurate Mass

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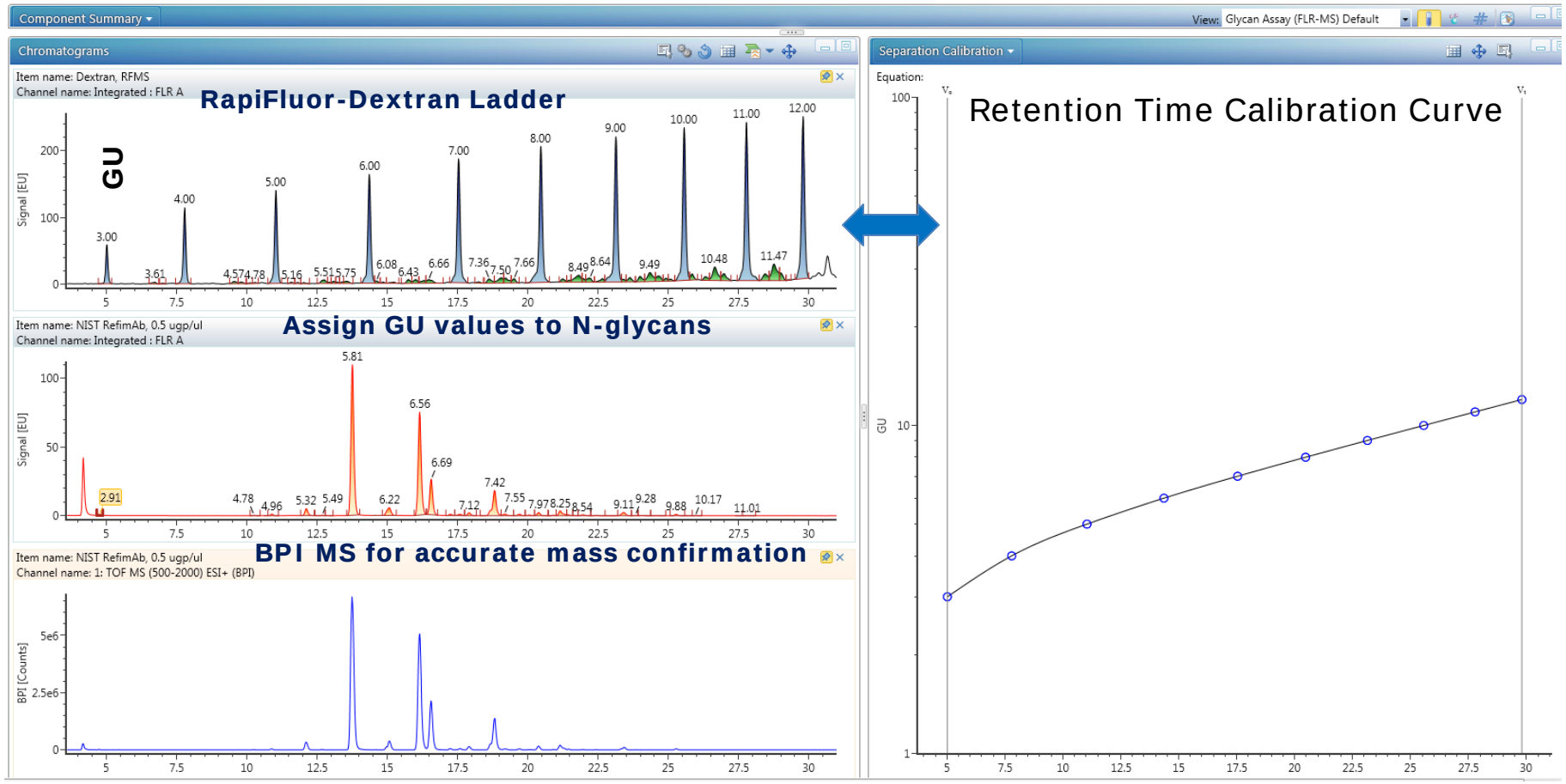
Method Robustness and Transferability, Confident Assignments



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

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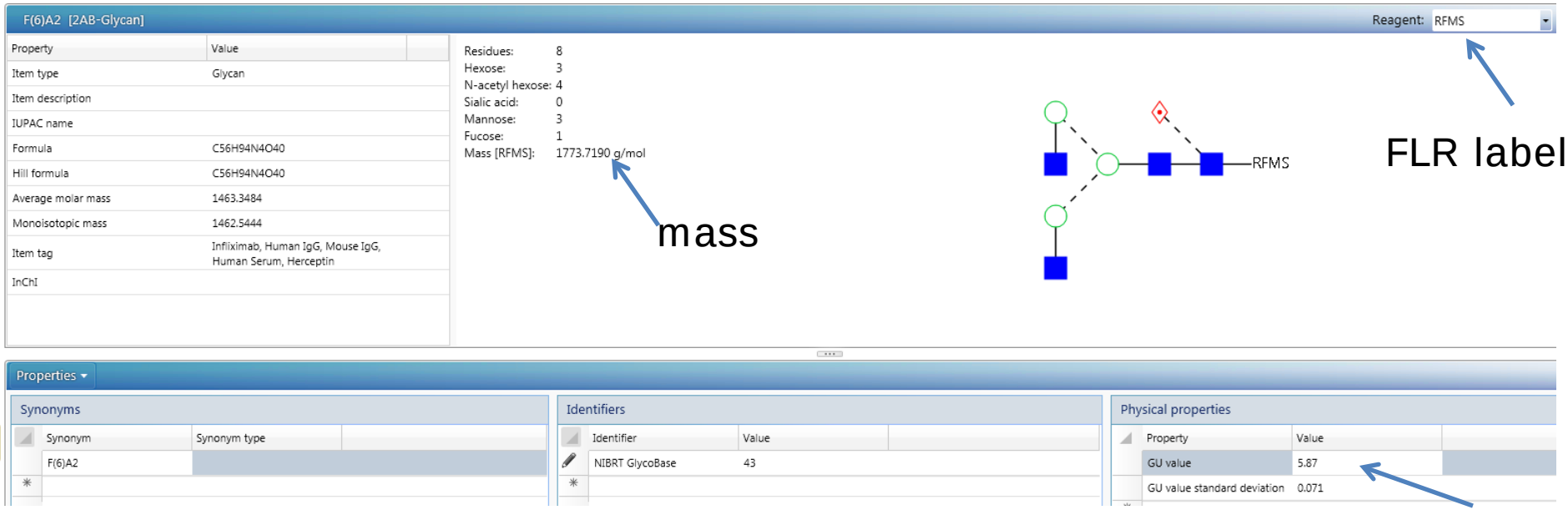

Waters Glycan GU Library:

- 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

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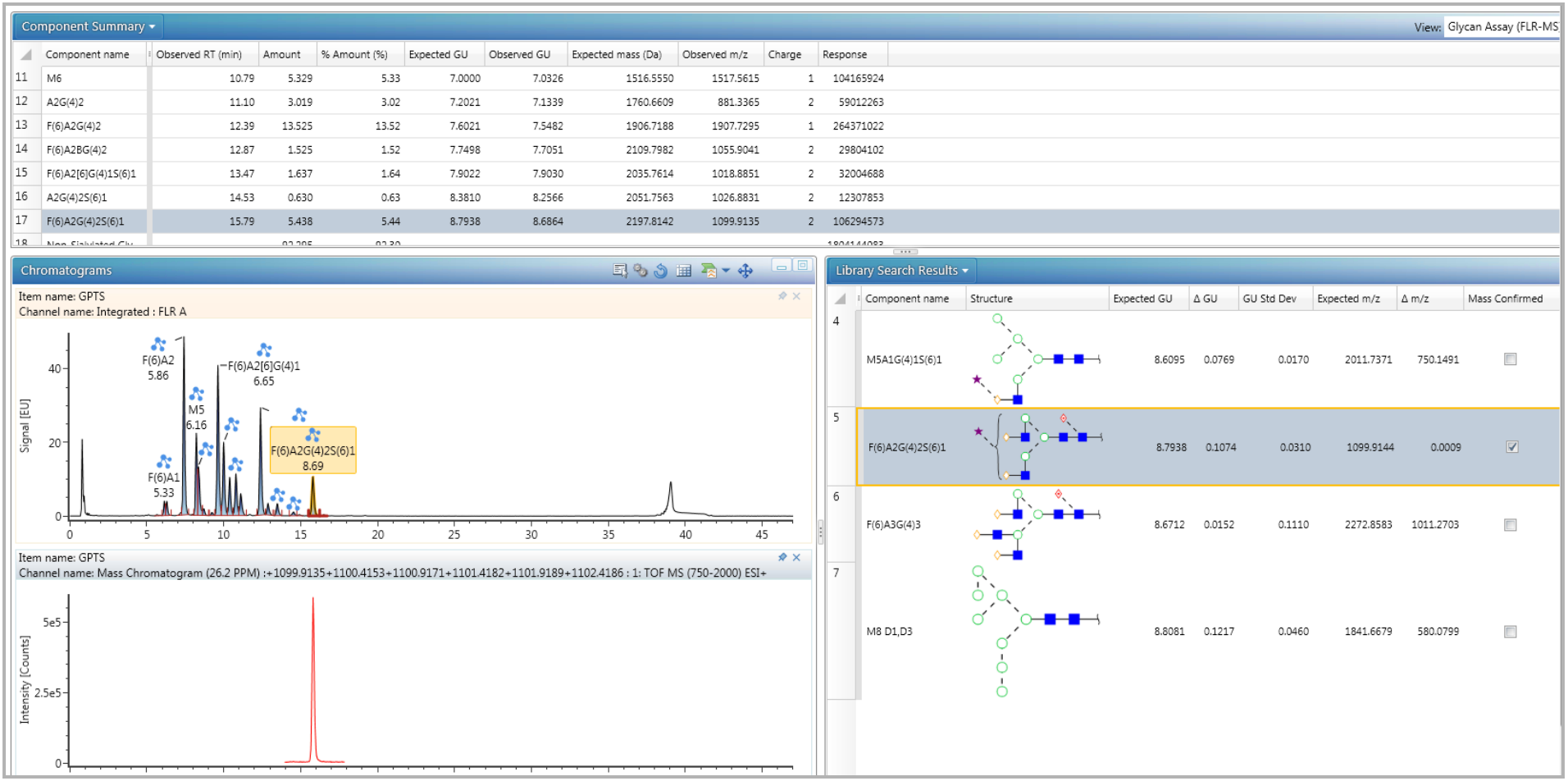

Powerful UNIFI® Reporting Architecture Simplifies Communication of Results

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Example

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	8LM	Unknown	1	1:B,5	2.00	

Analysis Method


Analysis Method Report

Purpose

Item name: Infliximab_2nd data_Innovator Targeted
Description:
Instrument system type:
Analysis type: Glycan Assay (FLR with MS Confirmation)
Analysis type description: Discovers and Quantifies Glycans using peaks in FLR chromatograms, identifies them via Library Search and Confirms identification using MS data
Accurate mass: Accurate mass
Screen enabled: Yes

Quantify enabled: Yes
Compare enabled: No
Discover enabled: Yes
Matrix Factor Enabled: No
Separation enabled: Yes

Analyte class: Glycan
Analyte class description: Glycan Analyte

Summary Table

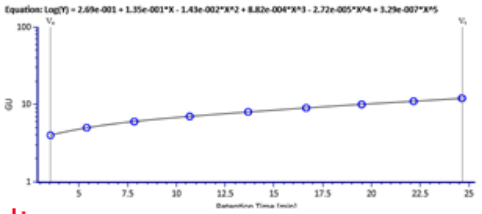

Summarized by: % Amount (%)

	Item name	A1	M3B	M4	F(6)A1	A2	M4A1	F(6)A2	M5	A2G(4)1	F(6)A2(3)G(4)1
1	9CM	1.29		0.15	3.17	1.99	0.57	47.11	4.58	0.44	2.38
2	9CM	1.28		0.13	3.16	2.01	0.57	47.17	4.56	0.43	2.34
3	9CM	1.28		0.14	3.15	2.00	0.57	47.17	4.55	0.44	2.32
4	8LM	1.08	0.11	0.28	2.68	1.90	0.58	44.09	4.46	0.48	2.30
5	8LM	1.08	0.11	0.26	2.66	1.88	0.57	44.09	4.45	0.47	2.32
6	8LM	1.08	0.11	0.28	2.67	1.92	0.57	44.10	4.44	0.48	2.30
7	08B	1.07	0.13	0.33	3.38	1.53	0.62	48.19	4.59	0.28	2.21
8	08B	1.08	0.14	0.36	3.38	1.53	0.61	48.18	4.55	0.29	2.15
9	08B	1.08	0.14	0.34	3.38	1.54	0.61	48.18	4.57	0.29	2.19
Mean		1.147	0.124	0.252	3.069	1.810	0.586	46.477	4.528	0.402	2.279
% RSD		8.99	14.64	35.45	10.29	11.78	3.51	3.96	1.30	22.08	3.44
Std dev		0.103	0.018	0.089	0.316	0.213	0.021	1.841	0.059	0.089	0.078

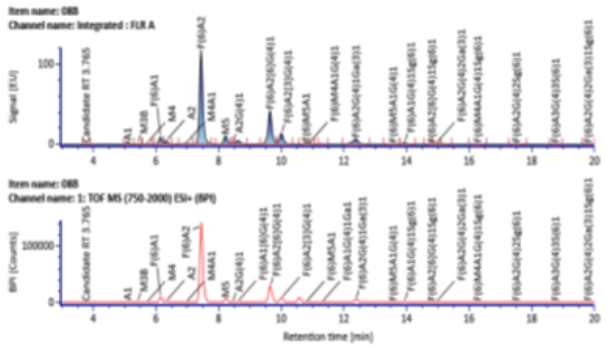
UNIFI

Report

RT Calibration Curve

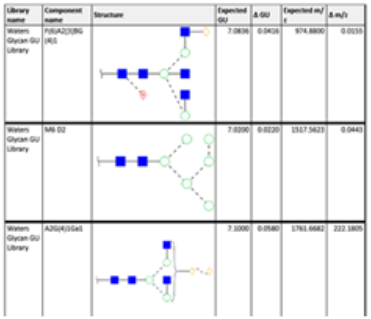


Processed UPLC/FLR/MS Chromatogram and Result Table



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
F(6)A1	6.15	5.33	3.38

Library Search Result



UNIFI® Glycan DDA Workflow

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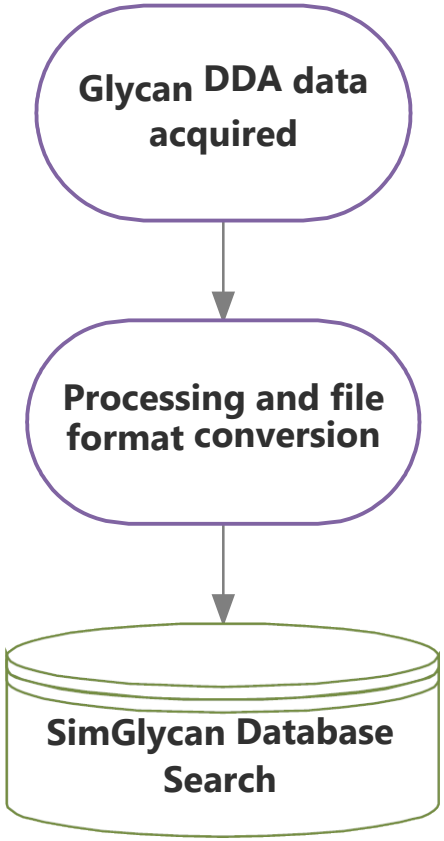

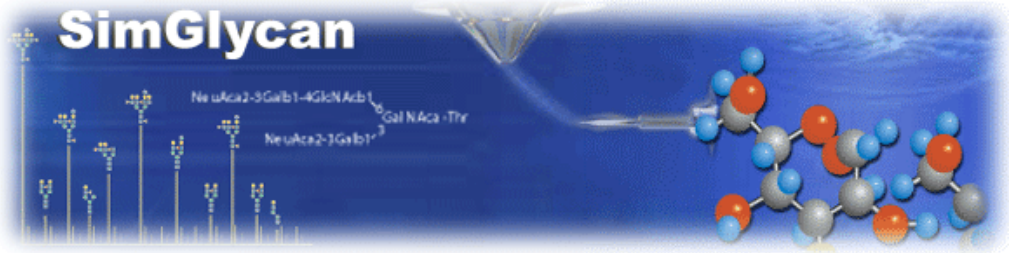

Optional UNIFI® Export to SimGlycan for MS/MS Database Search

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A2G2S1

Scan35@1122.43_1 (20141031_YEA439_yqy_Human IgG_DDA_01)

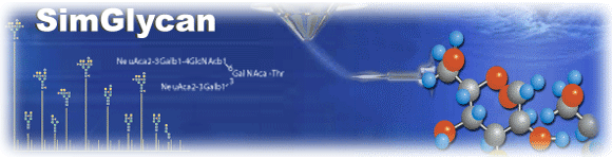
MS Profile | Search Results | Annotated Peaklist

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



Enhanced MS/MS with *Rapi*Fluor-MS™ Labeling

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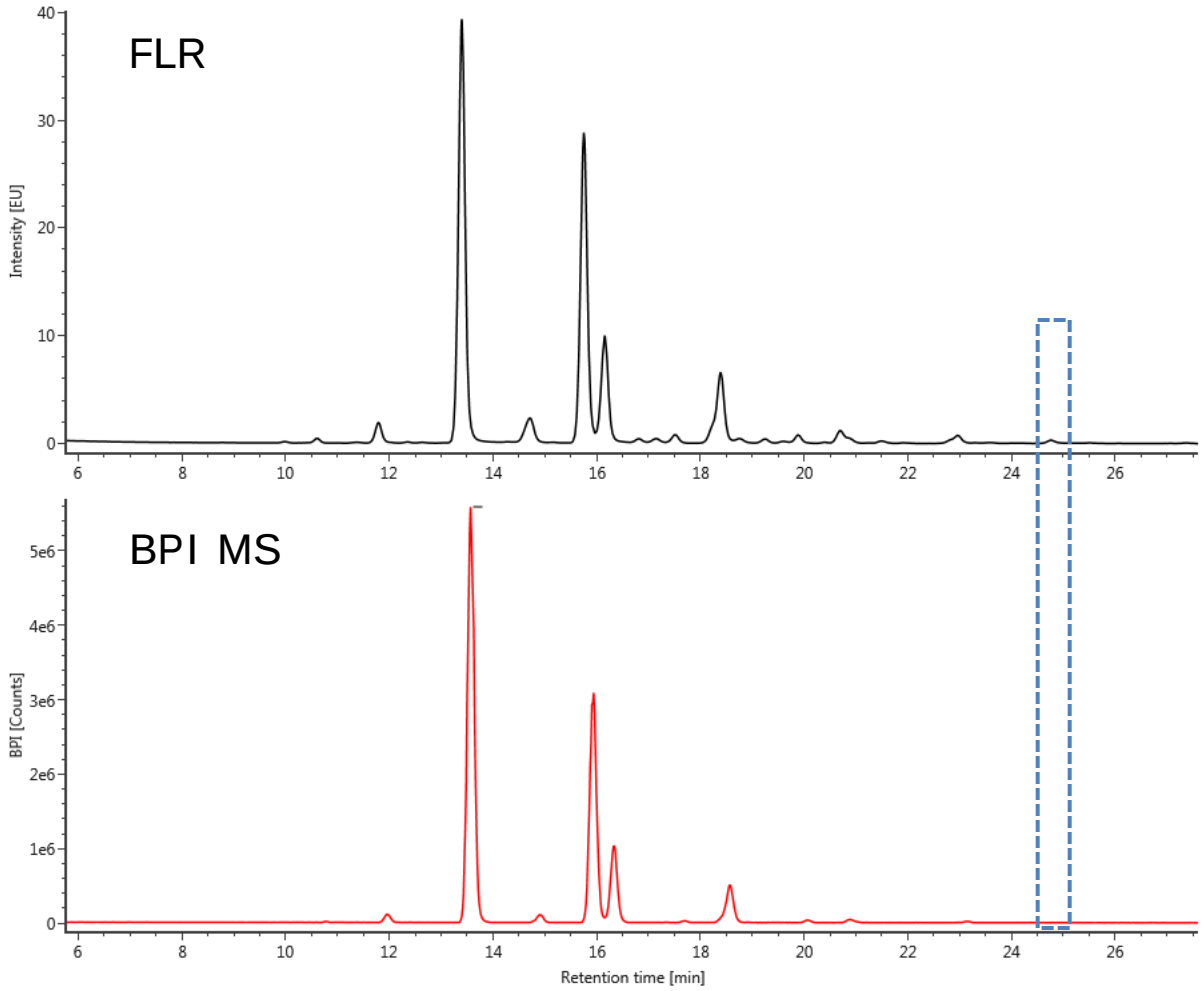
MS performance extends to fragmentation data



MSMS

Enhanced MSMS with *Rapi*Fluor-MS Labeling

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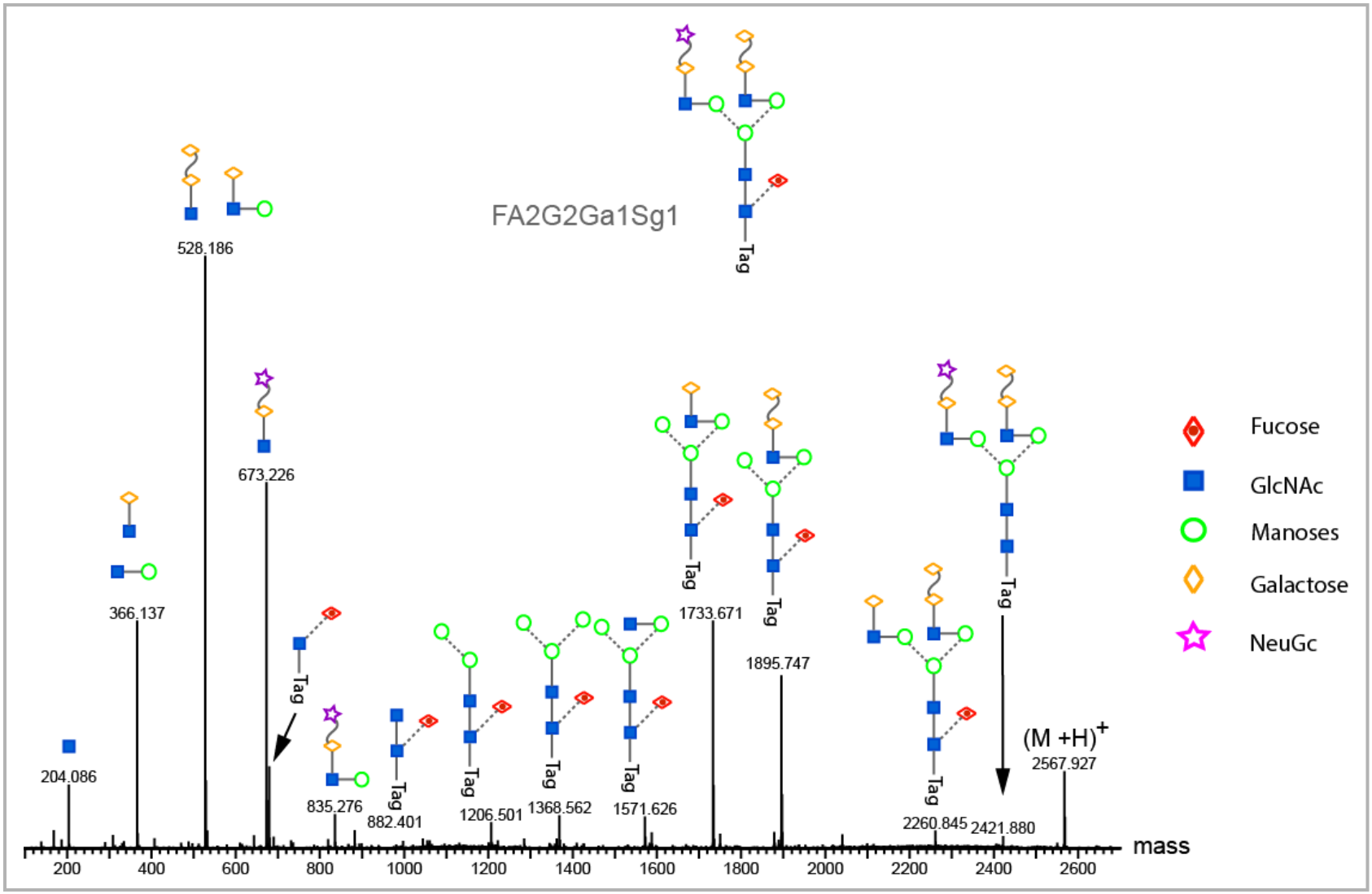
MS performance extends to MS/MS fragmentation data



Poster

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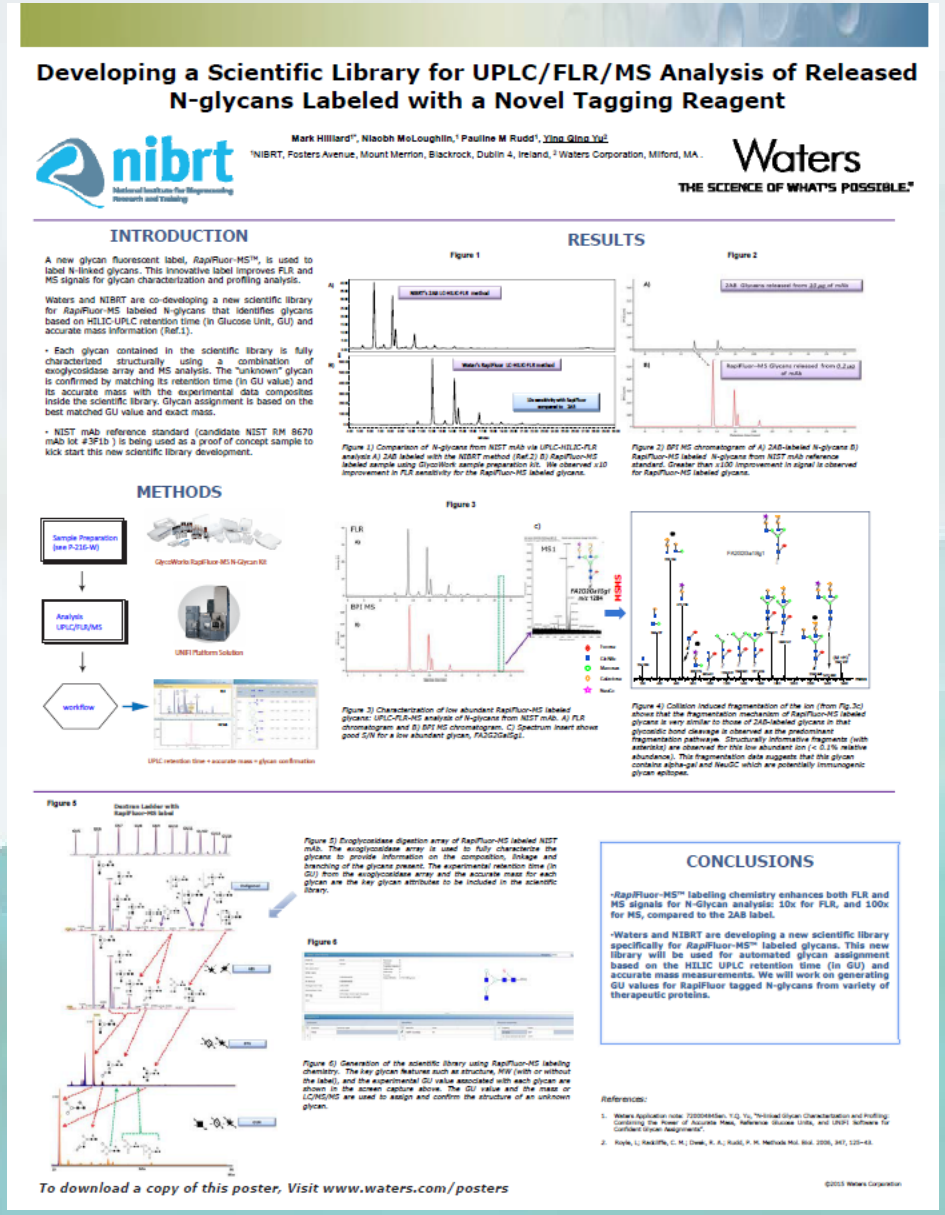

Developing a Scientific Library for UPLC/FLR/MS Analysis of Released N-glycans Labeled with a Novel Tagging Reagent

Mark Hilliard[1*], Niaobh McLoughlin,[1] Pauline M Rudd[1], Ying Qing Yu[2]
[1]NIBRT, Fosters Avenue, Mount Merrion, Blackrock, Dublin 4, Ireland, [2]Waters Corporation, Milford, MA.

INTRODUCTION

A new glycan fluorescent label, *Rapi*Fluor-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 *Rapi*Fluor-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.

METHODS

RESULTS

CONCLUSIONS

- *Rapi*Fluor-MS™ labeling chemistry enhances both FLR and MS signals for N-Glycan analysis: 10x for FLR, and 100x for MS, compared to the 2AB label.
- Waters and NIBRT are developing a new scientific library specifically for *Rapi*Fluor-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 measurements. We will work on generating GU values for RapiFluor tagged N-glycans from variety of therapeutic proteins.

To download a copy of this poster, Visit www.waters.com/posters

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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*Rapi*Fluor-MS: Enhanced Workflows for Glycan Monitoring

Transferability between UPLC® and HPLC

RapiFluor-MS™ Labeled Glycans

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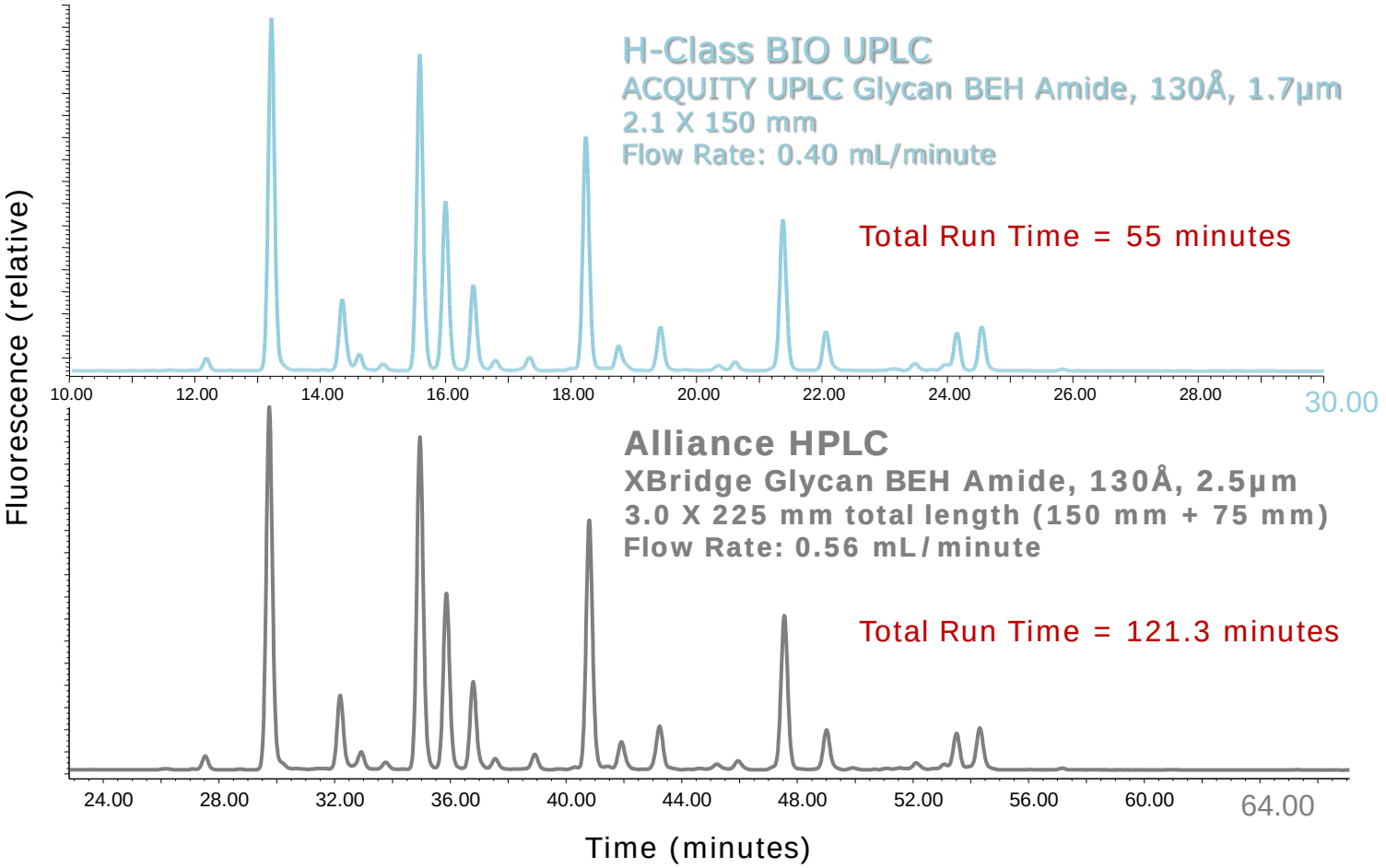

- Comparable sensitivity and resolution – 3x sample load / 2x increase in time
- Transfer between labs with different LC equipment capabilities

Glycan Monitoring

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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™ *Rapi*Fluor-MS™ N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column

ACQUITY® QDa® Mass Detector

A breakthrough product with mass appeal

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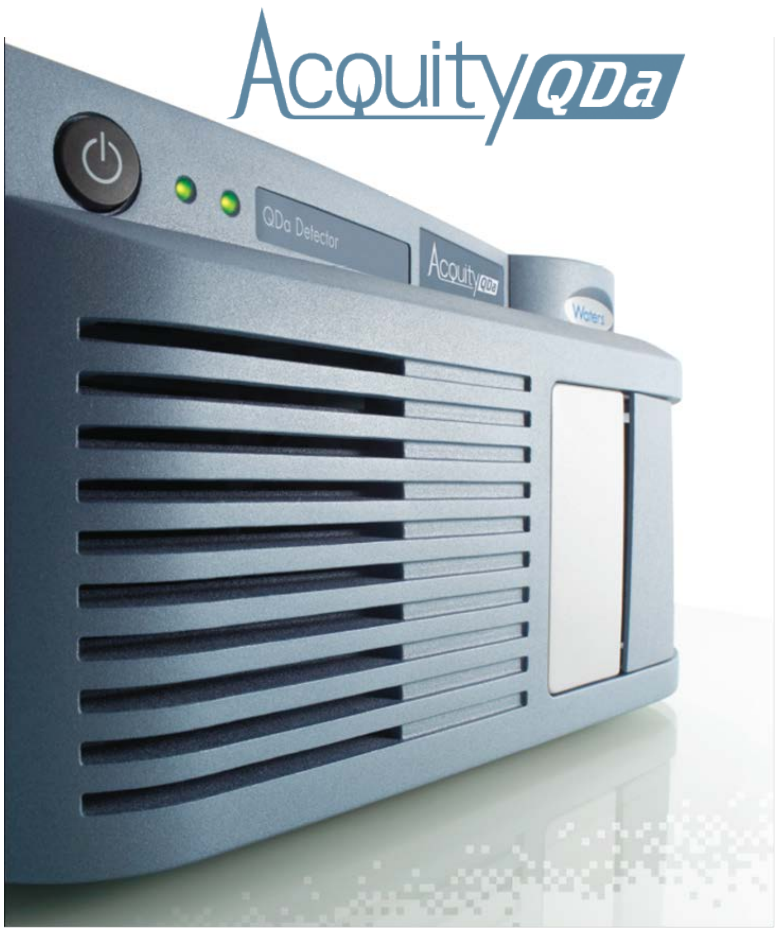

- Revolutionary innovative design focused on ease of use for analysts
- Empowering analytical chemists everywhere with orthogonal mass detection – added information with every sample
- Compact, robust and affordable: Built for constant use with a wide variety of chromatographic conditions
- Seamlessly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

Automated Start Up Provides Robust, Reproducible Performance

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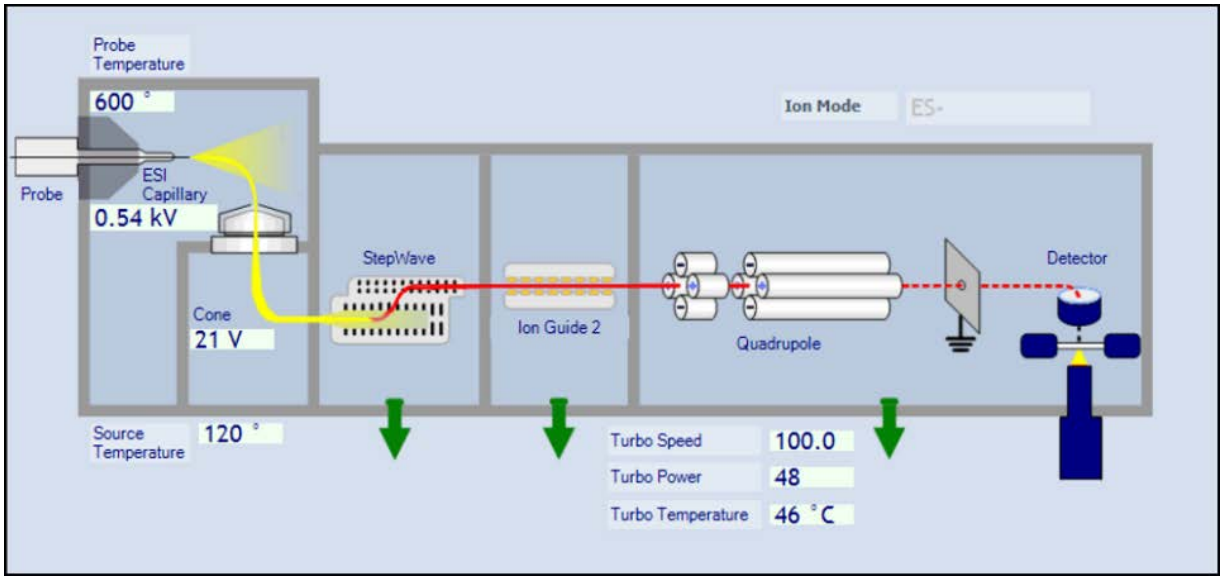
- **Automated resolution and calibration** occurs with each start-up ensuring mass information is accurate and precise
- **ESI interface optimized** for UPLC® performance to ensure chromatographic resolution, sensitivity and throughput is preserved
- **Disposable** sample aperature and capillary for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

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



Routine N-Glycan Detection with Comparable FLR and MS response

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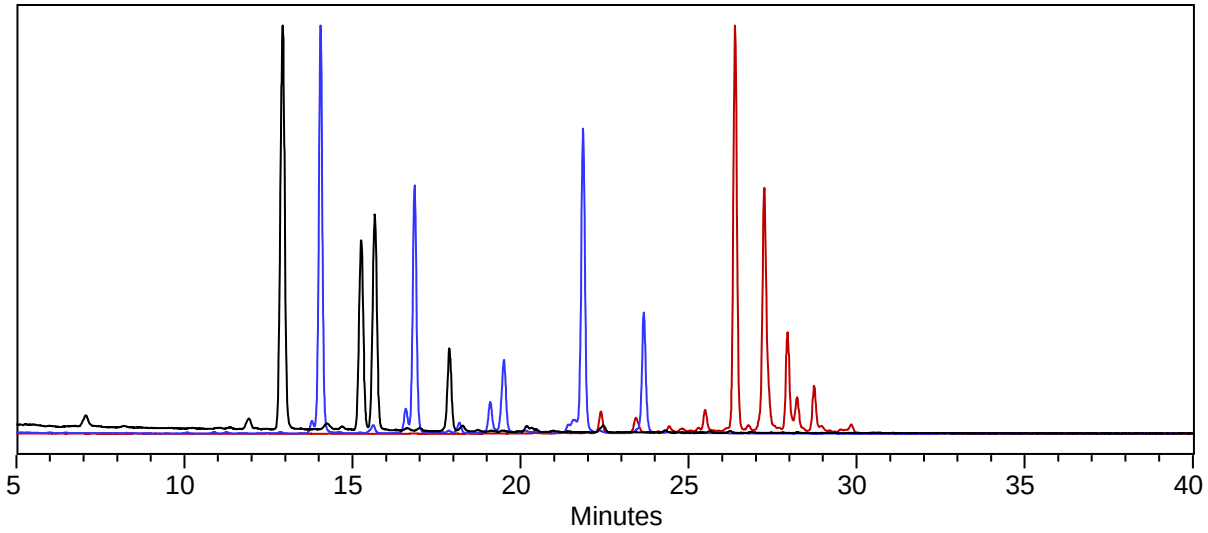

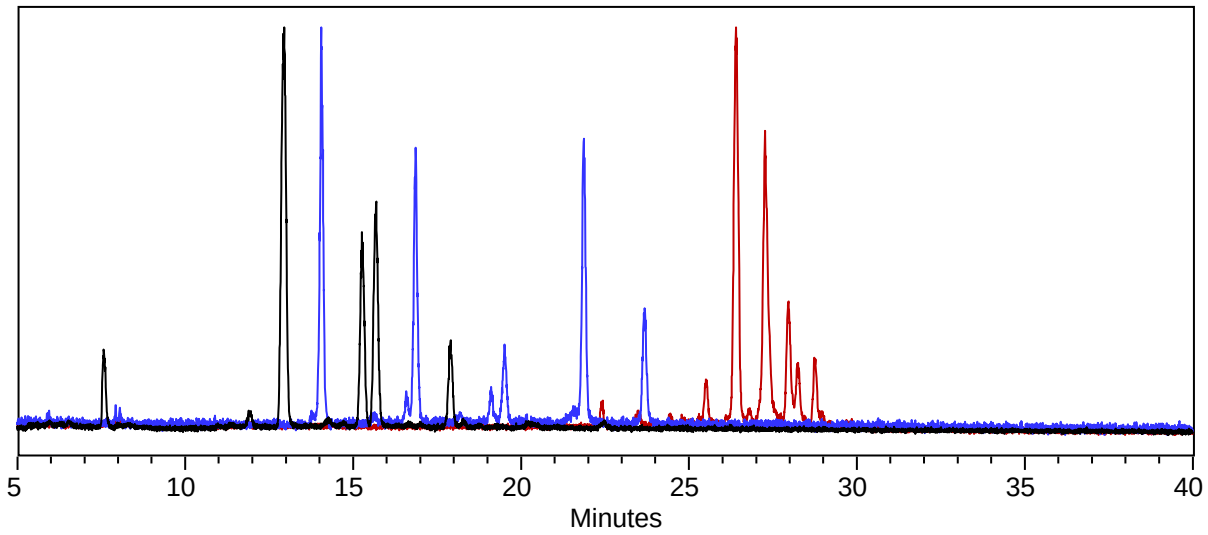

Detection across a broad range of glycoforms:

IgG
Simple bi-antennery structures

RNase B
High mannose structures

Fetuin
Large, complex structures

IgG Glycan Profile and Structure Confirmation Using ACQUITY® QDa®

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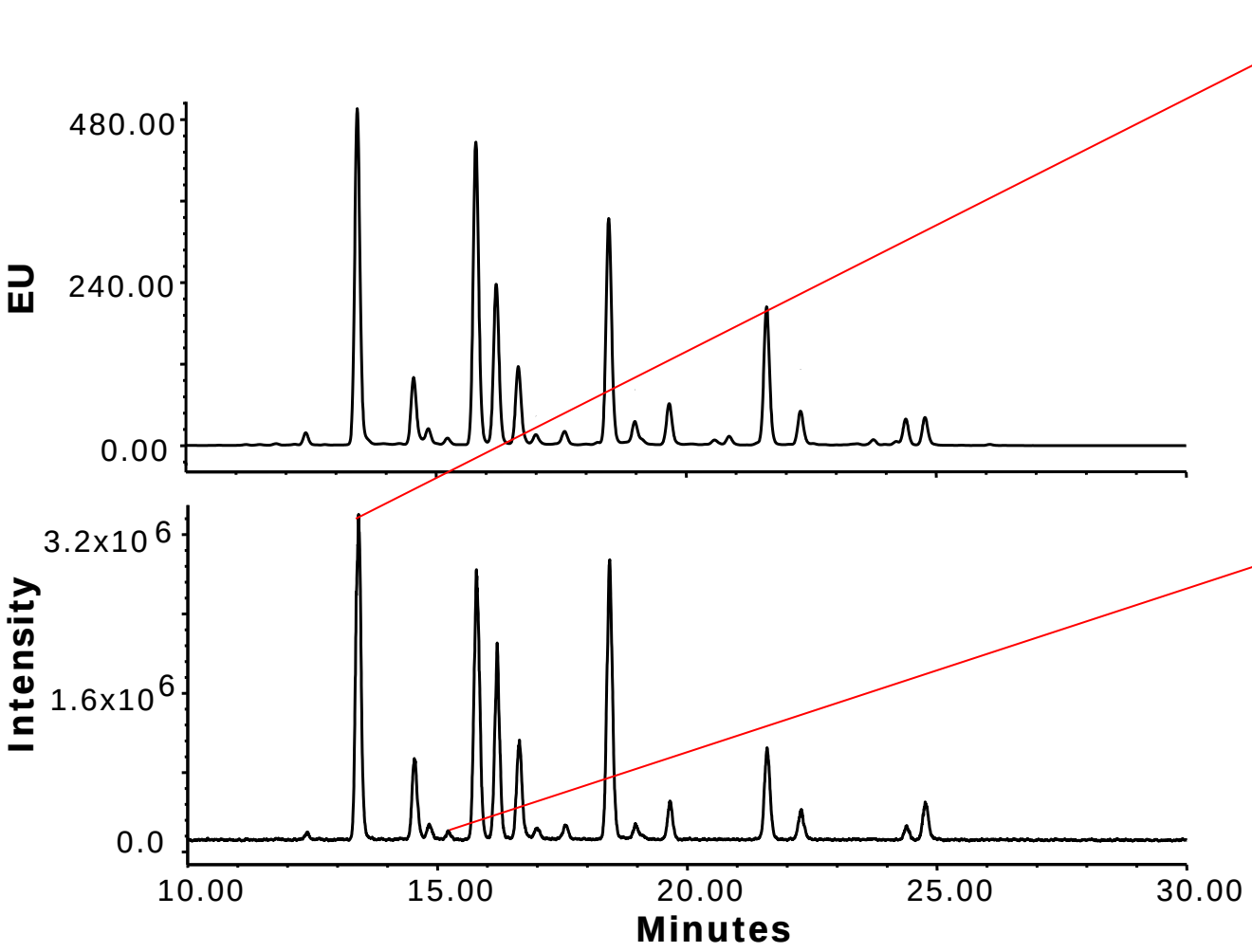

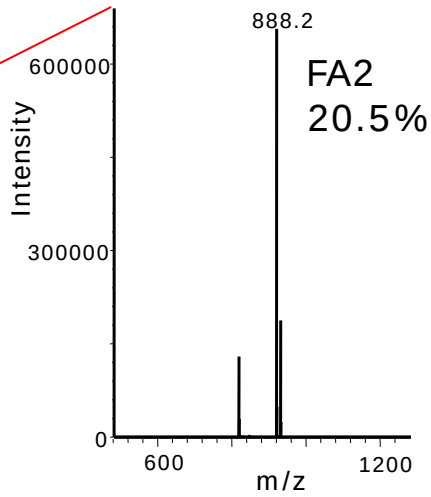

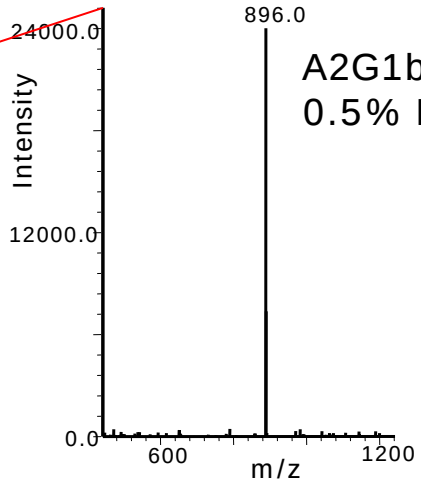

<u>Key take away</u>: Large dynamic range – can clearly see most abundant and least abundant glycoforms.

Intuitive GMP compliant Reporting

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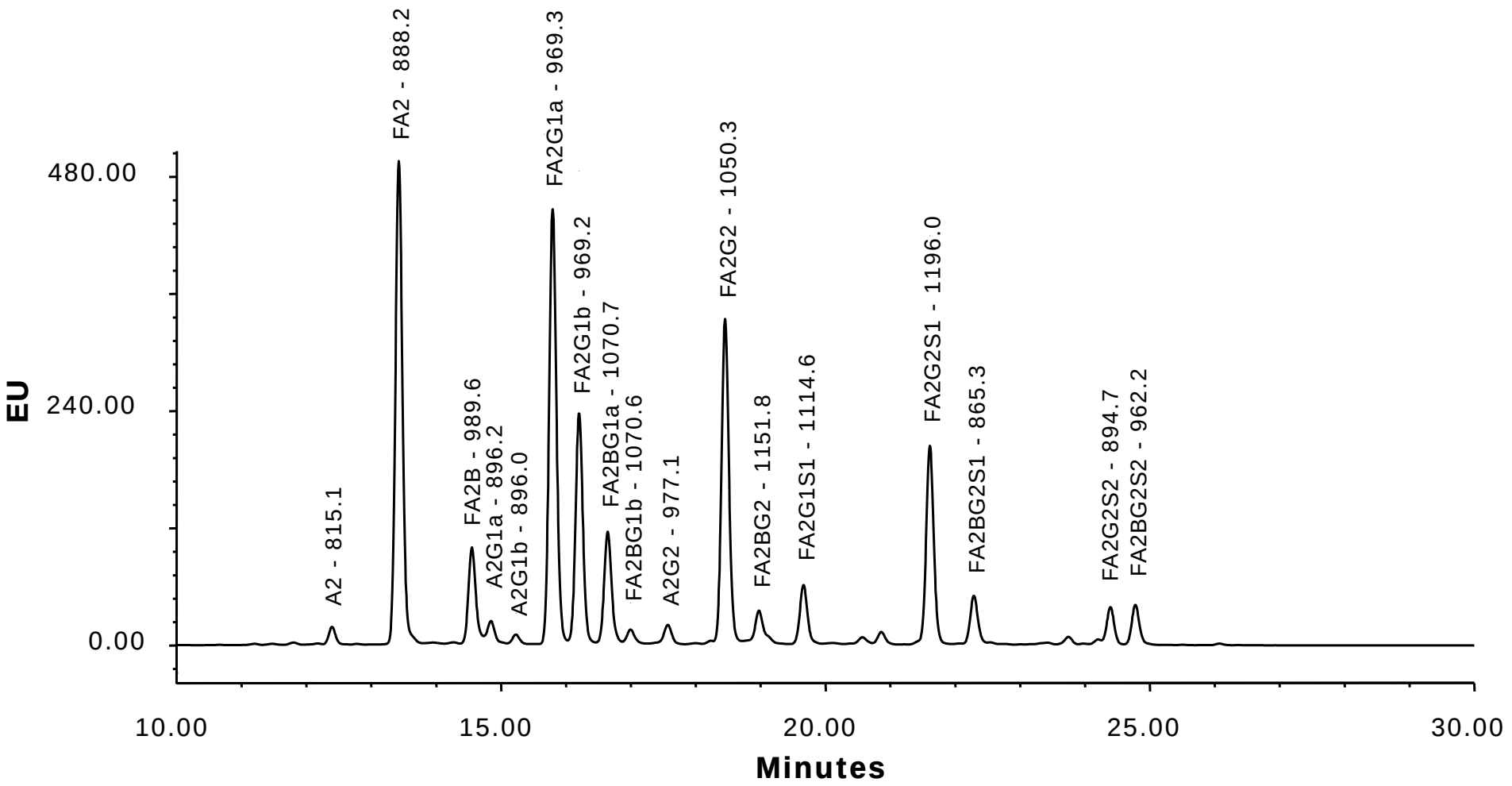
Empower® integration enables annotation of peaks with names and *m/z*



Developing a Rapid Method for Glycan Analysis

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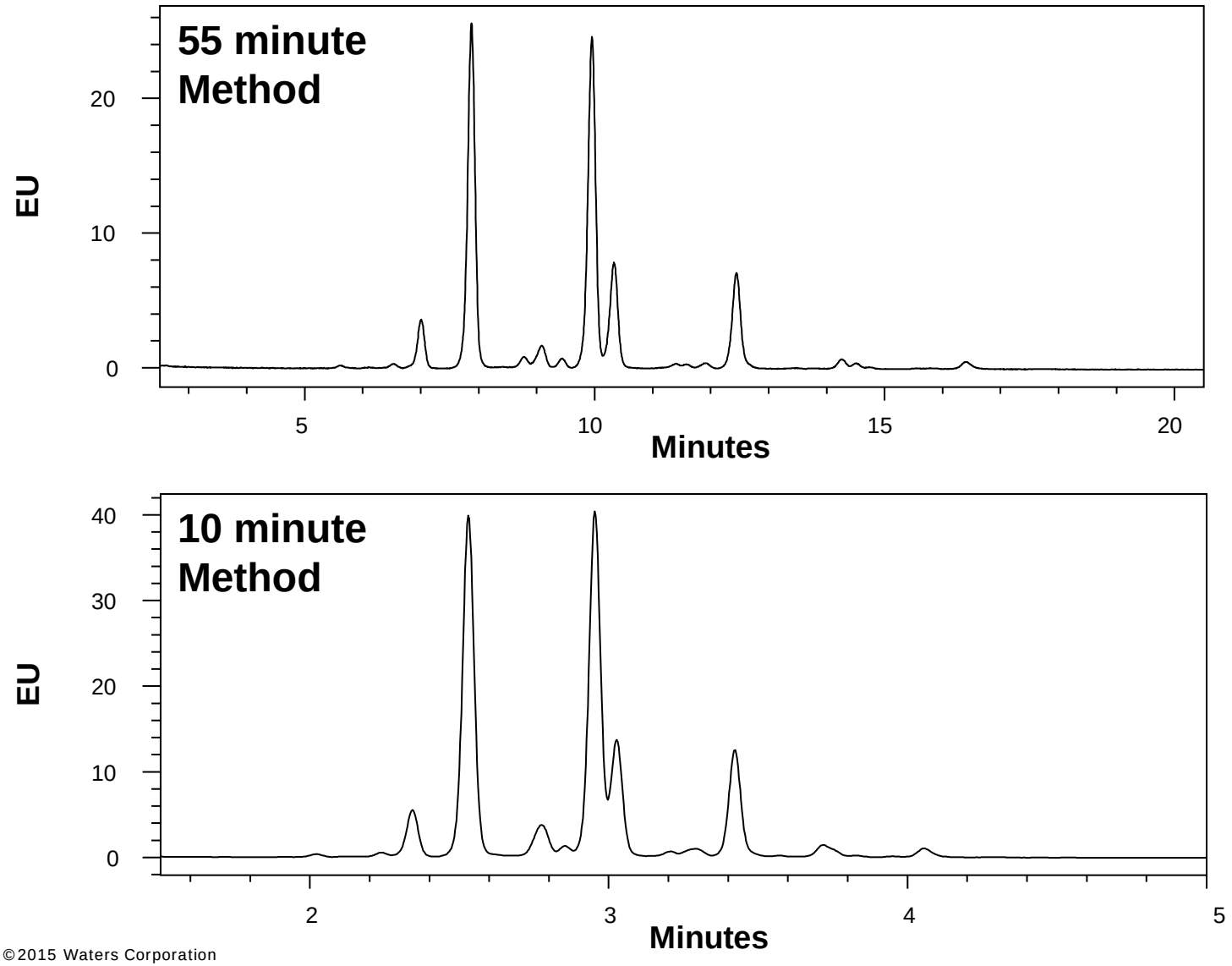

Rapid Screening Process for Development Samples

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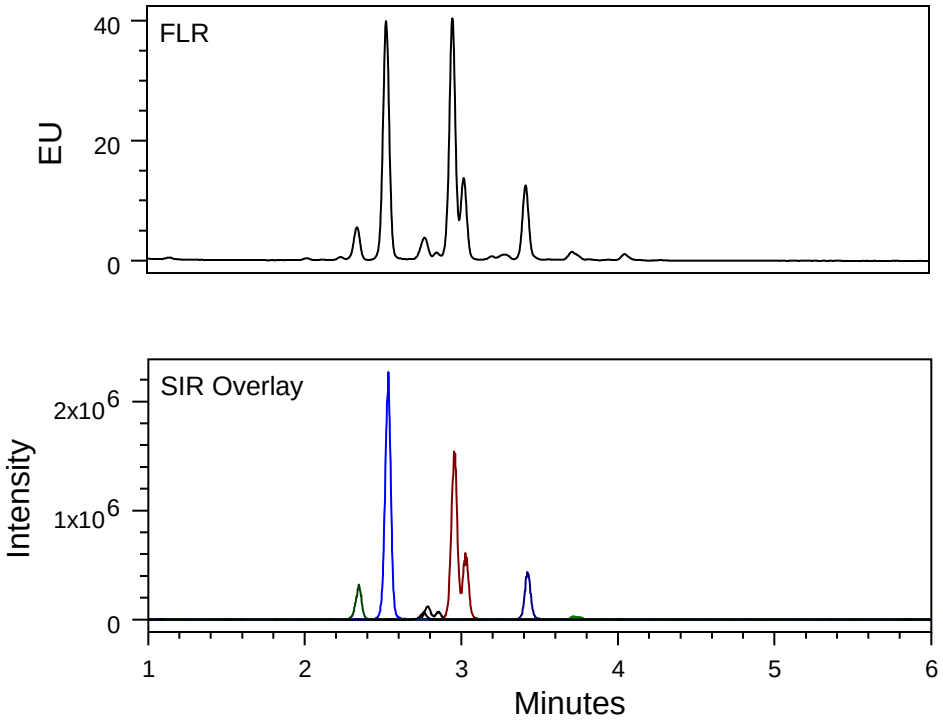
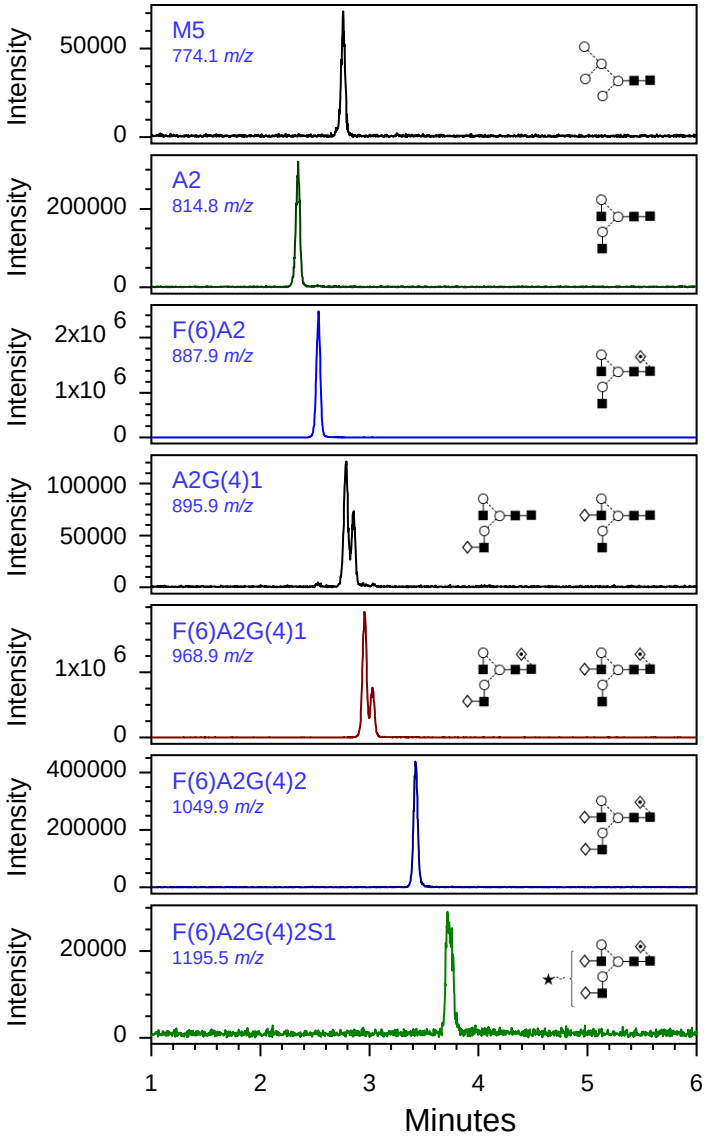
Trastuzumab N-Glycan Analysis

***Rapi*Fluor-MS™ labeled glycans: 10 minute method**





Monitoring Glycan Ratios

Keeping Tabs on Mannose 5

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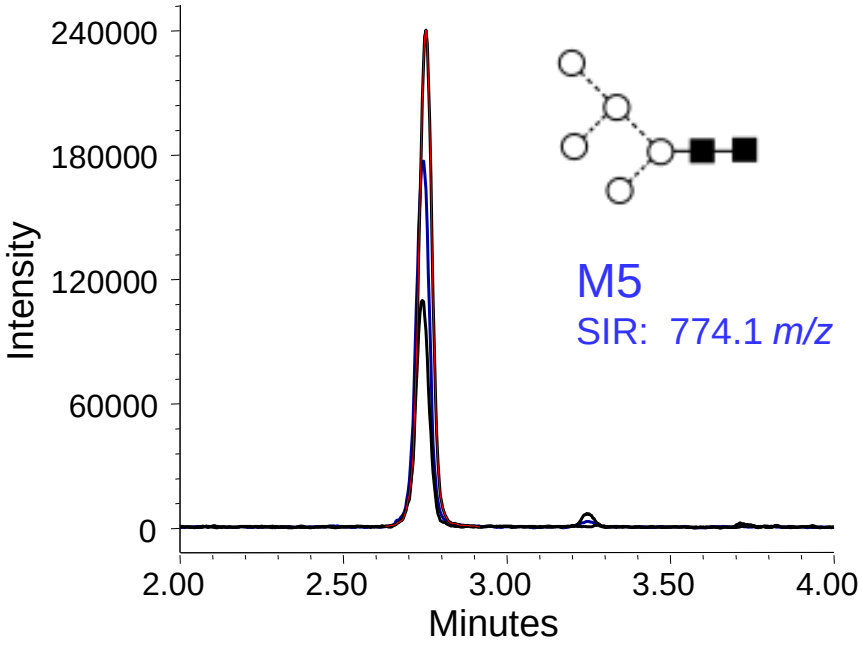

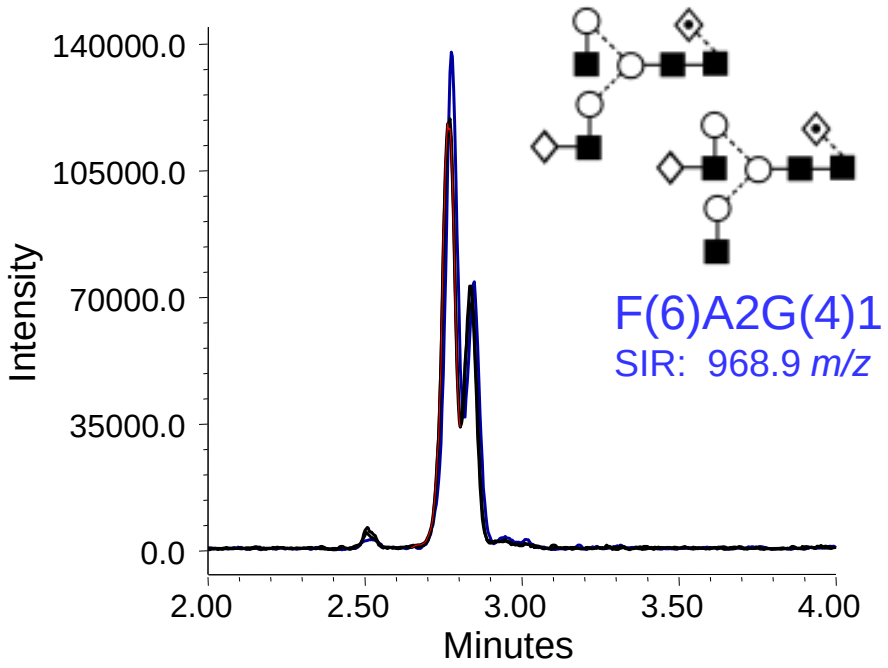

Man5: F(6)A2G(4)1 Ratio

	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

Waters
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SAMPLE PREP

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

Deglycosylation, Labeling and Clean-up in 30 min

Unmatched sensitivity for FLR and MS detection

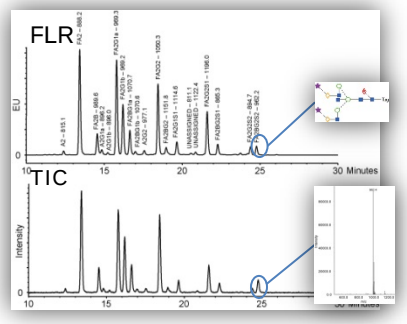
SEPARATION

ACQUITY UPLC®
Glycan BEH
Amide Column

DETECTION & INFORMATICS

Glycan Monitoring

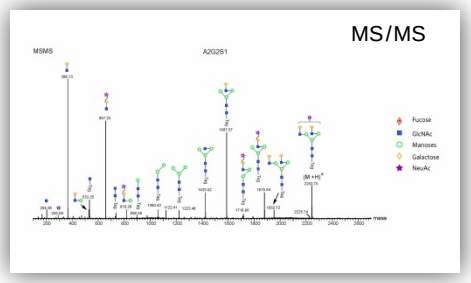
ACQUITY® FLR/QDa and Empower® 3 Software



FLR Quantification
GU Retention
MS Confirmation

Glycan Characterization

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



FLR Quantification
GU Retention
Accurate Mass Confirmation
MS/MS Fragmentation

Poster

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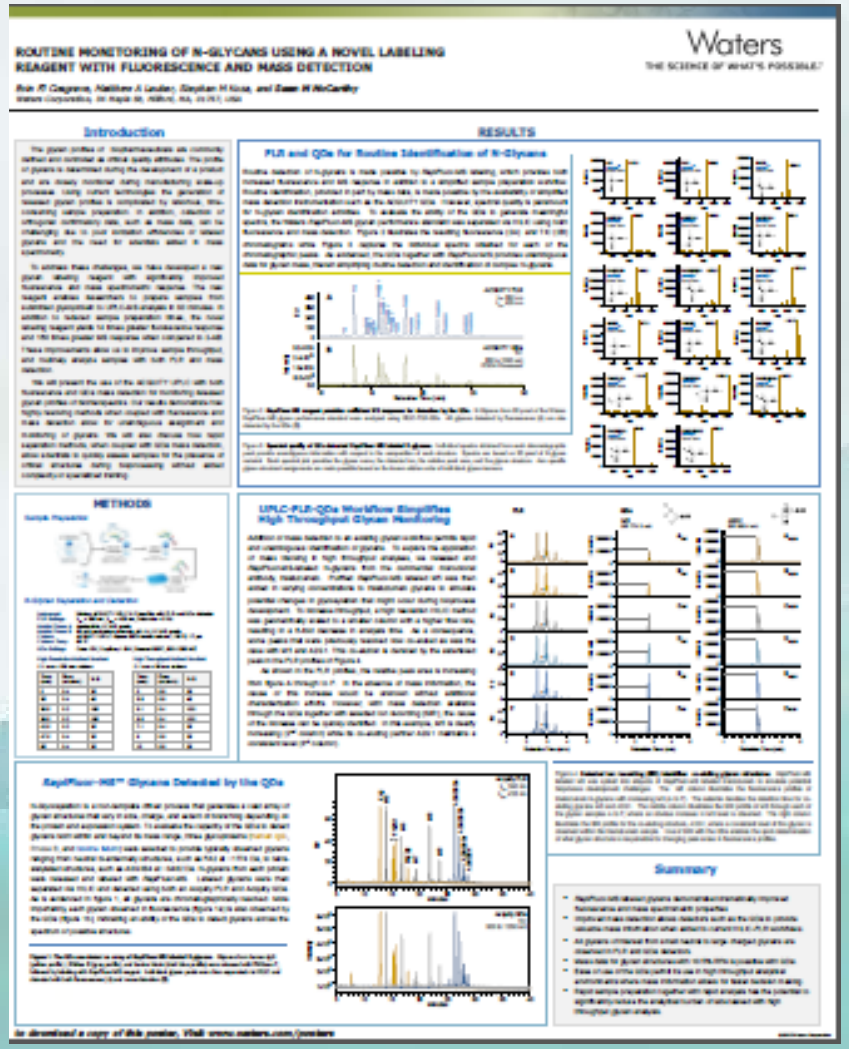

ROUTINE MONITORING OF N-GLYCANS USING A NOVEL LABELING REAGENT WITH FLUORESCENCE AND MASS DETECTION

Waters
THE SCIENCE OF WHAT'S POSSIBLE.

Introduction

RESULTS

FLR and QDa for Routine Identification of N-Glycans

METHODS

UPLC-FLR-QDa Workflow Simplifies High Throughput Glycan Monitoring

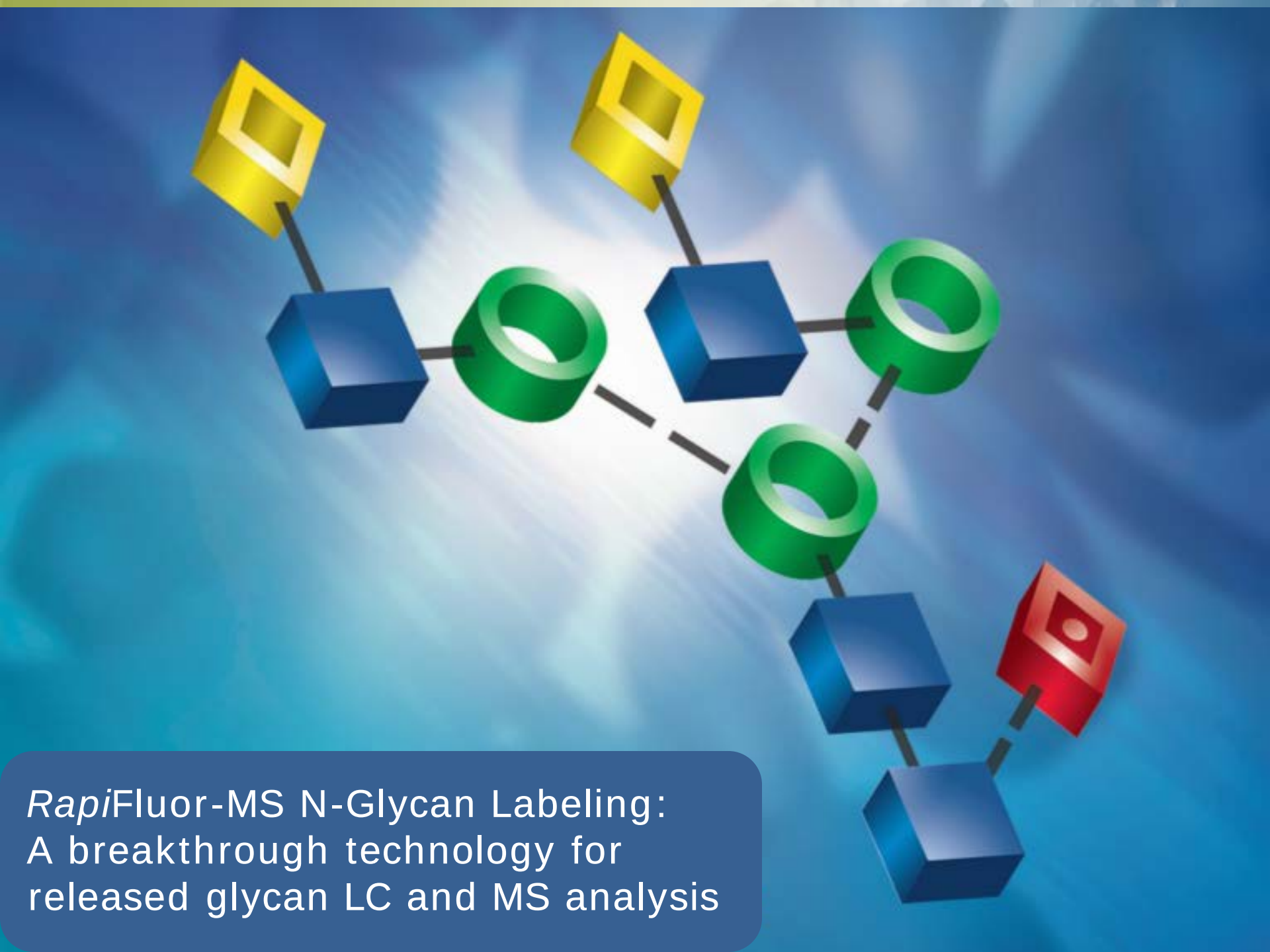
RapiFluor-MS™ Glycans Detected by the QDa

Summary

WCBP 2015 **Poster P-206-W**

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

Download pdf



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