# N-linked Glycan Characterization and Profiling: Combining the Power of a Novel Labeling Reagent and a Streamlined Analytical Workflow

Ying Qing Yu Ph.D

Waters Corporation

Glycobiology World Congress 2015

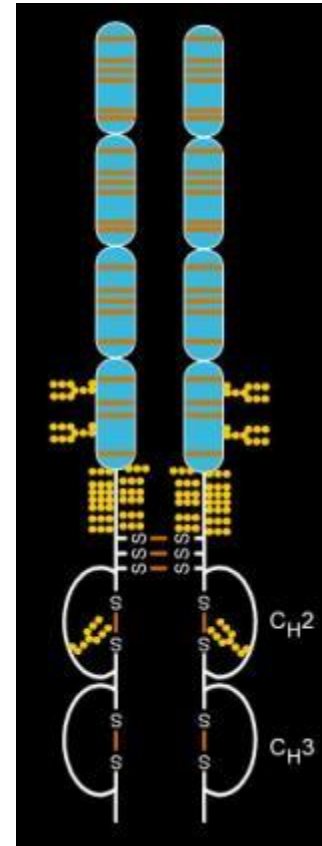# Glycosylation is a Key Critical Quality Attribute

The International Conference on Harmonization Guideline Q6B requires the analysis of carbohydrate content, structural profiles, and characterization of the glycosylation site(s) within the polypeptide chain(s).
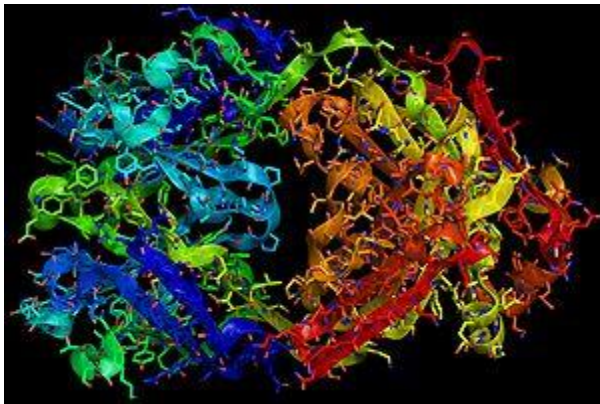
TrastuzumAb,
1 N-linked site
150 KDa

Erythropoietin
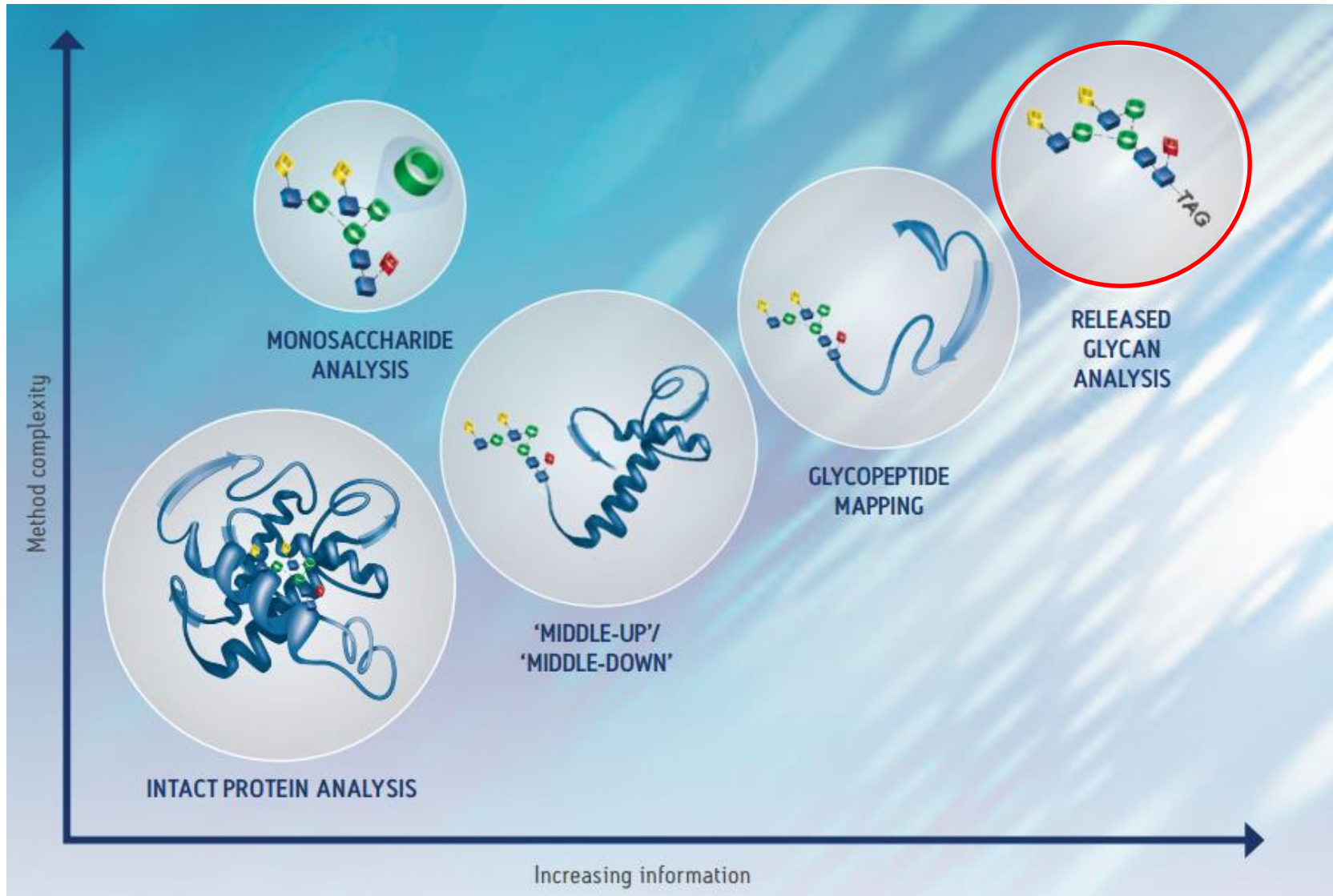*3 N-linked sites*
1 O-linked site
34 KDa

Entanercept
3 N-linked sites
13 O-linked sites
51 Kda

figures are from Wikipedia
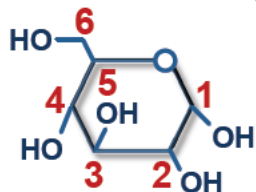
Glycoprotein Characterization
Multiple Strategies – Complementary Information

Method complexity

MONOSACCHARIDE ANALYSIS

'MIDDLE-UP'/ 'MIDDLE-DOWN'

GLYCOPEPTIDE MAPPING

RELEASED GLYCAN ANALYSIS

INTACT PROTEIN ANALYSIS

Increasing information

Carbohydrate Complexity

A) Composition

Type of monosaccharide building block
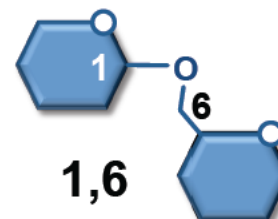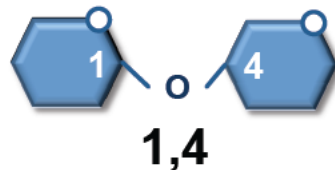
Glucose   Galactose   Mannose
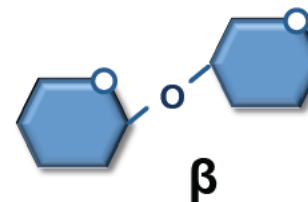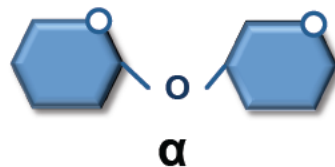
Often identical atomic composition

B) Connectivity

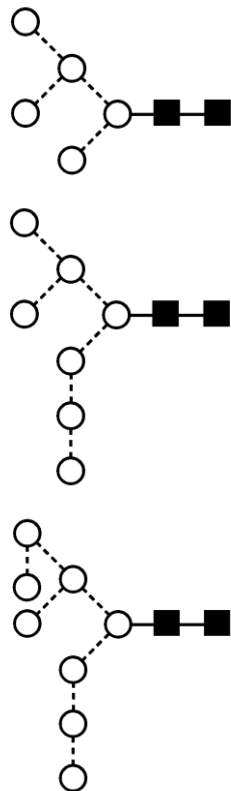Position of glycosidic bond

1,4

1,6

C) Configuration

Stereochemistry of glycosidic bond

α

β

31

Classes of *N*-Linked Glycans

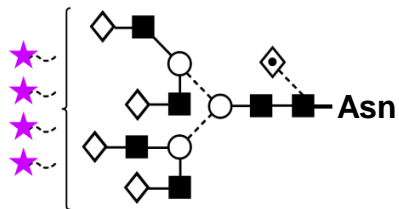**High Mannose**   **Complex**   **Hybrid**
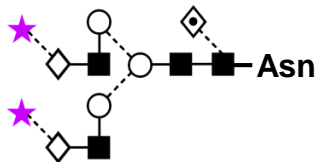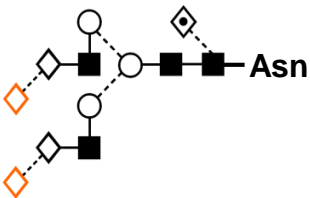
Influence on Biopharmaceutical Production
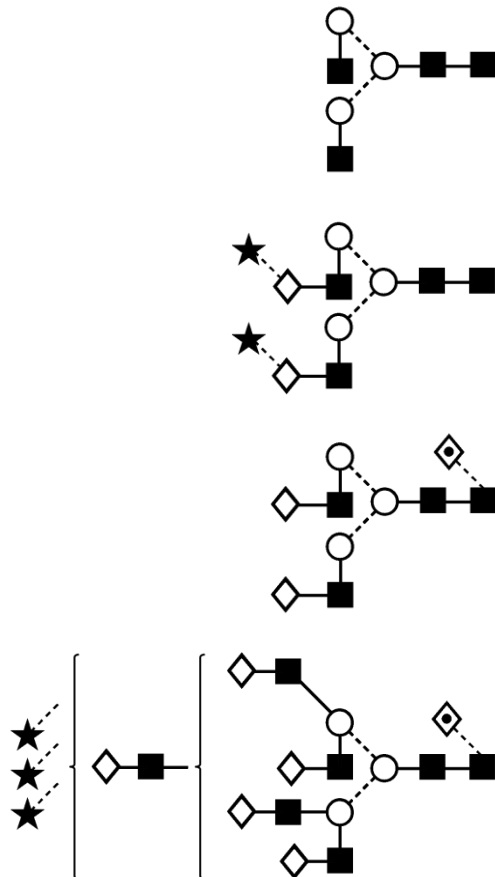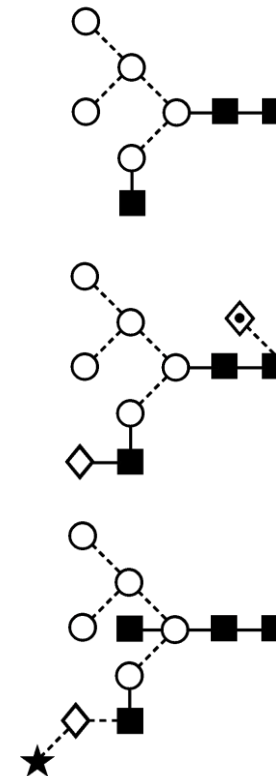
Loss of sialylation decreases EPO half-life from 2 h to 10 min
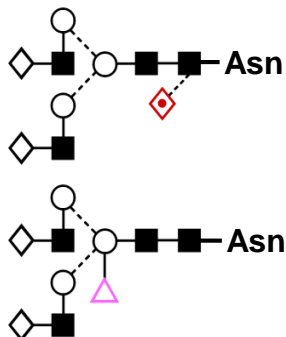Fukuda *et al* (1989). *Blood*; 73(1): 84-89

Desialylation of intravenous immunoglobulin abrogates its anti-inflammatory properties
Kaneko *et al* (2006). *Science*; 313(5787): 670-673

Presence of gal-α(1,3)-gal can induce anaphylaxis (shock) and can be present on biotherapeutics
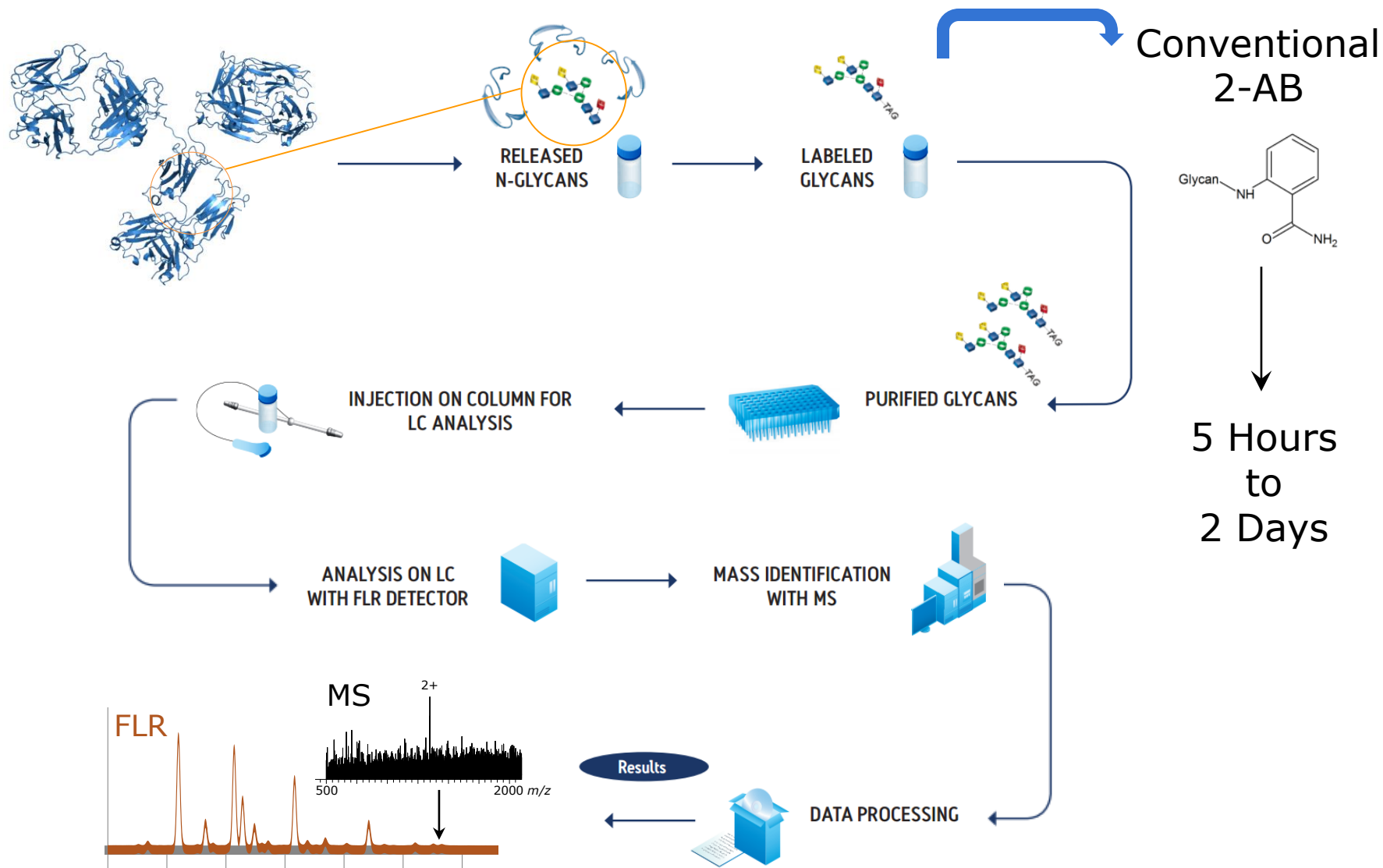Chung *et al* (2006). *N Engl J Med*; 358(11): 1109-1117

Half of all people contain antibodies against β(1,2)-xylose and α(1,3)-core fucose
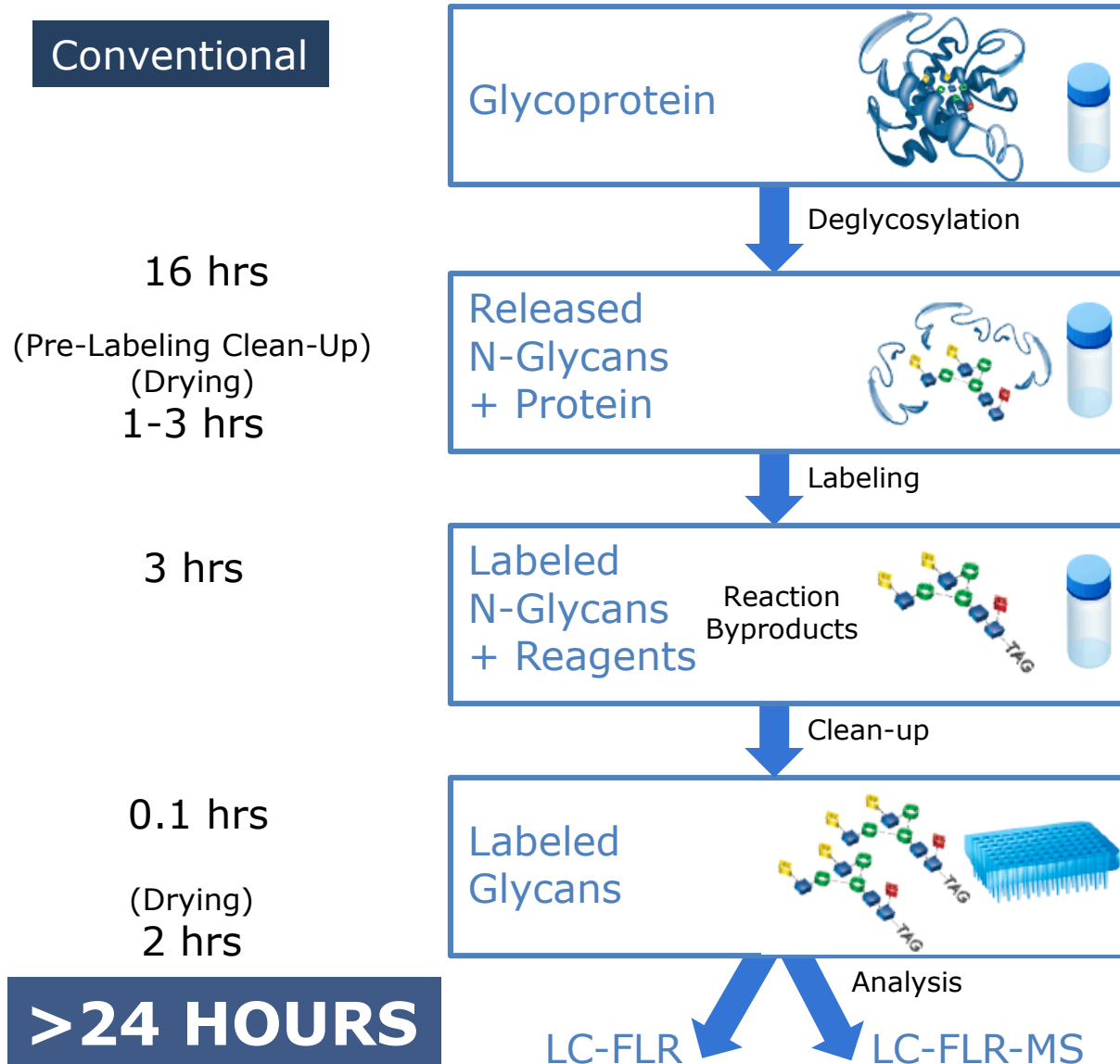Bardor *et al* (1995). *Glycobiology*; 13(6): 427-434

Released Glycan Analysis
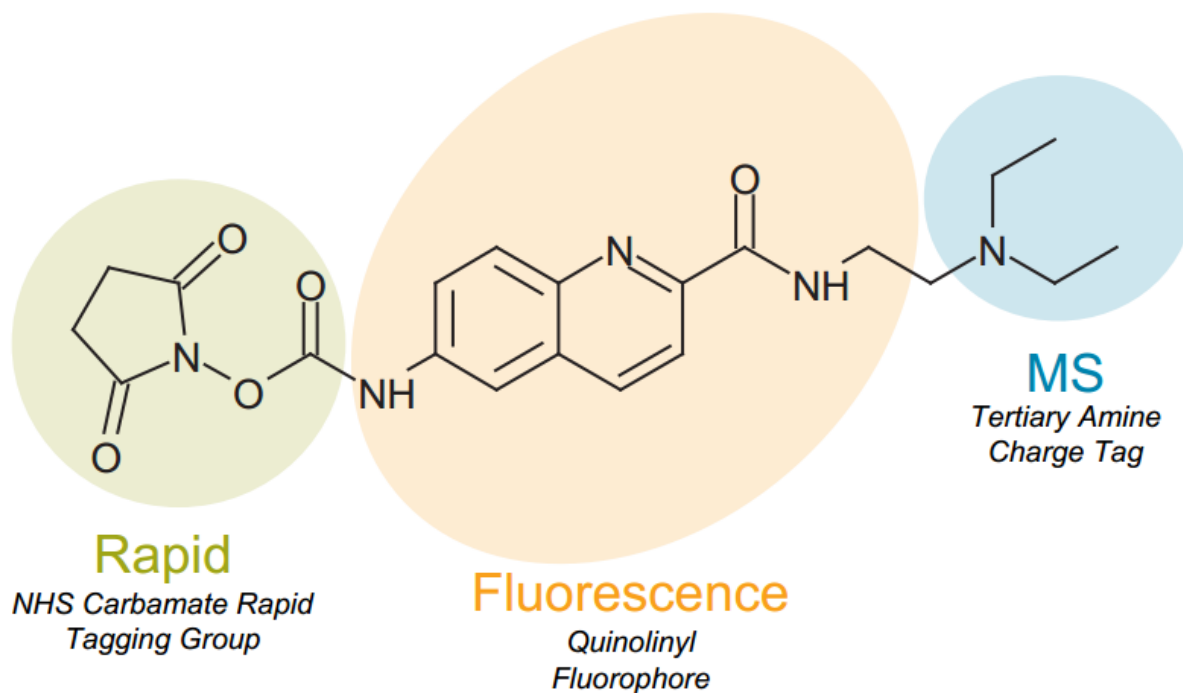## *HILIC Profiling*

Waters

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Conventional
2-AB

5 Hours
to
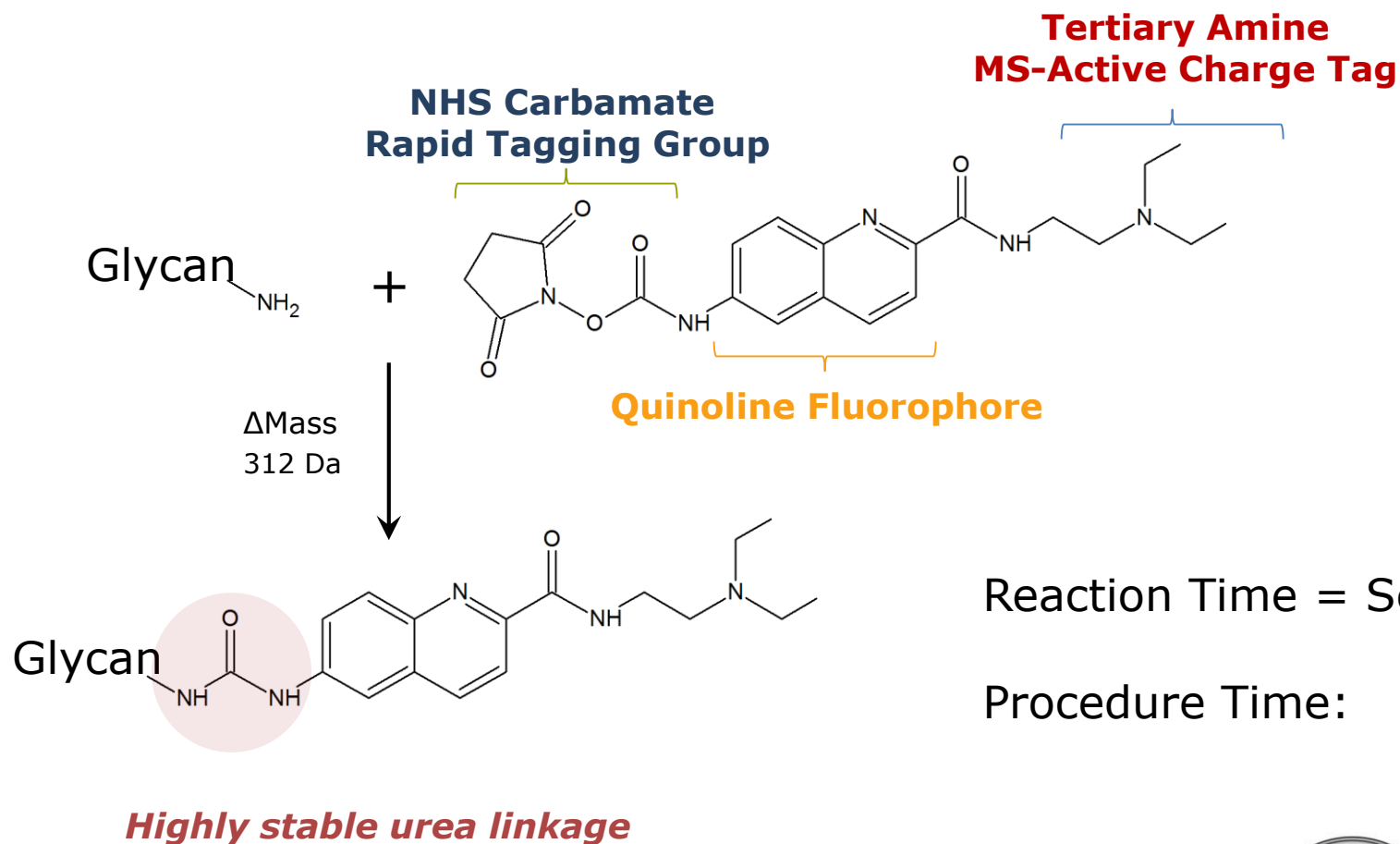2 Days

Conventional Workflow

Conventional

Glycoprotein

↓ Deglycosylation

16 hrs

(Pre-Labeling Clean-Up)
(Drying)
1-3 hrs

Released N-Glycans + Protein

↓ Labeling

3 hrs

Labeled N-Glycans + Reagents

Reaction Byproducts

↓ Clean-up

0.1 hrs

(Drying)
2 hrs

Labeled Glycans

↓ Analysis

>24 HOURS

LC-FLR   LC-FLR-MS

What is new?

Novel *Rapi*Fluor-MS™ (RFMS) Reagent

Rapid
NHS Carbamate Rapid
Tagging Group

Fluorescence
Quinolinyl
Fluorophore

MS
Tertiary Amine
Charge Tag

Patent Pending

*Rapi*Fluor-MS Reagent
Rapid Reaction Kinetics

NHS Carbamate Rapid Tagging Group

Tertiary Amine MS-Active Charge Tag

Quinoline Fluorophore

ΔMass 312 Da

Glycan—NH₂ +

Highly stable urea linkage

Reaction Time = Seconds

Procedure Time:

5min

Simplified Sample Preparation

Conventional

Glycoprotein
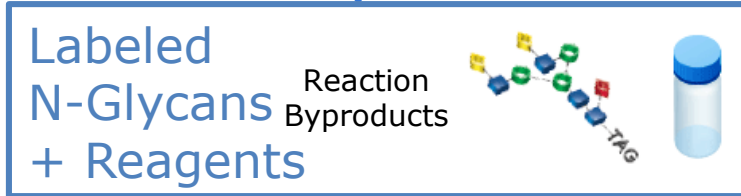
GlycoWorks RapiFluor-MS
N-Glycan Kit

NEW ENGLAND BioLabs Inc.

Deglycosylation

16 hrs

(Pre-Labeling Clean-Up)
(Drying)
1-3 hrs

**Released
N-Glycans
+ Protein**

<15 min

Labeling

3 hrs

**Labeled
N-Glycans
+ Reagents**

Reaction
Byproducts

5 min

Clean-up

0.1 hrs

(Drying)
2 hrs

**Labeled
Glycans**

10 min

Analysis

>24 HOURS

LC-FLR   LC-FLR-MS

30 min

*Patent Pending*11
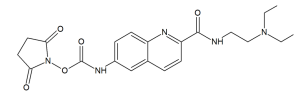
RFMS vs. 2AB for MS sensitivity comparison

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Greater than **100x MS** response over 2AB labeling

2AB Glycans released from *10 µg* of mAb

RapiFluor-MS Glycans released from *0.2 µg* of mAb
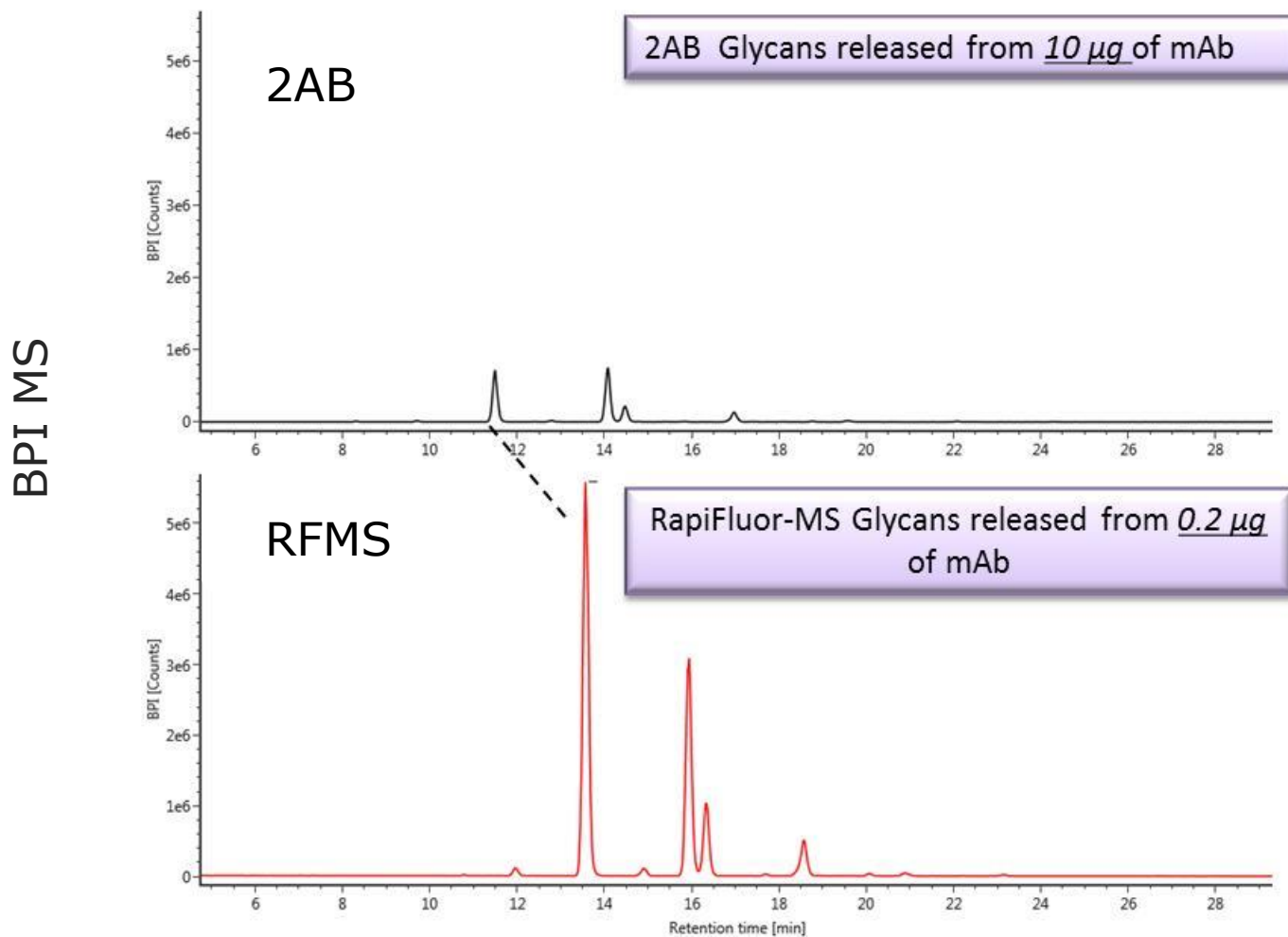
Sample: NIST RM 8670 mAb lot #3F1b

Glycan Characterization with RFMS Labeling
Comparable FLR and MS response across a broad range of glycans

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IgG

Simple bi-antennery structures

Fetuin

Large, complex structures

FLR

BPI MS

FA2

A3G3S3

A3S1G3S3

FA2G2S1

Fucose
GlcNAc
Manoses
Galactose
NeuAc

Xevo G2-XS QTof

13
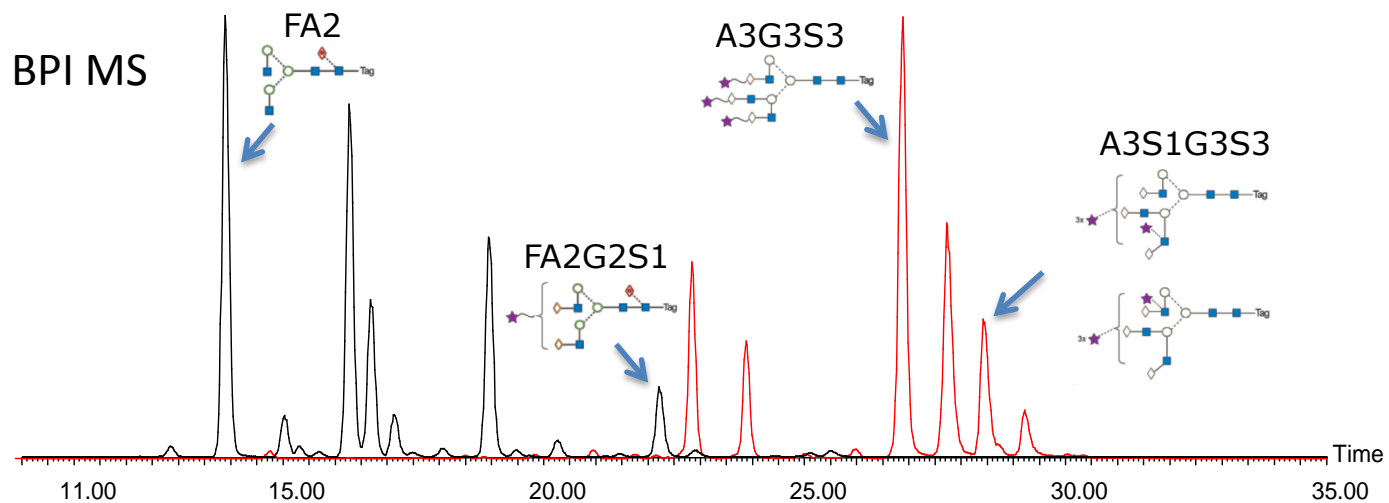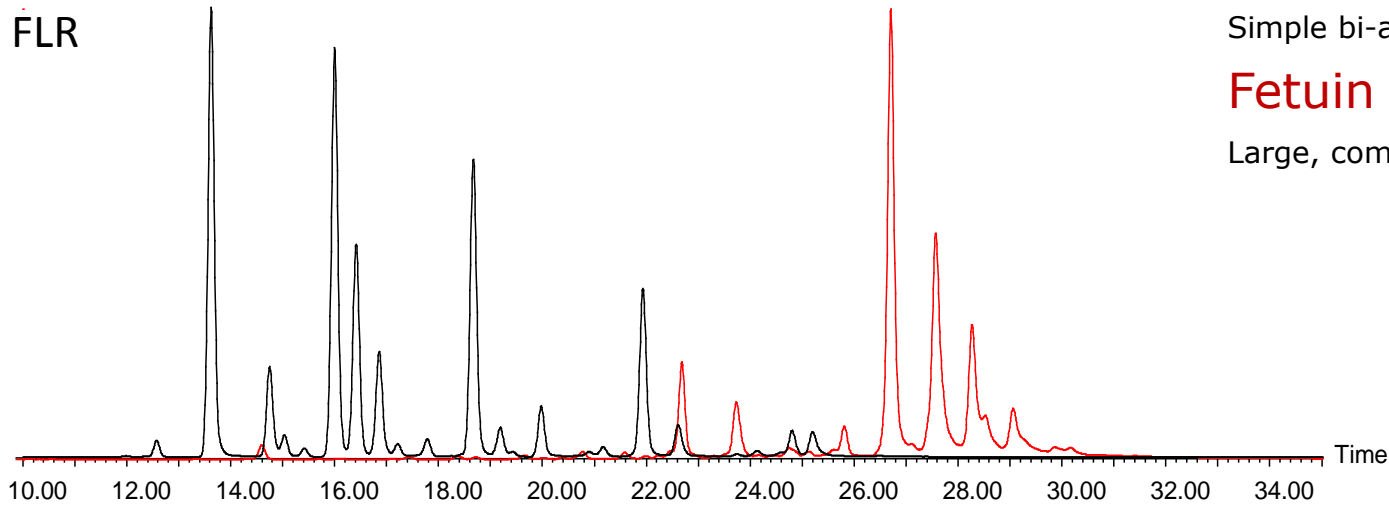
Detailed MS Profiling of a Wide Dynamic Range of Glycans labeled with RFMS
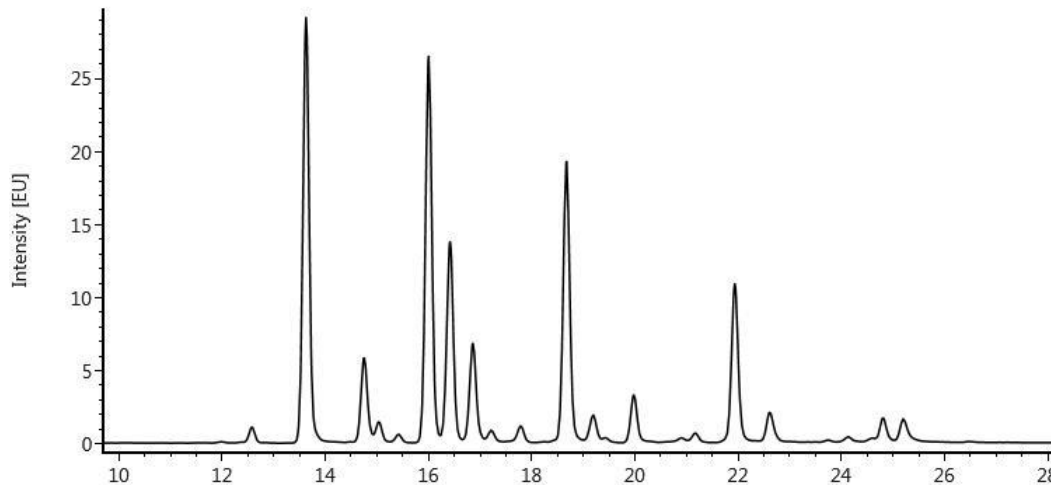
Fetuin

FLR
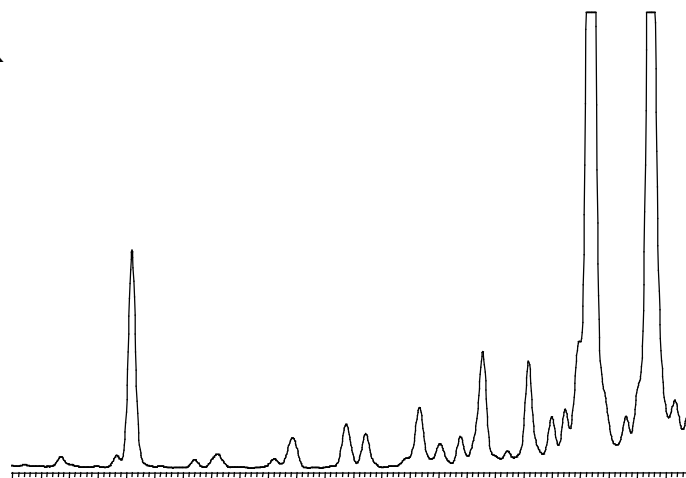
FLR
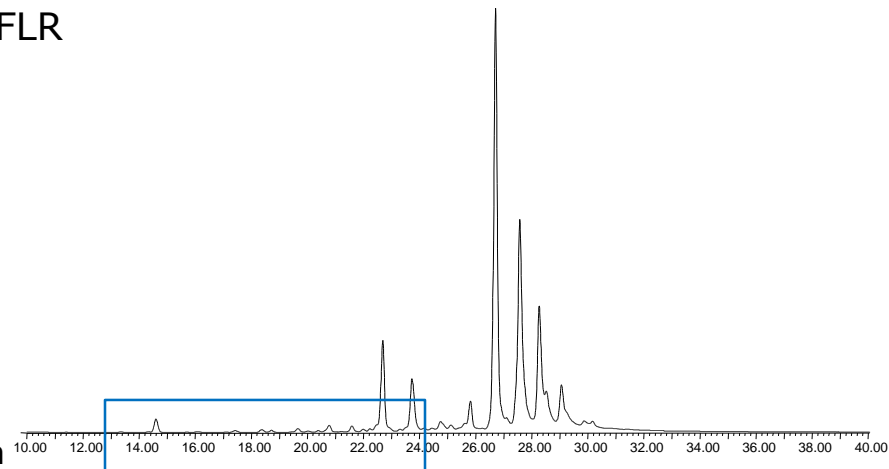
Zoom In ~20x

BPI MS

BPI MS

14
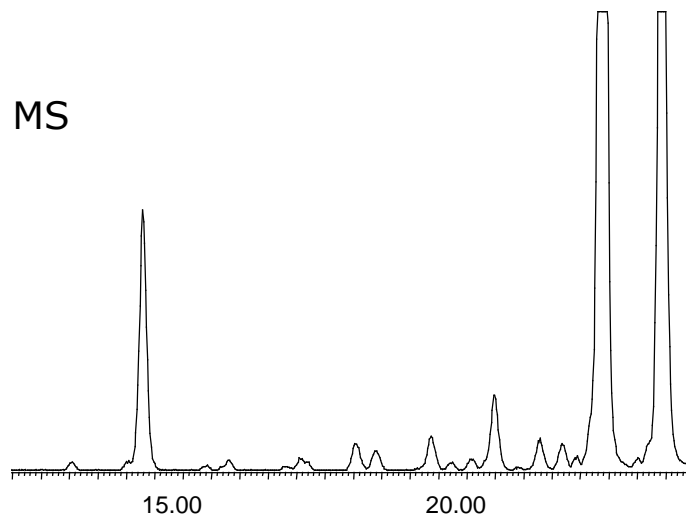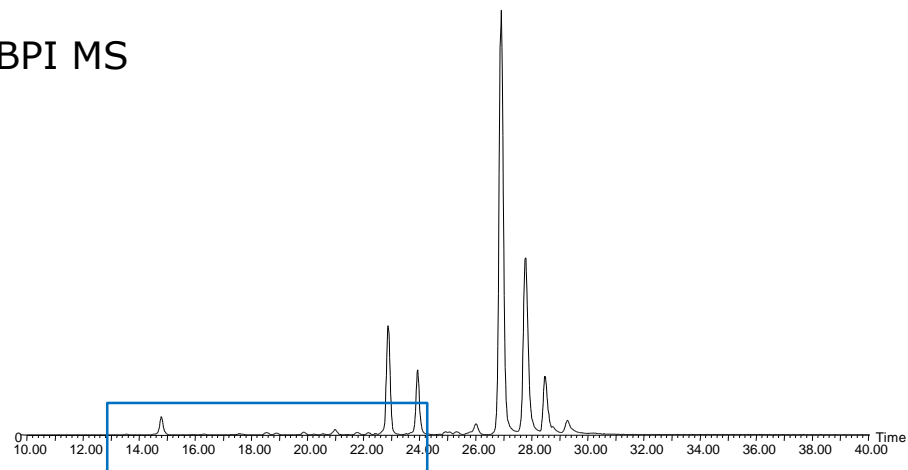
RFMS Labeling Enables MS Detection of Very Low Abundance Glycans

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Human IgG
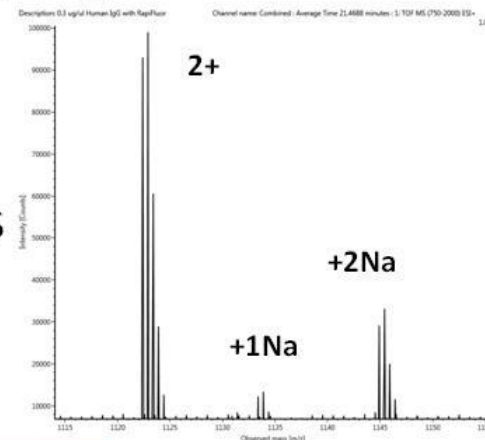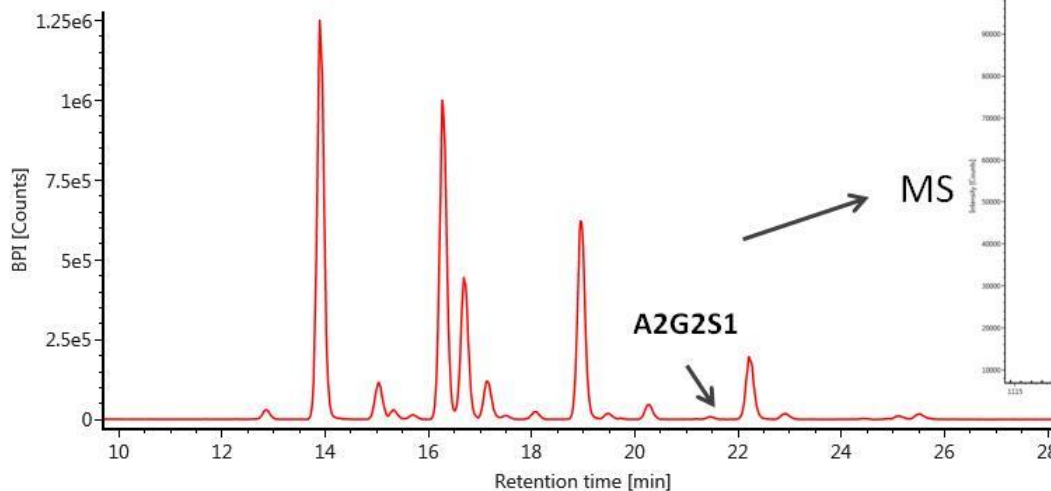
RFMS extends MS enhancement throughout the glycan fragmentation pattern
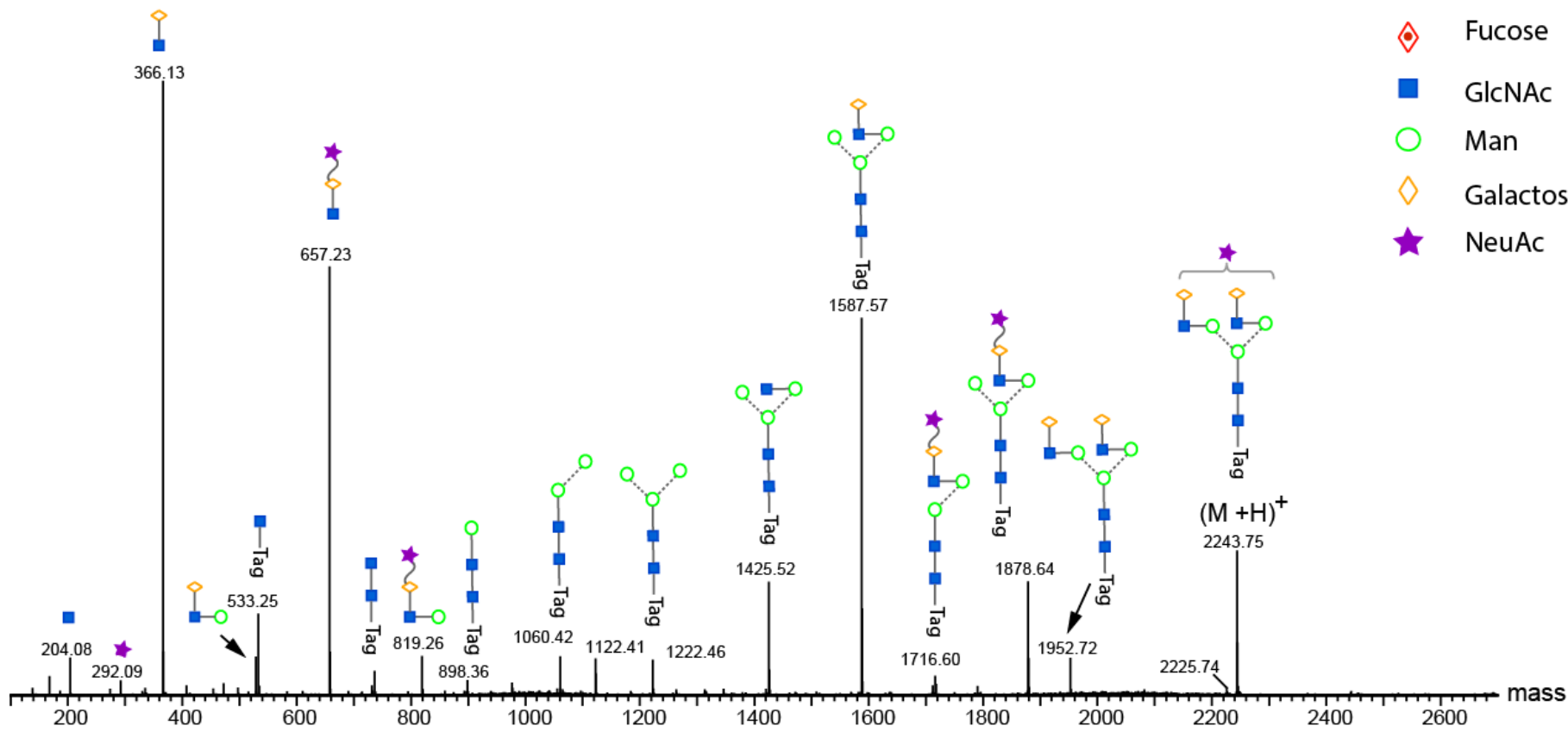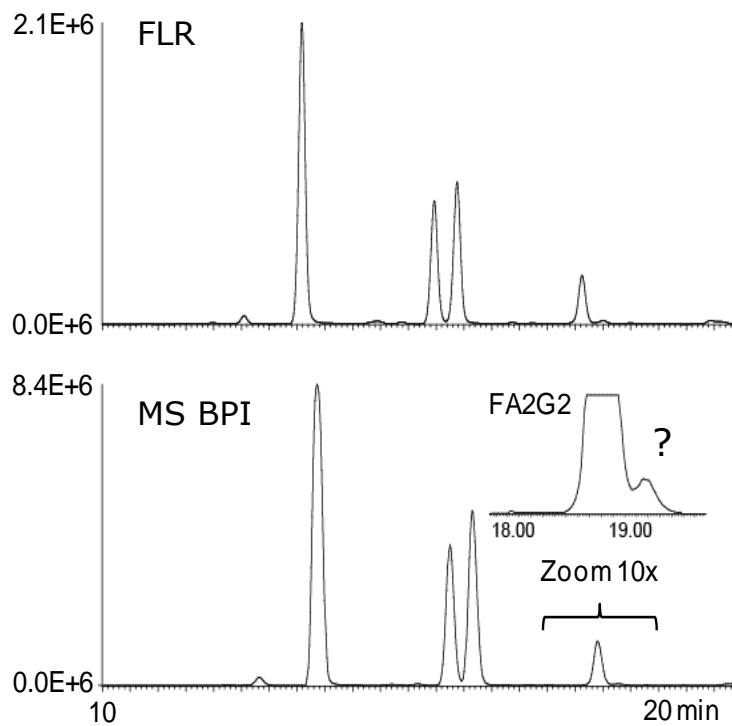
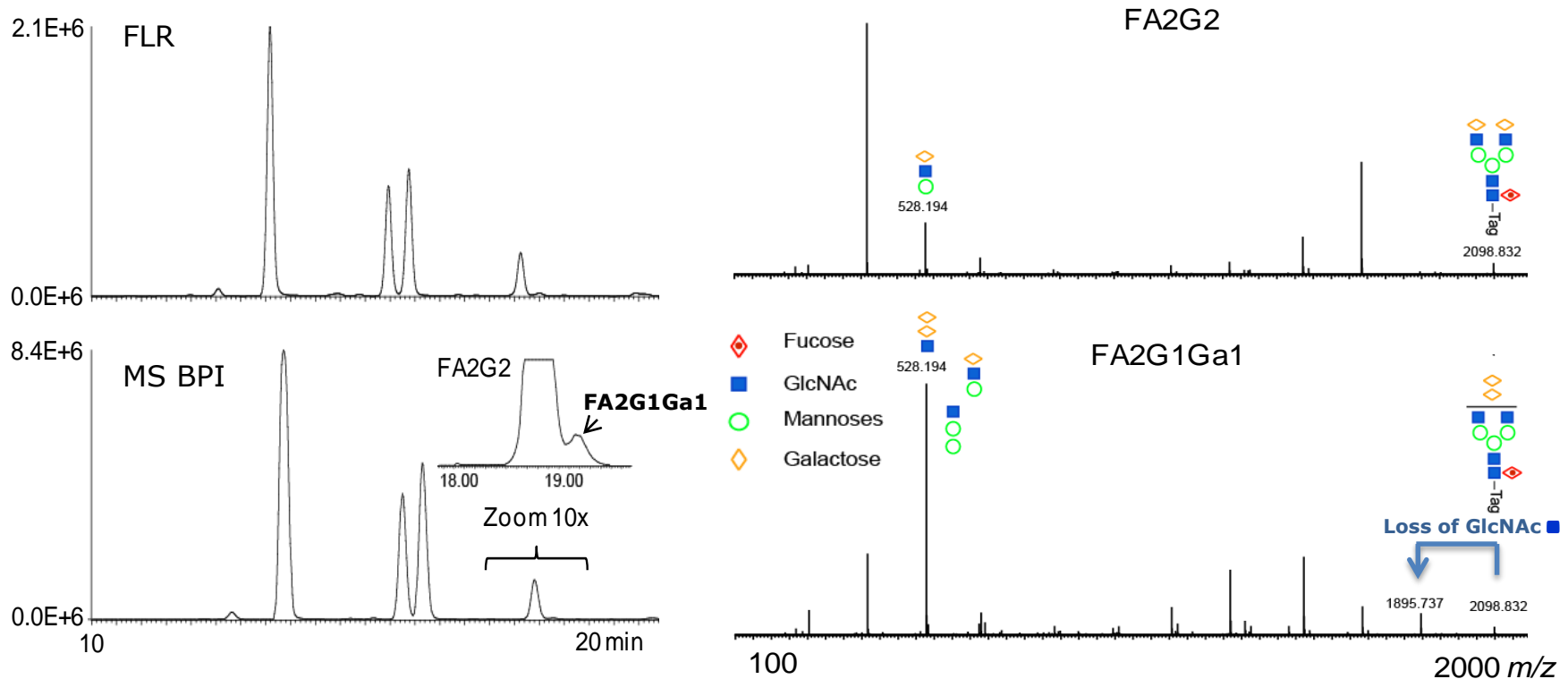# RFMS enables easy assignment of two isobaric glycans

RFMS labeled N-glycans from a murine IgG1 mAb

- The two isobaric glycans, FA2G2 and a minor shoulder peak, partially resolved by HILIC
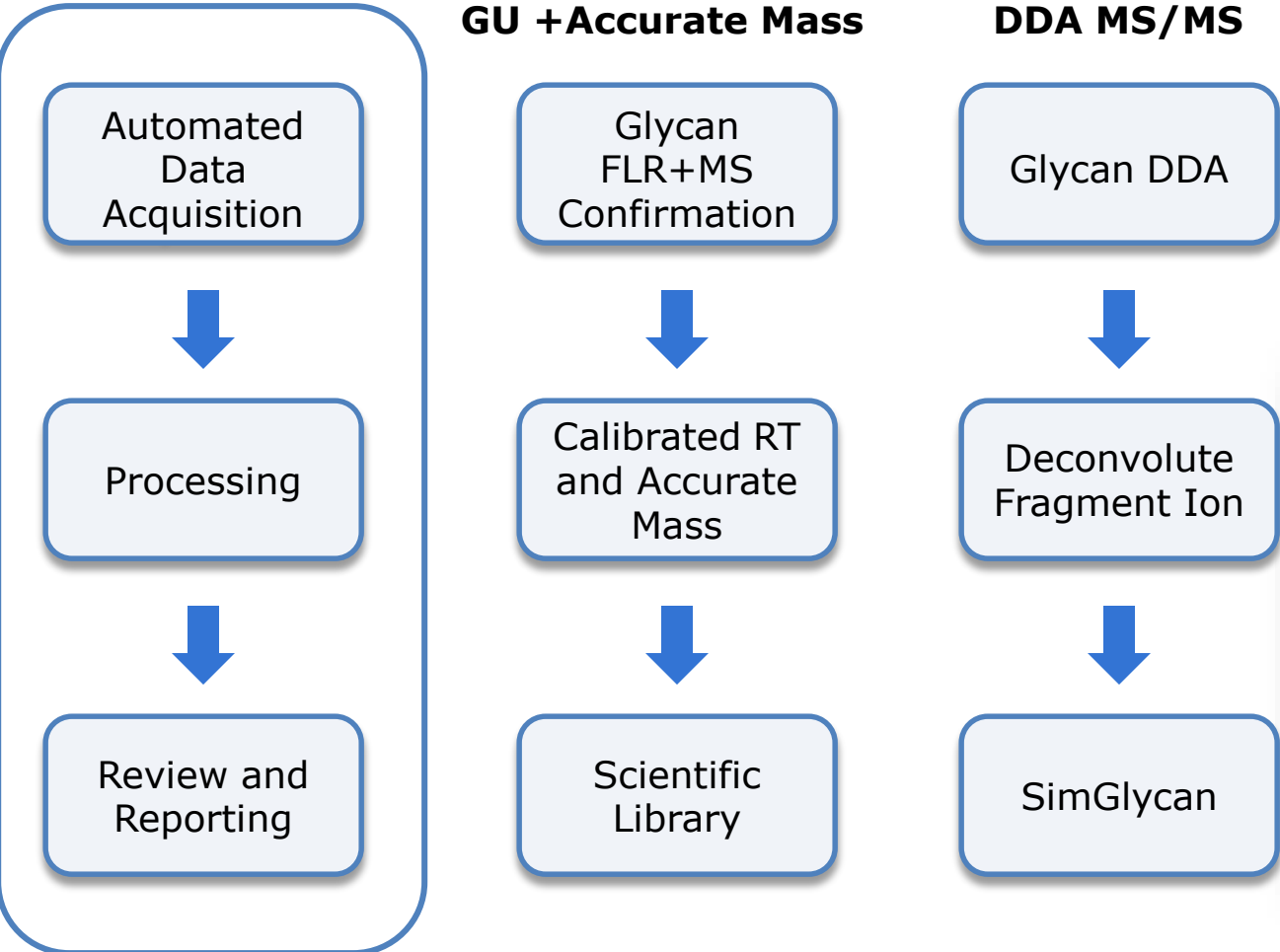- The minor peak represents only 0.7% of total FLR signal

RFMS enables easy assignment of a minor shoulder peak of FA2G2 as FA2G1Ga1

RFMS labeled N-glycans from a murine IgG1 mAb

- The two isobaric glycans, FA2G2 and a minor shoulder peak, partially resolved by HILIC
- The minor peak represents only 0.7% of total FLR signal
- Structurally diagnostic ions: 1) predominant 528 m/z ion and 2) prominent GlcNAc loss
- High sensitivity and information rich fragmentation data support the identification of the isobaric, lower abundance species as an α-Gal containing FA2G1Ga1.

UNIFI Glycan Application Workflows

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**HILIC FLR
GU +Accurate Mass**

**HILIC FLR
DDA MS/MS**

Automated Data Acquisition

↓

Processing

↓

Review and Reporting

Glycan FLR+MS Confirmation

↓

Calibrated RT and Accurate Mass

↓

Scientific Library

Glycan DDA

↓

Deconvolute Fragment Ion

↓

SimGlycan

The Utility of GU Values

What is a GU Value?

- GU stands for Glucose Unit

- A GU value is a **normalized glycan structure retention time** observed in HILIC for glycan peaks**,** obtained using a dextran ladder calibration

Why is the GU approach useful?
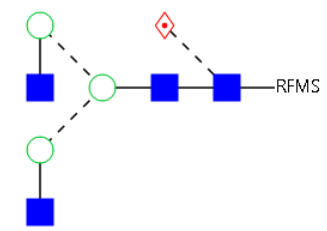
- GU Values assist in normalizing glycan retention time across days, instruments and laboratories, so data can be compared and shared easily.

- GU Values facilitate more routine glycan assignments by enabling the creation of a single glycan GU retention library.

Generating Waters RFMS GU Glycan Libraries

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FLR label

mass

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

HILIC FLR GU + Accurate Mass Workflow

Method Robustness and Transferability, Confident Assignments
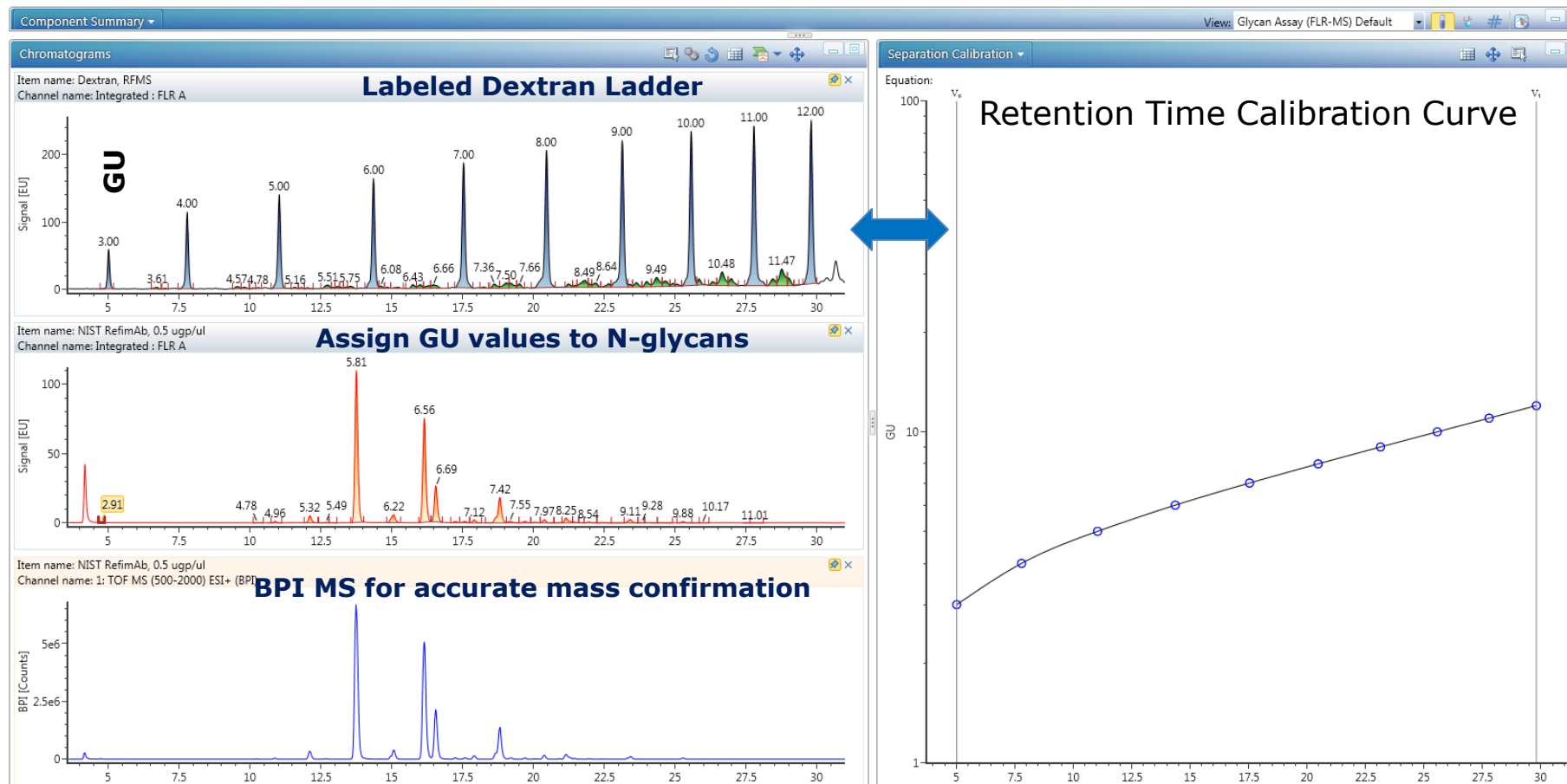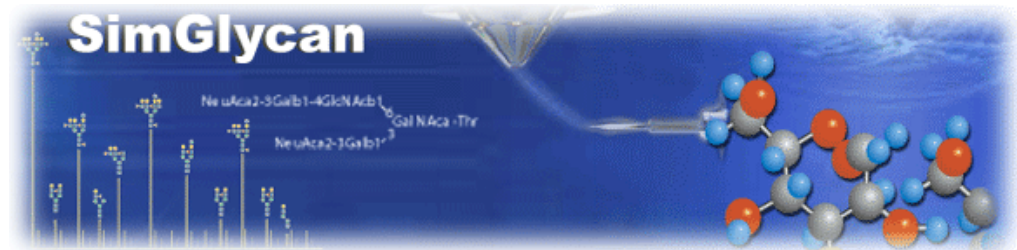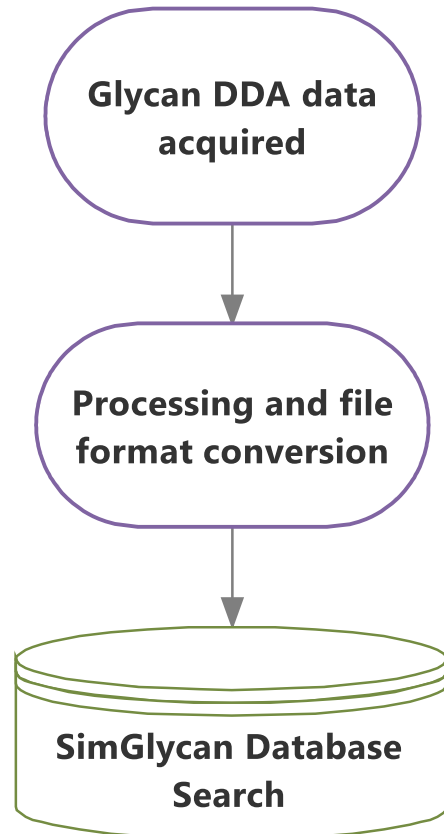
Glycan GU Scientific Library Search for Confident Glycan Assignments
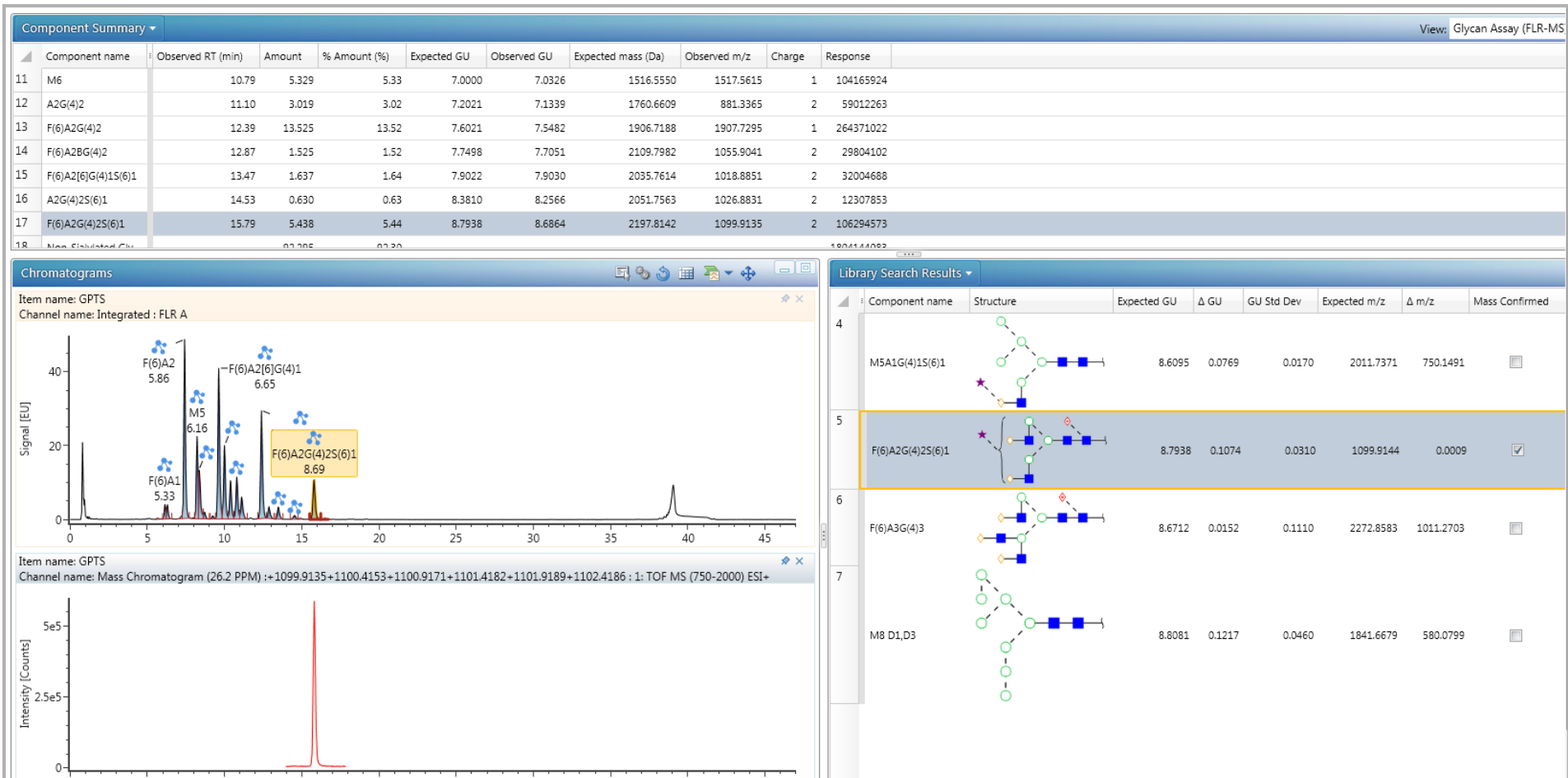
Both 2-AB and RFMS labeled glycan performance test standards are now available to support this workflow
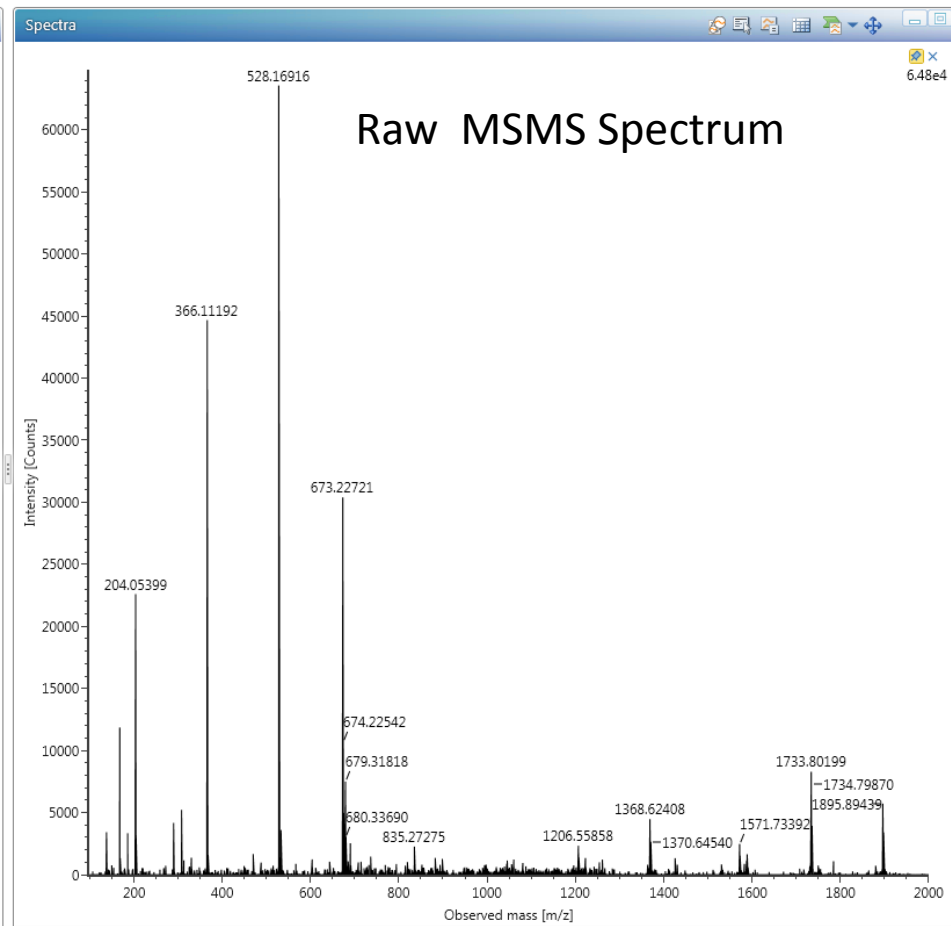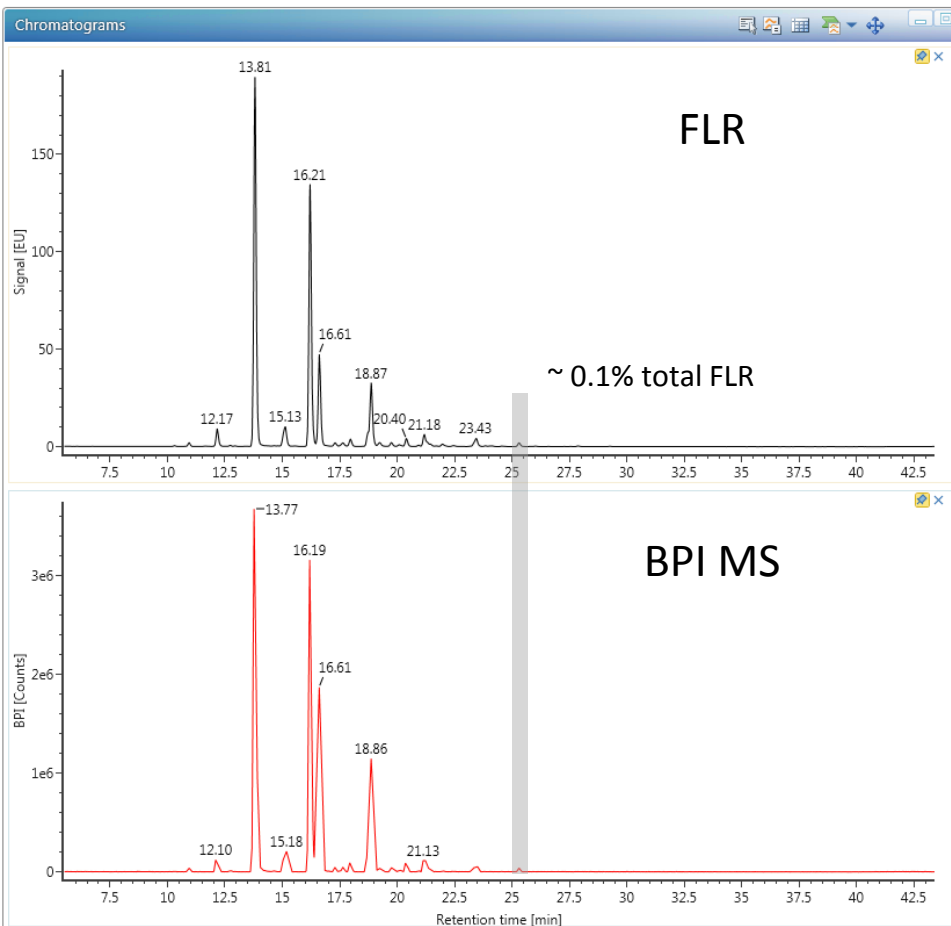
UNIFI Glycan DDA Workflow

Glycan DDA data acquired

↓

Processing and file format conversion

↓

SimGlycan Database Search

UNIFI Acquire and Process Glycan DDA Data
RFMS labeled N-glycans from mouse IgG

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MSMS fragmentation of a highlighted minor glycoform was displayed

FLR

~ 0.1% total FLR

BPI MS

Raw MSMS Spectrum

Deconvolute & deeisotope
Export as .LCS (or mzml) format

Automated Glycan Identification using .LCS Files with SimGlycan

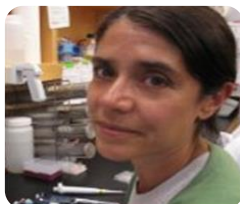## The minor glycoform was correctly identified as FA2G2Ga1Sg1

Work in progress

- Developing a scientific library for RapiFluor-MS labeled glycans for automated glycan assignment

- Applying RapiFluor-MS label for more complex glycosylated proteins

Acknowledgements

Waters
Matt Lauber   PhD

New England Biolabs
Paula Magnelli PhD

- Professor Pauline Rudd (COI)
  - Mark Hilliard (UNIFI Glycan Scientific Library)
  - Giogio Carta (UNIFI Glycan Scientific Library)