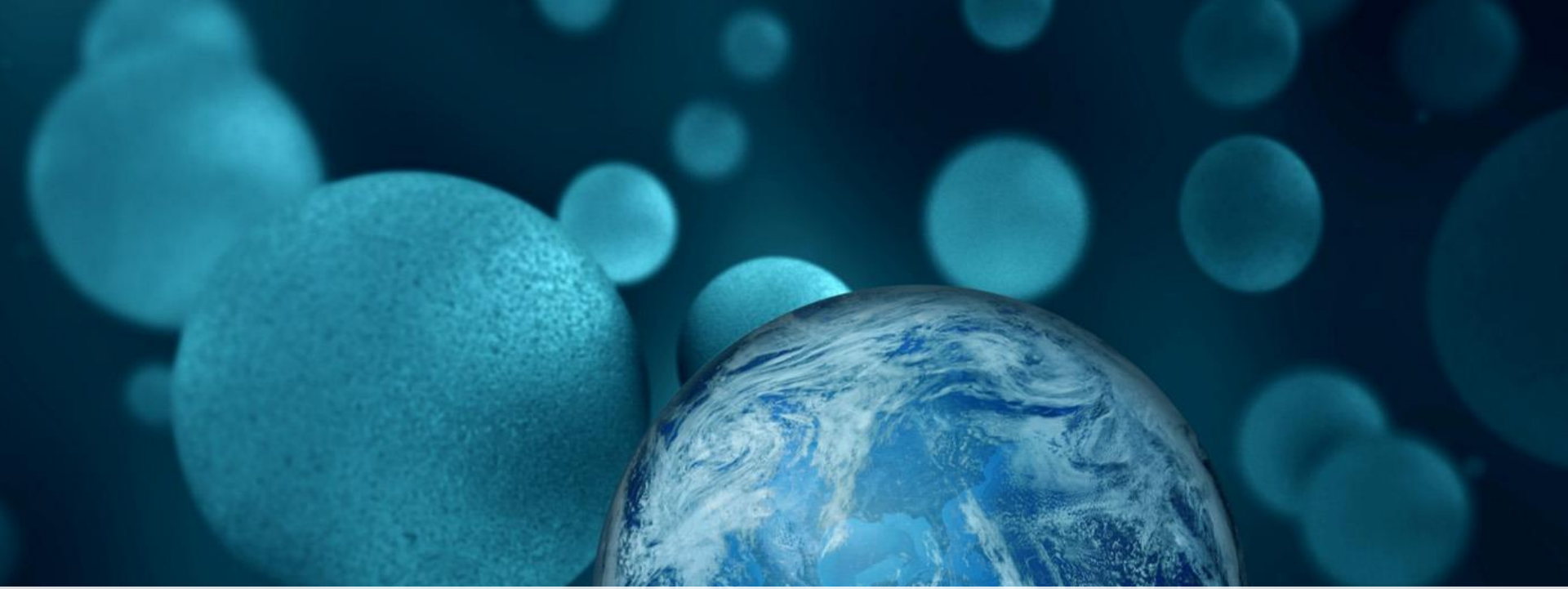

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Rapid and Robust *N*-Glycan Profiling Workflow for Therapeutic Proteins with Multicapillary CE & UHPLC

Anahita Eckard, Ph.D.
Staff Scientist
Pharma Analytics, Bioproduction Division
South San Francisco, California, USA

Agenda

Introduction

- Major category of Biotherapeutics approved by FDA
- When to monitor glycosylation

Thermo Fisher GlycanAssure Platform

- Introduction to traditional sample prep
- Introduction to Multicap CE3500xL system
- Introduction to GlycanAssure APTS sample prep. kit

Sample Data Sets

- Quality of rapid deglycosylation
- Quality of in-solution dye labeling
- Quality of clean up module
- Workflow Scalability
- Capability of sample analysis with low protein input

Sample Data Sets

- Comparison of glycoprofiling between UHPLC & CE3500xL
- Variation of workflow on both platforms

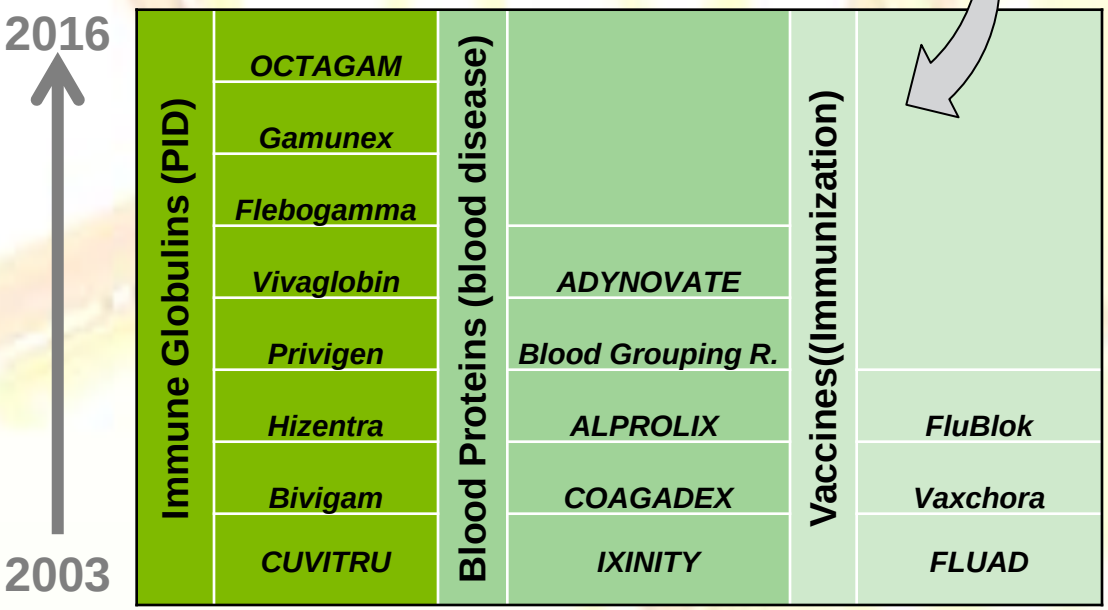
Biotherapeutics

- **Major Category of Biotherapeutics DP Approved by FDA Every Year:**

  -Primary Immune system (5 classes of Ig's)
  -Growth hormones
  -Lysosome enzymes
  -Blood agents & proteins

~>65% are mAb's

Selected Approved Biologics by FDA



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Glycosylation is Monitored at Various Stages

High throughput

Clone Selection

Product Development

Product Optimization

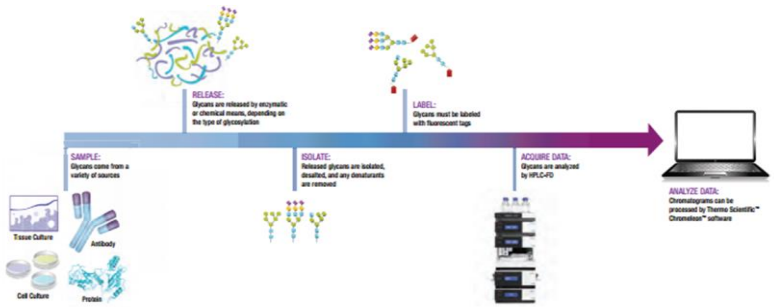
BLA (Comparability)

Process Monitoring

Lot Release

Medium/ Low throughput

Glycan Profiling



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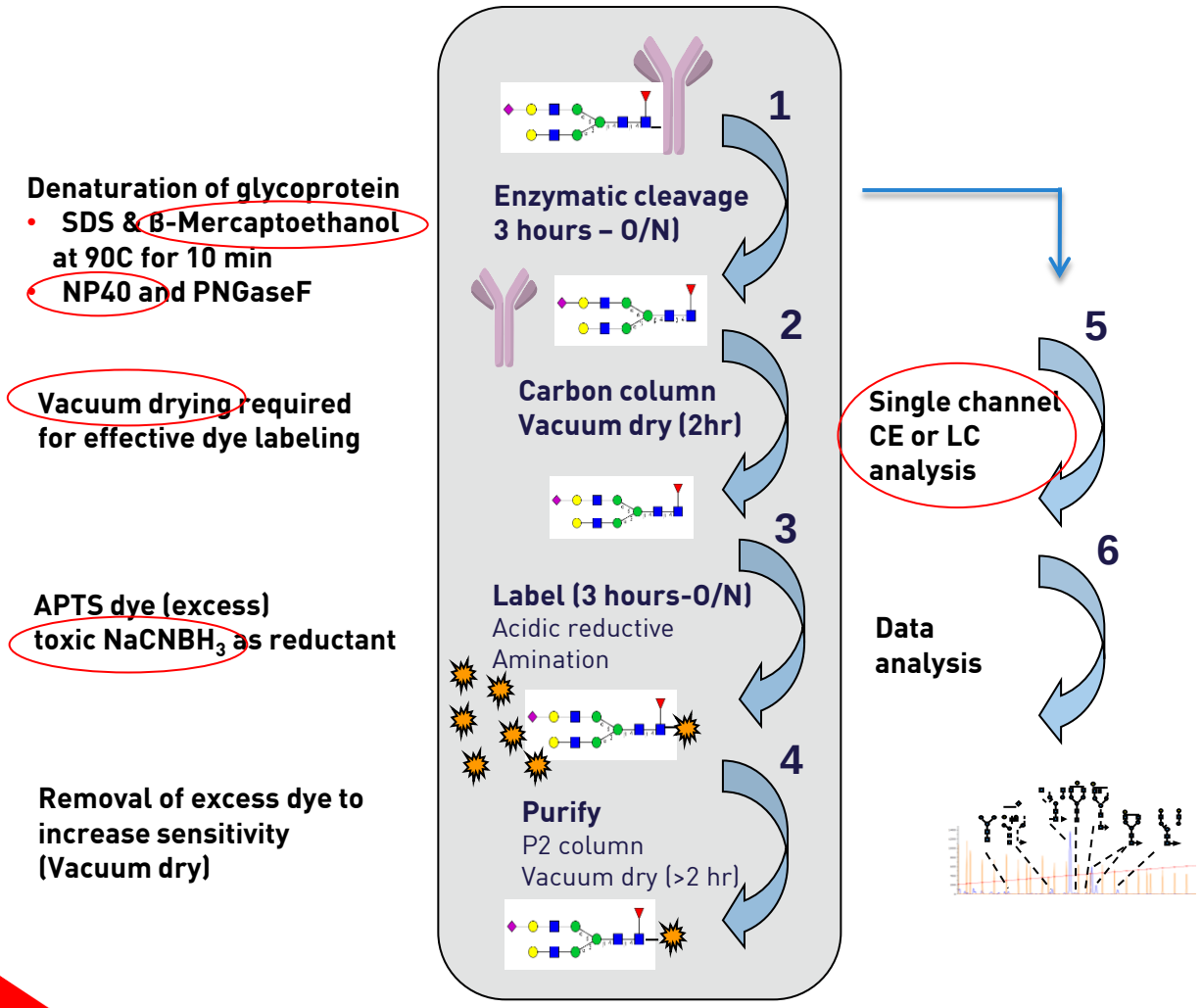
Long and Labor Intensive Traditional Workflow



Total Time: 1– 3 days

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GlycanAssure™ Analysis Platform

APTS Kit, P/N A28676

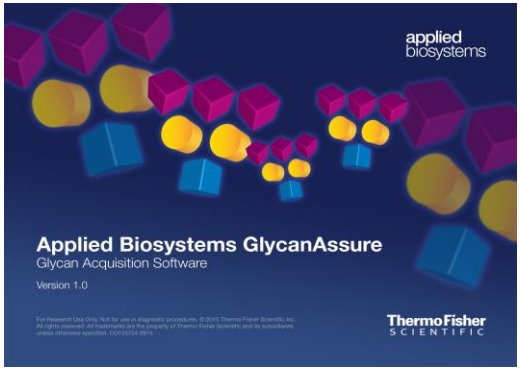
Teal Kit, P/N A28677

Turquoise Kit, P/N A28678

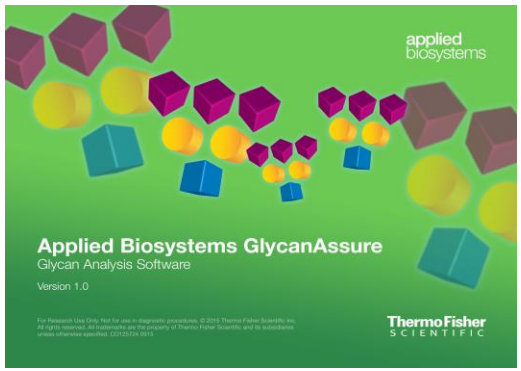
Multicapillary CE

Applied Biosystems™
3500 & 3500XL
P/N A30467 & A30556

First Fully Integrated System Combining Throughput & Data Quality



Data Acquisition Software
P/N A30750



Data Analysis Software
P/N A30751

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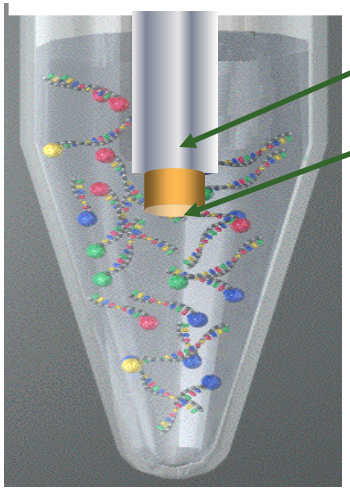
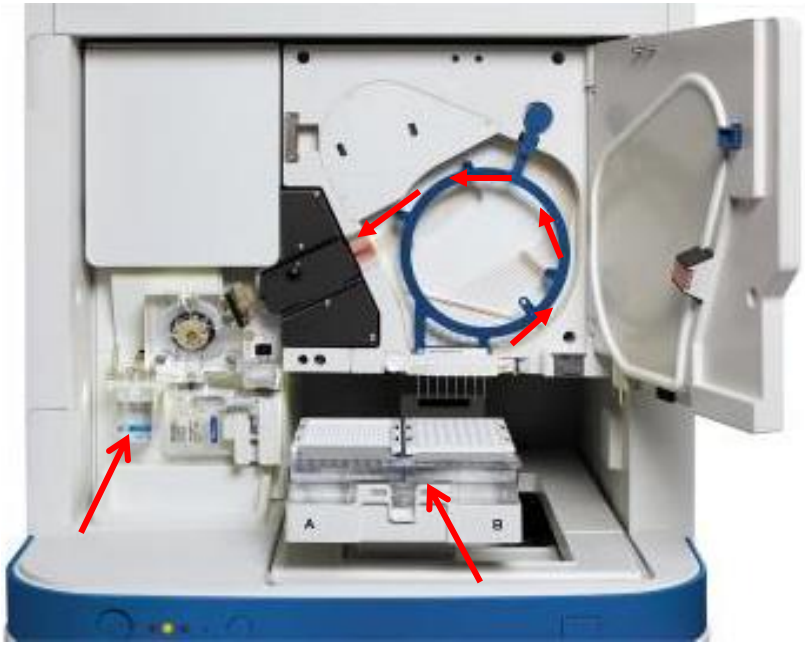
Benefits of Applied Biosystems™ CE 3500xL Platform

- ✓ 1 µl sample volume at min protein input (~2 ug)
- ✓ Multi-capillary option
- ✓ Fast Analysis (2 min/sample)
- ✓ Good Reproducibility
- ✓ High separation efficiency

Highlight of Applied Biosystems™ CE 3500xL

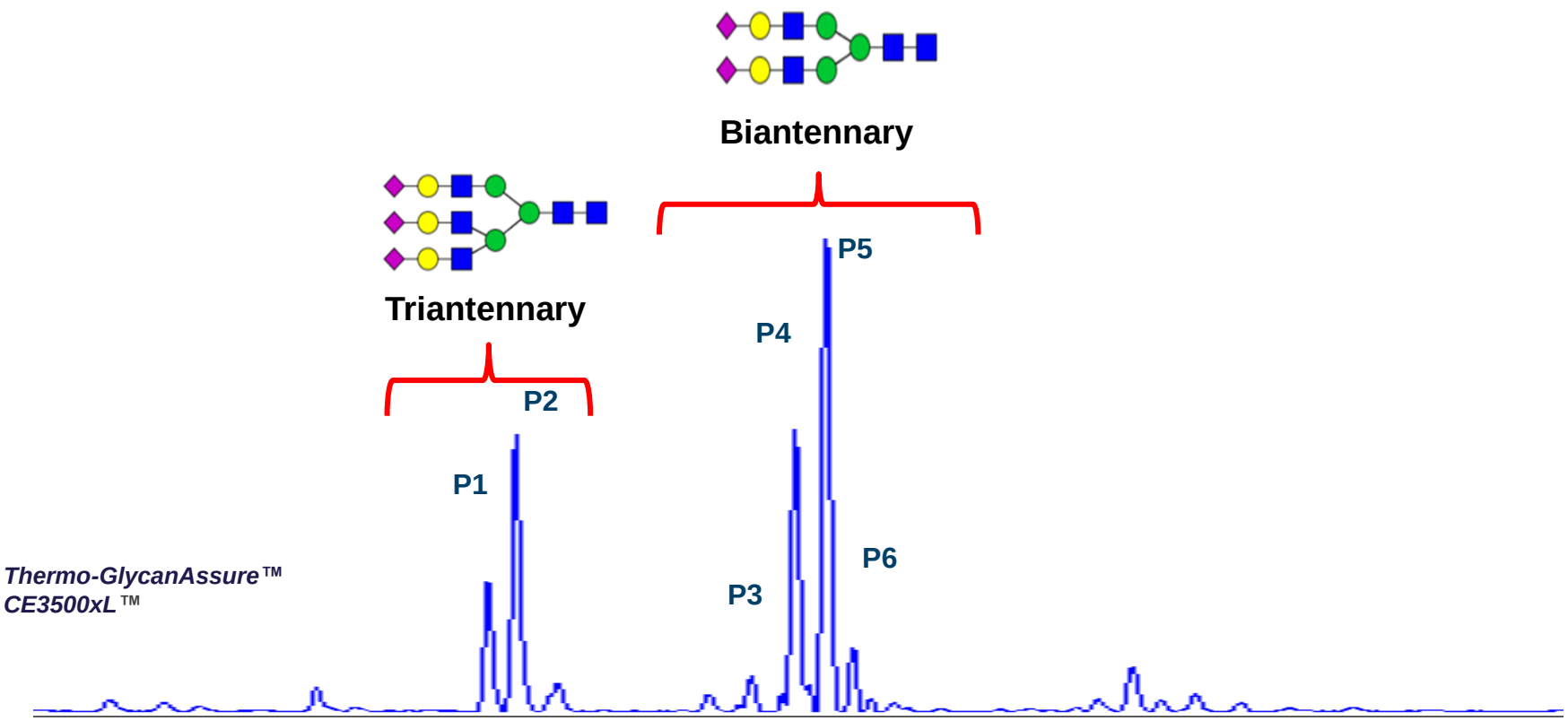




- Capillary and electrode (cathode) are placed into the sample
- Voltage is applied for a specified time
- Negatively-charged glycans enters the capillary
- Capillary is removed and placed into conductive buffer fluid for EOF
- Glycans migrates toward the positively-charged electrode (anode) at the other end of the capillary

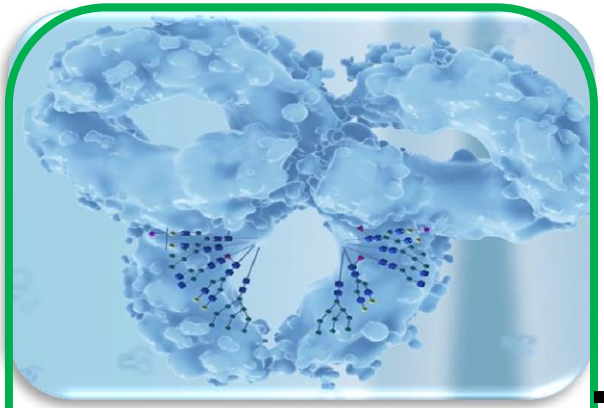
https://www.youtube.com/watch?v=wSqLVxQS0pk

Improved Resolution of High Sialylated Glycans Analysis



- A superior resolution between Trisialylated Glycans of P (1,2) and Disialylated glycans of P (3,4), P (4,5) and P (5,6) were observed compared to similar results published by leading glycan LC kit and leading single capillary CE

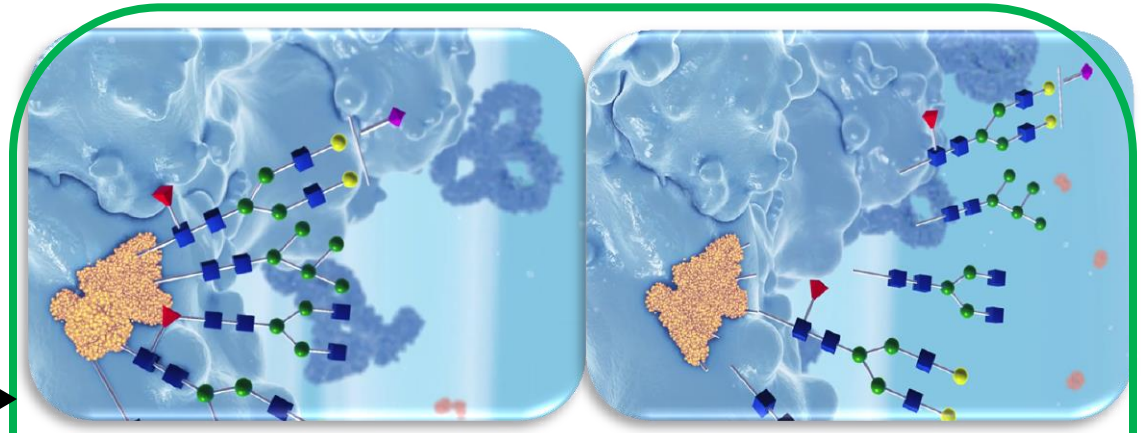
GlycanAssure™ Workflow (TTR: <2.3 h)



Denaturation

-5 min, at 80ºC
- 6µl LC-MS friendly denaturant
& 1.2 µl denaturant buffer
- ~2-50 µg protein input

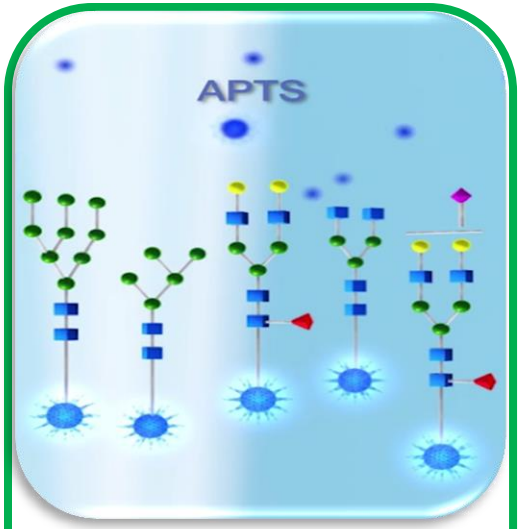
-No BME or NP40
-Compatible with digestion, labeling & MS eliminating the need for glycan clean up



Rapid Enzymatic Deglycosylation

-10 min, 50ºC
-1.5 µl PNGase-F digestion
- 3 µl enzyme buffer

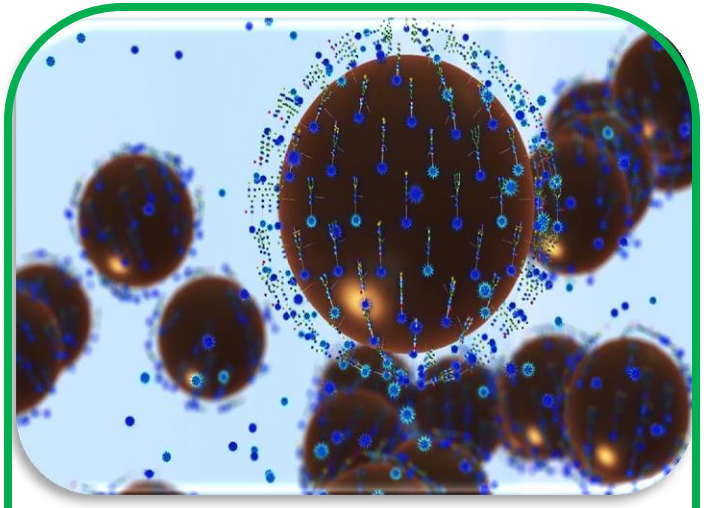
GlycanAssure™ Workflow (TTR: <2.3 h)



Dye Labeling

-60 min, 50ºC
-APTS, Borane Reductant

-No vacuum centrifugation steps
-No use of Sodium cyanoborohydride



Dye Removal

-20 min
-1x ACN. Wash
-2x Wash buffer
-Elution

CE Separation

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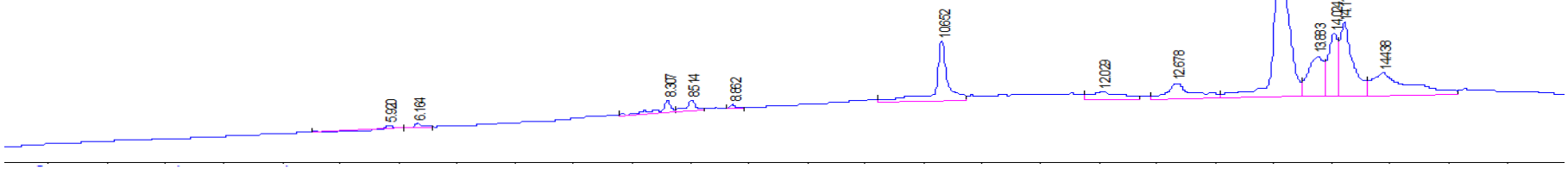
Completeness of Rapid Deglycosylation Assured- Fetuin

Glycosylated

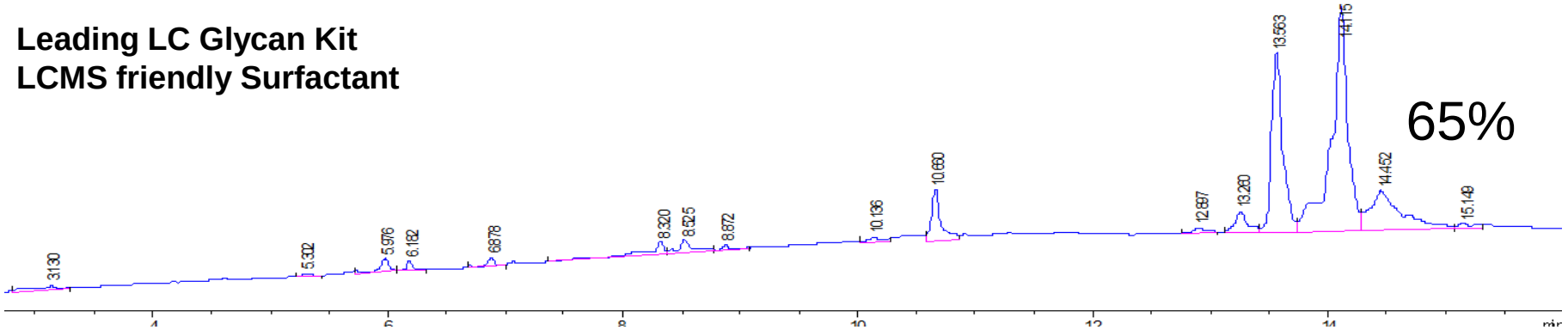
DeGlycosylated

No Denaturation-Only Enzymatic Deglycosylation



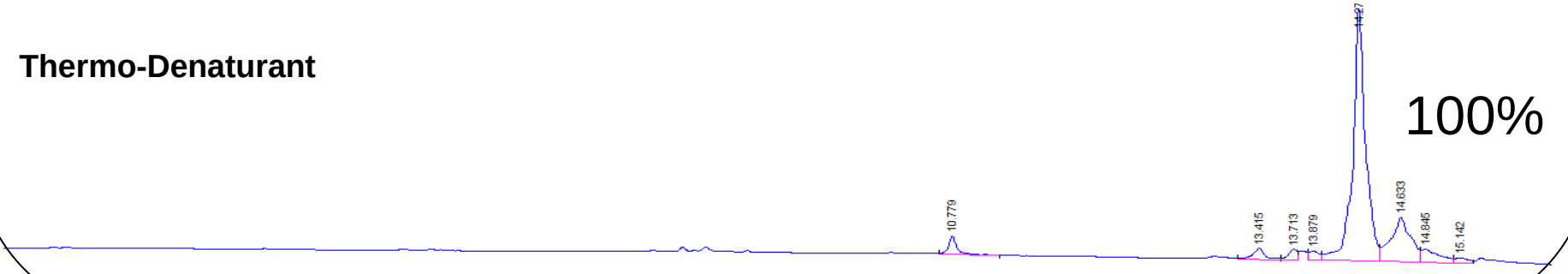
Leading LC Glycan Kit
LCMS friendly Surfactant

65%



Thermo-Denaturant

100%



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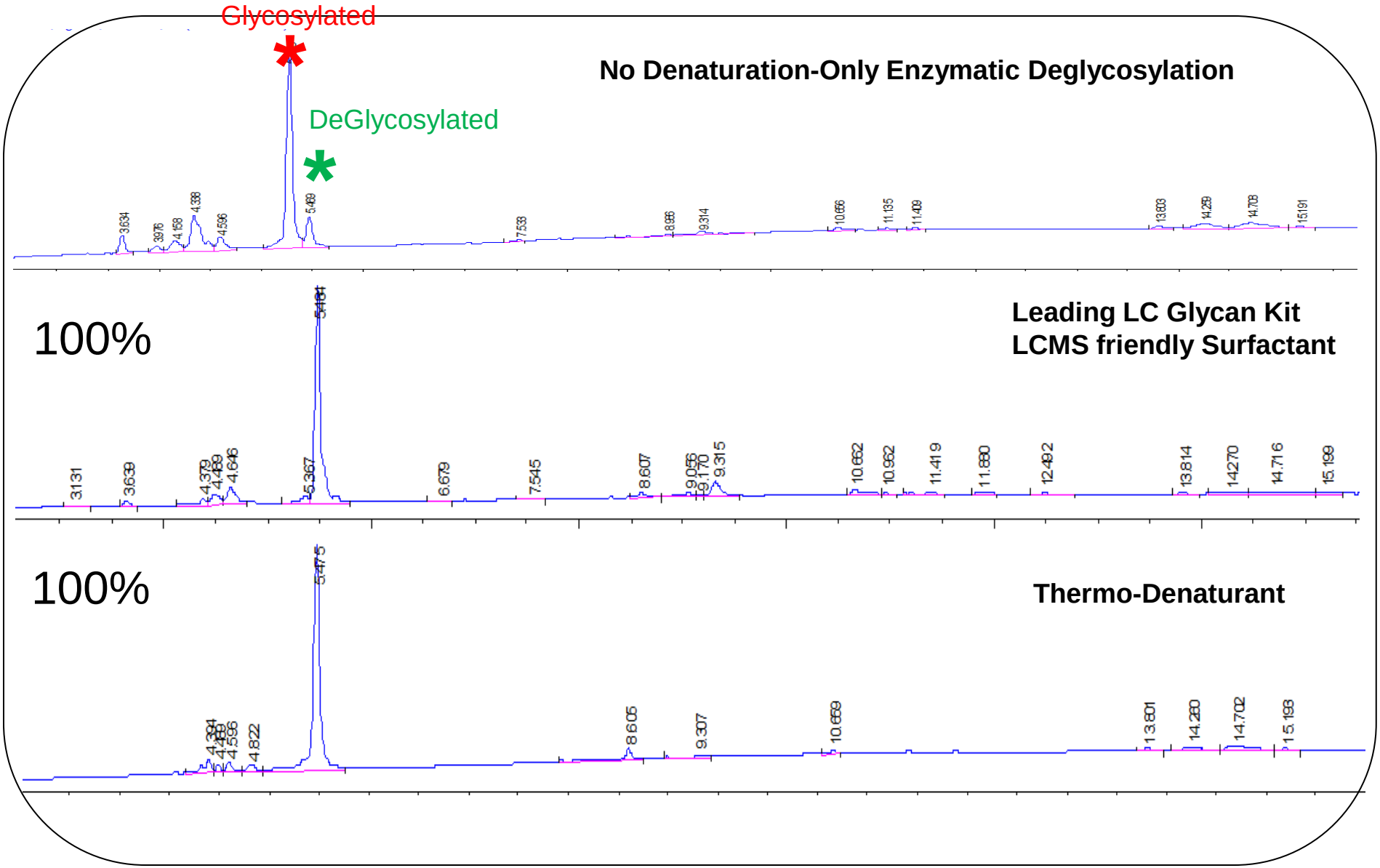
Completeness of Rapid Deglycosylation Assured- Rnase B



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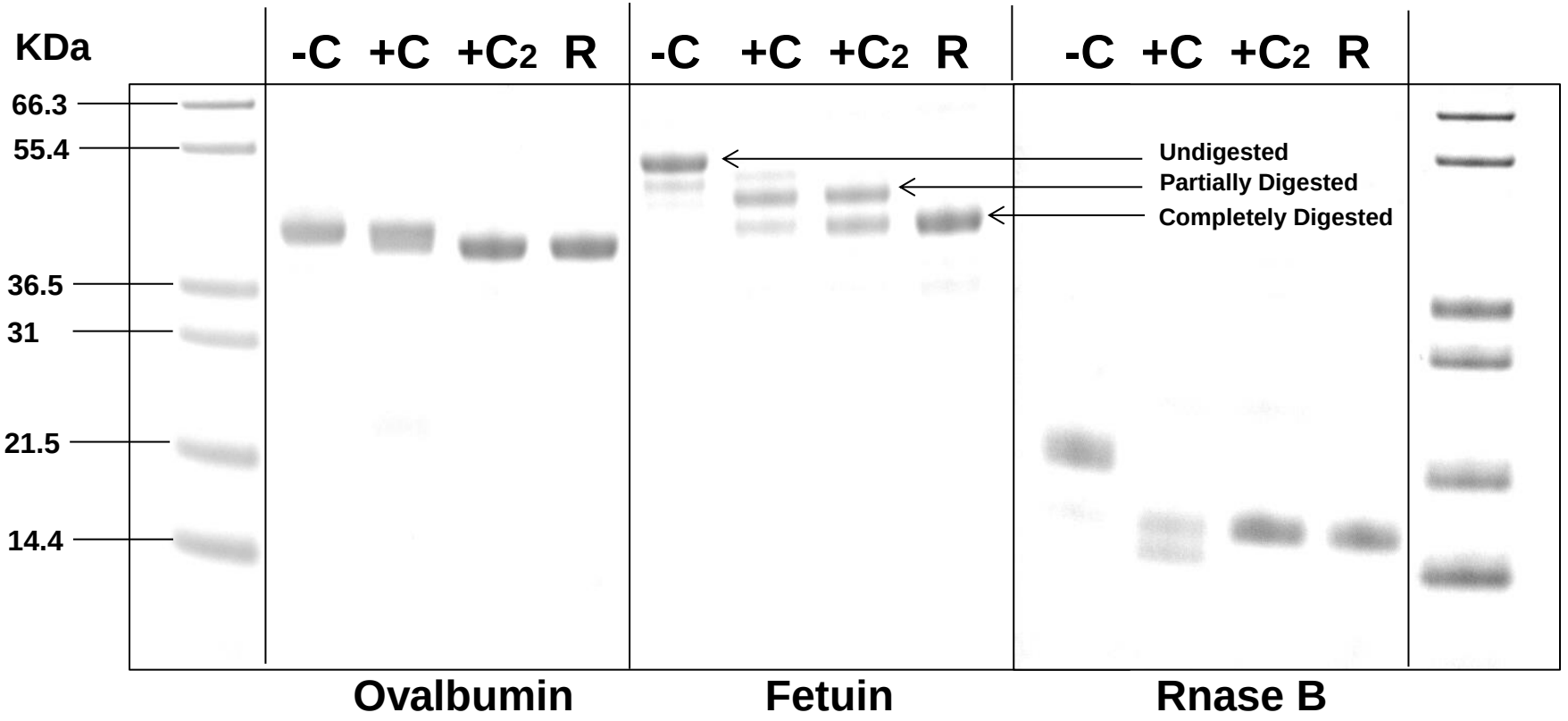
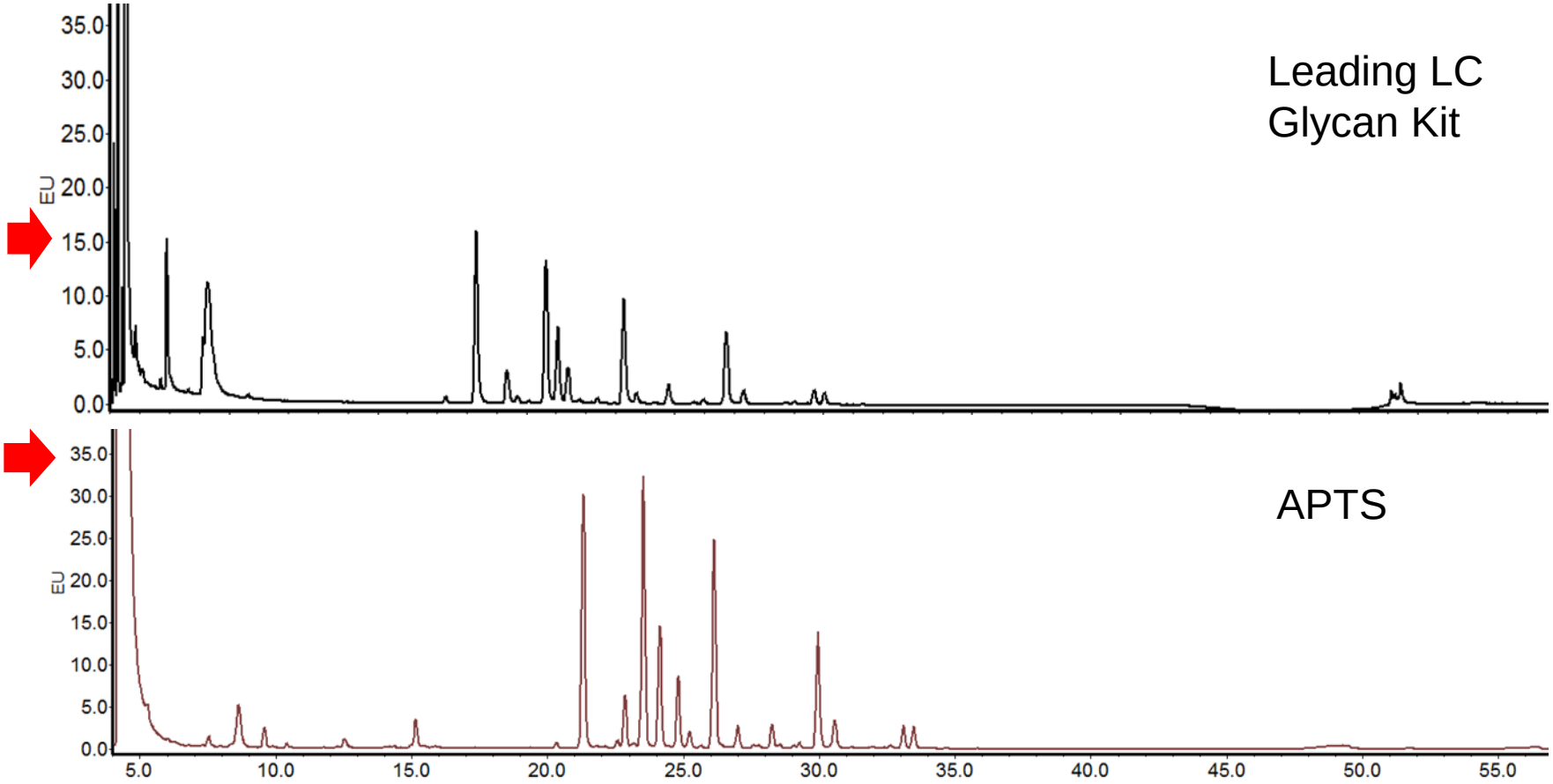
Rapid Deglycosylation:
Completeness of Deglycosylation Measured by Gel Shift Assay



- **-C** represents sample with no enzyme
- **+C** represents standard condition: denaturation at 95ºC, 2 min + 38 mM final DTT
- **+C2** represents leading LC Glycan Kit condition: denaturation at 90ºC, 5 min, 6 µl (LC-MS friendly surfactant)
- **+R** represents Thermo protocol: denaturation at 80ºC, 5 min, 6 µl Thermo Denaturant, 1.2 µl denaturant buffer
- All enzymatic de-glycosylations were performed for 10 min with 1.5 µl of GlycanAssure PNGase-F (Cat#A28404)

Optimized In-Solution Labeling:
Superior FLR Signals without Vacuum Drying



- At 50 ug protein input, our optimized in- solution APTS labeled glycans generates superior FLR signal over that labeled with leading LC Glycan Kit in the market
- LOQ of relative area **0.1%-0.2%**

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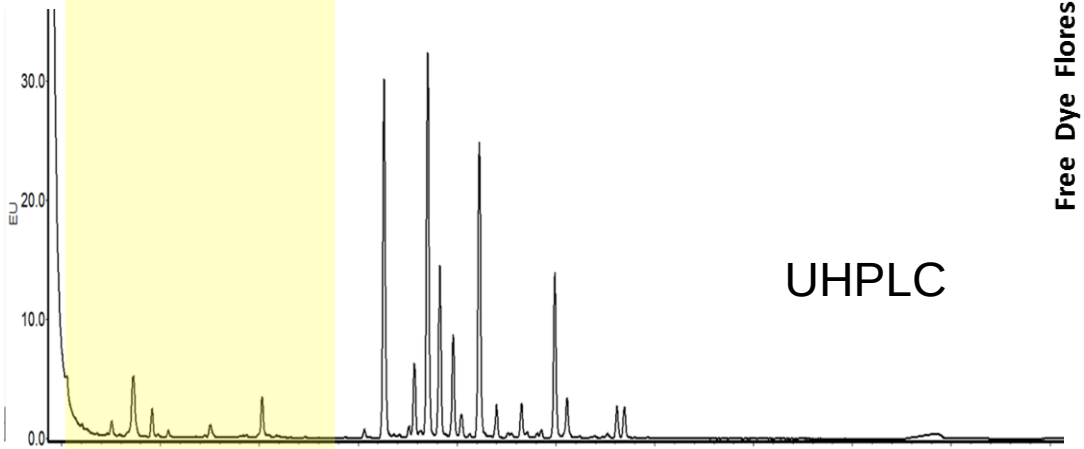
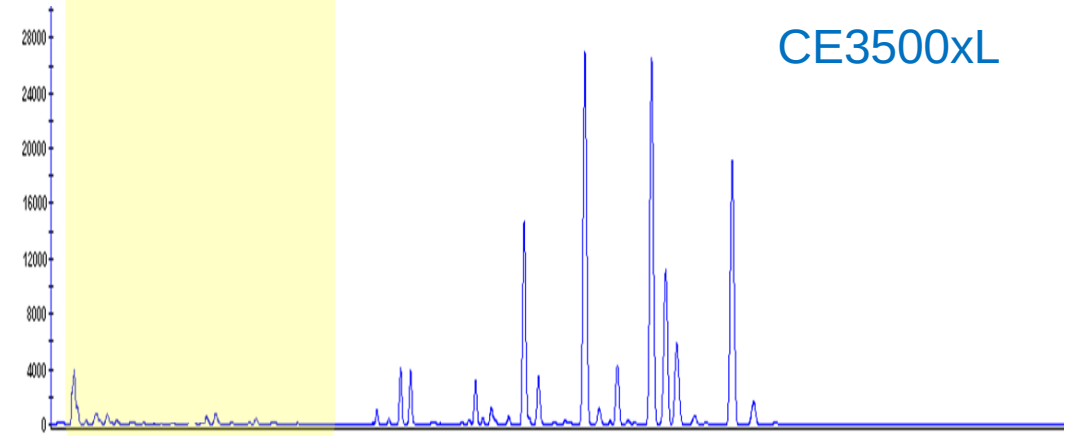
Optimized Clean Up Module for Minimum Free Dye

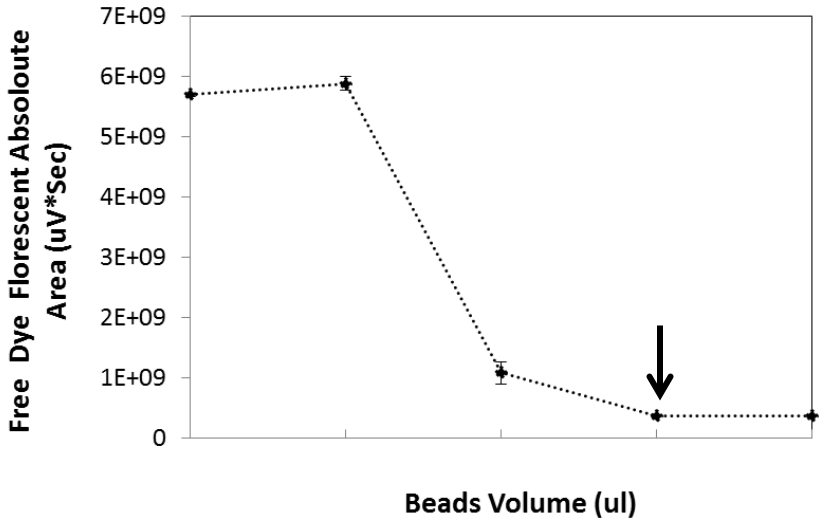
Effective Free Dye Removal



UHPLC



CE3500xL



- Optimized bead volume eliminates interferences from free dye very efficiently for a quality profile on both CE & LC

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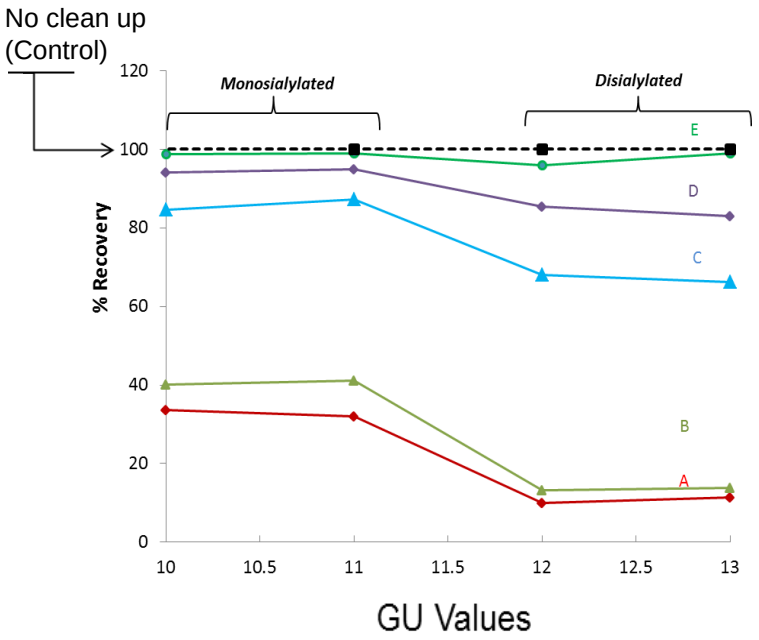
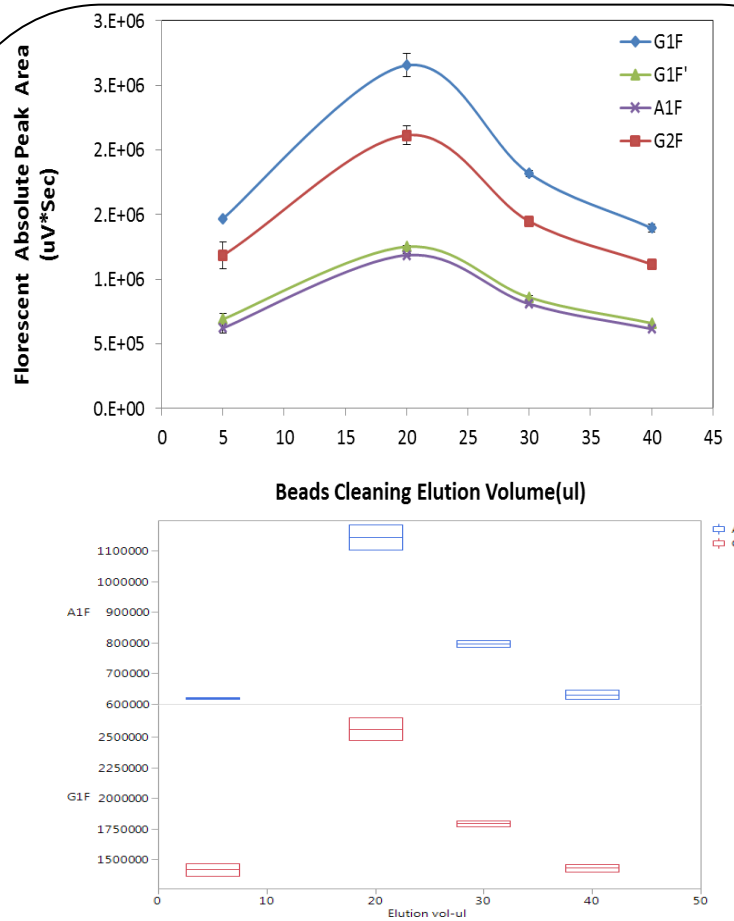
Optimized Magnetic Beads Elution Volume & Composition



- **Elution buffer composition** significantly impacts the bias of glycan profile with Sialylated glycans being most susceptible to lack of recovery
- Percent recoveries of mono and disialylated glycans has been screened for various elution buffers (A-E) relative to no clean up
- GlycanAssure™ uses elution buffer E



- **Elution buffer volume** to reach a maximum glycan recovery was evaluated.
- Any elution volume larger than optimum only dilutes the glycans, reducing the FLR signals
- Selected elution volume significantly increased total glycans

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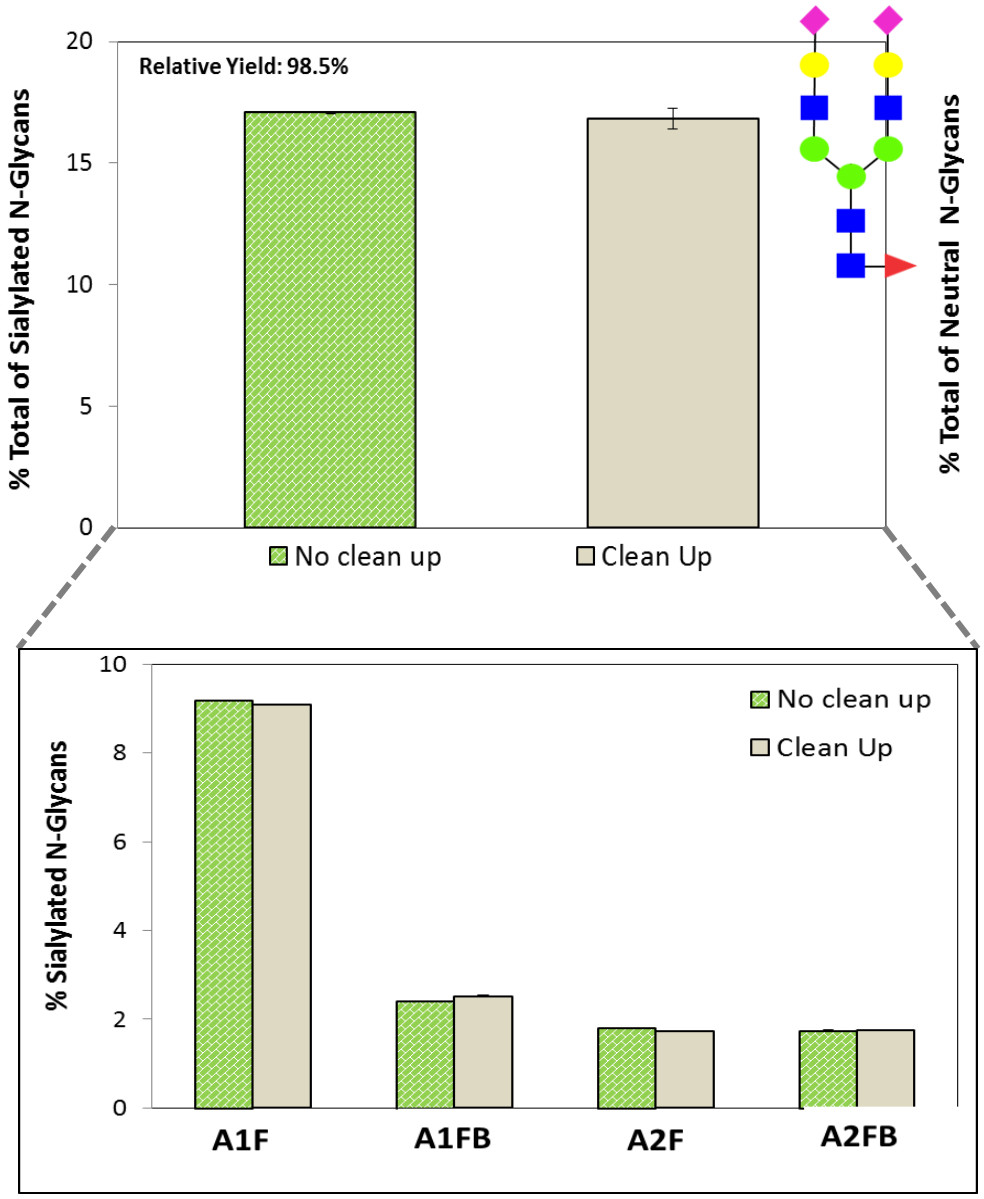
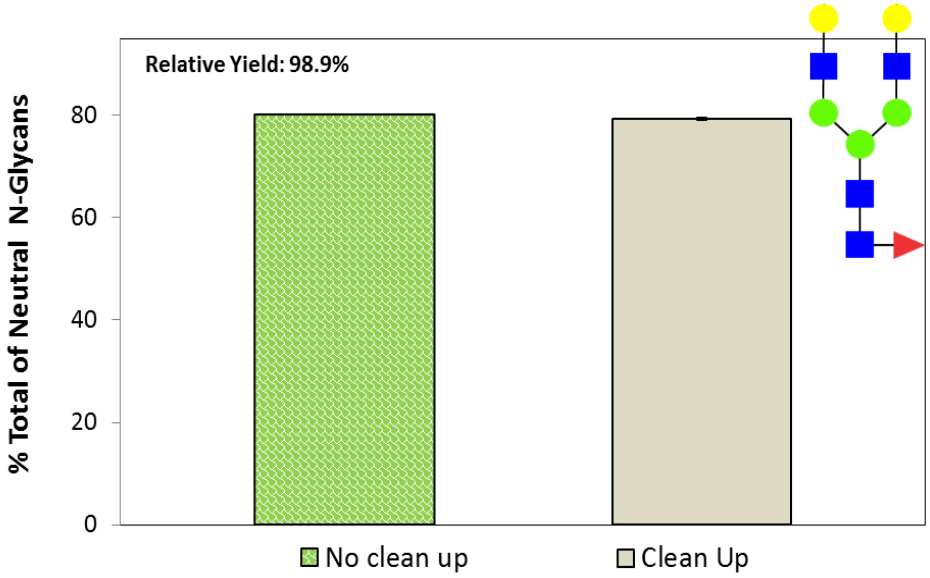
High Recovery, No Biased Clean Up Module- ~99% Yield





- Magnetic bead cleaning module preserves sialylated and neutral glycans with high recovery
- No bias is observed between sialylated and neutral glycans

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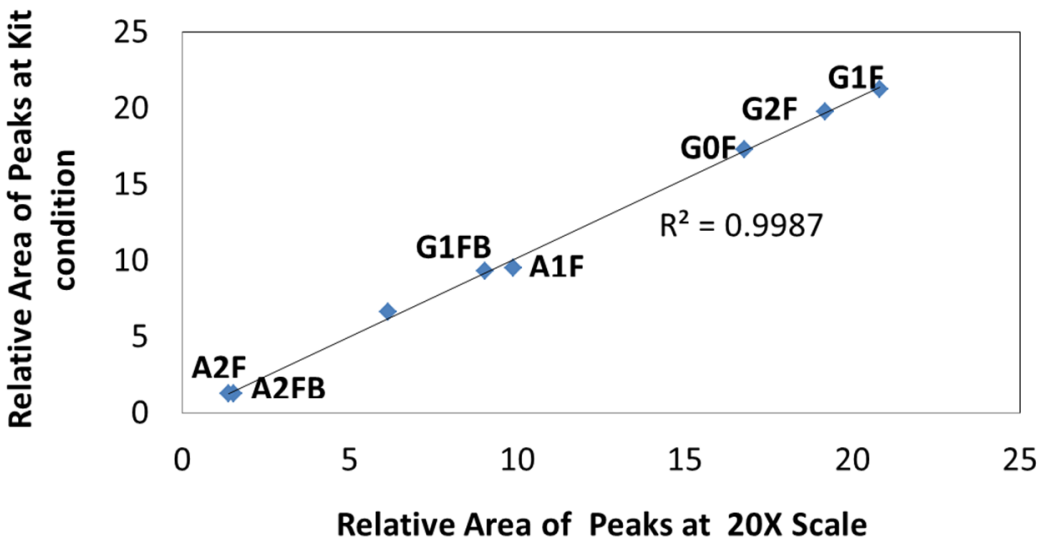
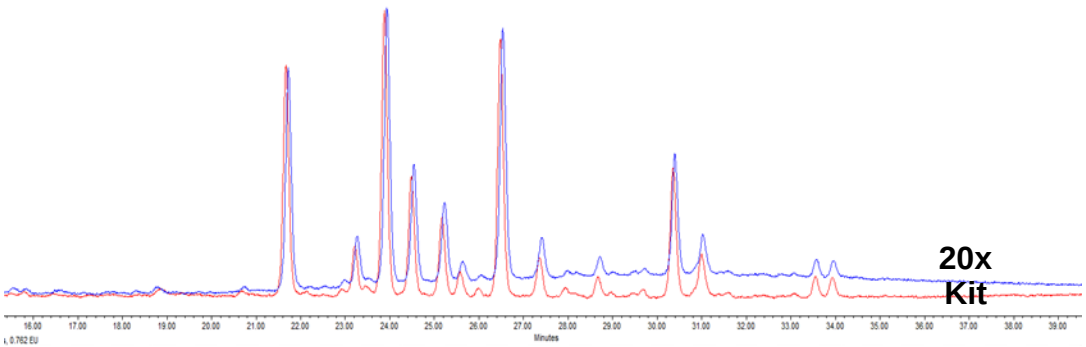
Good Sample Preparation Scalability

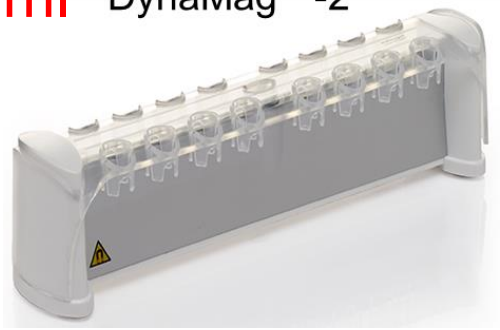




- Sample preparation at total volume of 0.2 ml was compared with 4 ml
- This is a initial total sample volume of 40 µl vs. 800 µl (after de-glycosylation)

5ml DynaMag™-5

2ml DynaMag™-2



0.2ml DynaMag™-96 bottom

Scale up

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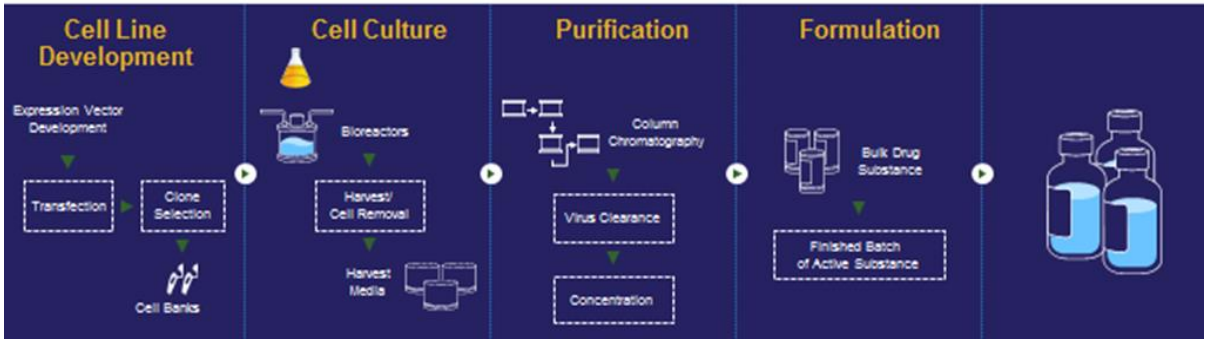
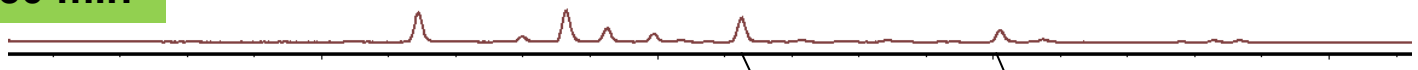
Sensitivity Comparison of Glycoprofiling with CE3500xL vs. UHPLC



UHPLC

At 1.8 ug Input

~60 min

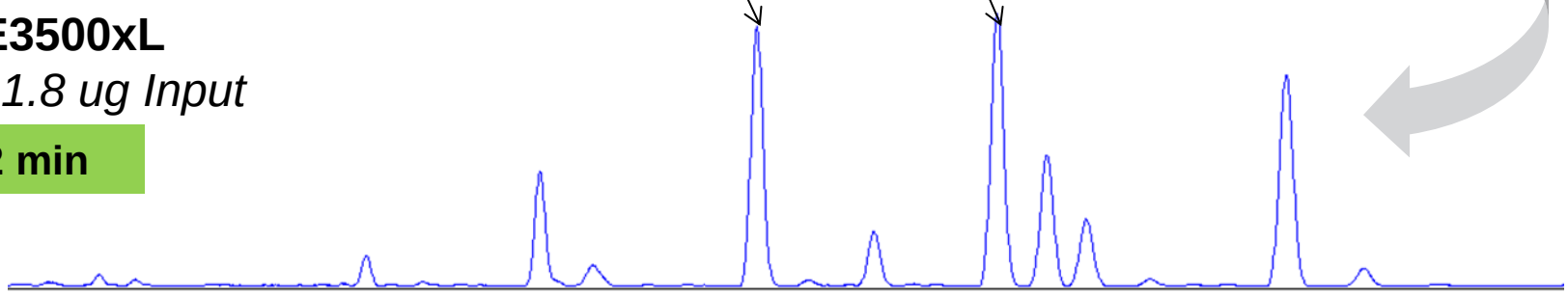


>12,000X Sensitivity

CE3500xL

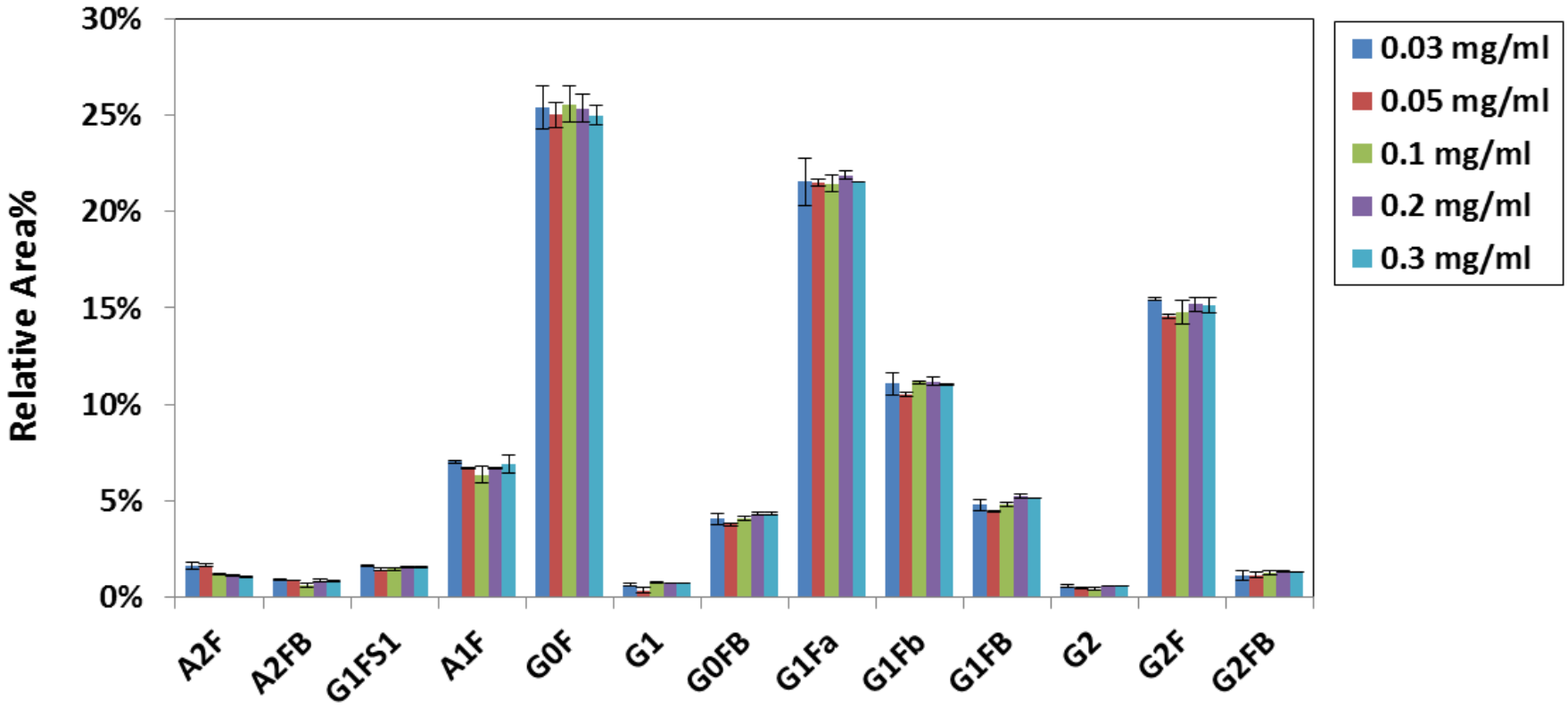
At 1.8 ug Input

2 min



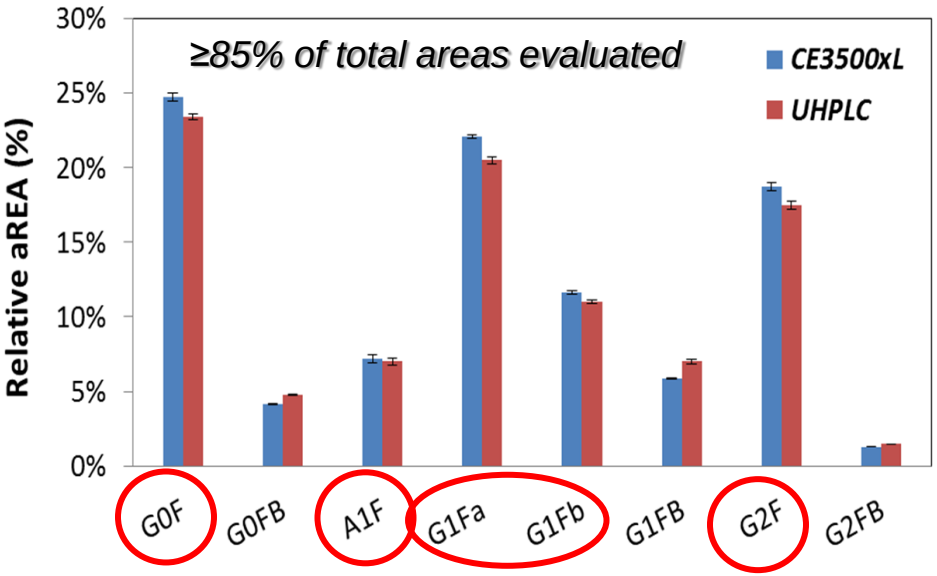
- CE3500xL's several thousand-fold greater sensitivity allows for glycoprofiling of samples with low protein concentration, without the need for time-consuming sample concentrating

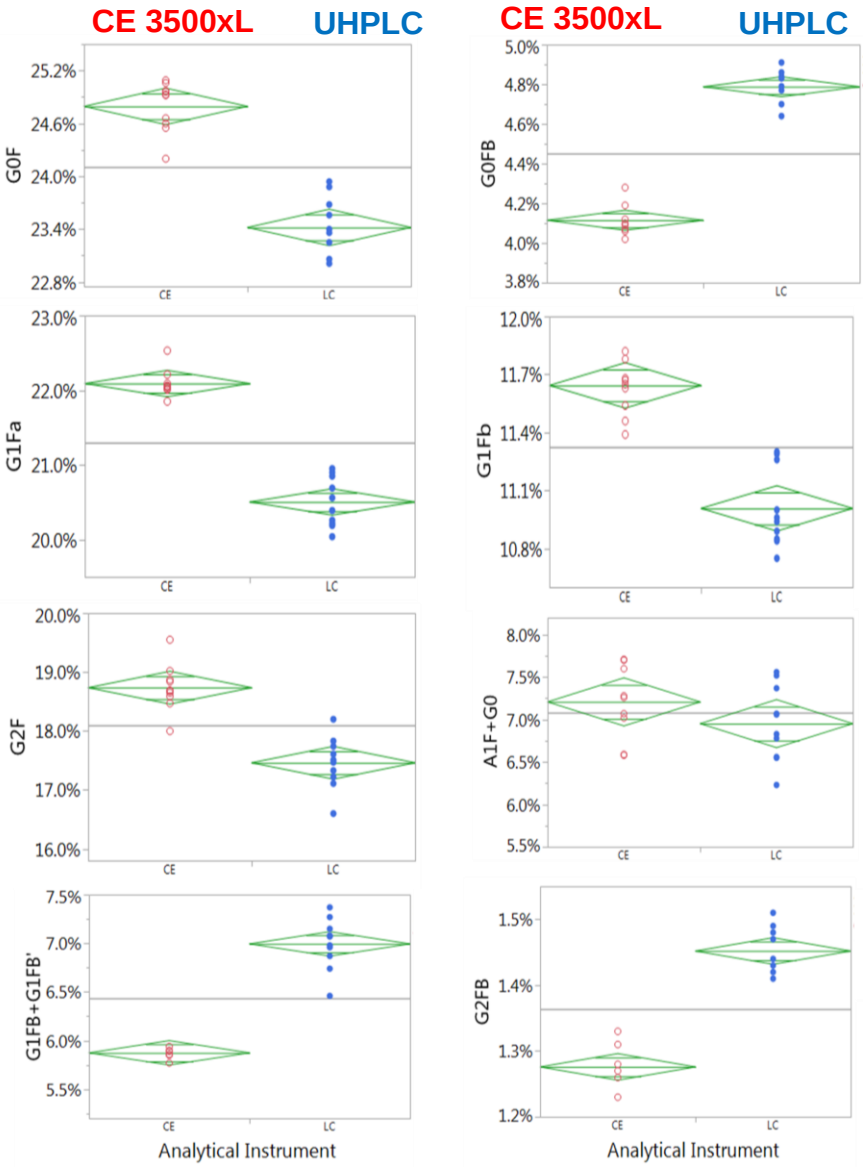
Data Consistency From Varying Glycoprotein Inputs



- Samples at 1.8 ug protein input show similar relative area to those with 10x higher concentration
- Data indicate a reliable system for highly sensitive analysis of *N*-glycan profiling in samples with low concentrations

Comparison of Relative Area of hIgG Glycan on CE 3500xL & UHPLC





- Major glycans of G0F, G1F1a, G1F1b, G2F and A1F in hIgG, were found to be very comparable in relative area between the two systems.

- Suggesting a significant improvement in resolution of CE 3500xL, comparable to UHPLC

- Because of the low variation in each set of data, relative peak areas with only by 0.1-2% difference between CE and LC shown to be significantly different

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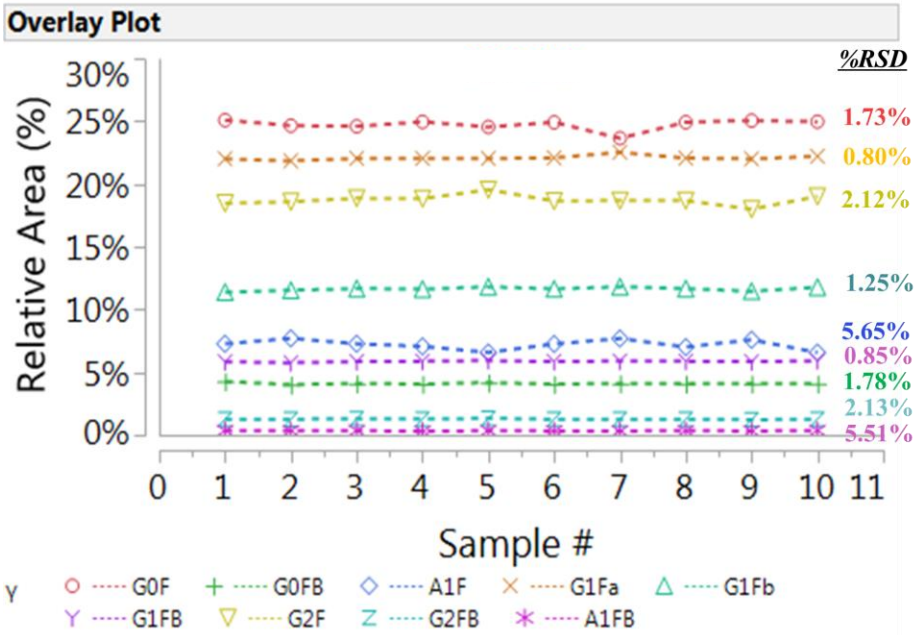
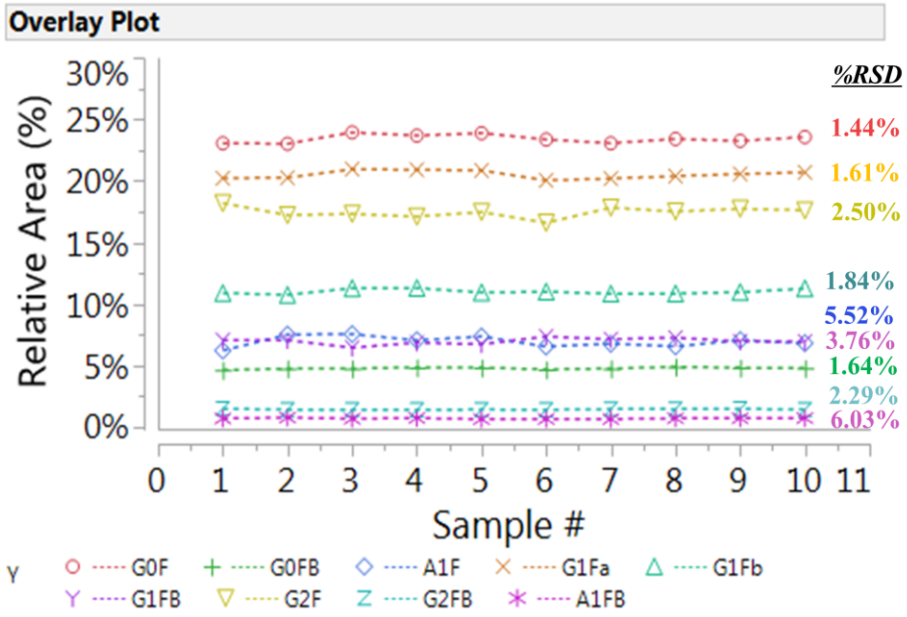
Variation of Sample Preparation on CE 3500xL & UHPLC

CE 3500xL



UHPLC



- ***Low Variation between samples from workflow on both CE and UHPLC Platforms***

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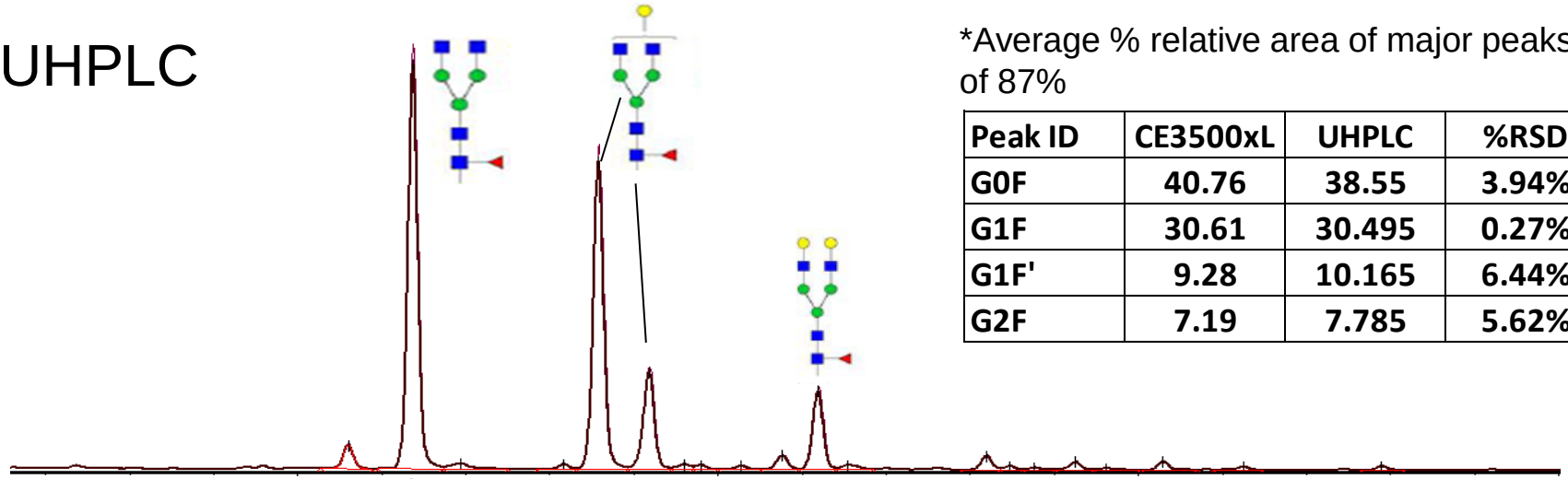
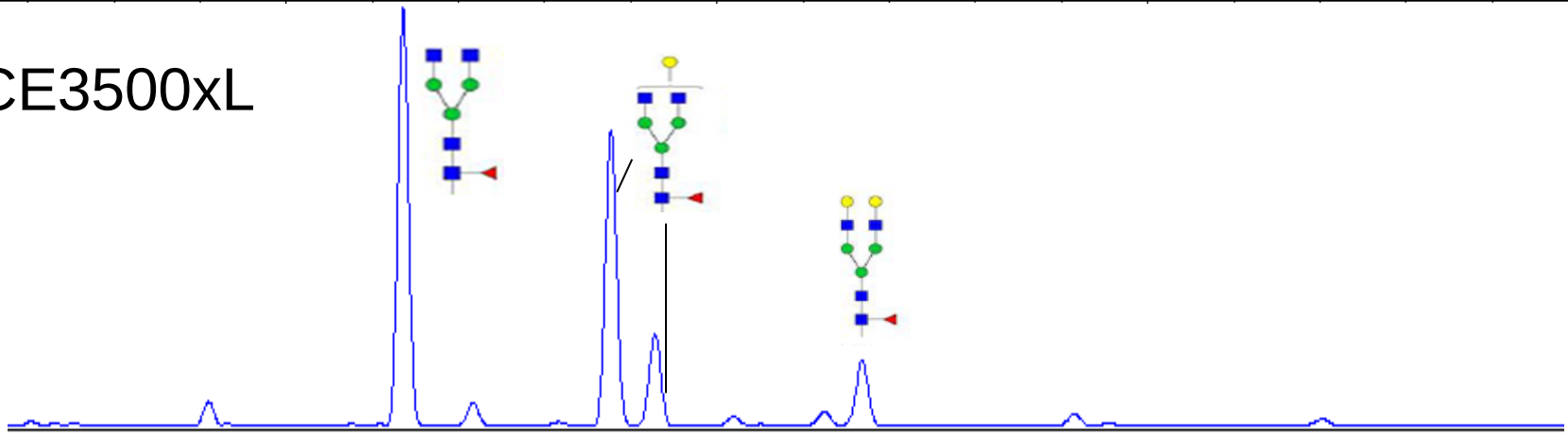
NIST Antibody Analysis on CE3500xL & UHPLC

UHPLC



CE3500xL



*Average % relative area of major peaks a total of 87%

Peak ID	CE3500xL	UHPLC	%RSD
G0F	40.76	38.55	3.94%
G1F	30.61	30.495	0.27%
G1F'	9.28	10.165	6.44%
G2F	7.19	7.785	5.62%

- Only peaks >0.2% were integrated (LOQ of 0.1-0.2%)
- 17 peaks (CE) and 20 peaks (LC) >LOQ were integrated with 4 major peaks shown in profiles
- ~0.2-2% of difference in relative areas were observed between two platforms

Take Home Message

Easy Sample Prep

- Hands-on-time <1 hours for 24 samples
- LC-MS friendly and low-medium temperature denaturant
- No BME or NP40 during deglycosylation
- No use of sodium cyano borohydride
- No vacuum drying

Throughput

Sample prep & data of 24 samples (TTR) in <2.5 hours

Sensitivity

Low glycoprotein input

Resolution

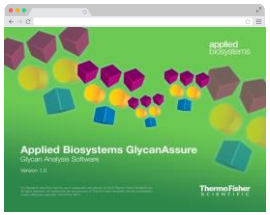
Good resolution between glycan peaks

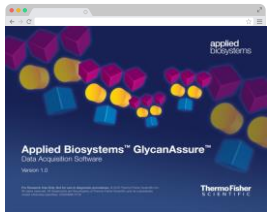
Software

Data acquisition and analysis software with novel features

No Injection failure or software error

Such as pressure injection and errors associated with software is eliminated





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Acknowledgements

- Baburaj Kunnummal
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- Michelle Yannetti
- Johnie Young
- David Dupont
- Shaheer Khan
- Jen-Kuei Liu
- Bharti Solanki

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Questions?

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- www.thermofisher.com/glycanassure

For more information, please visit thermofisher.com/glycanassure