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# GlycanAssure™: A High Throughput & High Resolution Glycan Analysis Platform

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# Agenda

- Introduction
  - Glycosylation and Glycan Diversity
  - Importance of Protein Glycosylation
  - Glycan Labeling Chemistry
  - Current Glycan Analysis Challenges
- Thermo Fisher GlycanAssure  Platform
  - Sample Prep
  - Instrumentation
  - Software
  - Multicap CE for Glycan Analysis – Literature
  - GlycanAssure Workflow
- Sample Data Sets
  - Magnetic Bead Based Workflow vs. Carbon Column
  - Consistency Across Varying Glycoprotein Inputs
  - Reproducibility of CE Separation
  - Variability Across Capillaries
  - Variability Across Instruments & Capillary Arrays
  - Glycan Spike Studies
  - Improved Glycan Separation Using Thermo Fisher Dyes
  - Thermo Fisher 3500 Vs. Competition
- Summary


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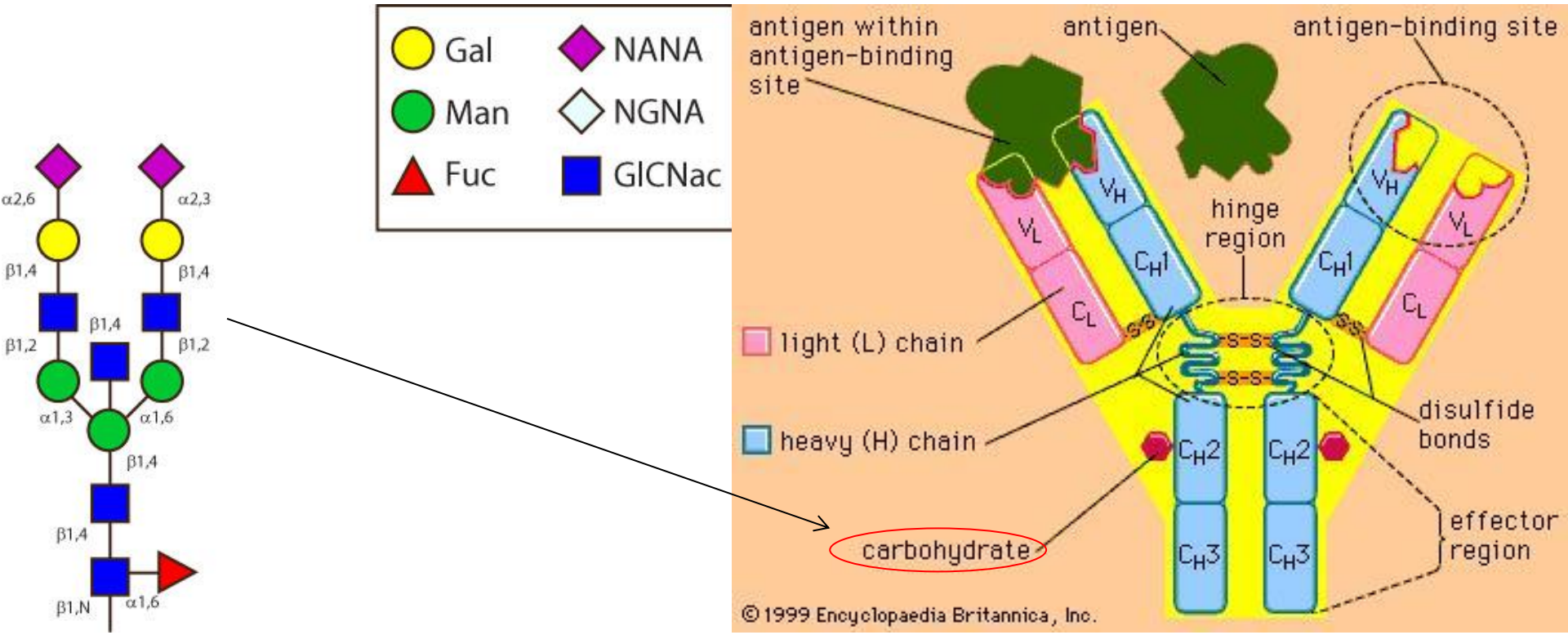
# Glycans - Introduction




- Glycosylation – Attachment of Glycans (Carbohydrates or Oligosaccharides) to proteins
- Glycans are made up of Monosaccharides
- More than 70% of biotherapeutics are glycosylated
- Glycans are important for protein stability, folding, assembly, signaling, etc.
- Glycosylation is a critical quality attribute (CQA) of biotherapeutics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# Glycan Diversity Poses an Analytical Challenge

| Oligomer | Composition | Peptide | Oligosaccharide |
|---|---|---|---|
| Dimer | AA / AB | 1 / 2 | 11 / 20 |
| Trimer | AAA / ABC | 1 / 6 | 120 / 720 |
| Tetramer | AAAA / ABCD | 1 / 24 | 1424 / 34560 |
| Pentamer | AAAAA / ABCDE | 1 / 120 | 17872 / 2144640 |

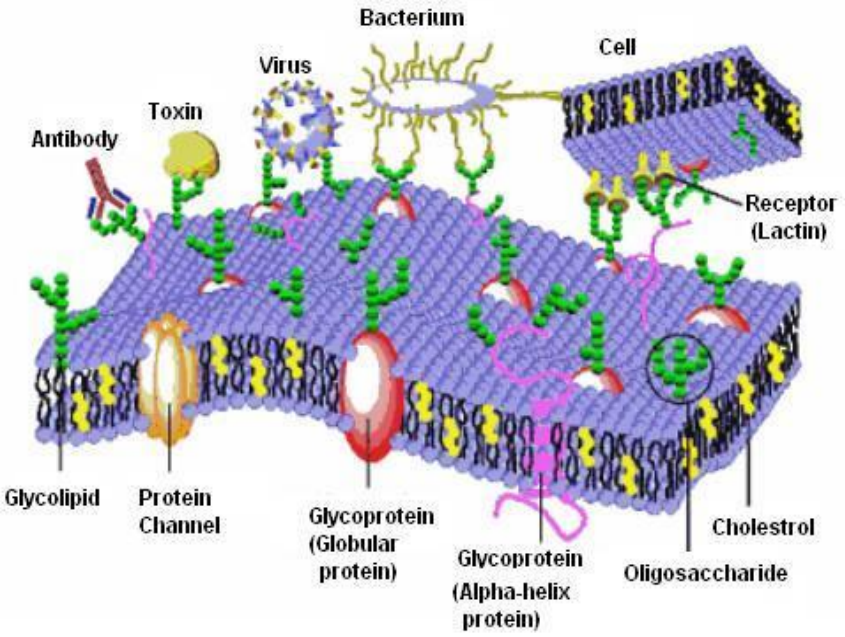
Number of Possible Isomers

Glycoconjugate Biosynthesis

- Glycans are not templated
- Glycan structure is determined by sequential glycosyltransferase action

More than 50% of Human Proteins are Glycosylated

- Glycosylation is Heterogeneous
  - Structures of Sugars Attached
  - Sites To Which They are attached
  - Anomericity





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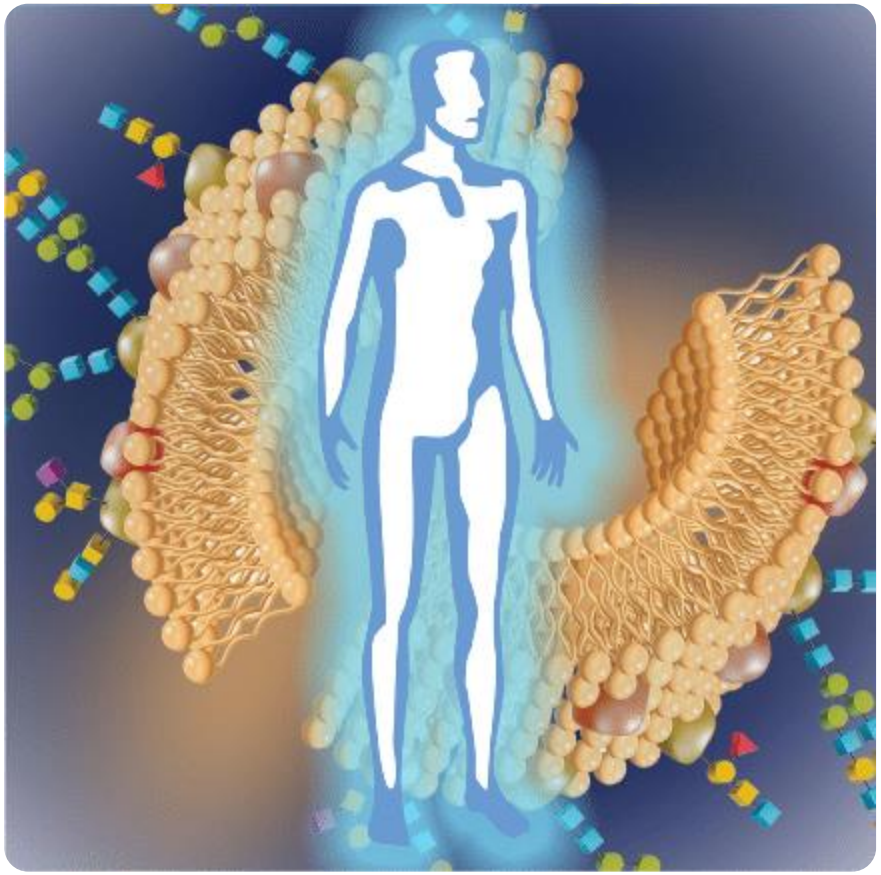
# Many Conditions Affecting Humans Involve Glycans

## Pregnancy
N-glycosylation of human serum transcortin and thyroxine-binding globulin

## Aging
Outer arm galactosylation of human serum IgG

## Alcoholism
Increase in relative number of serum asialo-tranferrin glycoforms



## Cancer
Unique oligosaccharides
Such as CA 125 (Ovarian)
CA 19-9 (Colon)

## Liver Disease
Increase in number of sialic acid residues on fibrinogens

## Tuberculosis
agalactosyl IgG

Ref: Dwek et al., Ann. Rev. Biochem., 57 (1988) 785-838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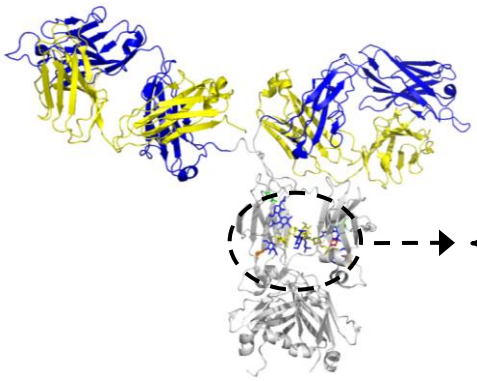
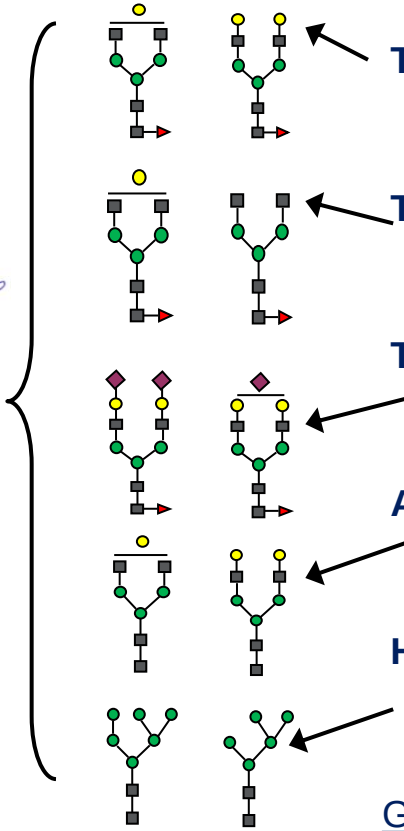
# Biotherapeutics: Glycosylation is a CQA





| | |
|---|---|
| **Terminal Gal** | **Increase in CDC immune response** |
| **Terminal GlcNAc** | **Increase in ADCC immune response** |
| **Terminal Sialylation** | **Decrease in ADCC immune response** |
| **Absence of core Fuc** | **Increase in ADCC immune response** |
| **High Man structures** | **Increase in ADCC immune response** |

Glycan changes effect efficacy, immunogenicity & clearance of biotherapeutics

- **Protein function & stability are tightly linked to glycosylation structure**
- **Majority of the biotherapeutics are glycosylated**
- **For the success of biosimilars it is critical to match innovator glycan profile**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# Current Regulatory Guidelines

**Ensures glycans/glycosylation is well characterized in biotherapeutics**

- **FDA (USA) and EMA (Europe) define the regulatory requirements**
- **Biotherapeutic manufactures are legally obligated to comply with these**

US FDA, November 3, 2010

Many complex biologics have sugar chains that impact their efficacy and safety, and industry experts have noted that the FDA has made several statements over the years suggesting a primary concern that biosimilars have the same sugar structure for interchangeability and those sugar structures do not change with time.

EMEA Guidelines 2009

On the development, production, characterization and specifications for monoclonal antibodies and related products ‘glycan structures should be characterized, and particular attention should be paid to their degree of of mannosylation, galatosylation, fucosylation and sialylation’.

European Medicines Agency
EMEA/CHMP/BWP/157653/2007 (2009)


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# Draft Guidance for Analysis of Biosimilars

**As monoclonal antibody molecules come off patent, biosimilars will start entering the market.**

FDA (USA) and EMA (Europe) consider the degree of glycosylation to be a critical factor in determining the degree of "similarity" to the original approved drug

**Similar**: Additional analytical data or other studies are necessary to determine if observed differences are within an acceptable range to consider the proposed biosimilar. For example, the agency says that "glycosylation plays an important role in the PK of certain protein products. Manufacturing process conditions may impact glycosylation. Comparative PK and PD studies of the proposed biosimilar product and the reference product help resolve that some difference in **glycosylation identified in analytical studies would be within an acceptable range to consider the proposed biosimilar product to be highly similar to the reference product**."

**Reference:**
**Draft Guidance for Industry - Clinical Pharmacology Data to Support a Demonstration of Biosimilarity to a Reference Product**
U.S. Department of Health and Human Services, Food and Drug Administration
Center for Drug Evaluation and Research (CDER) & Center for Biologics Evaluation and Research (CBER)
May 2014 Biosimilars

European Medicines Agency EMEA/CHMP/BWP/157653/2007 (2009)


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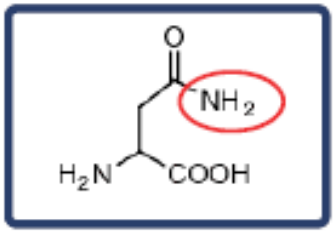
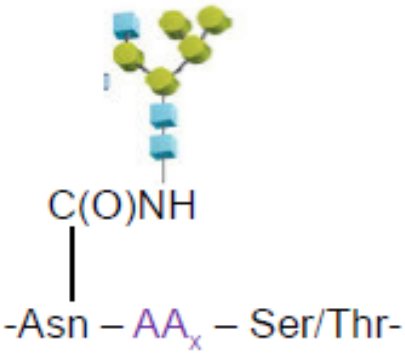
# Which Glycans are Analyzed?

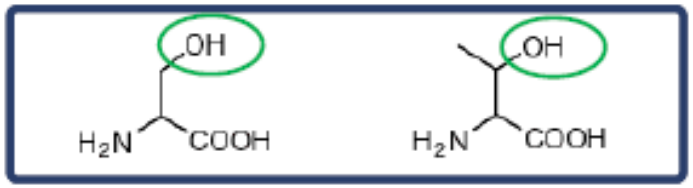
- *N*-linked glycans
  - Attached to the amide side chain of Asparagine in peptide sequence: Asn- $AA_x$-Ser/Thr
- *O*-linked glycans
  - Attached to the hydroxyl group on Serine or Threonine residues
- Other forms (less studied)
  - Glycophosphatidylinositol anchors
    - Attached to protein C-terminus
  - C-glycosylation on tryptophan residues
  - S-linked glycosylation
    - Linked through S atom in Cys and Met residues




Asparagine (Asn)

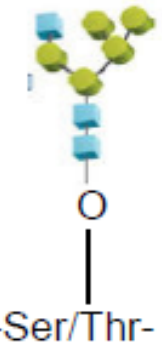
*N*-Linked




Serine (Ser) Threonine (Thr)

*O*-Linked




Our method (and almost all current CE/HPLC methods) analyze ONLY N-glycans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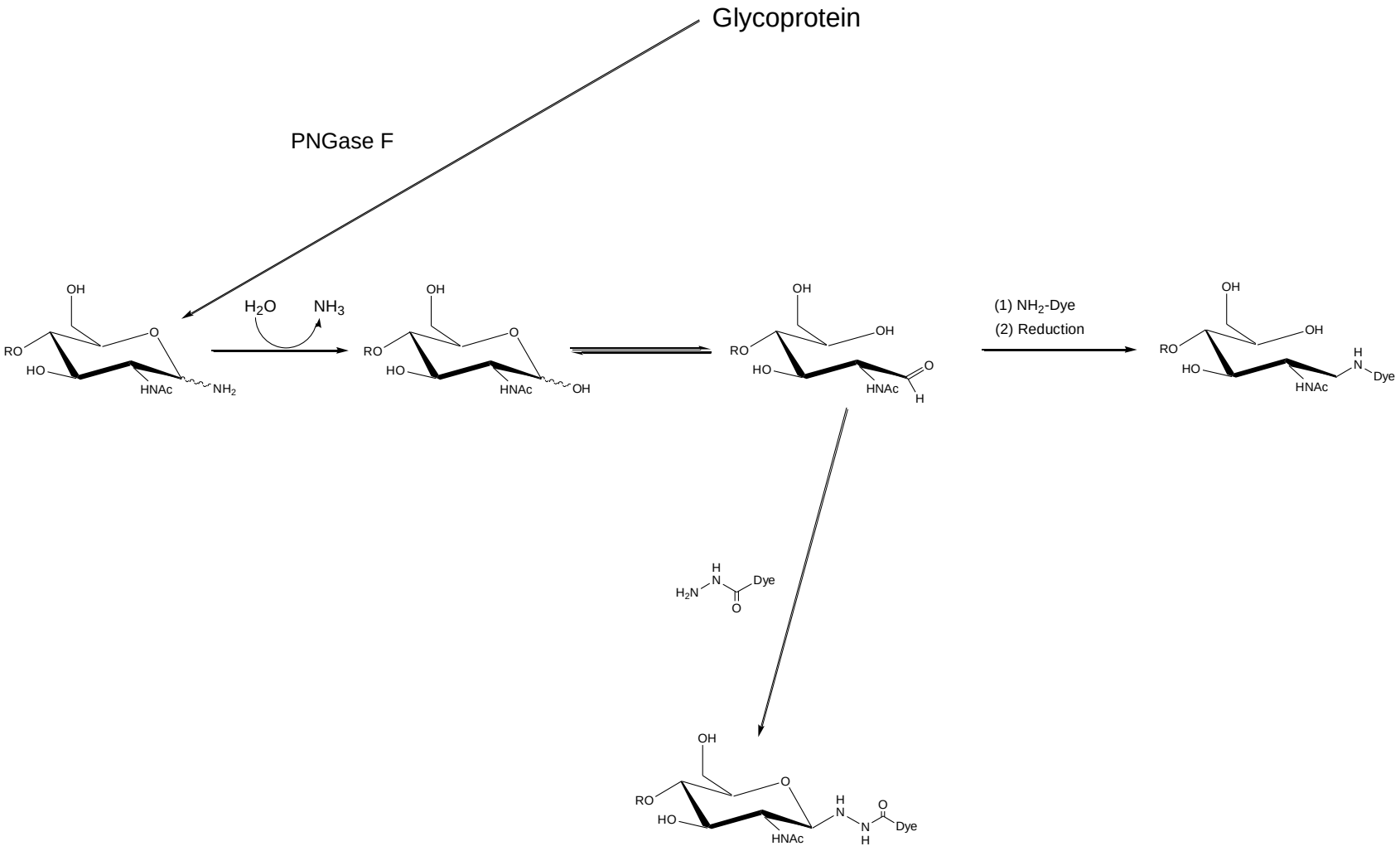
# Glycan Labeling Chemistries





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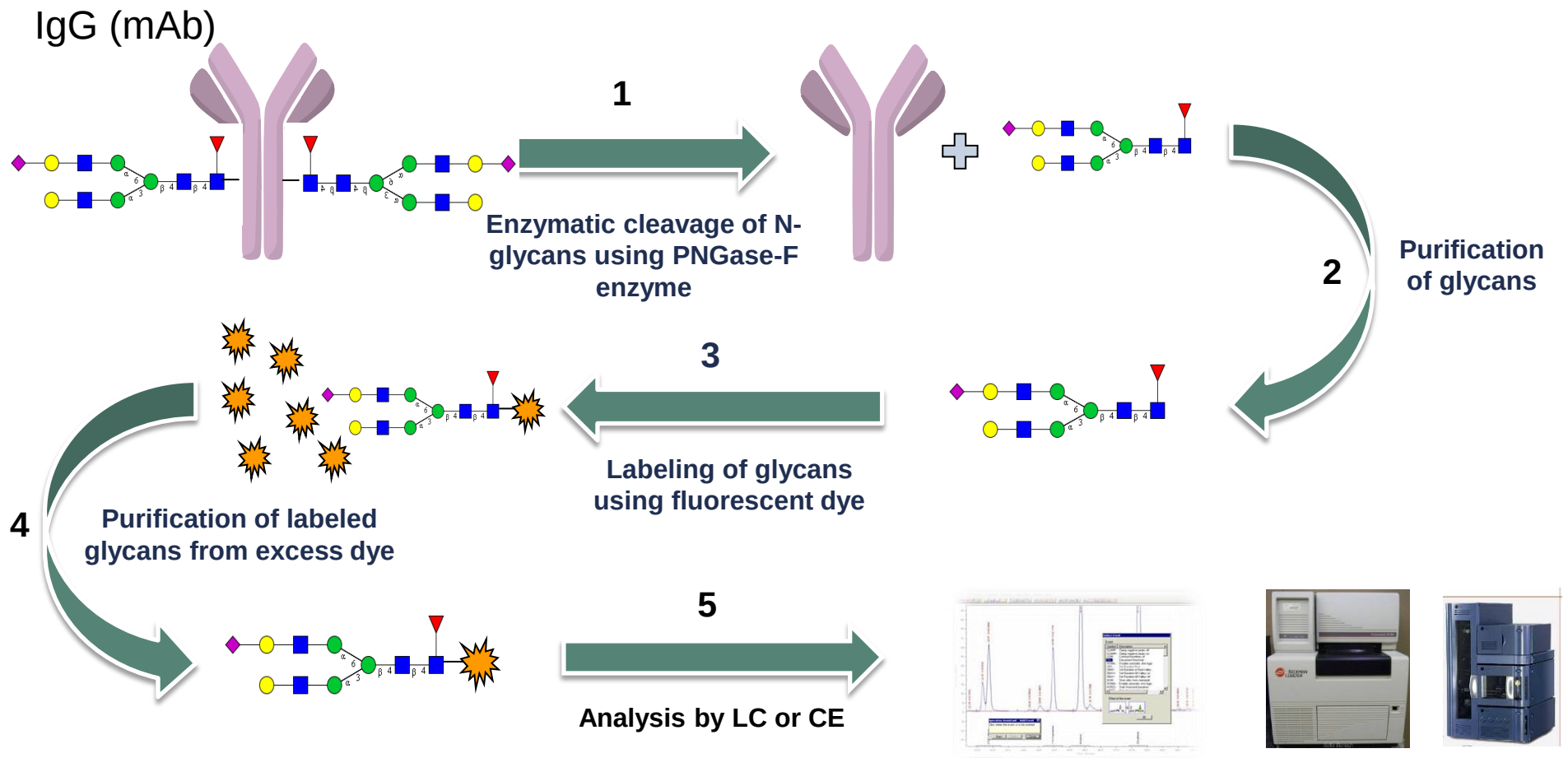
# How N-Glycans are Analyzed?





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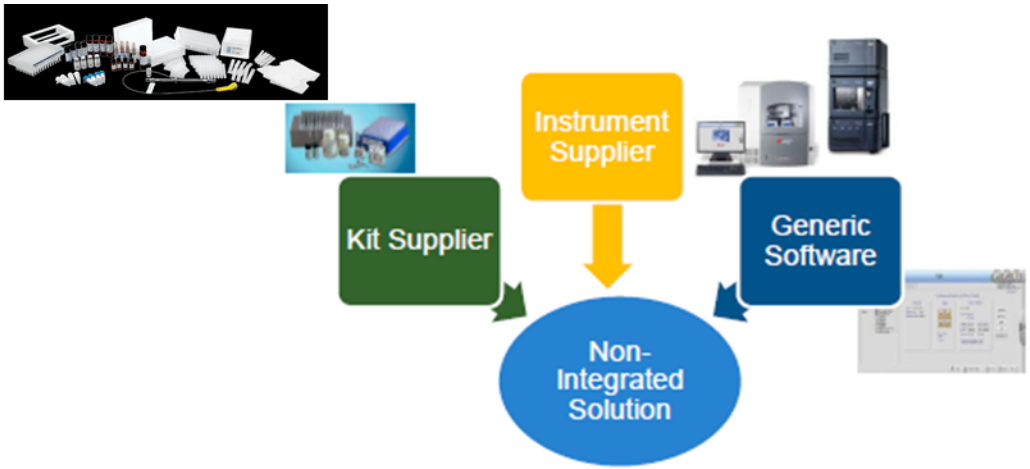
# Current Glycan Analysis Challenges




- Single channel separation with low throughput (HPLC/UHPLC & single cap CE)
- Poor data quality of high throughput methods (Chip based instruments)
- Labor Intensive workflow (multiple pipetting steps, use of spin columns, etc.)
- Use of toxic Sodium cyanoborohydride chemistry
- Use of lengthy vacuum centrifugation steps
- Generic software taking long analysis time
- Non-integrated solution from multiple vendors
- Commercial sample prep kits with high cost per sample
- No validation support for result/solution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# Thermo Fisher GlycanAssure

- Very high throughput (96 samples/7-9hrs)
- No compromise in CE separation time
- Easy magnetic bead based workflow
- Fewer pipetting steps & hands-on time
- Multiple fluorescent dyes for glycan labeling
- No use of Sodium cyanoborohydride
- No vacuum centrifugation steps
- Parallel analysis of 24 samples on AB DNA Analyzer
- Fit for use “app” style software
- Complete integrated solution with sample prep kits, CE instrument & software
- Reduced cost per sample

**First Fully Integrated System Combining Throughput & Data Quality**


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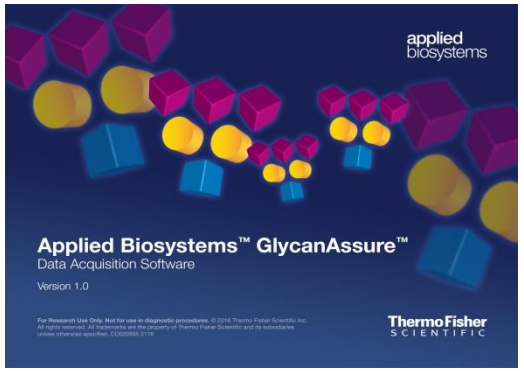
# Product Overview

**APTS Kit**
**PN: A28676**

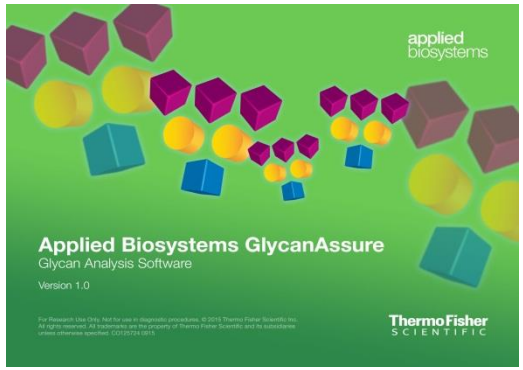
**Turquoise Kit**
**PN: A28678**

**Teal Kit**
**PN: A28677**

**3500 & 3500XL**
**PN: A30467 & A30556**




**Data Acquisition Software**
**PN: A30750**




**Data Analysis Software**
**PN: A30751**



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# GlycanAssure Value Proposition

## General

First in class glycan analysis platform that combines both high throughput and data quality

## Specific*

- Faster
- Simpler
- High Quality
- Cost-effective
- Integrated solution

*Compared to other Commercial & Homebrew Products


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# GlycanAssure Sample Prep Kits

APTS Kit

Teal Kit

Turquoise Kit

- Three fluorescent dyes with distinct properties
- Traditional APTS and two Thermo proprietary dyes
- Faster glycan labeling with Teal & Turquoise dyes (30min)
- Magnetic beads for glycan purification and excess dye removal
- No need for excess dye removal with Teal (96 samples in 7hrs)
- Each kit with PNGase-F enzyme and beads for 96 samples

9-Aminopyrene-1,4,6-trisulfonic acid (**APTS**)


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# GlycanAssure CE Instrumentation

## Key Features:

• 8 & 24-capillary systems for medium & high throughput analysis

• Single-line, 505 nm, solid-state, long-life laser that utilizes a standard power supply and requires no heat-removal

• Powerful, integrated data acquisition and analysis software that provides real-time assessment of data

• Radio frequency identification (RFID) technology that tracks key consumables data and records administrative information

• Advanced multiplexing capabilities for glycan analysis with up to six unique dyes

• Unrivaled application flexibility—one array and one polymer are used for most applications

• Simple setup, operation, and maintenance


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# GlycanAssure CE Instrumentation

- Industry “Gold Standard” CE instrument
- Parallel analysis of 8 or 24 samples **(< 2min/sample)**
- Robust capillary array
- Calibration across capillaries using internal standard


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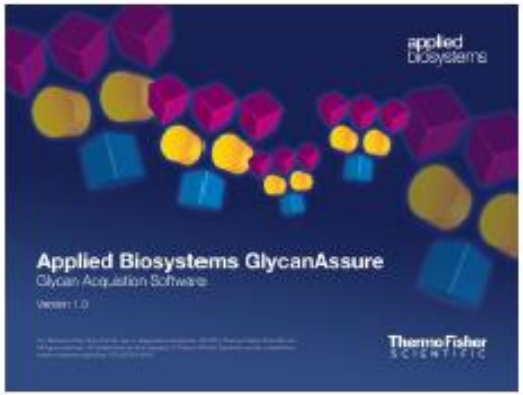
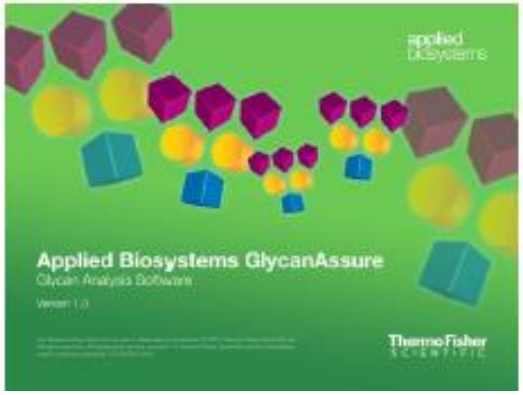
# GlycanAssure Software

- **Acquisition Dashboard**
  - Quick access to instrument status and data
- **Run Setup**
  - Simplified and Intuitive chevron based, step by step workflow
- **Reports**
  - Easily create reports with choice of templates
- **Library**
  - Stores historical information for plates, experiments, methods and reference
- **Analysis Dashboard**
  - Quick view of analyzed and un-analyzed samples. Allows to create data analysis projects
  - Quick access to Favorite Projects
- **Data Processing**
  - Quick comparison of analysis methods, alignment, normalization, and smoothing
  - Library of analysis methods and recommendations for glycan analysis
- **Analysis**
  - Simplified graphics interface for manual integrations





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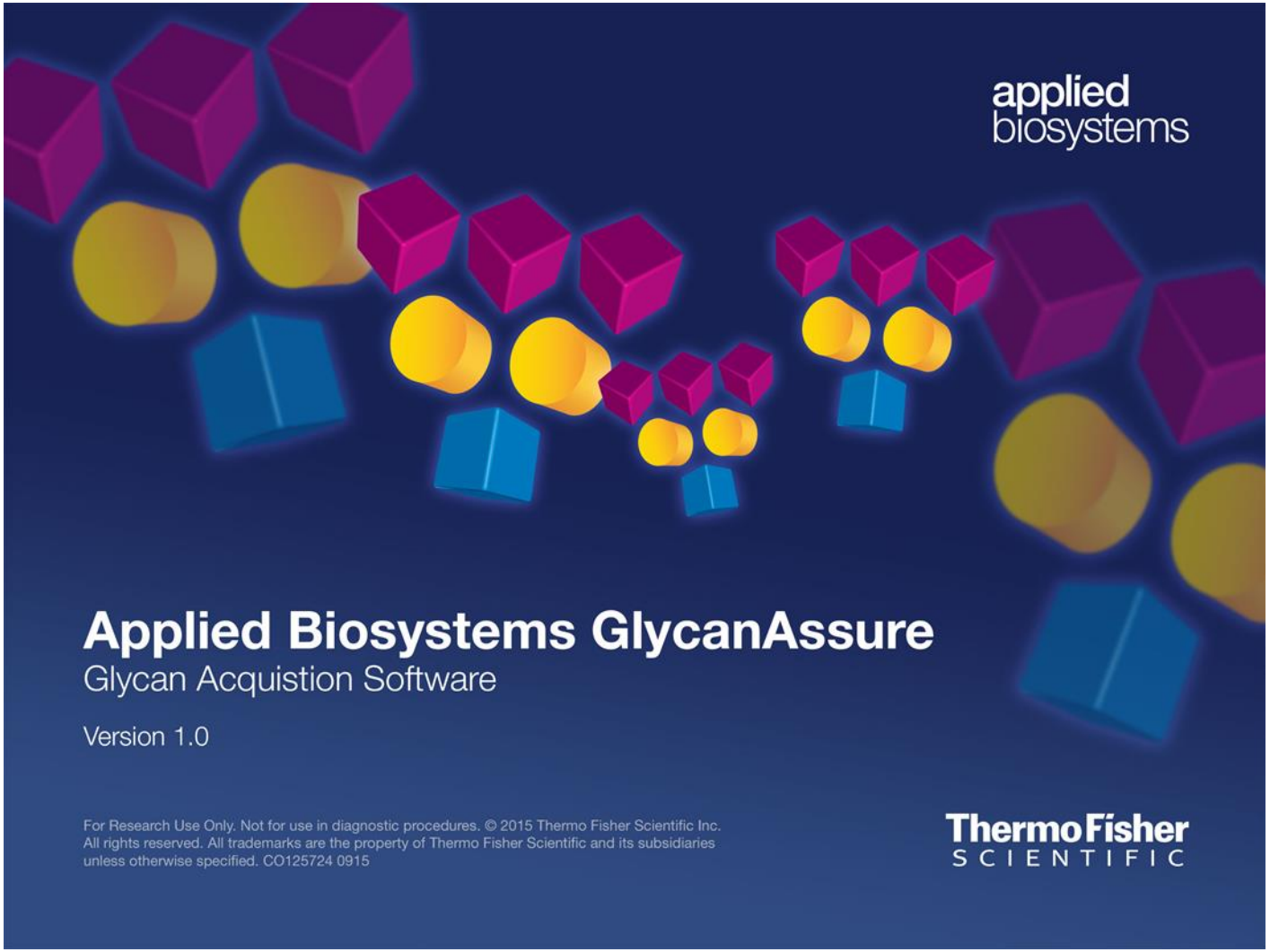
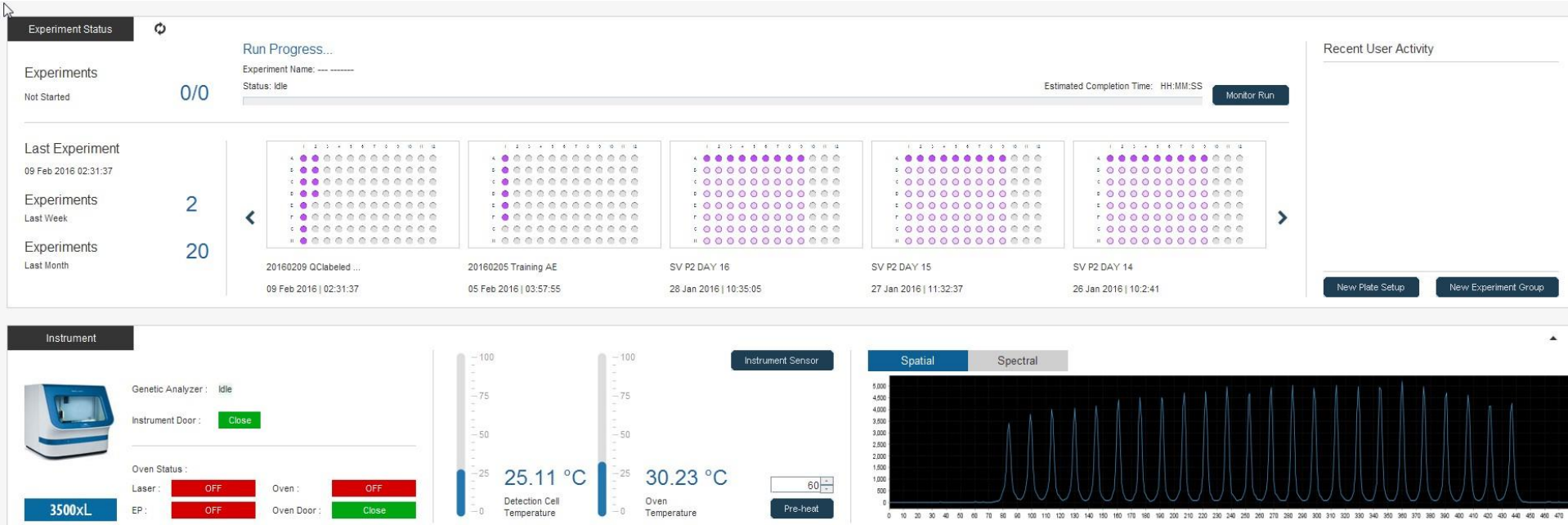
# GlycanAssure: Data Acquisition Software

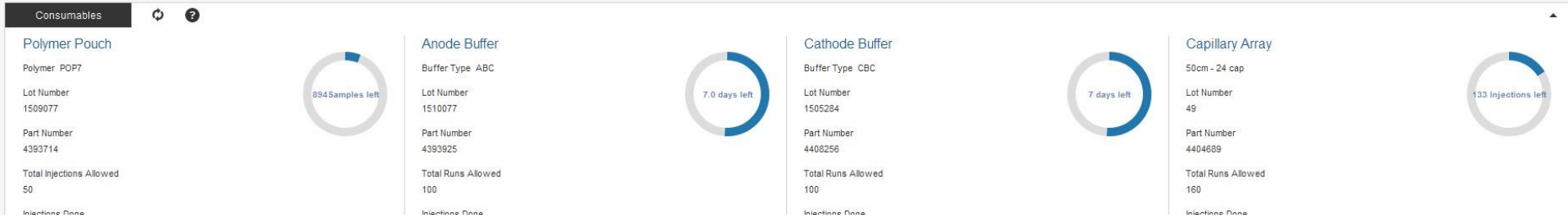


# GlycanAssure: Data Acquisition Software











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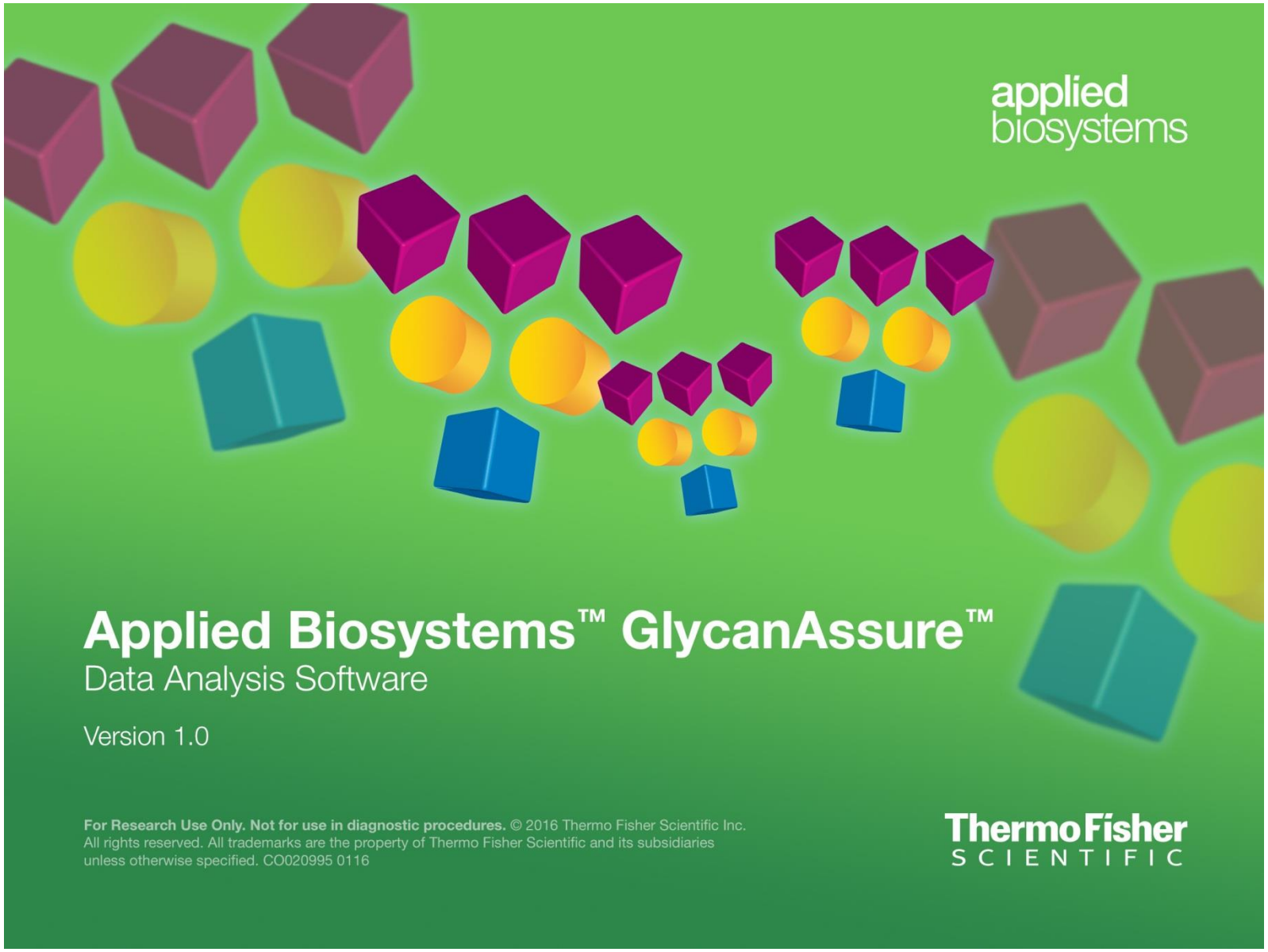
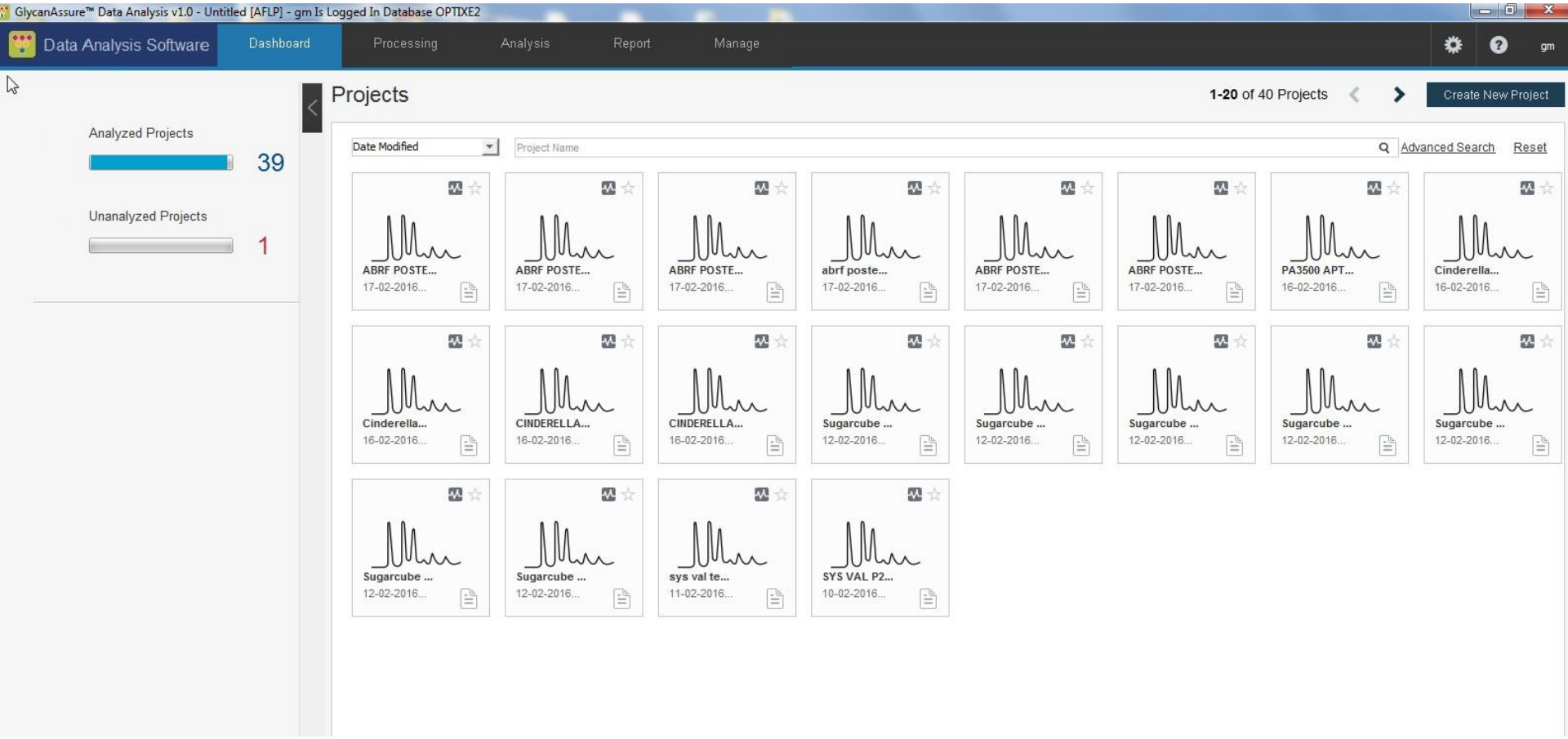
# GlycanAssure: Data Analysis Software



# GlycanAssure: Data Analysis Software





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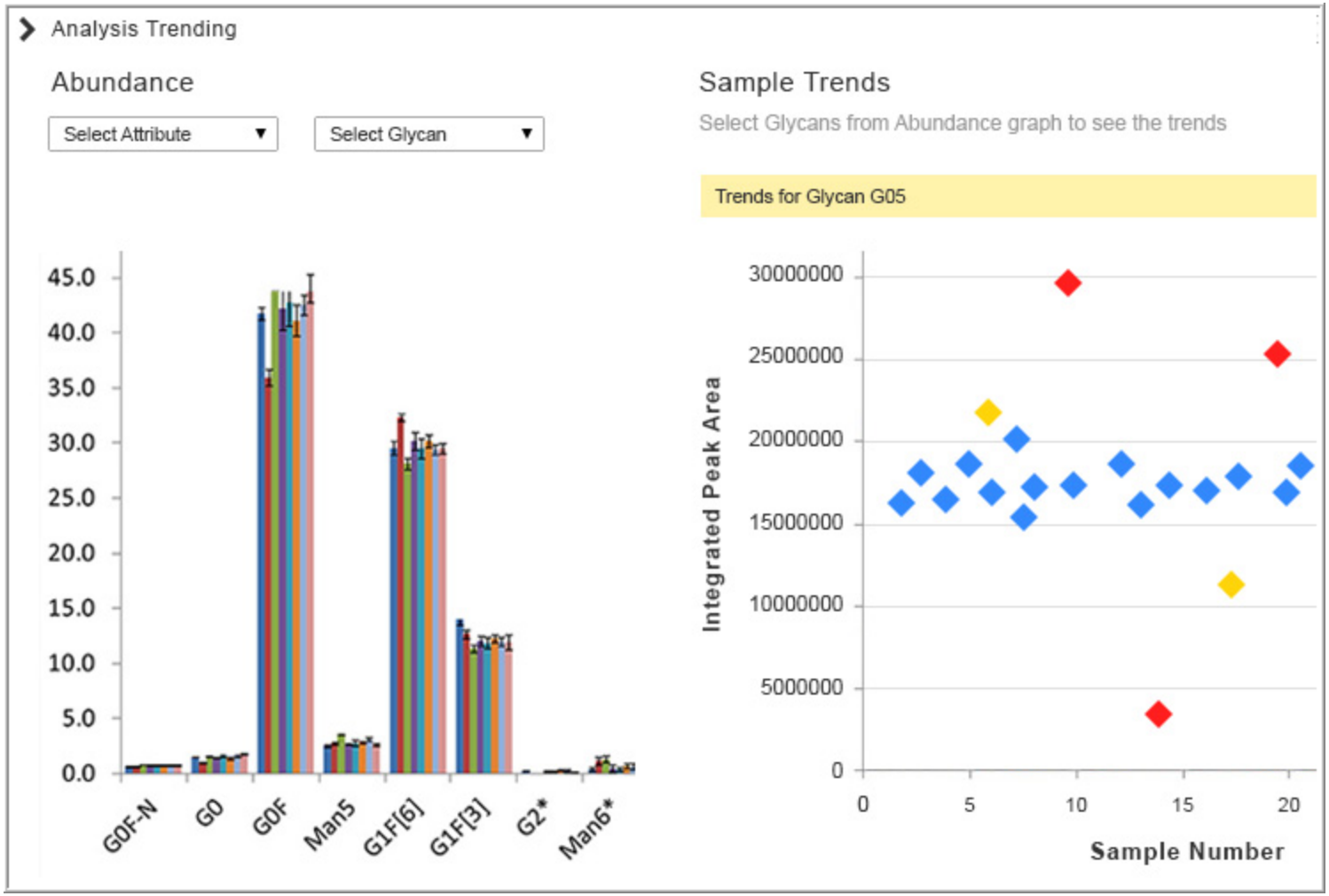
# GlycanAssure: Data Analysis Software





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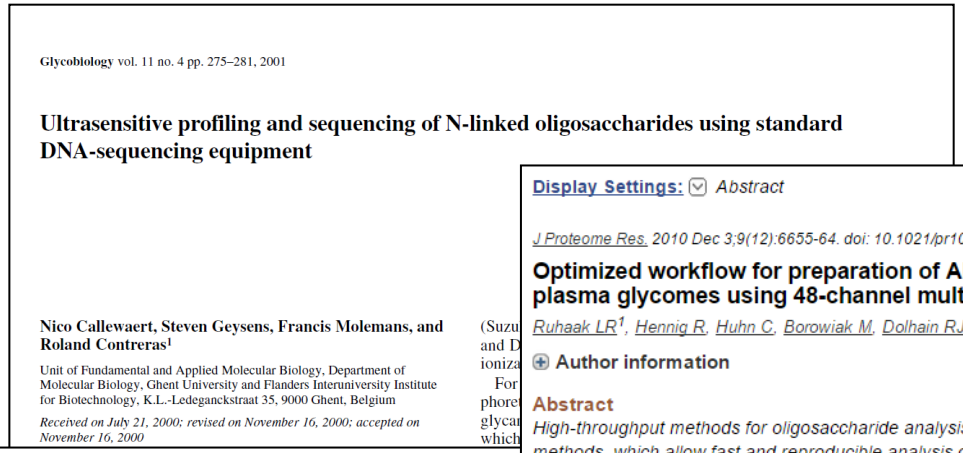
# Reports of AB Multi Capillary CE for Glycan Analysis



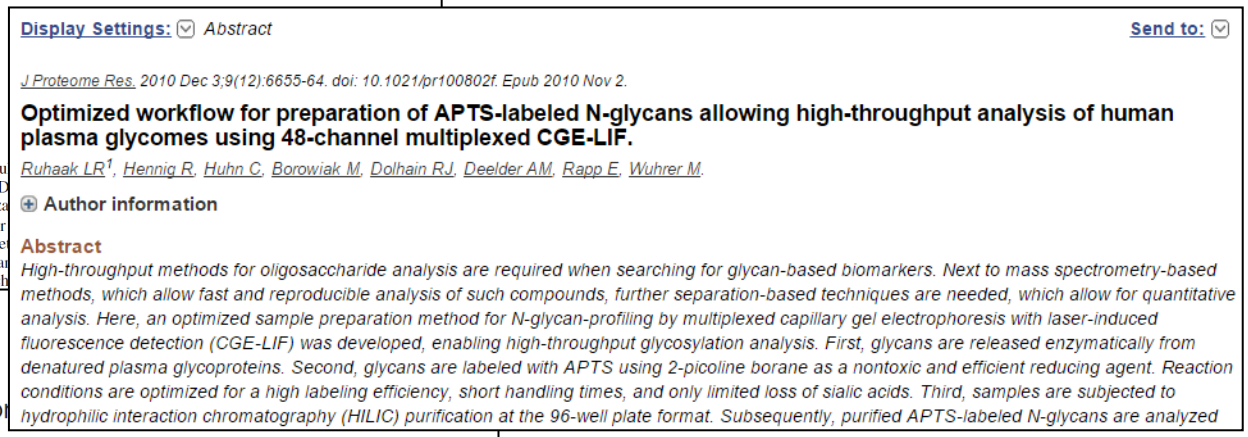
Glycobiology vol. 11 no. 4 pp. 275–281, 2001

**Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment**

**Nico Callewaert, Steven Geysens, Francis Molemans, and Roland Contreras[1]**

Unit of Fundamental and Applied Molecular Biology, Department of Molecular Biology, Ghent University and Flanders Interuniversity Institute for Biotechnology, K.L.-Ledeganckstraat 35, 9000 Ghent, Belgium

*Received on July 21, 2000; revised on November 16, 2000; accepted on November 16, 2000*



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*J Proteome Res.* 2010 Dec 3;9(12):6655-64. doi: 10.1021/pr100802f. Epub 2010 Nov 2.

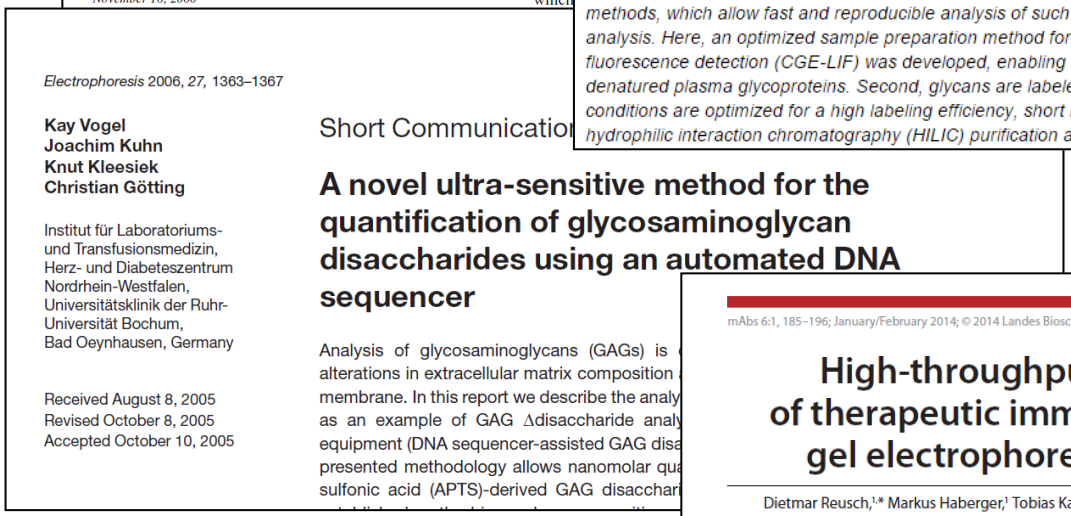
**Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF.**

*Ruhaak LR[1], Hennig R, Huhn C, Borowiak M, Dolhain RJ, Deelder AM, Rapp E, Wuhrer M.*

Author information

**Abstract**

*High-throughput methods for oligosaccharide analysis are required when searching for glycan-based biomarkers. Next to mass spectrometry-based methods, which allow fast and reproducible analysis of such compounds, further separation-based techniques are needed, which allow for quantitative analysis. Here, an optimized sample preparation method for N-glycan-profiling by multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF) was developed, enabling high-throughput glycosylation analysis. First, glycans are released enzymatically from denatured plasma glycoproteins. Second, glycans are labeled with APTS using 2-picoline borane as a nontoxic and efficient reducing agent. Reaction conditions are optimized for a high labeling efficiency, short handling times, and only limited loss of sialic acids. Third, samples are subjected to hydrophilic interaction chromatography (HILIC) purification at the 96-well plate format. Subsequently, purified APTS-labeled N-glycans are analyzed*



*Electrophoresis* 2006, *27*, 1363–1367

**Kay Vogel**
**Joachim Kuhn**
**Knut Kleesiek**
**Christian Götting**

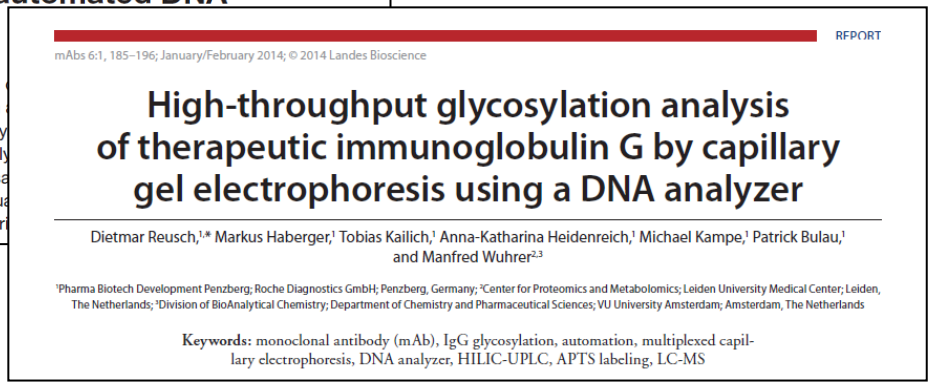
Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Bad Oeynhausen, Germany

Received August 8, 2005
Revised October 8, 2005
Accepted October 10, 2005

Short Communication

**A novel ultra-sensitive method for the quantification of glycosaminoglycan disaccharides using an automated DNA sequencer**

Analysis of glycosaminoglycans (GAGs) is
alterations in extracellular matrix composition
membrane. In this report we describe the analy
as an example of GAG Δdisaccharide analy
equipment (DNA sequencer-assisted GAG disa
presented methodology allows nanomolar qua
sulfonic acid (APTS)-derived GAG disacchari



REPORT

mAbs 6:1, 185–196; January/February 2014; © 2014 Landes Bioscience

**High-throughput glycosylation analysis of therapeutic immunoglobulin G by capillary gel electrophoresis using a DNA analyzer**

Dietmar Reusch,[1,*] Markus Haberger,[1] Tobias Kailich,[1] Anna-Katharina Heidenreich,[1] Michael Kampe,[1] Patrick Bulau,[1] and Manfred Wuhrer[2,3]

[1]Pharma Biotech Development Penzberg; Roche Diagnostics GmbH; Penzberg, Germany; [2]Center for Proteomics and Metabolomics; Leiden University Medical Center; Leiden, The Netherlands; [3]Division of BioAnalytical Chemistry; Department of Chemistry and Pharmaceutical Sciences; VU University Amsterdam; Amsterdam, The Netherlands

**Keywords:** monoclonal antibody (mAb), IgG glycosylation, automation, multiplexed capillary electrophoresis, DNA analyzer, HILIC-UPLC, APTS labeling, LC-MS



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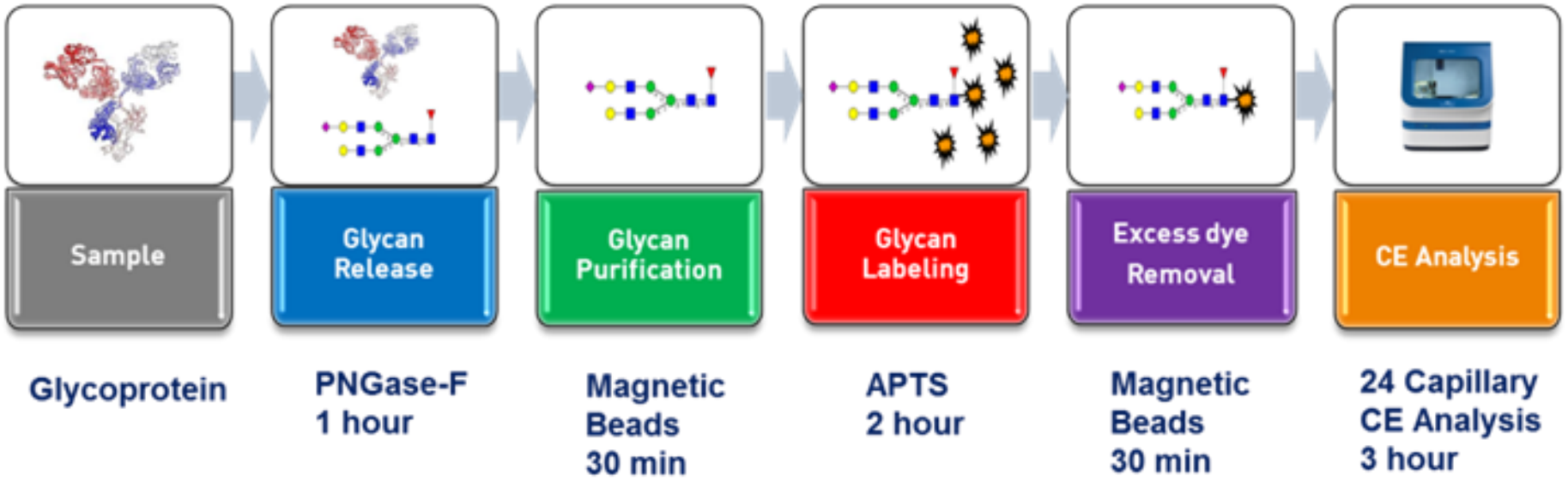
# GlycanAssure APTS Workflow

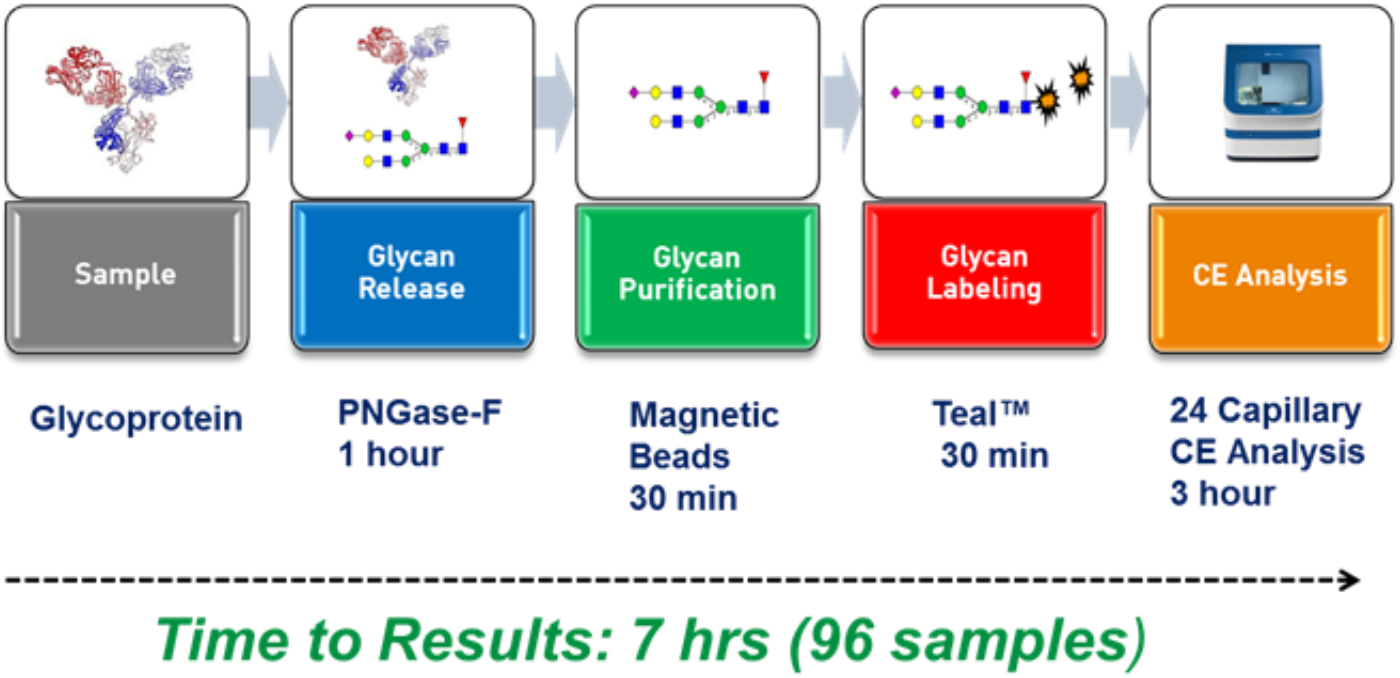



***Time to Results: 9 hrs (96 samples)***

*Total 9hrs taking into account initial glycoprotein prep time and time between steps.
Total time of the workflow is only 7hrs. CE takes 45min to analyze 24 samples


*Proprietary & Confidential*

# GlycanAssure: Teal Workflow




*Total 7hrs taking into account initial glycoprotein prep time and time between steps.
Total time of the workflow is only 5hrs. CE takes 45min to analyze 24 samples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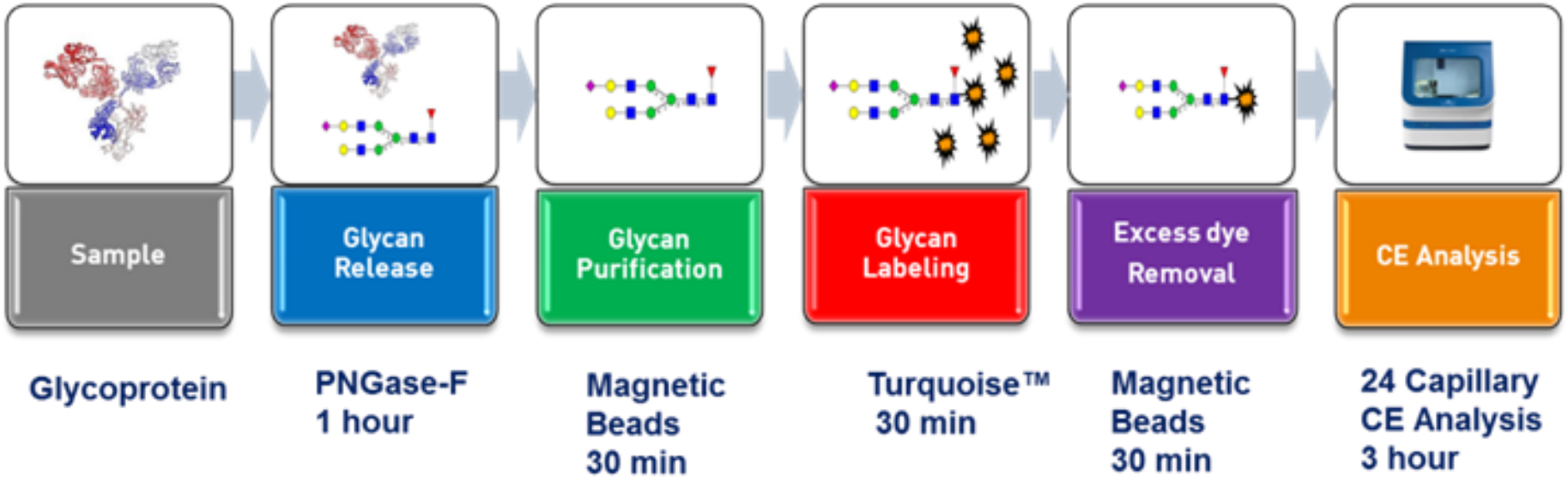
# GlycanAssure: Turquoise Workflow




***Time to Results: 8 hrs (96 samples)***

*Total 8hrs taking into account initial glycoprotein prep time and time between steps.
Total time of the workflow is only 5.5hrs. CE takes 45min to analyze 24 samples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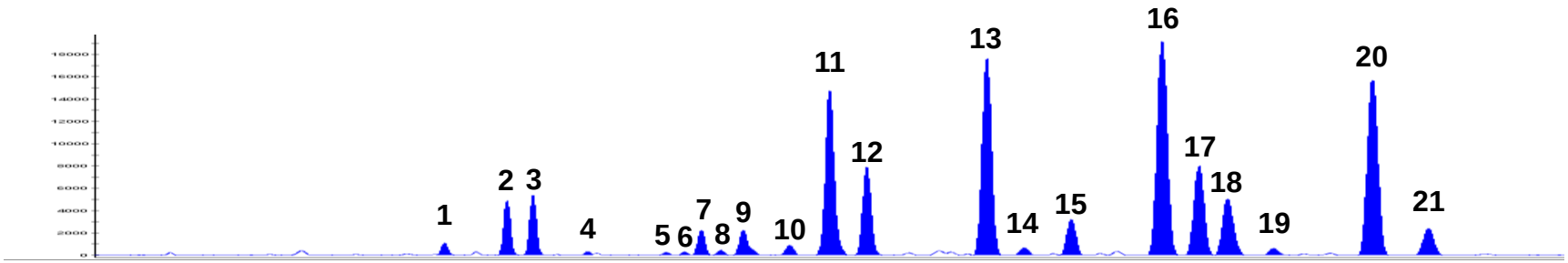
# Magnetic Bead Workflow Vs Carbon Spin Columns

- Glycans purified by *carbon column* or *magnetic beads* in triplicate
- Glycans were labeled with APTS for separation on 3500xL




| | Peak | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon column | Rel % Area | 0.69% | 3.11% | 3.54% | 0.20% | 0.16% | 0.16% | 1.55% | 0.29% | 1.96% | 0.68% |
| | CV% | 5.12% | 4.88% | 5.75% | 4.31% | 5.38% | 3.29% | 3.80% | 0.39% | 2.52% | 1.76% |
| Magnetic Beads | Rel % Area | 0.68% | 2.82% | 3.09% | 0.23% | 0.16% | 0.18% | 1.66% | 0.26% | 1.94% | 0.68% |
| | CV% | 1.91% | 3.63% | 4.13% | 1.69% | 1.17% | 1.52% | 0.50% | 2.28% | 1.37% | 1.12% |

| | Peak | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon column | Rel % Area | 12.28% | 6.49% | 15.64% | 0.51% | 2.80% | 18.15% | 7.58% | 5.25% | 0.56% | 15.95% | 2.43% |
| | CV% | 2.64% | 1.76% | 0.95% | 18.63% | 1.26% | 1.19% | 2.15% | 1.41% | 1.57% | 1.68% | 2.62% |
| Magnetic Beads | Rel % Area | 12.77% | 6.44% | 15.76% | 0.53% | 2.66% | 18.38% | 7.67% | 5.16% | 0.54% | 16.00% | 2.39% |
| | CV% | 0.88% | 1.46% | 1.07% | 1.53% | 0.85% | 0.59% | 1.00% | 0.46% | 2.69% | 0.60% | 0.64% |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# Consistent Glycan Quantitation

| | 10µg | | 50µg | | 100µg | |
|---|---|---|---|---|---|---|
| Peak # | %Area | %CV | %Area | %CV | %Area | %CV |
| 1 | 0.90% | 4.05% | 0.72% | 3.06% | 0.64% | 5.07% |
| 2 | 0.61% | 6.31% | 0.45% | 3.31% | 0.39% | 4.77% |
| 3 | 1.64% | 2.22% | 1.69% | 2.46% | 1.66% | 2.68% |
| 4 | 0.42% | 7.09% | 0.42% | 4.67% | 0.38% | 4.26% |
| 5 | 8.54% | 2.47% | 8.31% | 2.19% | 8.18% | 1.48% |
| 6 | 0.76% | 5.89% | 0.55% | 8.97% | 0.42% | 6.47% |
| 7 | 22.51% | 2.04% | 23.07% | 4.25% | 23.71% | 1.60% |
| 8 | 0.63% | 4.50% | 0.71% | 9.54% | 0.71% | 3.36% |
| 9 | 3.73% | 0.83% | 3.82% | 2.60% | 3.85% | 1.28% |
| 10 | 23.52% | 0.43% | 23.33% | 2.52% | 23.84% | 0.65% |
| 11 | 9.98% | 0.52% | 10.16% | 3.37% | 9.91% | 0.88% |
| 12 | 5.37% | 1.00% | 5.46% | 3.50% | 5.42% | 1.00% |
| 13 | 0.43% | 4.27% | 0.44% | 4.53% | 0.43% | 2.12% |
| 14 | 19.58% | 1.16% | 19.53% | 2.91% | 19.20% | 0.85% |
| 15 | 1.38% | 2.25% | 1.33% | 4.83% | 1.27% | 1.84% |


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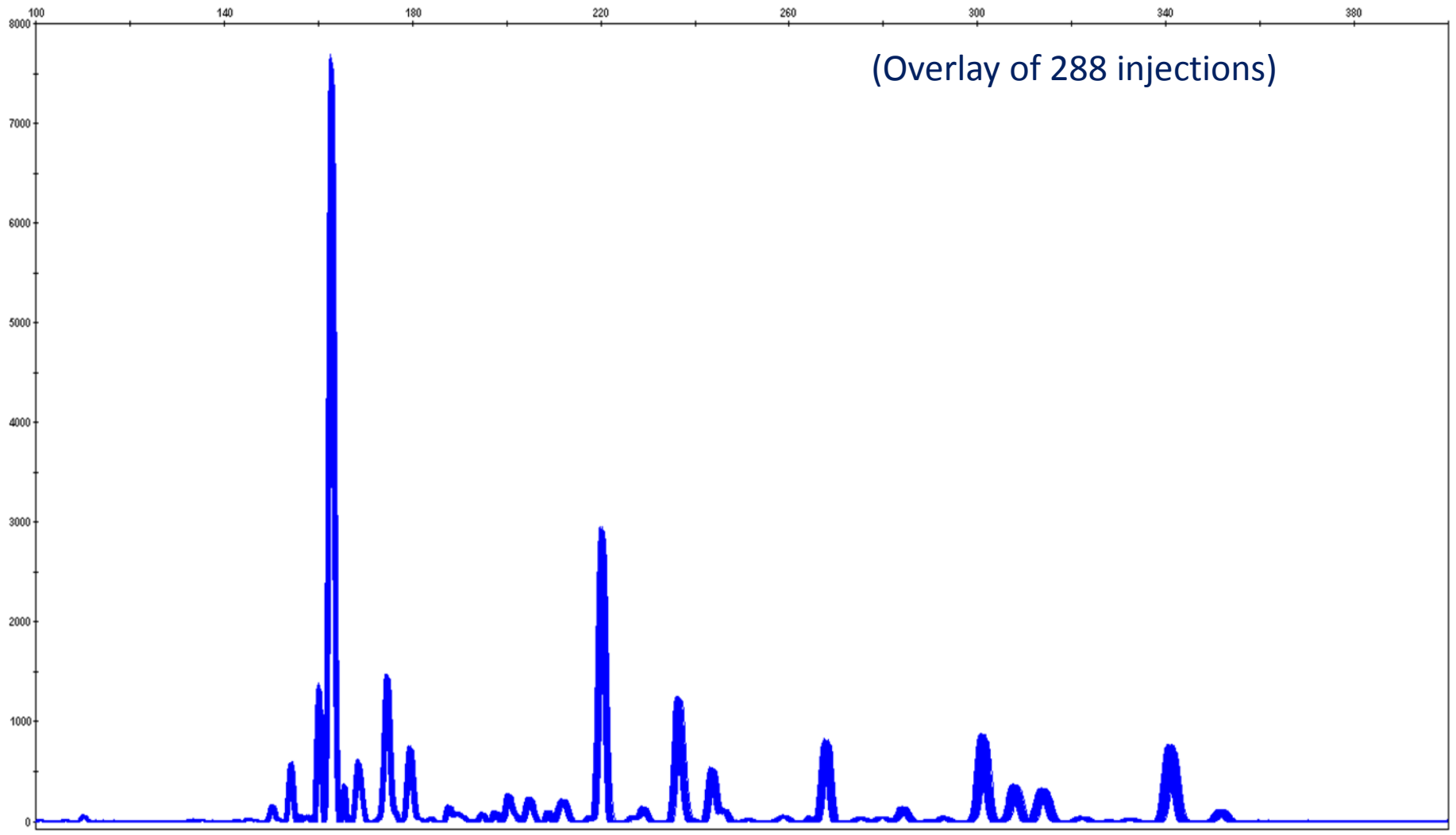
# Reproducibility of Glycan Separation





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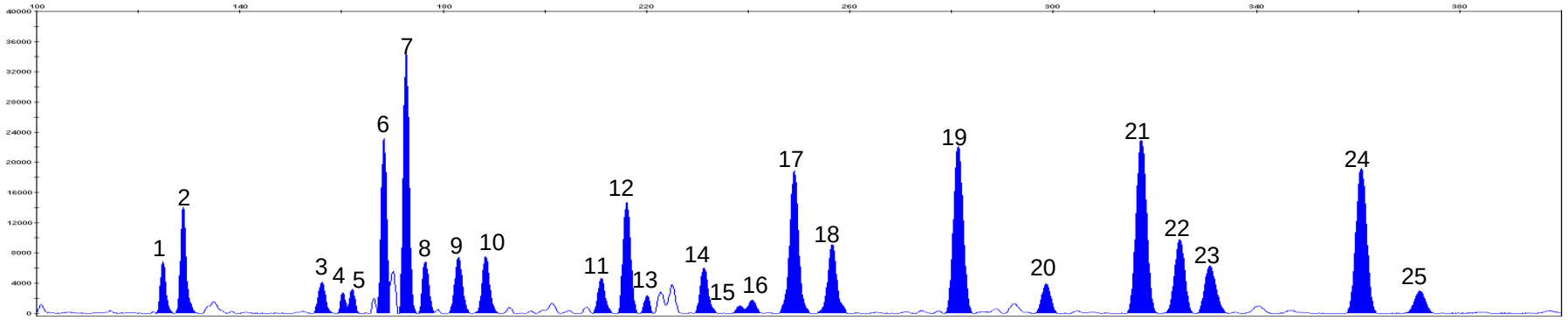
# Variability Across Capillaries of an Array

- Mixture of glycans from purified human serum IgG and bovine Fetuin
- Labeled with APTS & run on 3500xL CE (24 capillaries)




| | Average | STDEV | CV% |
|---|---|---|---|
| 1 | 1.8% | 0.03% | 1.5% |
| 2 | 3.9% | 0.08% | 2.1% |
| 3 | 1.4% | 0.02% | 1.5% |
| 4 | 0.6% | 0.02% | 3.1% |
| 5 | 0.8% | 0.02% | 2.3% |
| 6 | 5.8% | 0.43% | 7.4% |
| 7 | 9.1% | 0.19% | 2.1% |
| 8 | 2.3% | 0.22% | 9.8% |
| 9 | 2.7% | 0.02% | 0.8% |
| 10 | 2.7% | 0.02% | 0.7% |

| | Average | STDEV | CV% |
|---|---|---|---|
| 11 | 1.7% | 0.03% | 2.0% |
| 12 | 5.4% | 0.08% | 1.4% |
| 13 | 0.7% | 0.08% | 11.3% |
| 14 | 2.4% | 0.04% | 1.5% |
| 15 | 0.3% | 0.02% | 4.8% |
| 16 | 0.7% | 0.01% | 1.4% |
| 17 | 8.2% | 0.07% | 0.9% |
| 18 | 4.2% | 0.04% | 1.0% |
| 19 | 11.0% | 0.09% | 0.8% |
| 20 | 1.8% | 0.03% | 1.7% |

| | Average | STDEV | CV% |
|---|---|---|---|
| 20 | 1.8% | 0.03% | 1.7% |
| 21 | 11.7% | 0.08% | 0.7% |
| 22 | 5.0% | 0.05% | 1.0% |
| 23 | 3.6% | 0.03% | 0.8% |
| 24 | 10.6% | 0.09% | 0.9% |
| 25 | 1.7% | 0.02% | 1.1% |

*Data analysis was done using GeneMapper software that won't allow manual integration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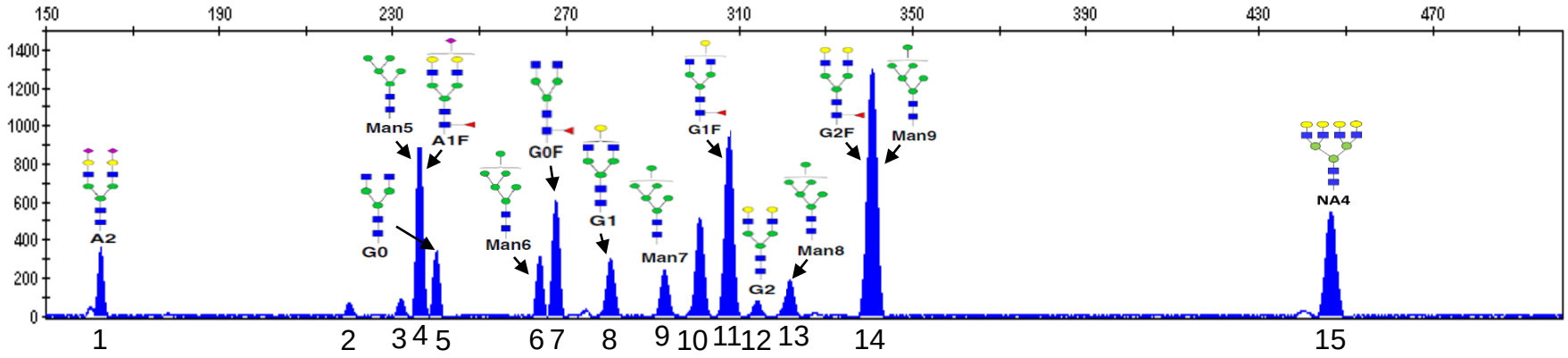
# Variability Across Multiple CE Instruments & Arrays




| | Peak | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1st 3500 32 Inj | Average | 3.4% | 0.8% | 1.0% | 10.5% | 3.3% | 3.6% | 8.4% | 4.4% | 3.7% | 8.0% | 15.3% | 1.0% | 3.0% | 23.5% | 10.2% |
| | CV% | 2.8% | 2.2% | 2.3% | 1.1% | 2.0% | 2.8% | 9.2% | 1.1% | 0.9% | 0.5% | 0.9% | 3.6% | 1.5% | 1.0% | 1.3% |
| 2nd 3500 24 Inj | Average | 3.3% | 0.8% | 1.1% | 9.7% | 2.8% | 3.7% | 8.1% | 4.5% | 3.7% | 8.2% | 15.7% | 1.0% | 3.1% | 24.0% | 10.4% |
| | CV% | 2.0% | 1.9% | 1.8% | 1.3% | 2.3% | 1.7% | 0.9% | 0.6% | 0.7% | 0.7% | 0.4% | 3.3% | 0.8% | 0.4% | 1.2% |
| 3rd 3500 24 Inj | Average | 3.5% | 0.8% | 1.0% | 11.1% | 3.6% | 3.6% | 7.9% | 4.4% | 3.6% | 7.9% | 15.1% | 1.0% | 3.0% | 23.3% | 10.1% |
| | CV% | 1.3% | 3.0% | 3.2% | 1.2% | 2.3% | 1.7% | 0.9% | 1.1% | 1.2% | 0.7% | 0.6% | 6.1% | 1.2% | 0.7% | 1.7% |
| Cap 1 | Average | 3.5% | 0.8% | 1.0% | 11.1% | 3.7% | 3.7% | 7.9% | 4.4% | 3.6% | 7.9% | 15.1% | 1.0% | 3.0% | 23.3% | 10.0% |
| | CV% | 1.5% | 3.8% | 2.7% | 0.9% | 1.9% | 1.3% | 0.6% | 1.0% | 1.1% | 0.7% | 0.3% | 3.0% | 1.1% | 0.5% | 0.9% |
| Cap 2 | Average | 3.4% | 0.8% | 1.1% | 10.9% | 3.6% | 3.8% | 7.9% | 4.4% | 3.6% | 7.9% | 15.2% | 1.1% | 3.0% | 23.3% | 10.1% |
| | CV% | 1.2% | 2.4% | 2.2% | 0.9% | 1.9% | 1.8% | 1.0% | 0.9% | 0.7% | 0.5% | 0.5% | 1.8% | 0.9% | 0.4% | 1.3% |
| Cap 3 | Average | 3.4% | 0.8% | 1.1% | 11.0% | 3.6% | 3.8% | 8.0% | 4.4% | 3.6% | 7.9% | 15.2% | 1.1% | 3.0% | 23.3% | 9.9% |
| | CV% | 1.5% | 3.8% | 2.1% | 1.2% | 2.3% | 1.2% | 0.6% | 0.6% | 0.8% | 0.6% | 0.3% | 2.8% | 1.2% | 0.5% | 0.7% |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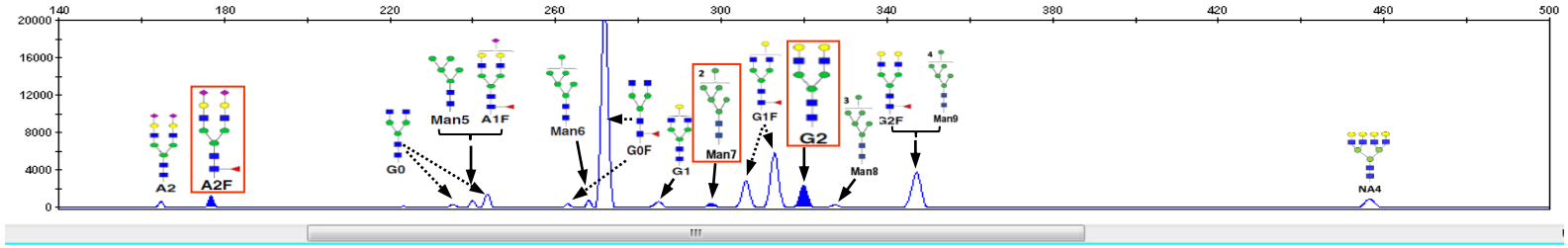
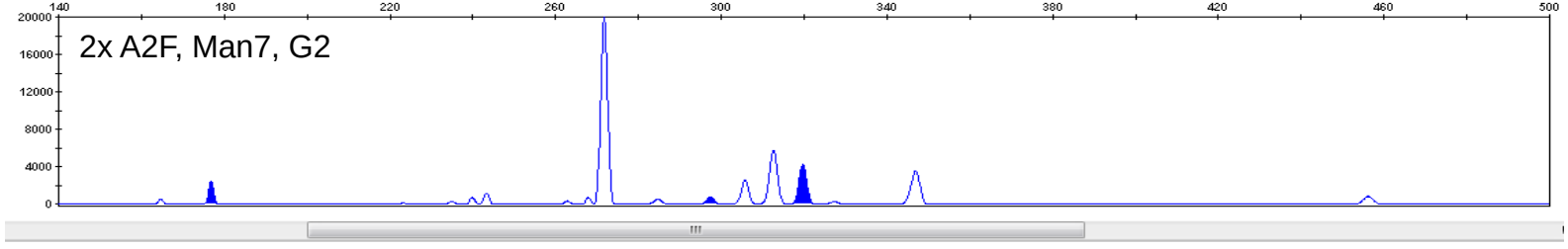
# Glycan Spike Recovery

- 15 diverse types of purified glycans labeled with APTS
- Mixed to create large difference in concentrations (50% to <1%)
- A2F (sialylated), Man7 (high mannose), and G2 (complex) glycans were increased serially by 2x

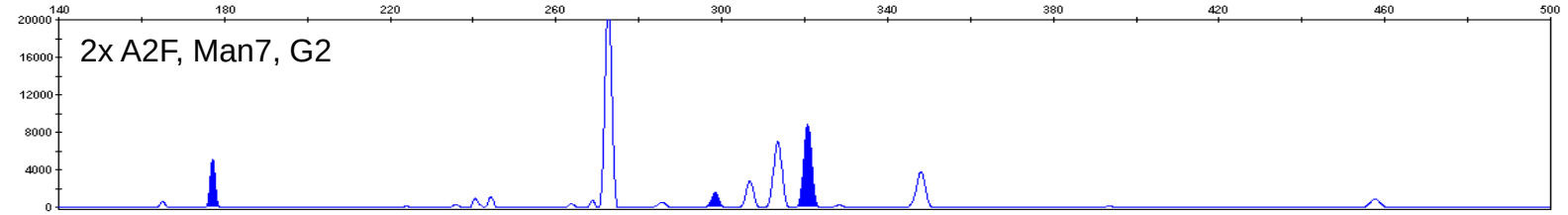
Pool 1




Pool 2




Pool 3




Increased signals of three specific glycans while the rest remain constant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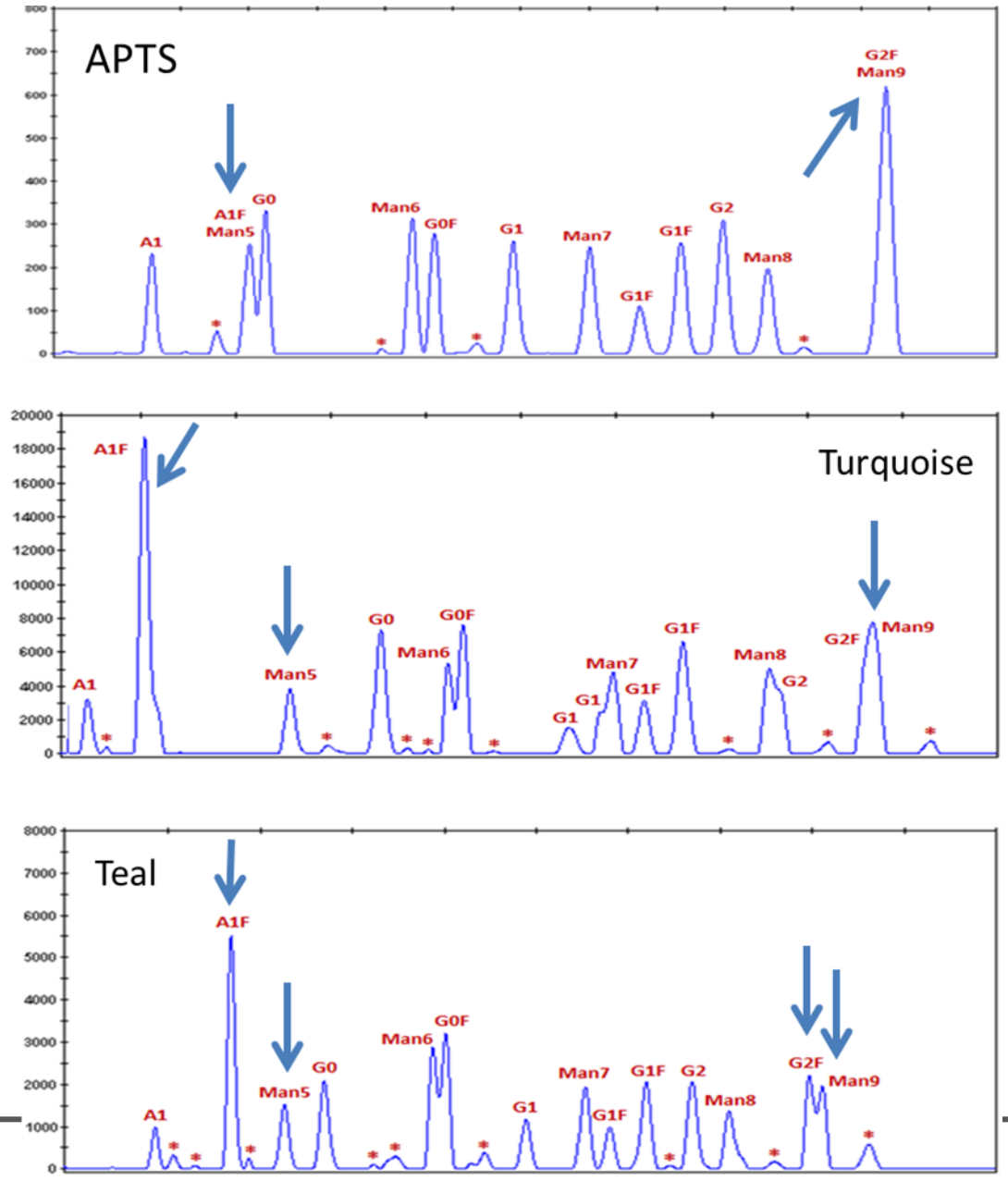
# Better Glycan Separation Using Thermo Fisher Dyes





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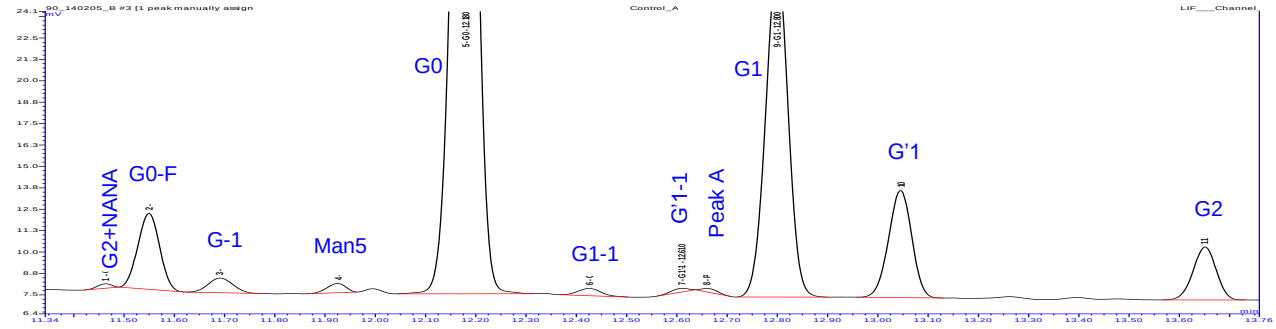
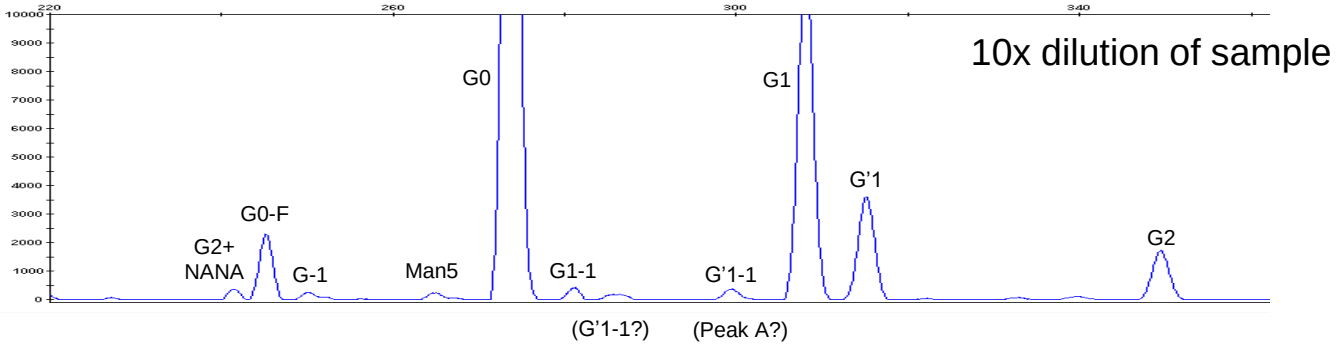
# Thermo Fisher 3500 Vs Leading Single Capillary CE

Single cap CE

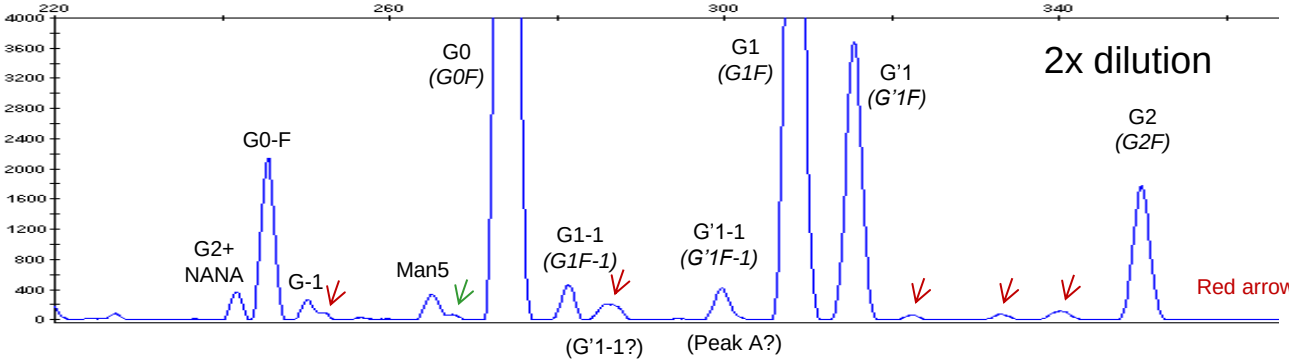



3500XL




10x dilution of sample

3500XL




2x dilution

Red arrows: new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# GlycanAssure: Summary

- **Easy sample prep**
  - Magnetic bead based sample prep
  - Hands-on-time <3 hrs for 96 samples
  - Less no. of pipetting steps
  - No use of Sodium cyano borohydride
  - No vacuum centrifugation steps
- **Throughput**
  - Sample prep & data of 96 samples in 7-9hrs
- **Resolution**
  - Sialyated glycans
  - Structural isomers
  - Fucose species
  - High Mannose species
- **Dye Labeling**
  - Multiple dyes with superior sensitivity
- **Sensitivity**
  - Low glycoprotein input
- **Low Cost of Analysis**
  - Robust instrument and capillaries with low running cost
- **Software**
  - Data Acquisition & Analysis software with novel features
- **Integration**
  - Full sample prep, hardware & software solution

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