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Creation, Implementation and Transferability of a Glucose Unit (GU) Library for HILIC-FLD Analysis of APTS-Labelled Released *N*-Glycans

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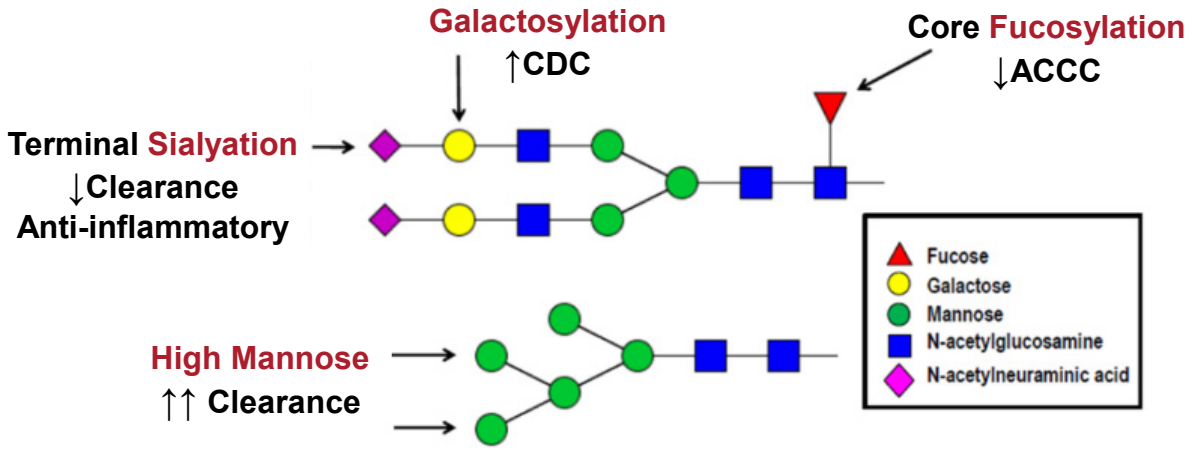
Overview

- *N*-linked glycosylation
  - Importance of glycans in biotherapeutics
  - Regulations
- Analysis of released *N*-glycans
  - Applied Biosystems™ GlycanAssure™ HyPerformance APTS kit
- Glucose Unit (GU) libraries
- Construction of APTS GU library
- Transferability of APTS GU library
  - eWorkflow

N-linked Glycans

- ~50% of biopharmaceuticals are glycosylated
  - *N*-glycan attached to Asn residues
    - Consensus sequence N-X-S/T
- Glycosylation can have an impact on
  - Immunogenicity
  - Biological activity, drug clearance and half-life
  - Stability
- Therefore is a Critical Quality Attribute (CQA)
  - ICH Q6B
- Microheterogeneity affected by manufacturing process
  - Both a manufacturing and analytical challenge



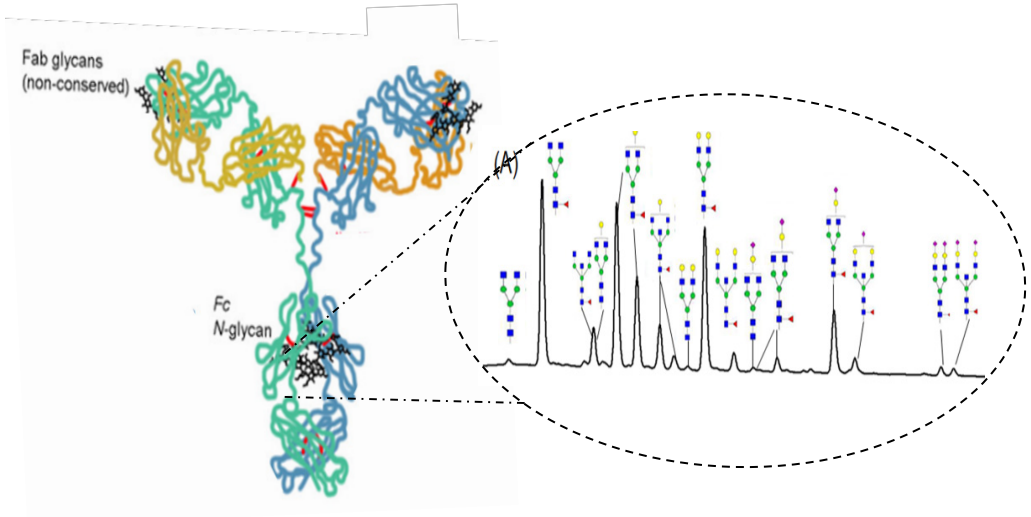
Reviewed in:

Liu, *J Pharm Sci.* 2015; 104: 1866-1884

Abes & Teillaud, *Pharmaceuticals* 2010; 3:146-57

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

CDC: Complement-Dependent Cytotoxicity



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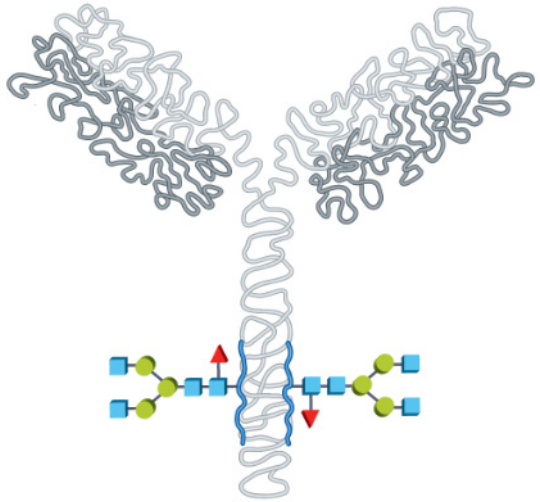
N-linked Glycans

ICH (Q6B) recommends 6 test approaches for characterization and confirmation of biological products:

- Amino acid sequence
- Amino acid composition
- Terminal amino acid sequence
- Peptide map
- Sulfhydryl group(s) and disulfide bridges
- **Carbohydrate structure**
  - *"For glycoproteins, the carbohydrate content and structure (neutral sugars, amino sugars, and sialic acids) is determined."*



Early development (High throughput clone screening) → QC (CQA monitoring)

Analysis of *N*-linked Glycans

Characterization /Analytical development

Biotherapeutic

Glycan pool characterization by MS and MS/MS

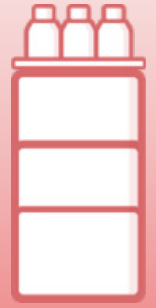
Critical Quality Attribute (CQA)

Method Transfer to LC-FLD

Quality control

Sequence and CQA identified

LC-FLD method development and validation

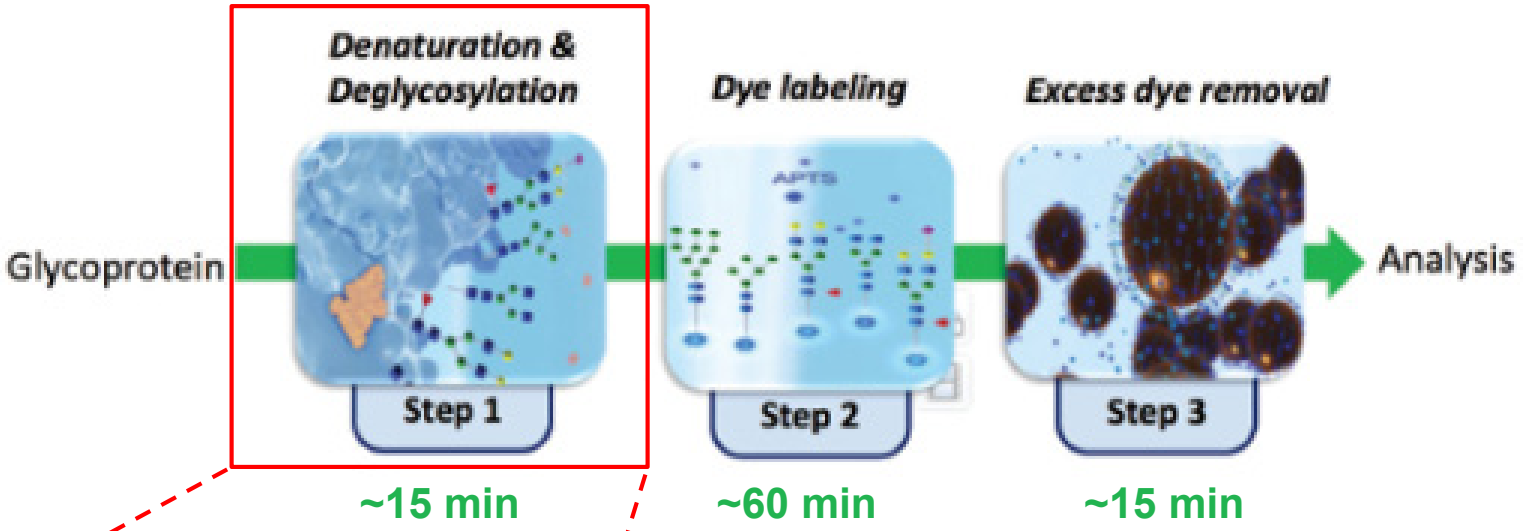


Glycan pool assessed, with comparison to reference sample

QA/QC

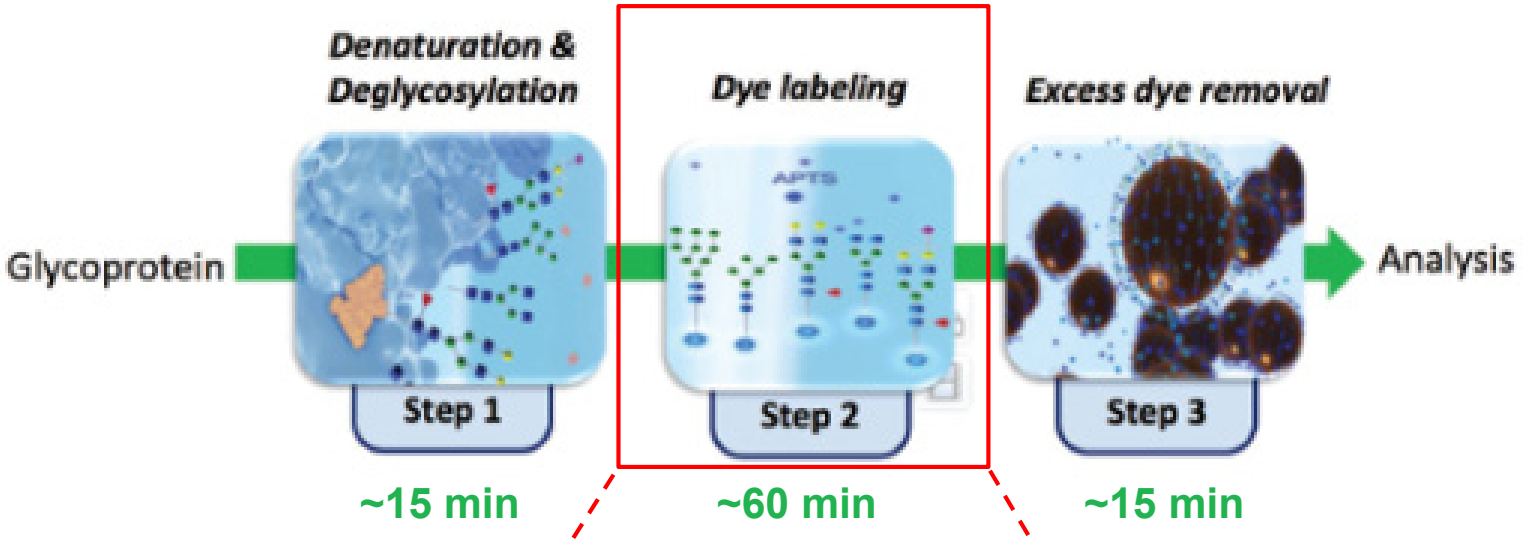
- **In-Process Control**
- **Batch Release**
- **Stability Studies**

GlycanAssure HyPerformance Kit – Denaturation and Deglycosylation



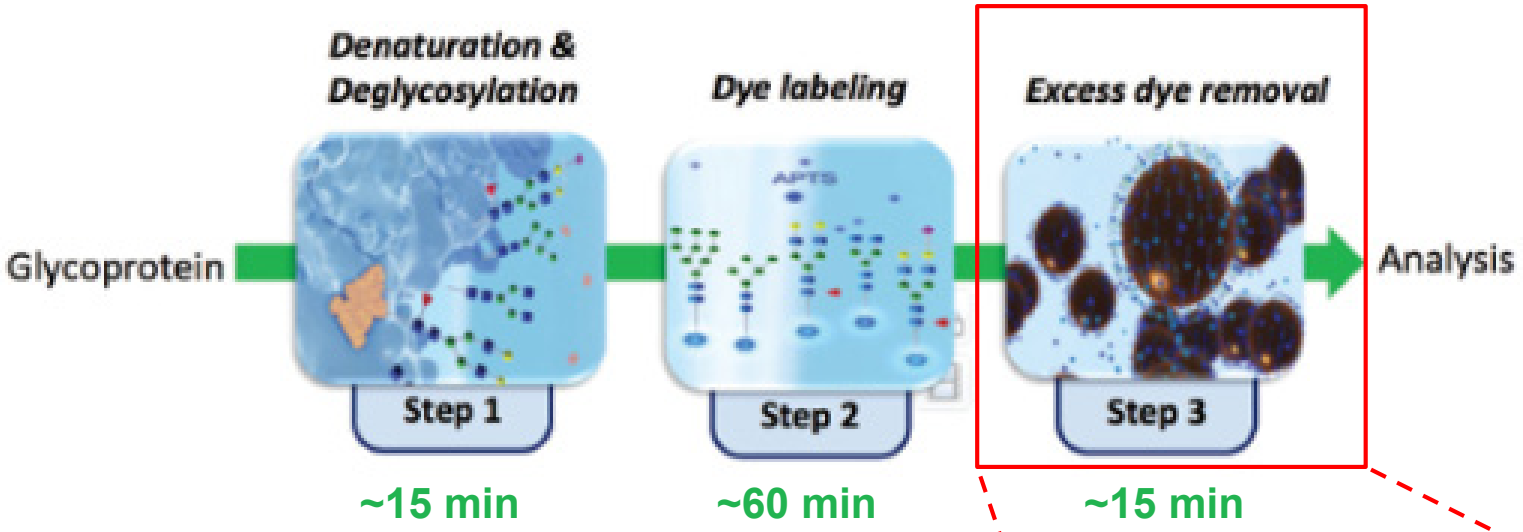
- Denatures and solubilizes protein
- Enables rapid and complete deglycosylation

GlycanAssure HyPerformance Kit – APTS Labelling



- In-solution labelling
- No use of sodium cyanoborohydride
- Retention of labile sialic acid

GlycanAssure HyPerformance Kit – Sample Clean-up



- Efficient clean-up of labelled glycans
- Magnetic bead-based
- Bind and elute workflow

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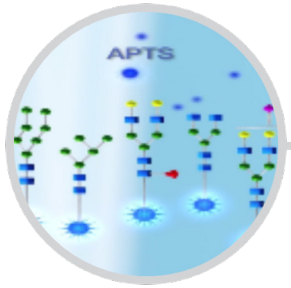
Overview of Glycan Labelling Using GlycanAssure

GlycanAssure
HyPerformance kit

AutoMate Express™
Forensic DNA
Extraction System

Hands-On Method



Labelled
Glycans

3500XL with
GlycanAssure Software

Mass Spectrometry

Thermo Scientific™ Vanquish™ Horizon UHPLC
With Chromeleon™ CDS Software

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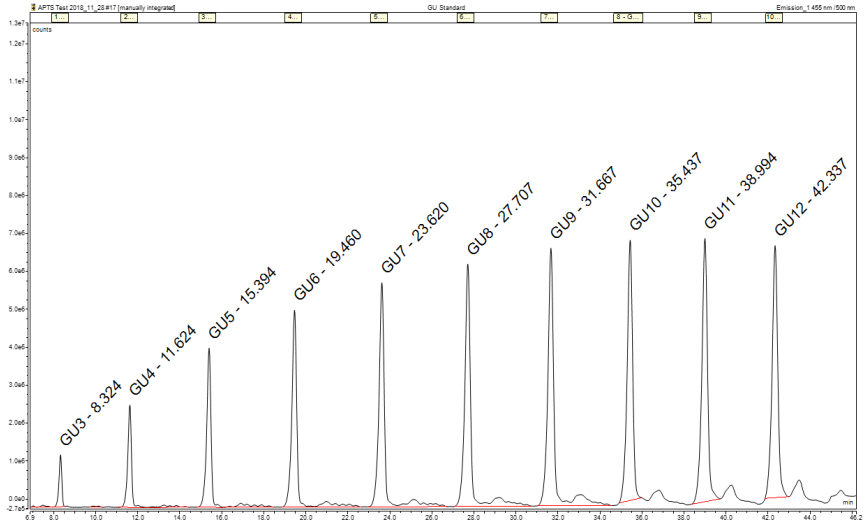
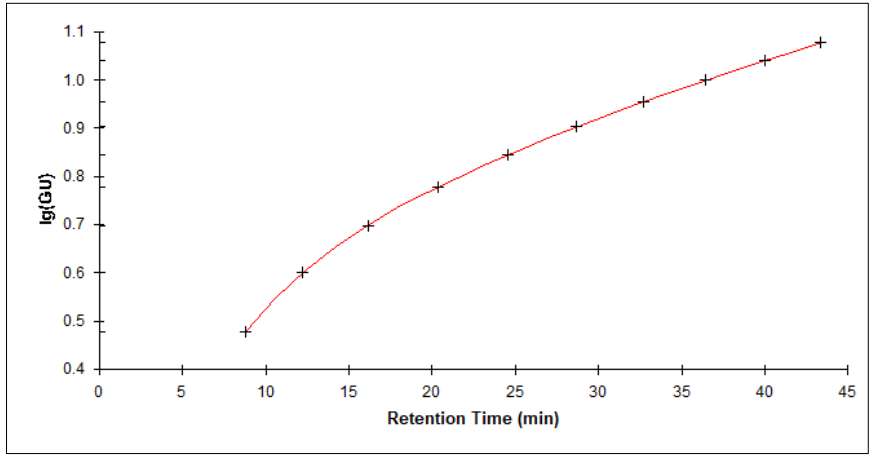
GU Libraries

- External retention calibration approach
- Uses labelled dextran (poly-glucose) ladder standard
  - Generates retention time calibration curve
  - Converts chromatographic time to GU value
- Critical for glycan library search robustness
  - Minimizes chromatographic variation associated with HILIC
    - System-to-system
    - Day-to-day
    - Site-to-site
- Requires method standardization



APTS-labelled dextran ladder (GU 3-12)



Retention time/GU calibration curve

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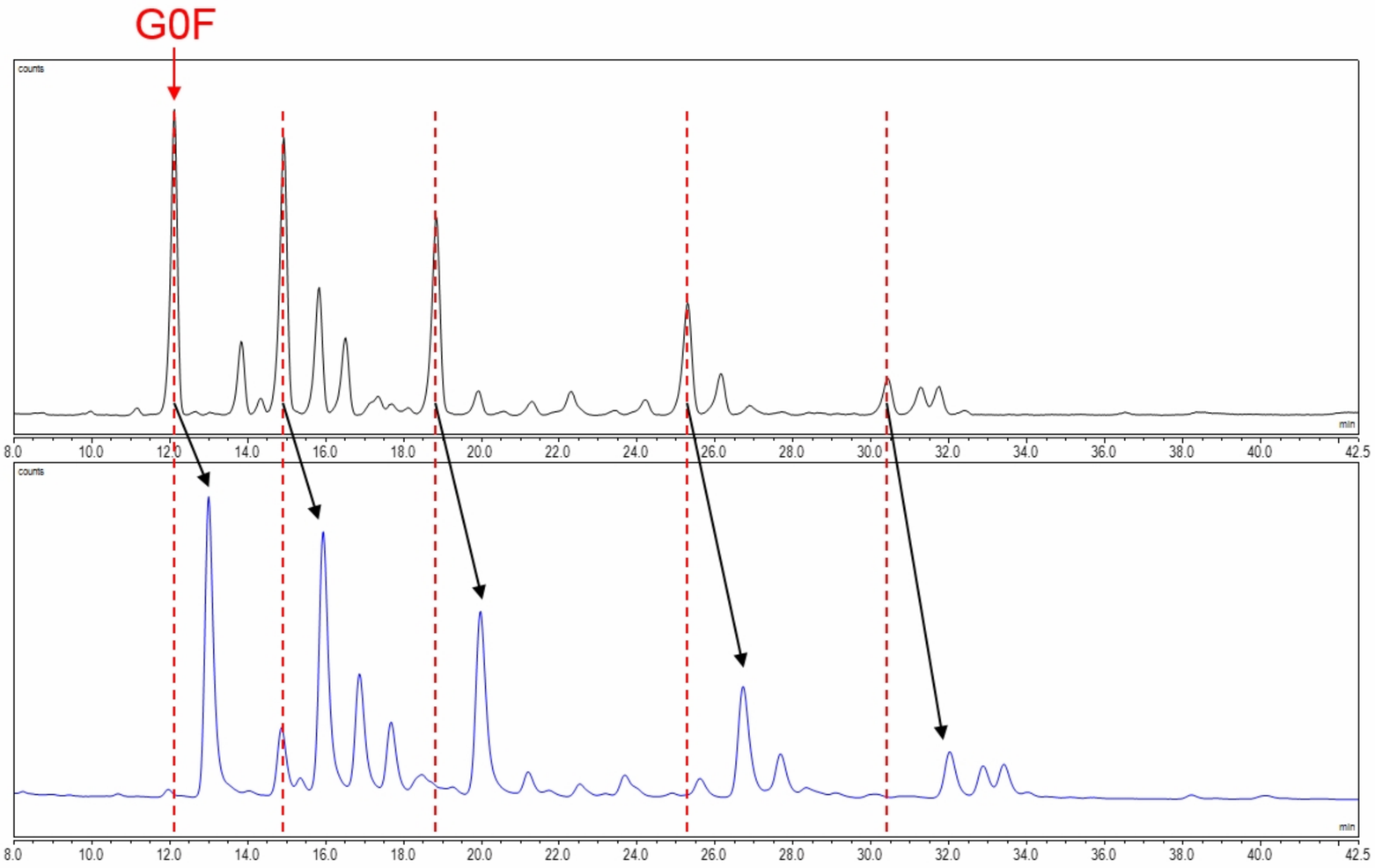
Importance of GU Calibration

- Human IgG – different
  - Analysts
  - Mobile phase
  - Sample preparation
  - Instruments
  - Columns…
- Retention times differ by ~1 min. across the run
- Dextran ladder calibration applied to correct this difference

  For G0F peak (n=12)

  RSD of RT = 3.5%

  RSD of GU = **0.9%**



Human IgG analysed across different sites

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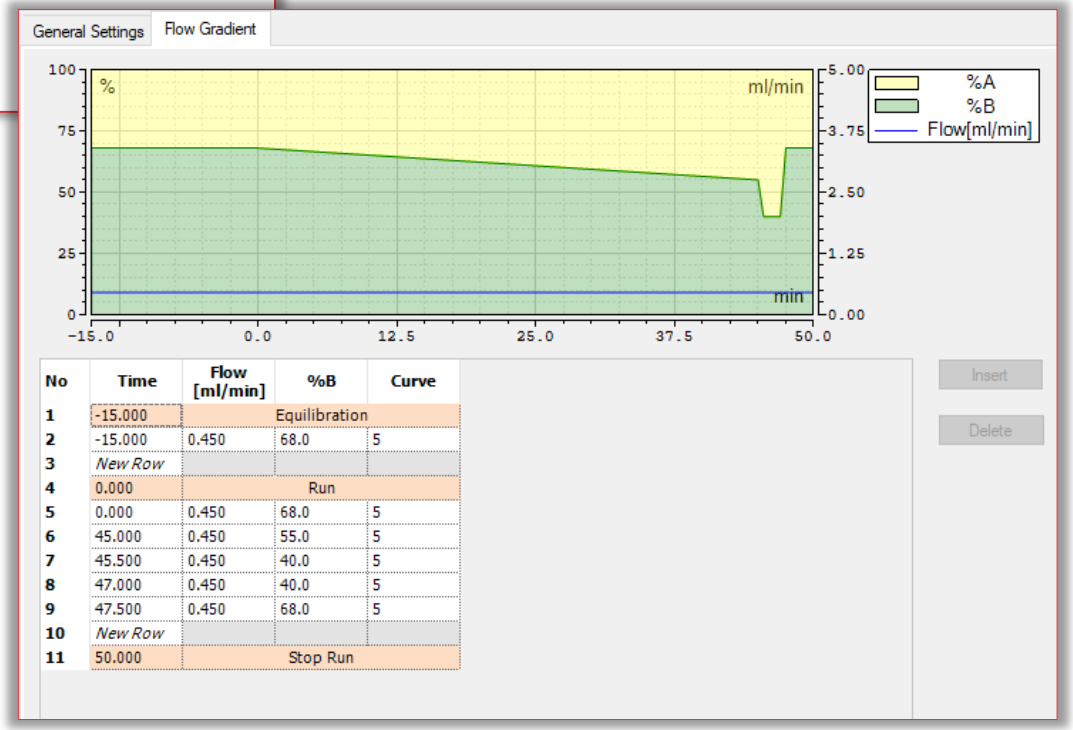
Method Parameters

Mobile phase A	100 mM ammonium formate, pH 4.4
Mobile phase B	Acetonitrile
Column temperature	50°C
Injection volume	2 µL
Excitation wavelength	455 nm
Emission wavelength	500 nm
Sensitivity	7
Lamp mode	High power
Data collection rate	10 Hz

Thermo Scientific™ Accucore™ 150 Amide column (2.1 x 150 mm, 2.6 µm)



No	Time	Flow [ml/min]	%B	Curve
1	-15.000	Equilibration		
2	-15.000	0.450	68.0	5
3	*New Row*			
4	0.000	Run		
5	0.000	0.450	68.0	5
6	45.000	0.450	55.0	5
7	45.500	0.450	40.0	5
8	47.000	0.450	40.0	5
9	47.500	0.450	68.0	5
10	*New Row*			
11	50.000	Stop Run		

Insert

Delete

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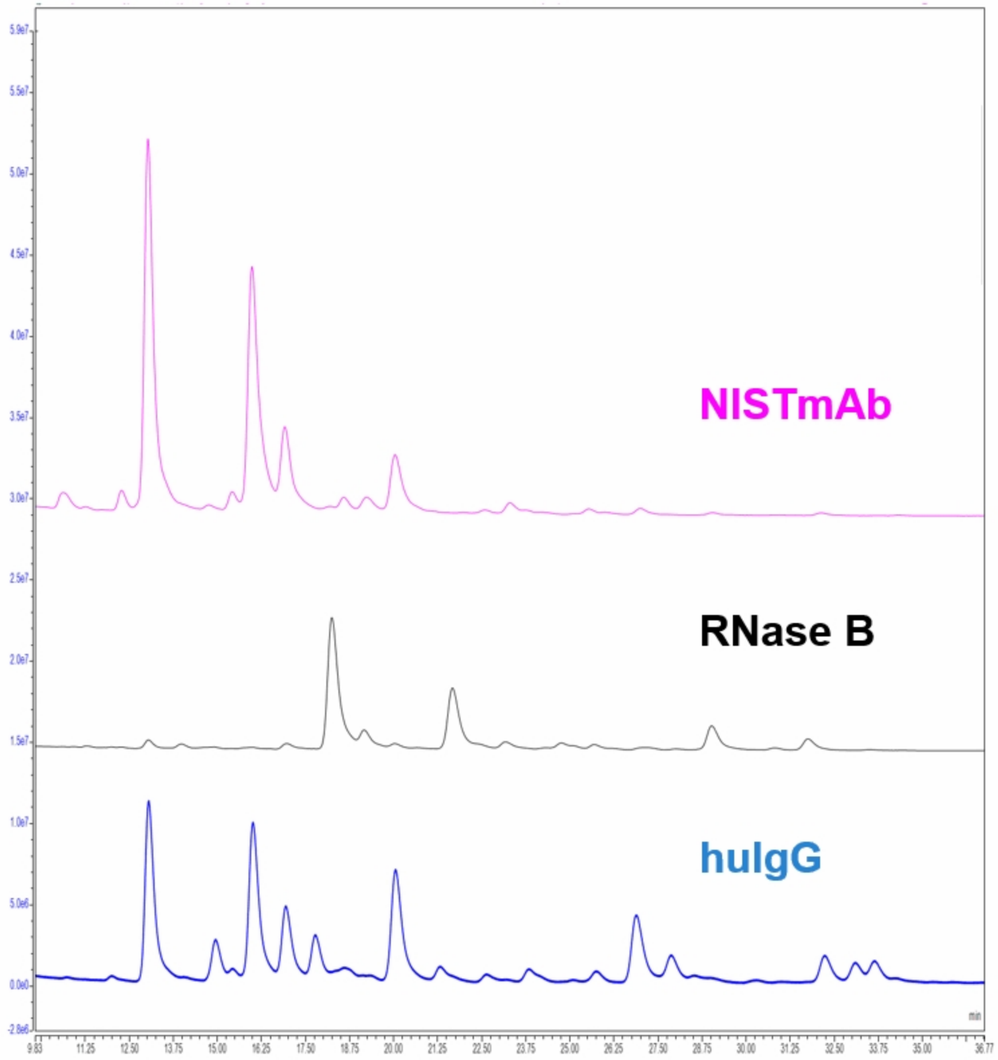
Overview of Library Construction

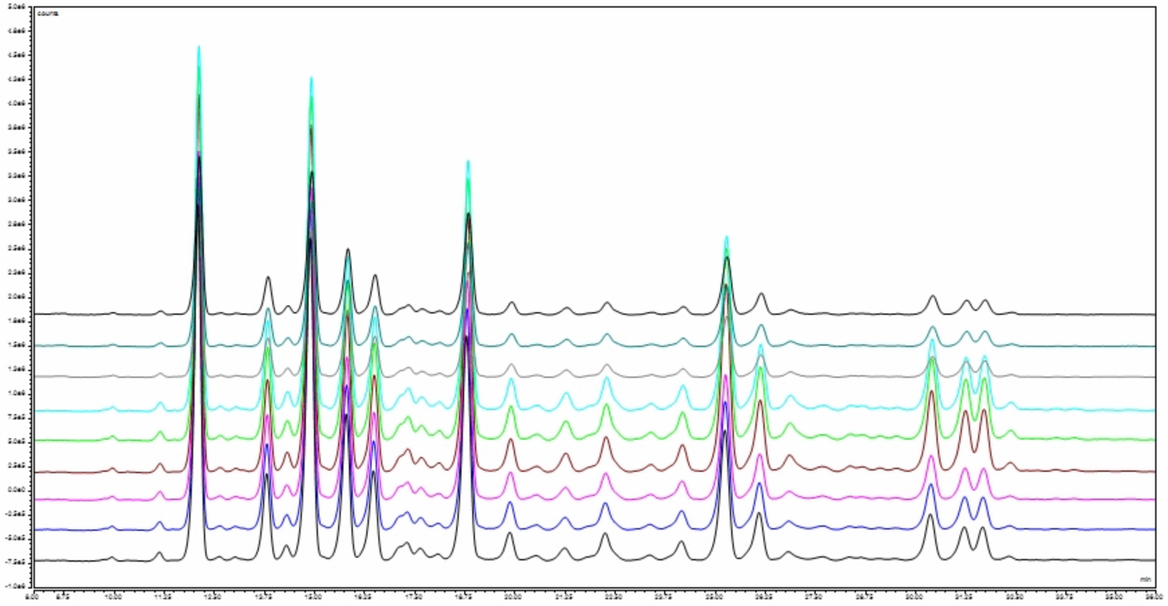
- Biopharmaceutical relevant samples
  - NIST mAb
  - RNase B
  - Human IgG
- Individually labelled, *N*-glycan standards
  - Supplement the sample glycan pools
  - Aid and confirm glycan identifications
- Two analysts; Two different sites; Two column lots
  - Different UHPLC instruments, mobile phase preparations, sample preparations

Typical Sample Chromatograms Used in Library Construction



NISTmAb, RNase B and hulgG representative samples

n=9 overlay of hulgG performed in the same analysis



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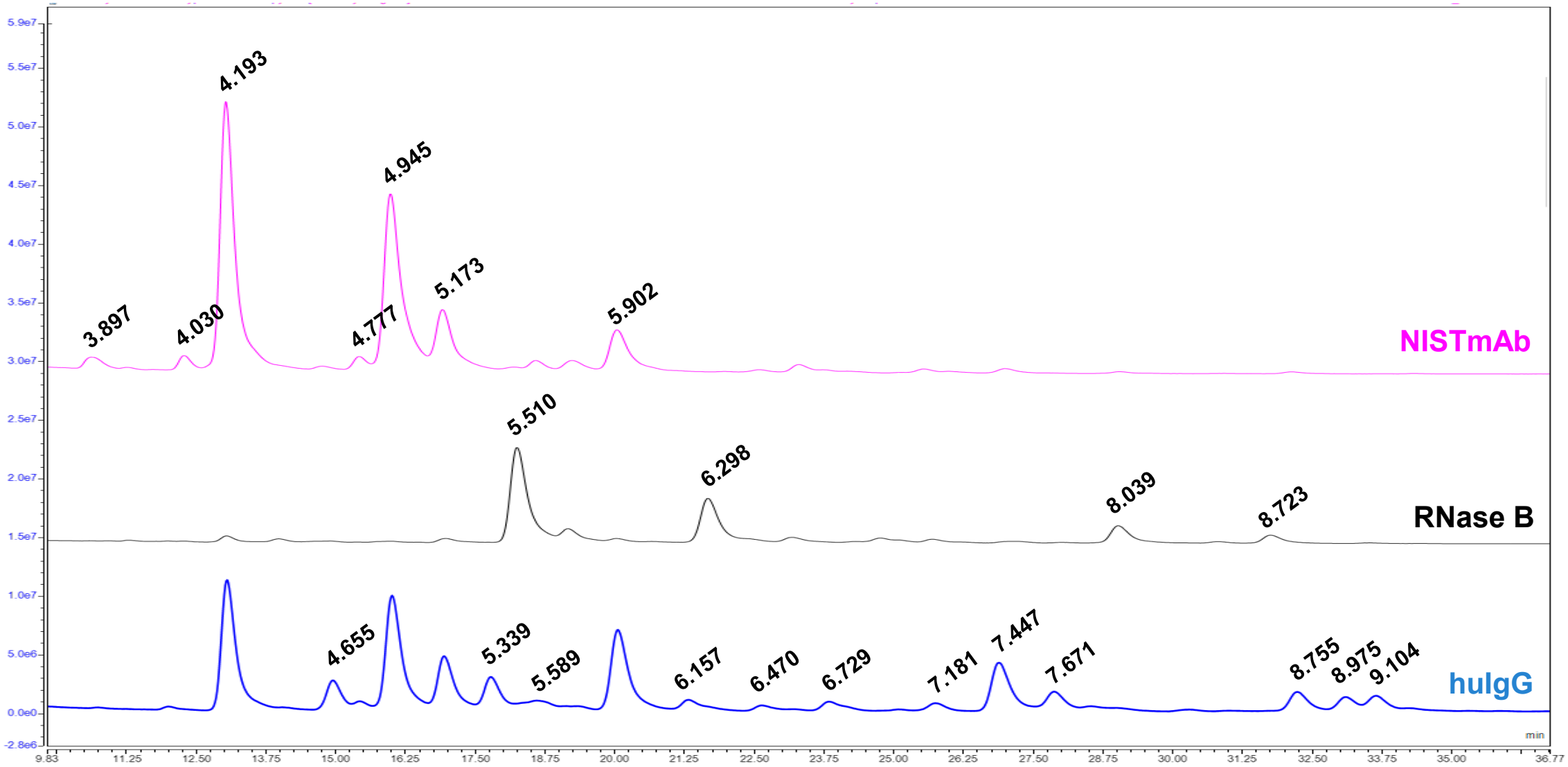
Glycans Incorporated into APTS GU Library

- APTS GU library contains 29 glycans
  - Biopharmaceutical relevant samples
  - Individually purified standards
- Average standard deviation (StDEV) for all glycans = 0.06
  - Robust and reproducible
  - Important for instrument-to-instrument and site-to-site transferability
  - Confident glycan assignments

Glycan	Average GU	StDEV
G0N	3.768	0.037
G0	3.897	0.057
G0N'	3.930	0.041
G0FN	4.030	0.031
G0FN'	4.171	0.042
G0F	4.193	0.060
G0FB	4.655	0.071
G1	4.777	0.065
G1'	4.891	0.050
G1Fa	4.945	0.061
G1Fb	5.173	0.059
G1FB	5.339	0.074
M5	5.510	0.040
G1FB'	5.589	0.051
G2	5.641	0.041
G2F	5.902	0.060
G2FB	6.157	0.074
M6	6.298	0.042
G1S1	6.470	0.066
G1FS1	6.729	0.081
M7	7.134	0.023
A1	7.181	0.078
A1F	7.447	0.082
A1FB	7.671	0.093
M8	8.039	0.046
M9	8.723	0.051
A2	8.755	0.098
A2F	8.975	0.101
A2FB	9.104	0.110

GU Annotated Chromatograms

Biotherapeutic samples annotated with library GU values



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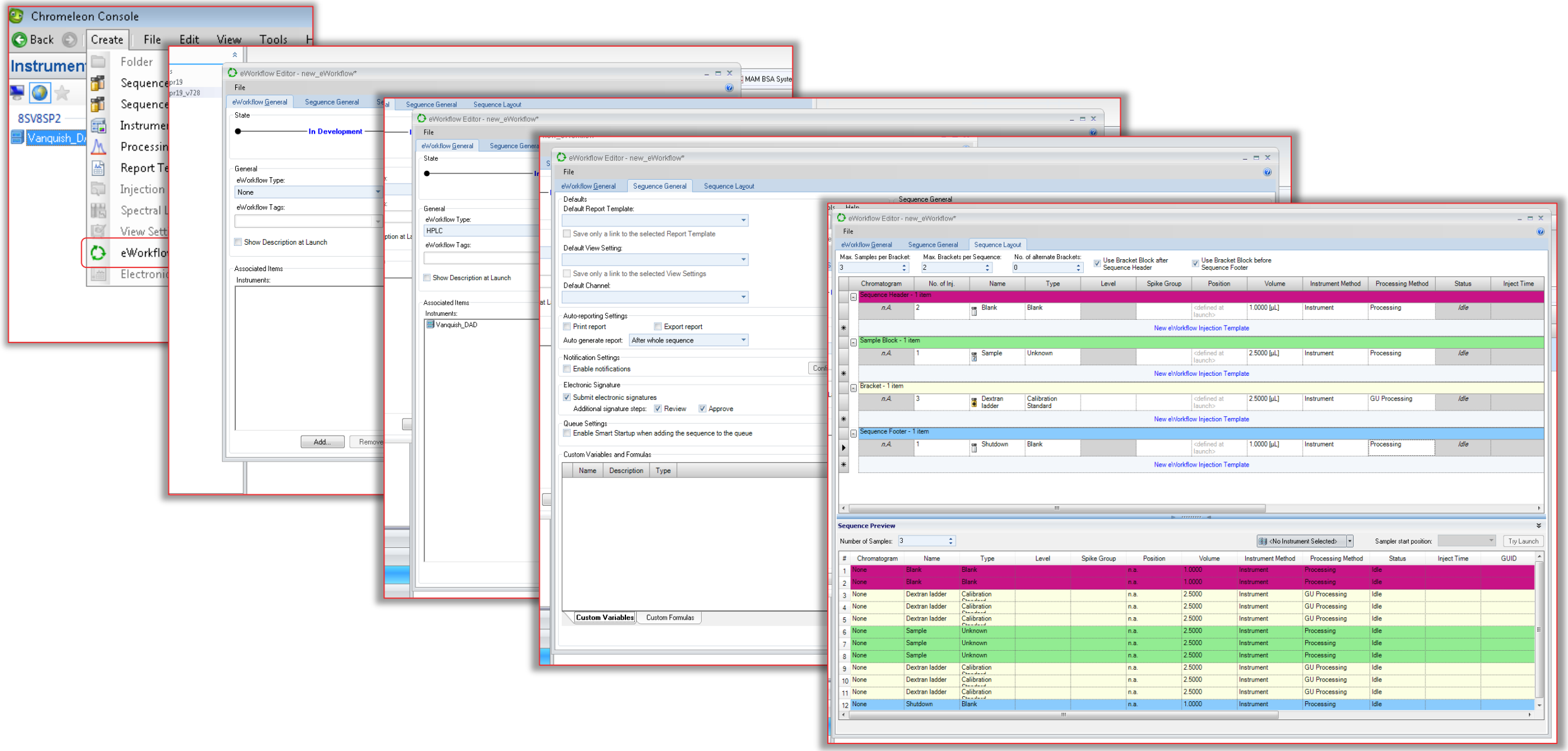
Preparation of eWorkflow



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Summary

- APTS GU library has been created
  - GlycanAssure HyPerformance Kit
- 29 biopharmaceutically relevant glycans incorporated
- Use of GU library increases robustness and confidence in glycan assignments
  - Without the requirement for mass spectrometry
- Library in the process of internal/external validation
- Creation of an eWorkflow
  - Seamless transfer of methods between laboratories/sites