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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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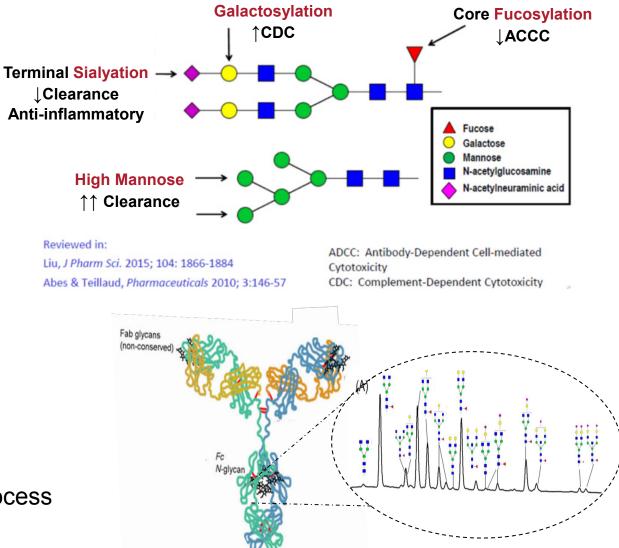
Overview

- 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



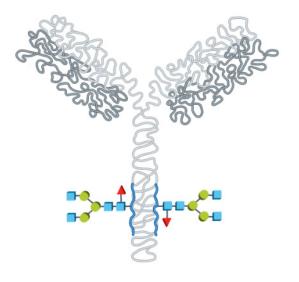


N-linked Glycans

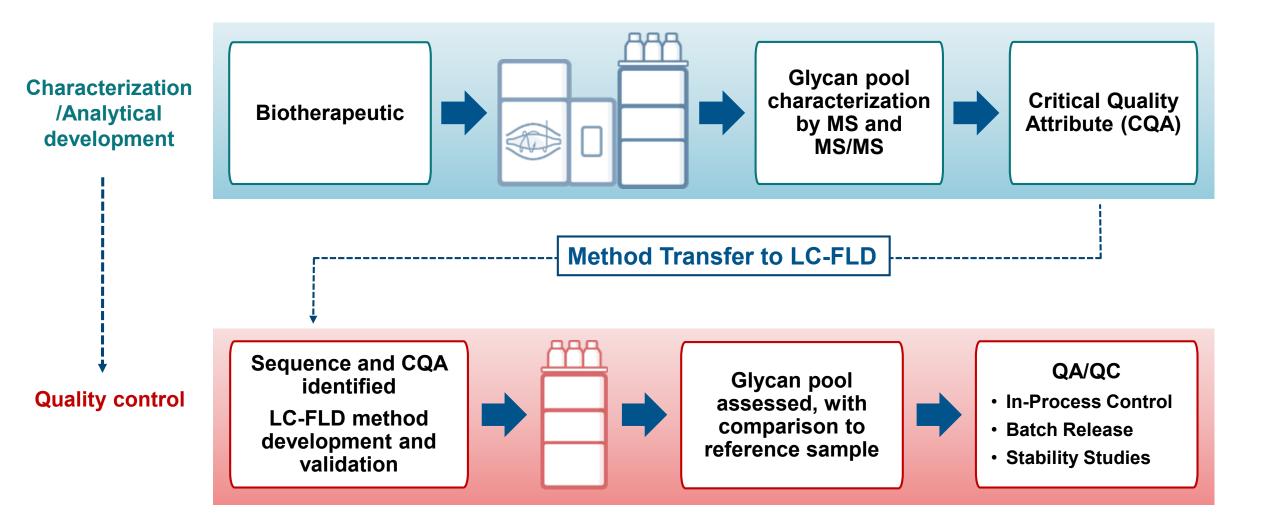
ICH (Q6B) recommends 6 test approaches for characterization and confirmation of biological products:

- 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) \rightarrow QC (CQA monitoring)

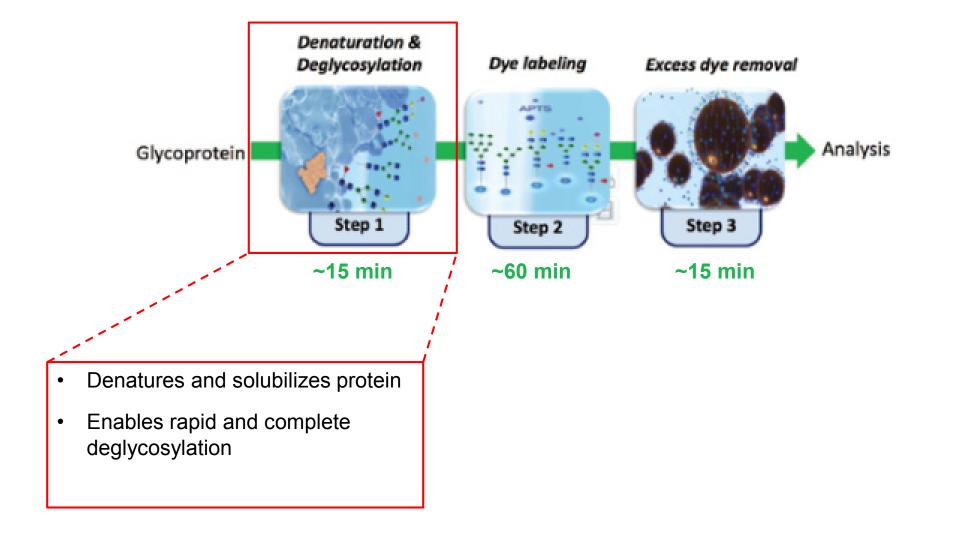






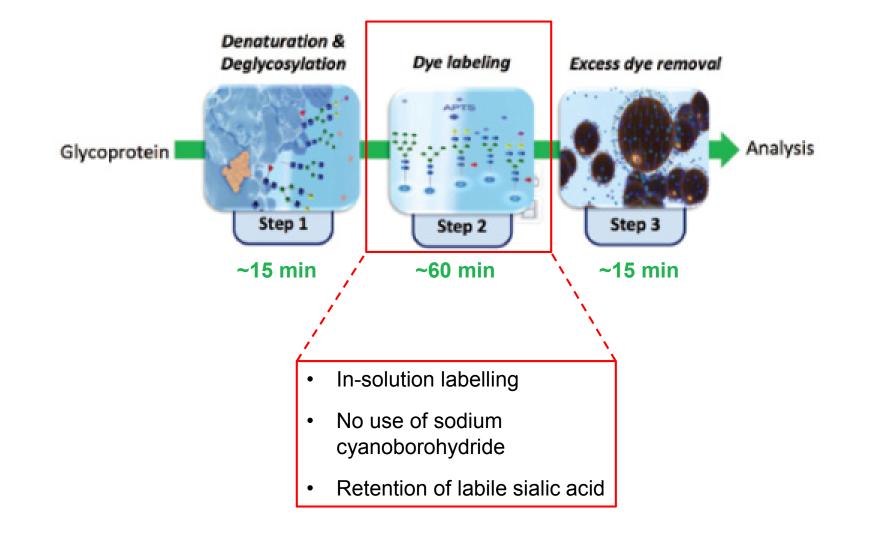


GlycanAssure HyPerformance Kit – Denaturation and Deglycosylation



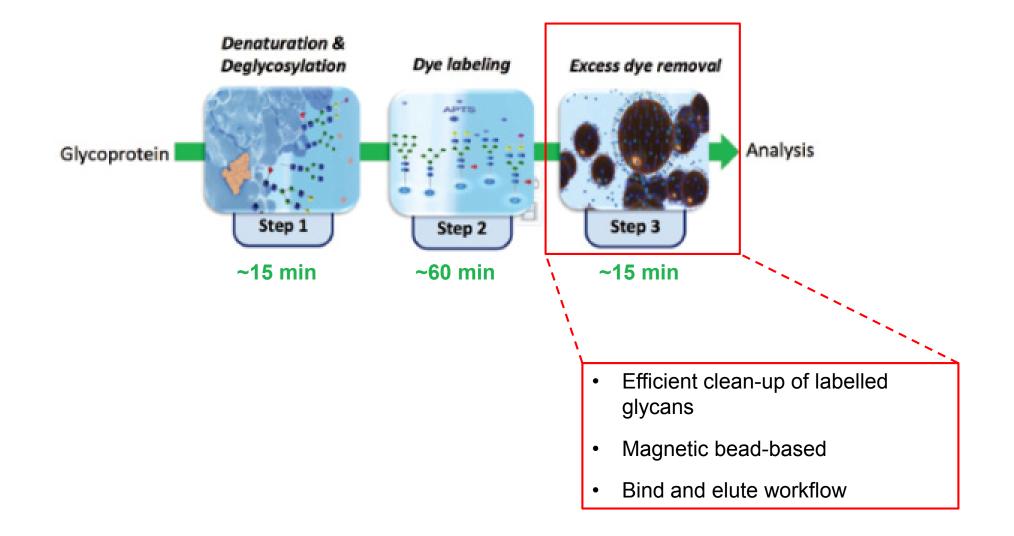


GlycanAssure HyPerformance Kit – APTS Labelling



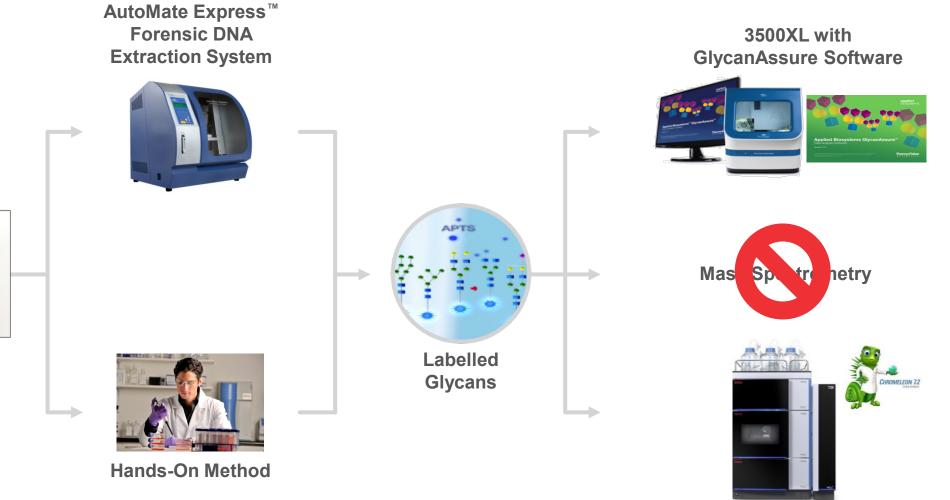


GlycanAssure HyPerformance Kit – Sample Clean-up





Overview of Glycan Labelling Using GlycanAssure



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

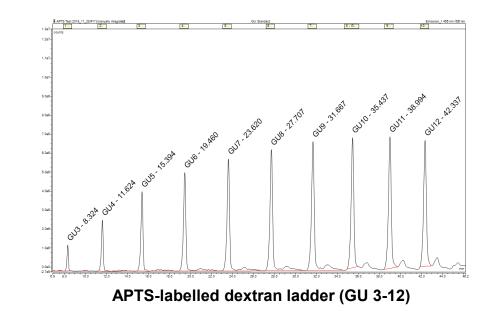


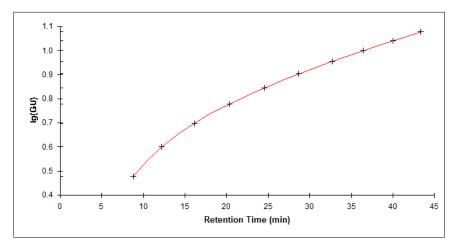


GlycanAssure HyPerformance kit

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





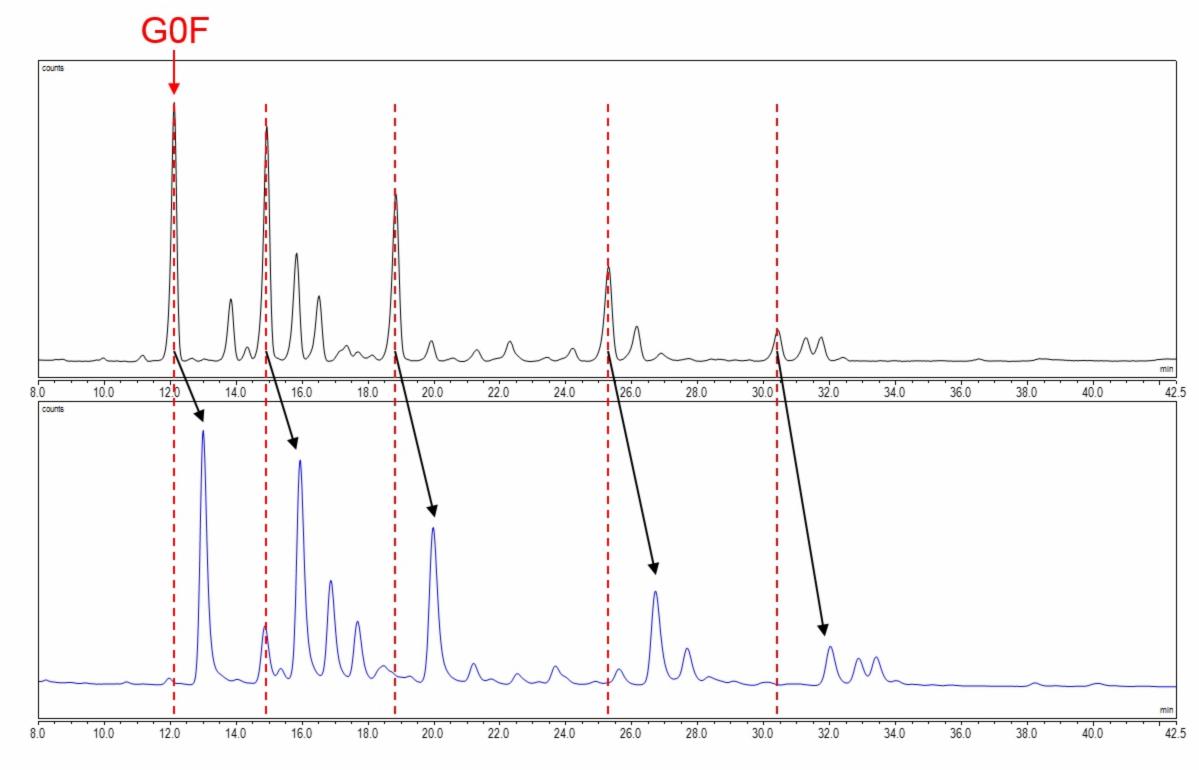
Retention time/GU calibration curve



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	Accucore™ 150 Amid	
Emission wavelength	500 nm	column (2.1 x 150 mn 2.6 µm)	
Sensitivity	7		75
Lamp mode	High power		50 25 1.25
Data collection rate	10 Hz		$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
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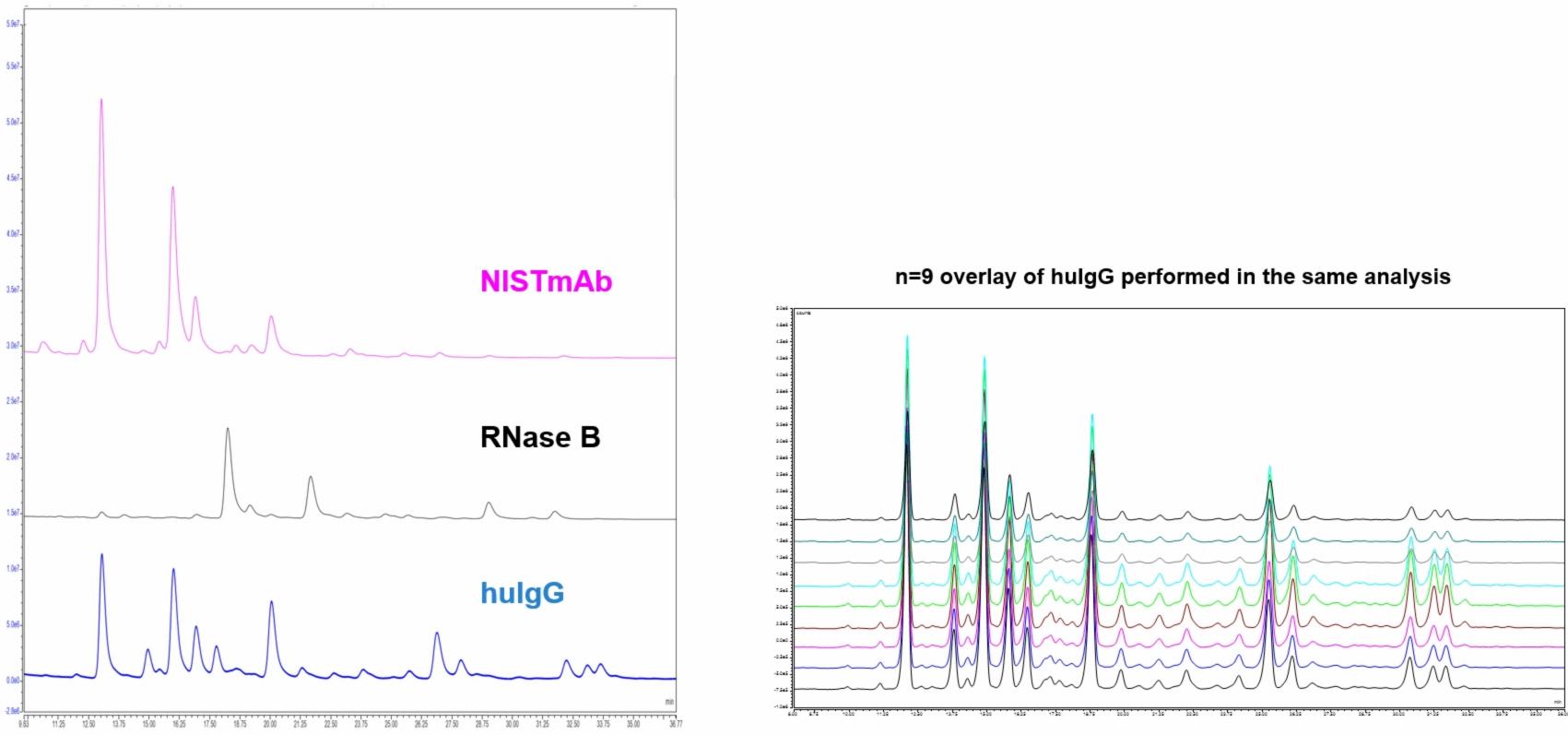


Overview of Library Construction

- 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



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-tosite 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

5.9e7 A.193 5.5e7 5.0e7 4.945 4.5e7 4.0e7 5.173 5.902 4.030 3.5e7 4.777 3.891 **NISTmAb** 3.0e7 5.510 2.5e7 6.298 8.0³⁹ 2.0e7 8.123 **RNase B** 1.5e7 1.0e7 7.181 7.447 5.³³⁹ 4.655 6.157 6.129 1.671 8.755 8.975.104 6.470 5.589 5.0e6 hulgG 0.0e0 min -2.8e6-9.83 16.25 23.75 25.00 26.25 27.50 28.75 30.00 36.77 11.25 12.50 13.75 15.00 17.50 18.75 20.00 21.25 22.50 31.25 32.50 33.75 35.00



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

