Strategic Implementation of Glycan Standards for Reliable Analysis of Glycomolecules

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Introduction

For most therapeutic glycoproteins the glycosylation patterns greatly influence clinical performance of the drug product, particularly its *in vivo* safety and efficacy profile.[1] In biological tissues glycosylation patterns can also correlate with the state of health or disease of the individual.[2] Given this, there is increasing interest in accurately characterizing glycosylation, for example monitoring glycosylation patterns of biopharmaceutical therapeutics throughout the product lifecycle as well as in glycan biomarker discovery for medical diagnostics.

Robust analytical strategies are required to meet the challenge of accurately and reliably characterizing glycosylation. There has been significant progress made in glycan analysis and the availability of commercial kits which contain the necessary reagents for release and labelling of monosaccharides, sialic acids, and *N*- and *O*- glycans have made it easier for laboratories to adopt technologies for glycan analysis. However, even with the advancement in glycan characterisation tools, multiple inter-laboratory studies have shown that there is still a lack in consistency of the data produced during glycan analysis.[3] These problems highlight the existing need for well-characterised glycan reference standards.

Glycan Standards

A key component in a well-designed analytical strategy is the inclusion of standards. Table 1 shows which standards can be used for best practice during the analysis of sialic acids, monosaccharides, *N*-glycans and/or *O*-glycans. These fall into the following categories;

1. **System suitability standards** enable an analyst to test the holistic functionality of an analytical system (e.g. chromatographic, mass spectroscopic and/or CE) and evaluate whether it is adequate for its intended use.

2. **Process standards or process controls** are used to verify that part of or an entire process has worked correctly. There are four main categories for processes standards in glycoanalysis: release, labelling, release followed by labelling and exoglycosidase sequencing.

3. **Reference standards** allow for characterisation by comparison. This can be accomplished by the direct comparison of the chromatographic or electrophoretic retention time of an unknown to that of a standard whose structure have been fully characterised. Additionally, primary assignment of unknown structures can be accomplished by comparison of their GU (Glucose unit) values (obtained using glucose homopolymer (GHP) standard) with glycans whose GU values are in databases or in the literature.

4. **Quantitative glycan standards** are used to determine the absolute amount of an analyte in a sample. They can also be used to quantify the efficiency of a process.

Ludger System Suitability Standards and Controls			Process positive control for release-labelling-analysis				Release Process positive control				Labelling Process positive control				MS System suitability		(u)HPLC System suitability				GU Calibration		WAX System Suitability		CE System Suitability	Structure Identification by retention time or m/z matching				Quantification				Exoglycosidase control	
Product Category	Ludger Code	Product Description	SA	MONO	N	O	SA	MONO	N	O	SA	MONO	N	O	N	O	SA	MONO	N	O	N	O	N	O	N	SA	MONO	N	O	SA	MONO	N	O	N	O
Glycoprotein/Glycopeptides	GCP-IGG	IgG Glycoprotein	●	●	●		●	●	●																										
	GCP-FET	Fetuin Glycoprotein	●	●	●	●	●	●	●	●																									
	BQ-GPEP-A2G2S2	GPEP-A2G2S2	●	●	●		●	●	●																	●	●	●		●	●				
Monosaccharide and Sialic Acid standards	CM-SRP	SRP Sialic Acid Reference Panel									●						●									●									
	CM-NEU-AC	Neu5Ac									●															●				●					
	CM-NEU-GC	Neu5Gc									●															●				●					
	CM-NEU5,9AC2	Neu5,9Ac$_2$									●															●				●					
	CM-MONOMIX	MonoMix										●						●									●				●				
	CM-XYL	Xylose										●															●				●				
Unlabelled N-Glycans	CN-x	Bi, Tri and Tetra-antennary N-glycans											●		●				●									●							
	CN-Man-x	High Mannose N-glycans											●		●				●									●							
	BQ-CHITOTRIOSE	Chitotriose											●	●	●	●			●	●												●			
	BQ-CN-MAN8	Man8											●		●				●									●							
	CLIBN-IGG	IgG N-glycan library											●		●				●									●							
	SA-MAB4	Mab 4 glycan ref panel											●		●				●									●							
	CLIBN-FETUIN	Fetuin N-glycan library											●		●				●						▲			●							
	CLIBO-FETUIN	Fetuin O-glycan library												●		●				●					▲				●							
2-AB labelled glycans	CAB-GHP	2-AB labelled Glucose Homopolymer																	●	●	●	●						●	●							
	BQ-CAB-CHI	2-AB labelled Chitotriose													●	●			●	●										●	●				
	CAB-IGG	2-AB labelled IgG N-glycan library													●				●									●						●	
	CAB-x	Bi, Tri and Tetra-antennary N-glycans													●				●						●			●						●	
	CAB-Man-x	High Mannose N-glycans													●				●									●						●	
	CAB-Cx-x	O-glycans														●				●					●				●						●
	CAB-AlphaGal	Alpha-Gal standard																																●	
2-AA labelled glycans	CAA-GHP	2-AA labelled Glucose Homopolymer																	●	●	●	●						●	●							
	BQ-CAA-CHI	2-AA labelled Chitotriose													●	●			●	●										●	●				
	CAA-x	Bi, Tri and Tetra-antennary N-glycans													●				●						●			●						●	
	CAA-Man-x	High Mannose N-glycans													●				●									●						●	
	CAA-AlphaGal	Alpha-Gal standard																																●	
APTS labelled glycans	CAPTS-IGG	APTS labelled IgG N-glycan library																	●								●							●	
	CAPTS-x	APTS labelled N-glycans																	●								●							●	
PROC labelled glycans	CPROC-GHP	PROC labelled Glucose Homopolymer													●				●	●	●	●						●	●							
	CPROC-IGG	Proc labelled IgG N-glycan library													●				●									●							●	
	CPROC-x	Bi and Tri-antennary N-glycans													●				●									●							●	
	CPROC-Man-x	High Mannose N-glycans													●				●									●							●	
Permethylated Glycan Standards	CPM-IGG	Permethylated IgG N-glycans													●													●							
	CPM-C13-IGG	Permethylated (^{13}C) IgG N-glycans													●													●						●	

SA: Sialic Acid / **MONO:** Monosaccharide / **N:** N-Glycans / **O:** O-Glycans
▲ requires labelling first

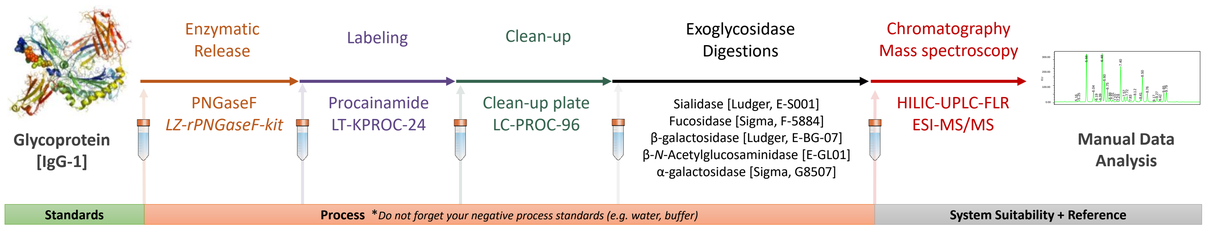
Table 1: *Standards Used for Glycan Analysis*

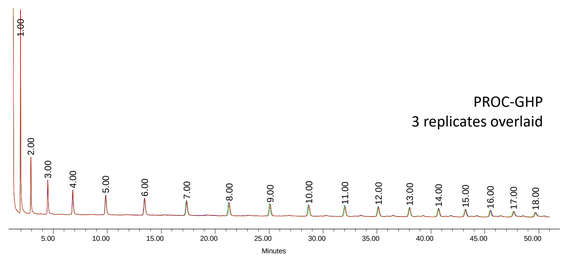
Detailed *N*-Glycan Analysis: A Case Study for Implementation of Standards

Here we show how various glycan standards work in concert to provide confidence in results. As a case study, we will illustrate how we use each type of standard to support reliable *N*-glycan analysis of an IgG-1 mAb containing the immunogenic Galα1-3Gal epitope.[4] The Galα1-3Gal disaccharide epitope is a high priority Glycosylation Critical Quality Attribute (GCQA) and is often present in small amounts (or even go unnoticed). However, due to the safety implications it must be monitored throughout the product's lifecycle. Here we explain our *N*-glycan characterisation and quantitation strategy which provides:

- A practical and reliable workflow suitable for QbD-based drug realisation with the appropriate and integrated process, reference and system suitability standards to provide confidence in data quality
- Compliance with emerging regulations from the FDA, EMA, KFDA and cFDA[5]

Workflow:

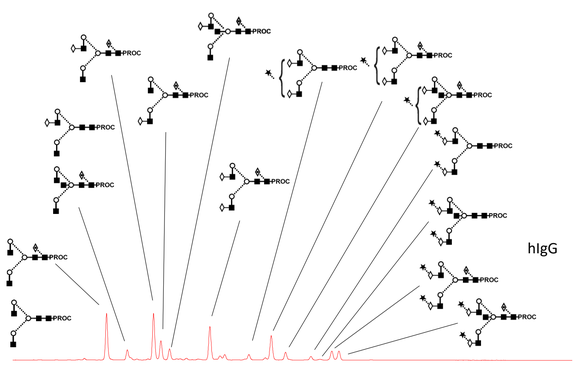




✓ **System Suitability Standard and Reference Standard for GU allocation**

Procainamide Labeled Glucose Homopolymer (GHP)
Provides GU values that can be used as a primary identification for glycans based on reported values in the literature and databases

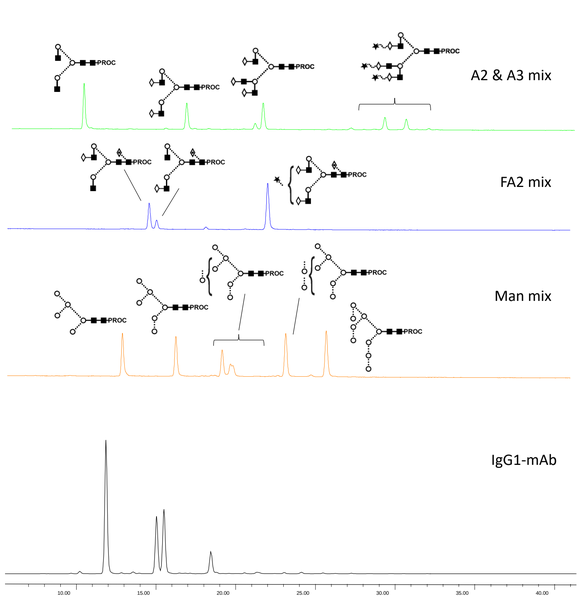
Acceptance Criteria: The peak width at half height for GU10 is less than 0.22 min. The profile should be similar to the profile shown in the Certificate of Analysis. The peaks should be symmetrical and well resolved. At least two runs of GHP should overlay without any drift in retention time



✓ **Process Control**

Ludger human IgG (hIgG) [Ludger product: GCP-IGG-100U] Well Characterised glycoprotein run in parallel with samples to assess the release, labelling, clean-up and analyses.

Acceptance Criteria: The profile of the procainamide labelled released *N*-glycans should be similar to the profile shown in the product certificate of analysis



✓ **Reference Standards**

Mixtures of *N*-glycans Common to mAb Samples
A2 & A3 mix [mixture of Ludger products: CPROC-NA3-01; CPROC-A3-01C; PROC-NGA2-01; and CPROC-NA2-01]
FA2 mix [mixture of Ludger products: CPROC-FA2G1-01 and CPROC-A1F-01]
Man mix [mixture of Ludger products: CPROC-Man5-01; CPROC-Man6-01; CPROC-Man7-01; CPROCMan8- 01 and CPROC-Man9-01]
Acceptance Criteria: The A2&A3 mix, FA2 mix and Man mix show peaks at expected GU values

❖ **UHPLC-FLR trace for IgG-1 mAb drug**
This drug contains a complex mixture of glycan structures, several of which co-elute on HILIC-LC and/or have the same mass composition. As a result, orthogonal methods were required: LC, ESI-MS/MS (data not shown) and exoglycosidase sequencing

Nomenclature

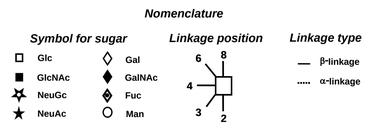


Figure 1: *HILIC-UPLC-FLR traces for IgG-1 Mab along with the process, reference and system suitability standards*

The relative proportion of glycans with the Galα1-3Gal epitope was determined by HILIC-UHPLC analyses of the *N*-glycan samples treated with various exoglycosidases. Key to detection of Galα1-3Gal was differential behaviour with α-galactosidase. The glycans are digested down until only structures bearing the Galα1-3Gal epitope remain (Figure 2A/B). This greatly simplifies identification and quantitation of this GCQA. A glycan standard process control is essential for this as it gives confidence in the function of the enzyme; being able to unequivocally and confidently identify an α- from a β-galactose in a glycan structure has major implications for the corresponding glycoprotein therapeutic (Figure 2C/D)

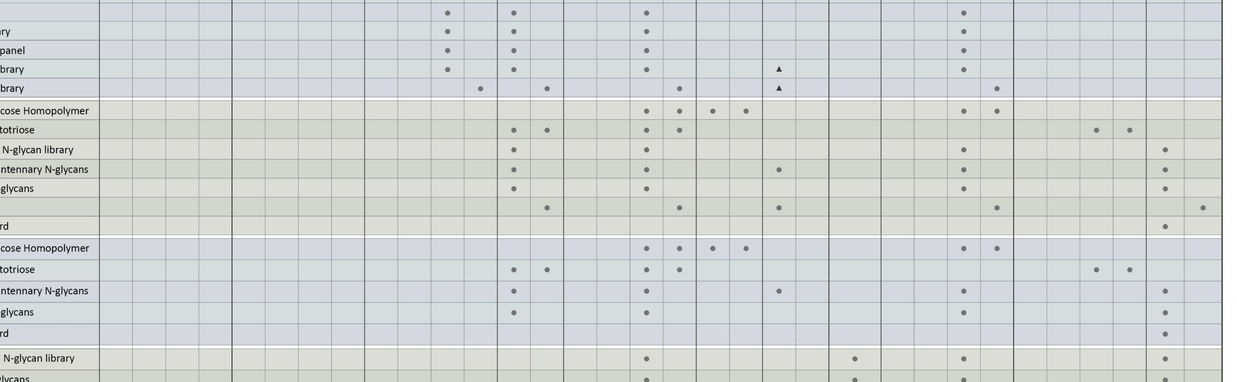


Figure 2: *HILIC-UPLC-FLR traces for (**A**) IgG-1 N-glycans after treatment with 4 glycosidases leaving Galα1-3Gal N-glycans (**B**) IgG-1 N-glycans after α-galactosidase confirming epitope (**C**) Galα1-3Gal trisaccharide process control (**D**) after α-galactosidase treatment*

✓ **Process Control**

Native or fluorescently labelled glycan [CAB-ALPHA-GAL-01]
Trisaccharide containing the Galα1-3Gal epitope
Acceptance Criteria: Shift in retention time corresponding to loss of the terminal α-linked galactose

Ludger Standards					IgG1													
					HILIC-UPLC		ESI-MS/MS						Degree of Certainty					
							Composition											
Structure	Oxford name	Average GU	Sample type	Ludger product name	Average GU	% area	Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)	Mass Found	Mass Calculated	GU	Exo	m/z at GU	MS/MS at GU	Structure	
	FA2G2	7.33	Human IgG N-glycan pool	CPROC-IGG-02	7.34	8.89	5	4	1	0	$[669.61]^{3+}$	$[669.61]^{3+}$	Y	Y	Y	Y	FA2G2	
			FA2 Mix standard	CPROC-A1F-01														
					7.46	0.56	5	4	1	0	$[669.61]^{3+}$	$[669.62]^{3+}$	Y	Y	Y	Y	FA2(3)G1Ga1	

Table 2: *Summary of GU, % area, ESI-MS/MS and digestion data from the IgG-1 mAb procainamide labelled N-glycans. Glycans bearing the Galα1-3Gal epitope are highlighted in red.*

The data obtained using orthogonal methods with the appropriate glycan and glycoprotein standards during this case study provides the following information vital for regulatory compliance (Table 2):

Analytical systems within specification

✓ System suitability standard GHP confirmed the holistic functionality of the analytical system and that it was adequate for its intended use

Experimental Processes performing as expected

✓ Process standard hIgG verified that the release, labelling, clean-up and exoglycosidase sequencing had performed as expected
✓ Process standard with Galα1-3Gal verified the function and specificity of the vital α-galactosidase

Detailed Glycan Characterisation and Quantitation

✓ GHP derived GU values provided preliminary characterisation by comparison of reported GU values for PROC-labeled glycans in the literature, in databases and with the reference standards
✓ Glycan composition and sequence from ESI-MS and MS/MS key fragment ions
✓ Glycan linkages and monosaccharide building blocks confirmed using exoglycosidases
✓ Relative % of glycan structures and glycans containing Galα1-3Gal epitope from HILIC-UPLC and exoglycosidase digestions

Galα1-3Gal Epitopes Strategy to assess RISK for IgG$_1$ mAb	
Can you see it?	✓
Is it really Galα1-3Gal?	✓
How much?	3.88%

References:

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2. G. Lauc, M. Pezer, I. Rudan, H. Campbell. Biochim Biophys Acta - Gen Subj, 2016, 1860, 1574-1582; B. Adamczyk, T. Tharmalingam, P.M. Rudd. Biochim Biophys Acta - Gen Subj, 2012,1820,1347-1353; A. Varki. Glycobiology, 2017,27,3-49.
3. S. Thobhani, C.T. Yuen, M.J.A. Bailey, C. Jones. Glycobiology, 2009,19,201; M. L. A. D. Leoz, D. L. Duewer, S. E. Stein, NISTIR 8186 NIST Interlaboratory Study on the Glycosylation of NISTmAb , a Monoclonal Antibody Reference Material June 2015 to February 2016, 2016.
4. TA Platts-Mills, AJ Schuyler, A Tripathi, SP Commins. (2015) 'Anaphylaxis to the carbohydrate side chain alpha-gal'. Immunol Allergy Clin North Am, 2015, 35:247-260.
5. Jennifer Fournier BioPharm International Volume 28, Issue 10, pg 32–37 and references therein.

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