# Glycan Standards as Key Tools for Robust and Reliable Analysis of Glycoproteins

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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.

| 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 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ludger Code | | 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 GHP | | | | | | | | | | | | | | | | | ● | ● | ● | ● | | | | | | ● | ● | | | | | | |
| | 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-C-x | O-glycans | | | | | | | | | | | | | | ● | | | | ● | | | | | ● | | | | | | | | | | ● |
| | CAB-AlphaGal | Alpha-Gal standard | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | ● | |
| 2-AA labelled glycans | CAA-GHP | 2-AA labelled GHP | | | | | | | | | | | | | | | | | ● | ● | ● | ● | | | | | | ● | ● | | | | | | |
| | 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 | N-glycans | | | | | | | | | | | | | | | | | ● | | | | | | | ● | | ● | | | | | | ● | |
| PROC labelled glycans | CPROC-GHP | PROC labelled GHP | | | | | | | | | | | | | ● | | | | ● | ● | ● | ● | | | | | | ● | ● | | | | | | |
| | 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 | | | | | | | | | | | | | ● | | | | | | | | | | | | | ● | | | | | | ● | |

▲ requires labelling first

**Table 1:** Standards Used for Glycan Analysis

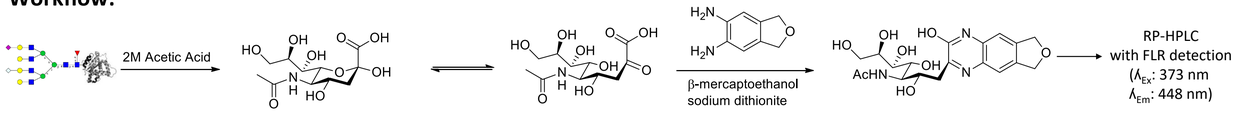
## Quantitative Analysis: Case Studies for Glycan Standards

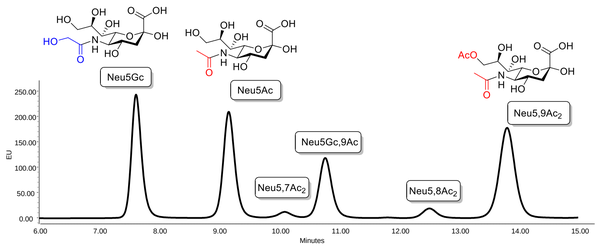
Here we show how various glycan standards work in concert to provide confidence in results. As case studies, we will illustrate how we use each type of standard to support reliable and consistent sialic acid analysis and monosaccharide analysis. These methods are used to quantify the total mass of specific monosaccharides within the glycan pool. Both methods are required to satisfy regulatory requirements for biopharmaceutical drug characterisation (e.g. ICH guideline Q6B).

## 1) Sialic Acid Analysis

Sialylation is integral for the structure and function of many glycoprotiens and is a glycosylation critical quality attribute (GCQA) for biopharmaceuticals. Sialic acid analysis provides data for both the abundance and the type of sialylation (including O-acetylation).

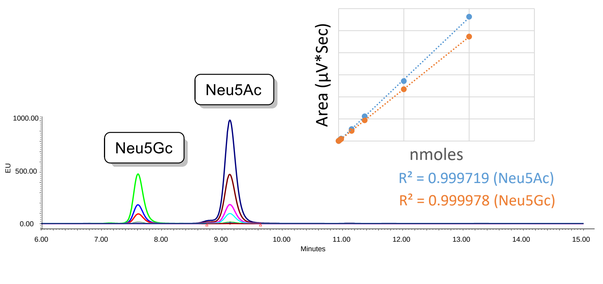
**Workflow:**



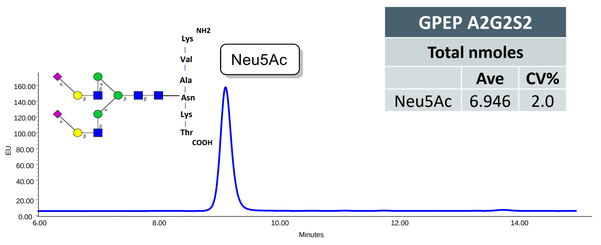

✓ **System suitability Standard and Reference Standard**

**Sialic acid reference panel (SRP**) - contains a mixture of sialic acids found in humans and animals.
Acceptance criteria: the HPLC profiles at the start and end of the sample set should overlap with minimal drift in retention time (e.g. ± 0.1 min.).




✓ **Quantitative Standards**

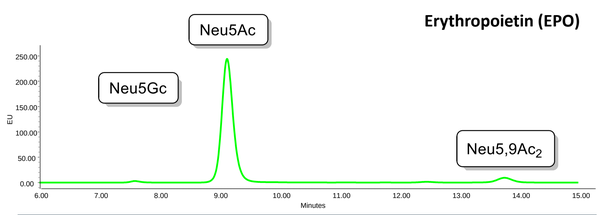
**Neu5Gc** and **Neu5Ac -** dispensed to approximately 1 nmole. Preparation of serial dilutions provide calibration curves.
Acceptance criteria: the calibration curves should give $R^2$ values of >0.99




| GPEP A2G2S2 | | |
|---|---|---|
| Total nmoles | | |
| | Ave | CV% |
| Neu5Ac | 6.946 | 2.0 |

✓ **Quantitative Process Standard**

**GPEP-A2G2S2** glycopeptide - contains a biantennary *N*-linked glycan terminating in two sialic acids and has been quantified using qNMR.
Acceptance criteria: Neu5Ac in the range of 5.6 to 8.4 nmol (which is the amount determined by quantitative NMR ± 20%)



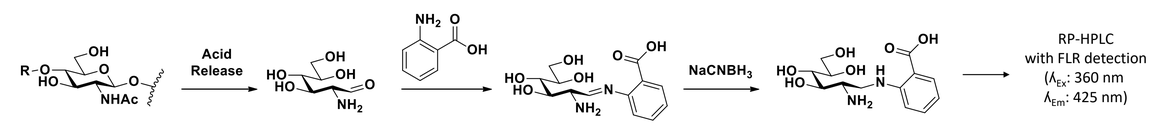

| EPO (50 µg Glycoprotein) | | | |
|---|---|---|---|
| Sialic Acid | Amount of Sialic Acids on Protein (nmoles/mg protein) | Amount of Neu5Gc and Neu5Ac (nmoles) | Average Relative Percent (%) of Sialic Acids from Peak Areas |
| Neu5Gc | 4.97 | 0.25 | 0.95 |
| Neu5Ac | 307.44 | 15.37 | 94.17 |
| Neu5,9Ac$_2$ | - | - | 4.88 |

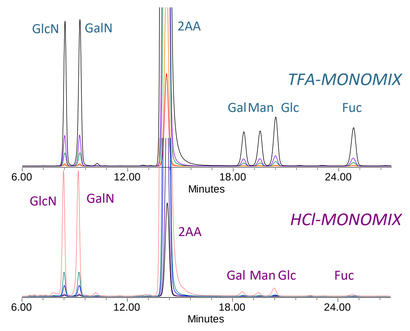
❖ **Reliable Data for Erythropoietin (EPO):** EPO is a highly glycosylated protein and its high level of sialylation and accompanying acetylation has a significant effect on its therapeutic properties.[4] The DMB labeled sialic acids from an EPO glycoprotein were analysed and the relative levels of the *N*-acetyl, *N*-glycolyl and *O*-acetyl sialic acids were calculated as a ratio of 94:1:5 respectively.

## 2) Monosaccharide Analysis

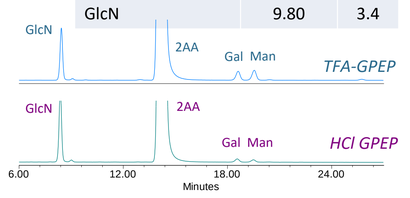
Monosaccharide analysis provides absolute or relative quantitation of the neutral (i.e. non-anionic) monosaccharides and information relating to the types of *N*- and/or *O*-glycans present on a glycoprotein.

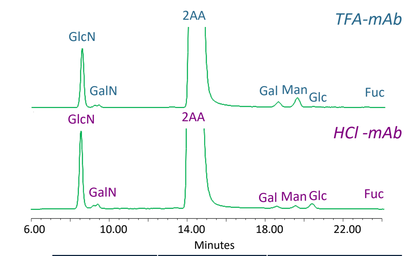
**Workflow:** Monosaccharides are released from the glycoprotein by acid hydrolysis using 2M trifluoroacetic acid (TFA) (for quantitation of mannose (Man), galactose (Gal), glucose (Glc), fucose(Fuc)) or 6M hydrochloric acid (HCl) (for quantitation of glucosamine (GlcN) and galactosamine (GalN))




| Monosaccharide (HCL & TFA Data) | Average (nmol) | %CV |
|---|---|---|
| GlcN | 15.46 | 1.0 |
| GlcN | 9.80 | 3.4 |




| HCL & TFA Release Data | nmol/mg protein | | # of monosaccharides per N-glycan site |
|---|---|---|---|
| Monosaccharide | Average | %CV | # per site |
| GlcN | 58 | 2.1 | 4.4 |
| GalN | 3 | 2.0 | 0.2 |
| Gal | 24 | 2.4 | 1.8 |
| Man | 41 | 1.6 | 3.1 |
| Glc | 5 | 9.2 | 0.4 |
| Fuc | 6 | 1.3 | 1.2 |

✓ **Reference Standard, Quantitative Standard, System Suitability Standard**

Monosaccharide mix (**MonoMix**) standard - contains GlcN, GalN, Gal, Man, Glc and Fuc dispensed to 10 nmole each. Preparation of serial dilutions provide calibration curves.
Acceptance criteria for quantitation: calibration curves should give $R^2$ values of >0.9
System suitability acceptance criteria: HPLC retention times of the monosaccharides should have less than 0.1 min difference when run at the start, middle and end of the analysis.

✓ **Quantitative Process Standards**

**GPEP-A2G2S2** glycopeptide or **Man-8** quantified glycan standards
Acceptance criteria for GPEP-A2G2S2: GlcN is in the range of 8.38 to 13.96 nmol (TFA) and 11.17 to 16.75 nmol (HCl).

❖ **Reliable Data for Monoclonal Antibody (mAb):** Glycosylation is a common post-translational modification in mAbs and has a critical role in antibody effector function.[5] The monosaccharide analysis of a mAb gave the following;

From the data for GlcN and GalN (from the HCl release):
- majority of the *N*-glycans are biantennary (with 4 GlcN)
- low percentage of *O*-glycans (low, but real GalN value)

From the data for Gal, Man, Glc & Fuc (from TFA release)
- Not all *N*-glycans have 2 Gal (1.8 per site).
- Complex *N*-glycans have 3 Man; indicates low % of high mannose structures present.
- The majority of the *N*-glycans are core fucosylated (1.2 per site)
- Glc is present as a background contaminant (detected in the negative controls)

## Acknowledgements and References

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