Overview of Ludger
Biopharmaceutical Glycoprofiling Services

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1. Introduction to Ludger Glycoprofiling Services

Ludger's glycoprofiling services are primarily for:

a. Companies developing new or follow-on biological therapeutics
b. Biopharmaceutical manufacturers

The services include: glycoprofiling for glycan characterization to support all stages of your drug development, regulatory submissions and patent protection; help to set up ICH-compliant glycoprofiling systems within your own laboratories; and training of your scientists in effective glycoanalysis techniques.

Our investment in glycoprofiling is to help you to develop safe, effective and profitable drugs enabling you to take them to market and manufacture them smoothly and efficiently.

QbD Framework for Biopharmaceutical Glycoprofiling

We use a QbD (Quality by Design) approach to biopharmaceutical glycoprofiling at Ludger. The main components of our QbD glycoprofiling framework are illustrated in figure 1 and outlined in Ref 1. The framework is based on the concepts developed in ICH Q8 and Q8 annex, ICH Q9 and ICH Q10 [Refs 2 – 4].

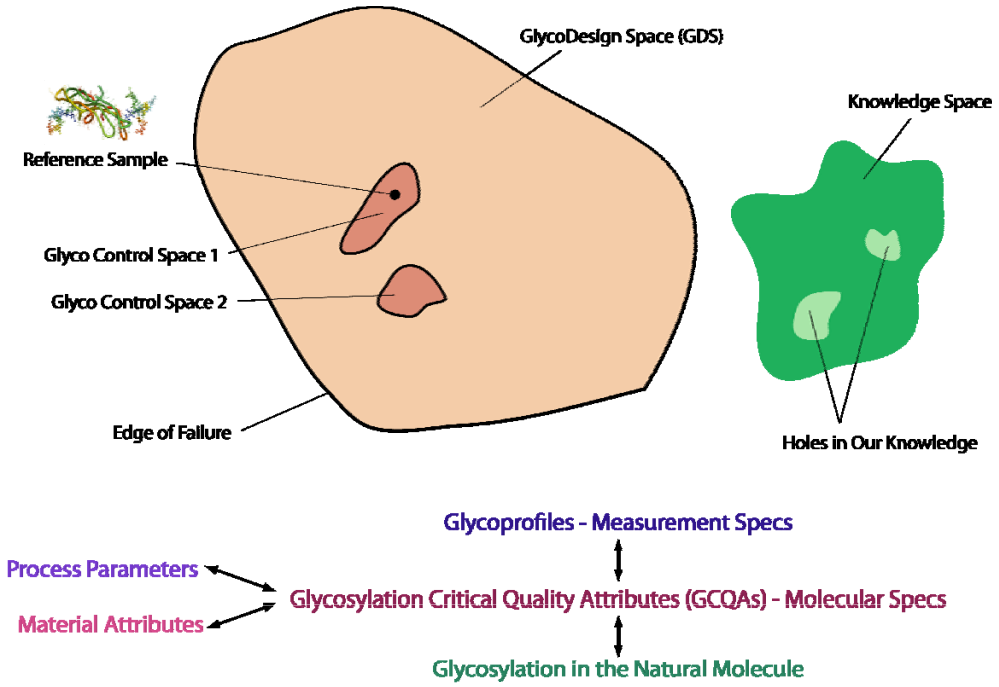


Figure 1: Main components of the Ludger QbD framework for characterizing biopharmaceutical glycosylation

Due to its complexity, characterization of biopharmaceutical glycosylation typically requires use of several orthogonal analysis methods to measure different glycosylation parameters. The data from these allows us to build profiles of the glycosylation pattern of the therapeutic. Figure 2 shows the main glycoanalysis modules used in the industry to characterize biopharmaceutical glycosylation and most of these are available through our glycoprofiling services.

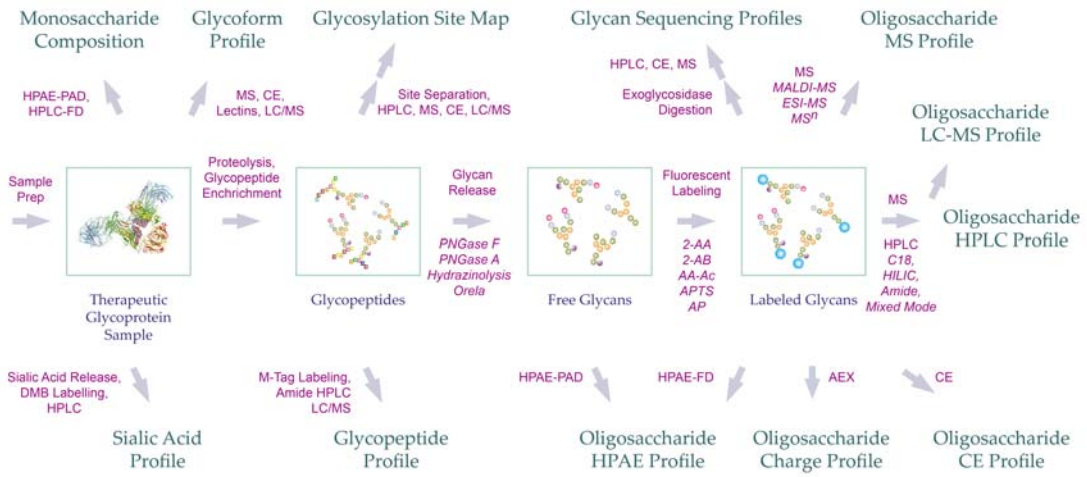


Figure 2: Analysis modules for characterization of biopharmaceutical glycosylation

The data from a subset of these modules are used at Ludger to build a profile of the glycosylation of the biopharmaceutical. We chose the glycoprofiling modules that are best suited to measure the particular Glycosylation Critical Quality Attributes (GCQAs) for the therapeutic. Note that there is no single universal glycoanalysis technique – the complexity of biopharmaceutical glycosylation makes it necessary to use several orthogonal analysis methods. Also, for practical reasons, different modules will be used at different stages in the drug life. [Ref 1]

We believe that a key to successful biopharmaceutical glycoprofiling is identification of the Glycosylation Critical Quality Attributes (GCQAs) relevant to the safety and efficacy of the therapeutic then selection of glycoprofiling methods that reliably measure those GCQAs.

For example, the GCQAs for a monoclonal antibody produced in a mammalian expression system and relying on Fc effector functions for activity, typically include the distributions of: charged glycans; galactosylation; bisecting GlcNAc; fucosylation; oligomannose; relative levels of human vs non-human sialylation; and relative levels of alpha-galactose containing glycans. Suitable glycoprofiles to measure those GCQAs include the sialic acid profile (NeuAc vs NeuGc) and glycan profiling using a set of orthogonal HPLC methods (including LS-N2 amide profile, LS-R1 C18 profile and LS-C3 AEX profile) plus mass spectrometry (including MALDI-MS and MS-MS) and exoglycosidase sequencing. In most cases, relative quantitation of glycan species is achieved by fluorescence HPLC with structure identification from: retention on HPLC compared to analytical glycan standards; mass spectrometry; and exoglycosidase sequencing. [Refs 1, 9].

2. Biopharmaceutical Glycoprofiling Modules at Ludger

The main biopharmaceutical glycoprofiling modules at Ludger are as follows:

Glycoprofiling Pilot Study (Module G-PS)

This module is to determine (a) how your biopharmaceutical samples behave during the standard glycoprofiling procedures and (b) give preliminary information on glycans structures and relative quantities. Module G-PS is usually a requirement for new glycoprofiling projects. During this module, one or two representative samples and a formulation buffer sample will be analyzed by a variety of methods in order to select the best conditions for detailed glycoprofiling work in the subsequent modules.

Typically, it will include the following work:

- Study to determine how the intact glycoprotein samples handle.
- Study to determine effect of formulation buffer on glycoprofiling procedures.
- Preliminary Level 1 glycoprofile of 2AB labeled glycans from the selected samples by a choice of techniques:
  a. LudgerSep-N2 HPLC profiling (figure 3)
  b. MALDI-MS
  c. LudgerSep-C3 HPLC charge profiling (figure 4),
  d. LudgerSep-R1 HPLC profiling (figure 5)
- Analysis of data from pilot studies with preliminary assignments of glycan structures where possible.
- If required, optimization of glycoprofiling procedures for your samples.

On completion of the pilot study we will have preliminary data on the glycosylation of your biopharmaceutical samples and information for selecting the best protocols for the subsequent detailed glycoprofiling work.

Level 1 Glycoprofiling (Module G-L1)

G-L1 is a key module for oligosaccharide profiling throughout the drug life cycle. It is particularly used during glycoengineering in early stage development, process optimization, characterization to support regulatory submissions, batch comparability studies, and product lot release QC.

During G-L1 we determine the overall glycan profile with classification and relative quantitation of the major glycan species for the therapeutic. Glycan quantities are expressed as molar % of the individual species - or groups of species if individual glycans cannot be fully resolved. For some therapeutics it will be important to know the relative quantities of glycans with specific structural features (e.g. core α1,6 fucosylation, sialylation, or oligomannosyl chains) and this information will be included in the specification for the G-L1 and G-L2

modules.

Module G-L1 builds on the module G-PS study (which is must be done before G-L1) and typically includes the following activities:

- Release of oligosaccharides from the glycoprotein. N-glycans can be released either enzymatically by endoglycosidase digestion (using PNGase F or PNGase A) or chemically (by hydrazinolysis). O-glycans are typically released by O-mode hydrazinolysis or Ludger's Orela™ chemical O-glycan release technology.
- Fluorescent labeling of released glycans by reductive amination followed by post-labeling cleanup. The most common glycan label used in this module is LudgerTag 2AB (2-aminobenzamide).
- Analysis of 2AB labeled glycans by LudgerSep-N2 amide, LudgerSep-C3 or LudgerSep-R1 HPLC.
- Analysis of the released biopharmaceutical glycan library by ion trap mass spectrometry.
- Comparison of the main therapeutic glycans and Ludger glycan standards on HPLC and MS.
- Preliminary identification of the main glycan species and relative quantitation from fluorescence HPLC data.
- The analyses are typically performed with triplicate glycan releases and triplicate HPLC analyses to provide suitable precision statistics.

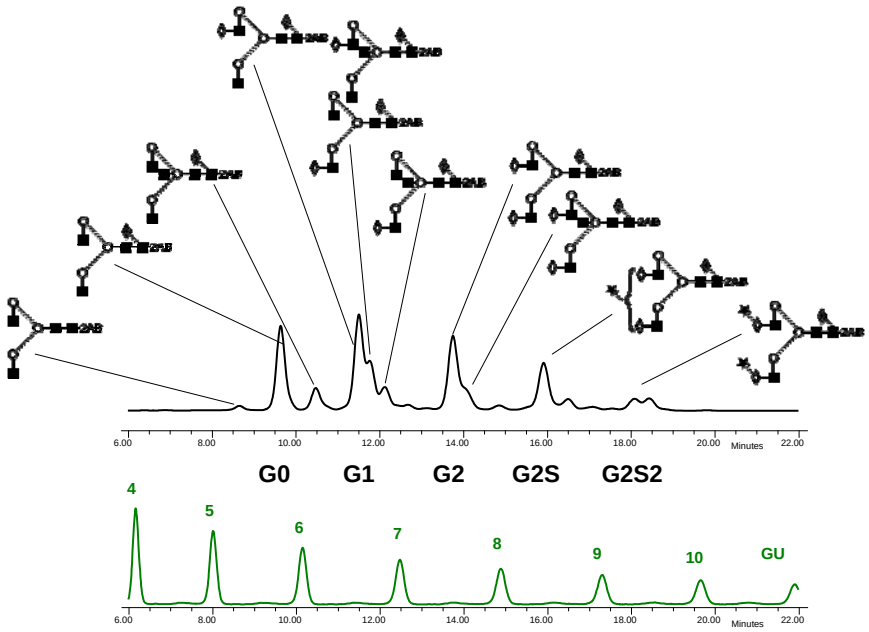


Figure 3: LS-N2 amide HPLC of 2AB labeled human antibody glycans.

Conditions: LudgerSep-N2 amide HPLC column (3 μm particles, 4.6 x 150 mm length) with a gradient from 65-35% (v/v) acetonitrile in 50 mM ammonium formate, pH 4.4 (aq) over 30 minutes. The bottom profile is for 2AB labeled glucose homopolymer (GHP) ladder which is used as an external standard. Retention times of oligosaccharides are expressed in terms of glucose units (GU) by reference to the GHP ladder. Glycan structures are drawn according to the system developed at the Oxford Glycobiology Institute [see Appendix 8 and Ref 10]

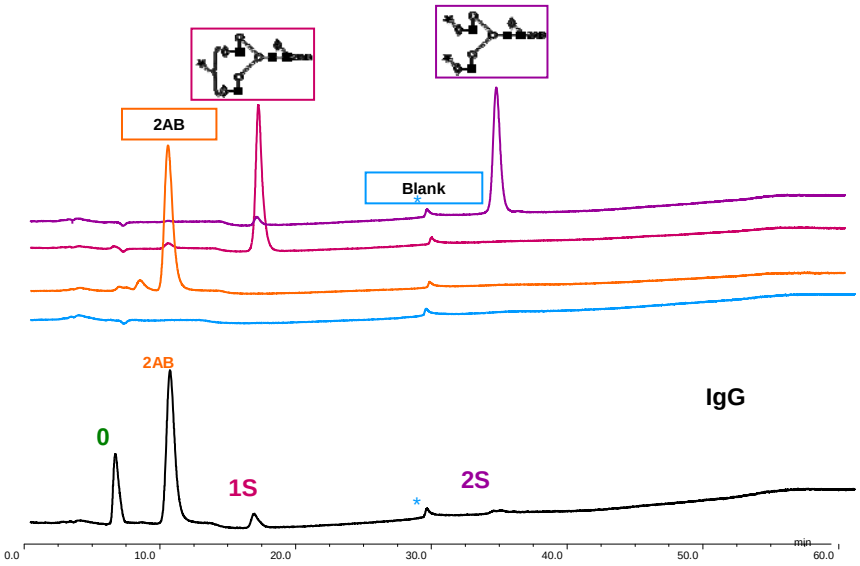


Figure 4: LudgerSep-C3 charge profiles of 2AB labeled glycans.

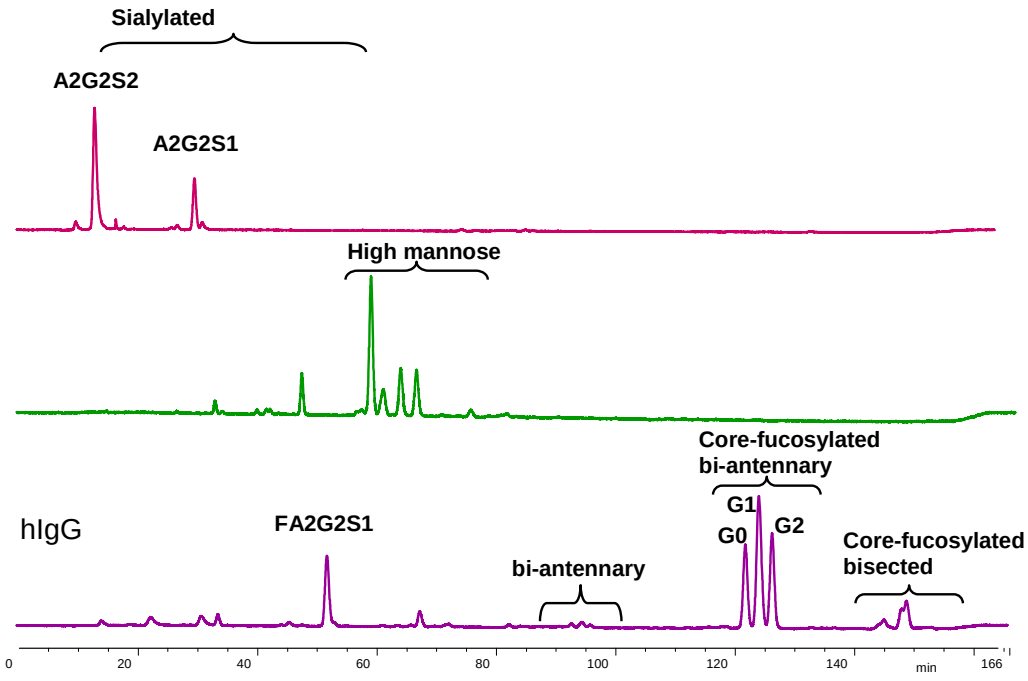


Figure 5: LS-R1 C18 HPLC profiles of 2AB labeled IgG oligosaccharides.

This illustrates the separation capabilities for different types of glycans. Sialylated glycans elute early in the run followed by high mannose glycans. The non-fucosylated, core-fucosylated and bisected bi-antennary glycans also elute in different regions of the chromatogram (G0, G1 and G2 are also separated within these

regions).

Level 2 Glycoprofiling (Module G-L2)

This is an extension of the Level 1 work to give more detailed information on the glycan structures. The G-L2 module is particularly useful for detailed characterization of glycosylation during mid-stage drug development and for regulatory submissions and patent work. It typically includes the following work:

- Sequencing of the fluorescently labeled therapeutic glycans by exoglycosidase digestions and LudgerSep-N2 HPLC.
- Further analysis of unlabeled glycans, labeled glycans and possibly methyl-esterified labeled glycans by ion trap mass spectrometry.
- Identification of glycans from HPLC, exoglycosidase sequencing, MS and MS fragmentation (where possible) (see figures 6, 7 and 8).

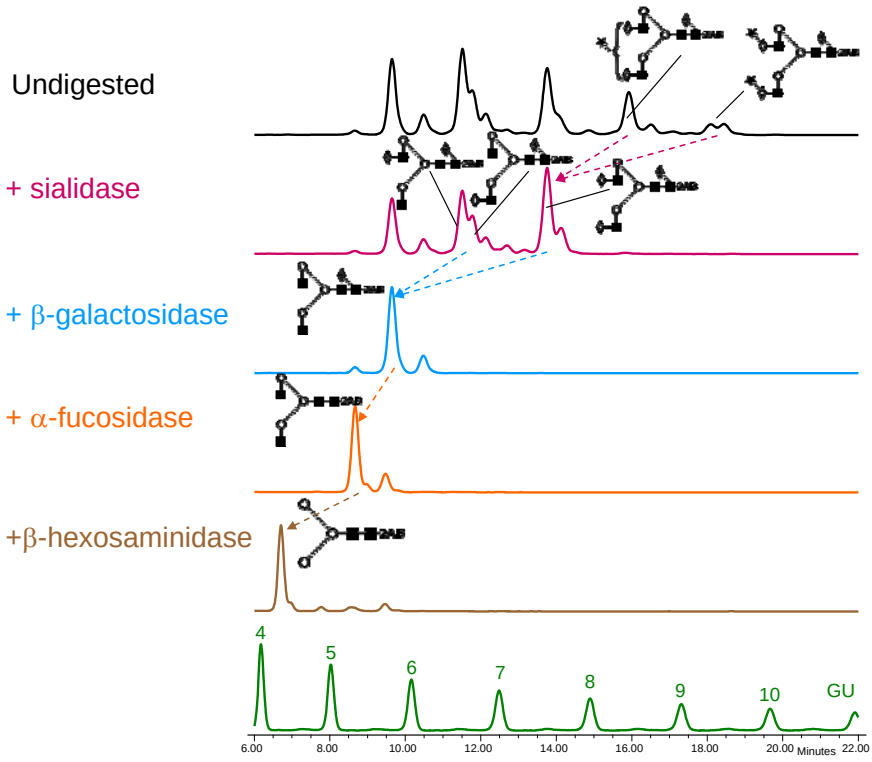


Figure 6: Exoglycosidase sequencing profiles for 2AB labeled glycans from human IgG

LuderSep-N2 amide HPLC profiles of 2AB labeled N-glycans from human IgG before and after exoglycosidase digestions. The bottom panel shows GU (glucose unit) values from the 2AB labeled glucose homopolymer (GHP) calibration standard.

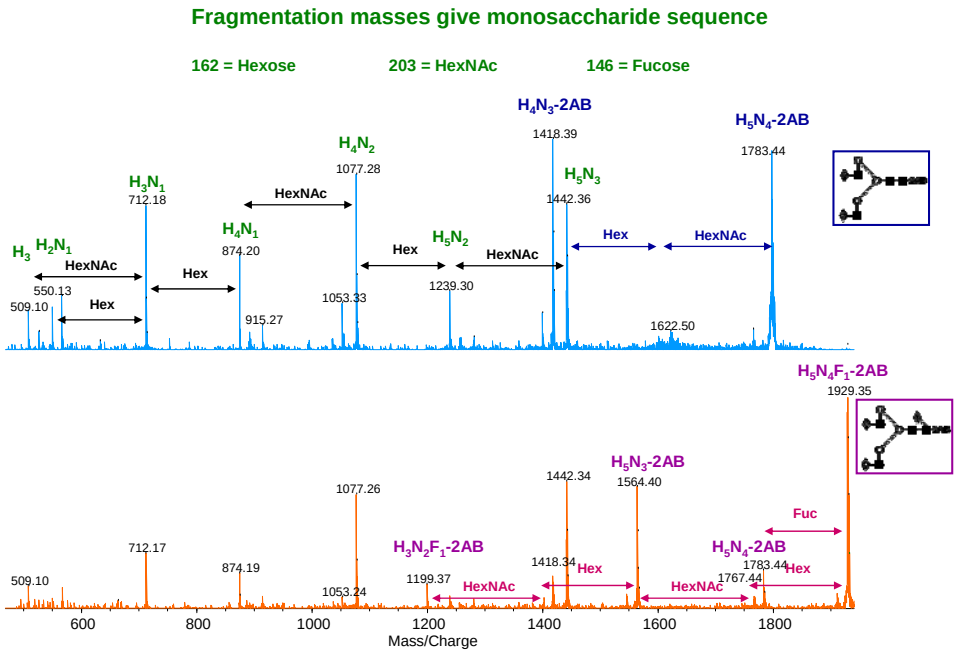
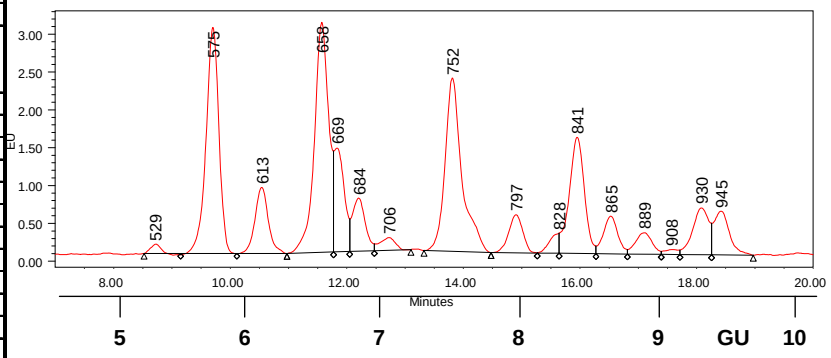


Figure 7: MALDI-MS/MS of 2AB labeled di-galacosylated biantennary oligosaccharides.

The top panel is the profile for non-fucosylated biantennary glycan, the bottom panel is for the core-fucosylated biantennary glycan.

Structure	STD GU	Ludger IgG	
		GU	%Area
A2	5.31	5.29	0.6
A2B	5.68		+
F(6)A2	5.77	5.75	17.1
F(6)A2B	6.15	6.13	5.2
A2[6]G(4)1	6.14		
A2[6]BG(4)1	6.14		
A2[3]G(4)1	6.43	6.58	+
A2[3]BG(4)1	6.56		+
F(6)A2[6]G(4)1	6.59		19.5
F(6)A2[3]G(4)1	6.70	6.69	6.2
F(6)A2[6]BG(4)1	6.85	6.84	4.1
F(6)A2[3]BG(4)1			+
A2G(4)2	7.08	7.06	1.1
A2BG(4)2	7.27		+
F(6)A2G(4)2	7.53	7.52	17.3
F(6)A2BG(4)2	7.67		+
A2G(4)2S1	7.98	7.97	3.2
A2BG(4)2S1		8.28	1.1
F(6)A2G(4)1S1	8.42	8.41	10.4
F(6)A2BG(4)1S1	8.67	8.65	3.2
A2G(4)2S2	8.91	8.89	2.1
A2BG(4)2S2	9.10	9.08	0.4
F(6)A2G(4)2S2	9.33	9.30	4.5
F(6)A2BG(4)2S2	9.47	9.45	4.0



+ indicates low levels of these structures co-eluting under major peak

Figure 8: Level 2 structure allocation of human IgG glycans.

The table lists the glycan structures with GU values and % areas.

Sialic Acid Profiling (Module G-SA)

This module is to determine relative levels of human and non-human type sialic acids (NeuAc and NeuGc respectively). This is important since some patients have high levels of anti-NeuGc antibodies which could lead to neutralization and rapid clearing of NeuGc containing biopharmaceuticals. The G-SA module typically includes the following work:

- Release of sialic acid residues from the glycoprotein by mild acid hydrolysis.
- Fluorescent labeling of released sialic acids with 1,2-diamino-4,5-methylenedioxybenzene (DMB).
- Relative quantitative analysis of DMB-labeled sialic acids by LudgerSep R1 HPLC (figure 9) and comparison with quantitative NeuAc standard series and NeuGc and sialic reference panel standards.

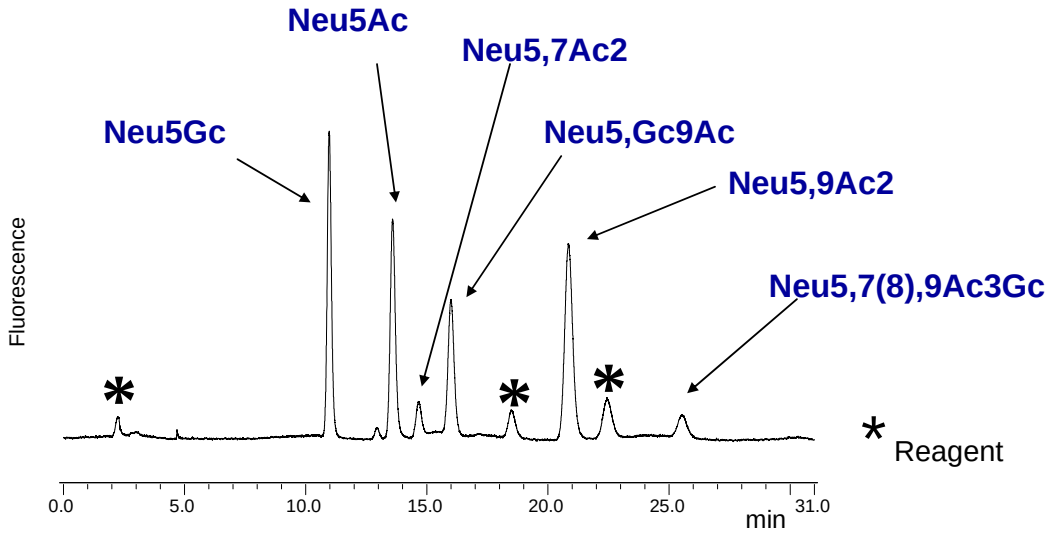


Figure 9: DMB labeled sialic acid reference panel profiled on an LS-R1 HPLC column

*This sialic acid reference panel contains Neu5Ac (human type sialic acid), Neu5Gc (*non-*human type sialic acid) plus acetylated variants.*

Determination of the Degree of Sialylation (Module G-SIANUM)

This builds on module G-L1 and is to determine the number of sialic acids per drug glycoprotein molecule (denoted γ_{NUMSIA} at Ludger). This replaces the former method for determination of sialylation patterns of the biopharmaceutical oligosaccharides using the Z-number method [Refs 7,8]. The Z-number has been widely used in the industry as a glycometric for therapeutics such as follicle stimulating hormone (FSH) and erythropoietin (EPO) variants where biological activity is related to the degree of sialylation. However, as a tool for determining comparability of drug sialylation, the Z-number should be used with caution since different

sialylation patterns can give the same Z-number [Ref 6]. Regulatory authorities such as the FDA and EMA seem generally more likely to ask for determination of γ_{NUMSIA} than for the Z-number for EPO, while the Z-number has been mainly used for patent work involving biosimilar or biobetter variants of Merck-Serono's FSH (which was protected by a patent on the Z-number for certain FSH sialylation patterns [Ref 8]).

At Ludger we use two orthogonal methods for determination of γ_{NUMSIA} – the 'Ratio method which involves absolute quantitation of both sialic acid and protein in a drug sample and the 'Distribution' method which involves determination of the average degree of sialylation at the glycosylation sites of the therapeutic.

Monosaccharide Composition for Glycoprotein - Pilot Study (Module G-MP)

This module is to determine (a) how your biopharmaceutical samples behave during the analysis procedures for neutral (non-anionic) monosaccharides and (b) give preliminary relative quantitation information on the neutral monosaccharides.

Module G-MP is a requirement before full monosaccharide analysis by modules G-MG or G-MR. During this module, one or two representative samples and a formulation buffer sample will be taken through one or more monosaccharide analysis procedures in order to select the best conditions for detailed analysis in the subsequent modules.

Typically, it will include the following work:

- Study to determine how the samples of glycoprotein and/or released glycans handle during the sample workup
- Release of neutral monosaccharides from intact glycoprotein and/or released glycans + buffer controls.
- Analysis of released monosaccharides *either* by 2AA fluorescent labeling with reverse phase HPLC (see fig 10), *or* HPAEC-PAD (high pH anion exchange chromatography with pulsed amperometric detection) (fig 11).
- Relative quantitation for monosaccharide composition will be done by comparison with traceable reference monosaccharides.

On completion of the pilot study we will have preliminary data on the monosaccharide composition of the biopharmaceutical samples and information for selecting the best protocol for the subsequent detailed repeatability analysis.

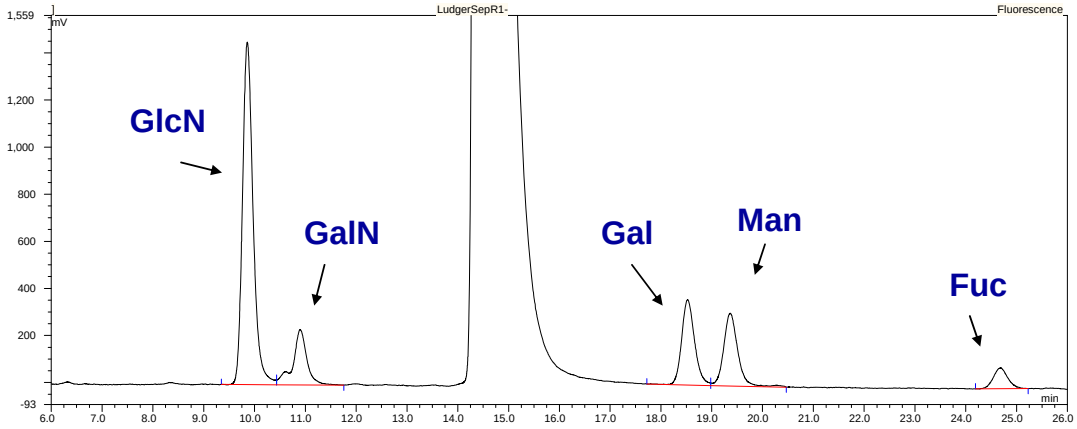


Figure 10: 2AA labeled monosaccharide analysis on a LudgerSep-R1 column

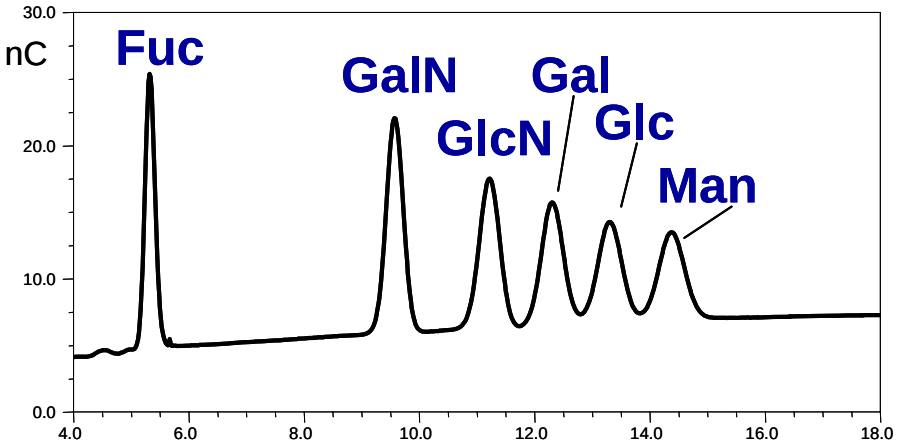


Figure 11: Monosaccharide profile by HPAE-PAD on a Dionex PA1 Carb column

Monosaccharide Composition Analysis of Glycoprotein (G-MG)

This module is to gain relative or absolute quantitative information on the neutral (i.e. non-anionic) monosaccharide composition of your therapeutic. The relative quantitation data for monosaccharides is a requirement for regulatory submissions.

Typically, it will include the following work:

- Release of neutral monosaccharides from intact glycoprotein + buffer controls, each in triplicate.
- Analysis of released monosaccharides *either* by labeling and reverse phase HPLC *or* HPAEC-PAD (depending on the results of the G-MP pilot study).
- Relative quantitation for monosaccharide composition will be done by comparison with traceable reference monosaccharides.

- For glycoprotein samples where accurate protein concentrations are known (you must provide us with the data on protein concentrations) then absolute quantitation for monosaccharide composition will be done by comparison with traceable quantitative reference monosaccharides.
- During this module, each glycoprotein sample will be taken through monosaccharide release in triplicate with triplicate HPLC analyses on each of the released monosaccharide daughter samples. Precision statistics will be performed on the resulting HPLC data.

Monosaccharide Composition Analysis of Released Glycans (Module G-MR)

This module is to gain relative quantitative information on the neutral (i.e. non-anionic) monosaccharide composition of glycans released from your sample. This can be used to compare the monosaccharide composition of released glycans (e.g. N-glycans released by PNGase F or O-glycans released chemically), with the monosaccharide composition of your intact glycoprotein. This can help identify glycation (non-enzymatic addition of glucose) on a sample and can be used to confirm stoichiometry of glycan release during Level 1 and Level 2 glycoprofiling studies. Module G-MR will follow on from modules G-L1 and G-MG.

Typically, it will include the following work:

- Release of neutral monosaccharides from glycans chemically or enzymatically removed from the glycoprotein.
- Analysis of released monosaccharides *either* by labeling and reverse phase HPLC *or* HPAEC-PAD (depending on the results of the G-MP pilot study).
- Relative quantitation for monosaccharide composition will be done by comparison with traceable reference monosaccharides.
- During this module, each glycoprotein sample will be taken through monosaccharide release in triplicate with triplicate HPLC analyses on each of the released monosaccharide daughter samples. Precision statistics will be performed on the resulting HPLC data.

Glycoprofiling Method Optimization (Module G-MO)

This is for clients who are considering using our standard glycoprofiling kits and reagents and who require optimization of glycoanalysis methods on their samples then transfer of the optimized methods into their laboratories. Typically, this involves the following work:

- Project specification (this will be a subset of the glycoprofiling planning process described in the next section).
- A glycoprofiling pilot study (module G-PS) on your samples.
- Selection of the glycoanalysis modules relevant to your needs
- Optimization of glycoprofiling methods

This is usually followed by module G-MT (transfer of methods to your labs and support for your scientists).

Glycoprofiling Method Development (Module G-MD)

This is for clients with applications requiring glycoanalysis technology beyond our existing glycoprofiling modules. The aim is to develop commercial grade ICH-compliant glycoprofiling methods tailored to your needs. The deliverable is an integrated system of kits and reagents together with protocols. Typically, we would validate these at Ludger on your samples then transfer the methods to our labs (as per module G-MT).

Production of Custom Kits and Reagents (Module G-KR)

For certain projects we can produce custom glycoprofiling kits and reagents. These include the following:

- Variations of existing Ludger kits (e.g. special pack sizes or modifications of certain components)
- Production of custom analysis kits to your specifications
- Production of custom analytical and system suitability standards

Transfer of Glycoprofiling Methods to Your Laboratories (Module G-MT)

We can provide this module for transfer of key glycoanalysis methods for biopharmaceutical research, development, process control and lot release to your laboratories. It includes the following work:

- Help you to select the glycoprofiles you need for your particular application.
- Specify the equipment and human resources you need to measure the glycoprofiles.
- Select the materials and protocols for the glycoprofiles.
- Provide initial training, troubleshooting and technical support for your scientists.
- Check glycoanalysis system reproducibility – compare results in your labs and Ludger's glycoprofiling labs.
- Ensure the system works smoothly within your facility.
- Help with troubleshooting
- Support your scientists with their in-house validation.

Technical Support (Modules G-TS1 and G-TS2)

We offer two levels of glycoprofiling technical support:

Level 1 Technical Support (module G-TS1) is for clients who are using our standard glycoprofiling kits and reagents for straightforward analyses. There is no charge for this service.

Level 2 Technical Support (module G-TS2) is for clients who need a higher level of scientific support for in-house glycoprofiling projects. The work will typically include elements from module G-MT but will be provided on a more *ad hoc* basis than the full G-MT module.

3. Glycoprofiling Project Planning at Ludger

The main steps for glycoprofiling project planning at Ludger are described below. The planning process is designed to ensure that what you want and what we do match, that the work goes smoothly on time and on budget.

1. Set Up a Confidential Disclosure Agreement and Material Transfer Agreement (CDA/MTA)

In most cases before any detailed discussions about your project we will set up a two-way confidential disclosure agreement and material transfer agreement between our companies. We can either use our standard mutual CDA/MTA (we will send a draft for you to check before signing) or we are happy to use your own legal documents.

2. Determine What You Need

We will then send you the following glycoprofiling project request document to fill in:

G-PJS-ludger-glycoprofiling-project-specification

This allows you to specify the types and number of samples you have, what glycoprofiles you need, why you need those glycoprofiles, and your timescales. The senior members of our glycoprofiling team will look at your information and then discuss your project. We will get back to you with any questions we have about your application and, if required, will arrange a teleconference call with you.

3. Project Proposal and Costs Spreadsheet

We will draw up then send you a detailed project proposal. This will contain the following:

- Summary of your application and the project aims
- Description of the samples you intend to have analyzed
- List of glycoprofiling modules relevant to your project.
- Quotation – prices for each glycoprofiling module and for any extra samples you submit
- Terms and conditions
- Signature pages for quotation and terms and conditions

Along with the proposal we will send you a costs spreadsheet giving you a detailed breakdown of the charges for each glycoprofiling module.

At this stage, get back to us if you would like to discuss any aspects of the proposal. Also, if there are any changes to your requirements then we can prepare an updated proposal for you.

4. Agree Project Proposal – Purchase Order

Once you have decided what you want then send us your official purchase order. This should refer to the relevant project proposal and list the glycoprofiling modules you want. We will acknowledge this by email.

5. Sample Submission Information

We will create a work order for your project then refine the information in the project specification document (G-PJS-ludger-glycoprofiling-project-specification). This document contains details of how to prepare and send your samples to us and inform you of the key contacts for your project at Ludger (including the Study Director). There are also sections for you to fill in with information on the samples you will be submitting for analysis and contact details of key people in your organization.

6. Study Plan

Once we have your completed sample submission document we will draw up and send you a study plan. This will outline the work to be done on your samples, summarize the number and type of samples you will be submitting, confirm the costs for the work.

7. Start of Glycoprofiling Work

When you are ready, send your samples to us (we will help you with the logistics for this) and we will start the work as scheduled.

8. Communication During Glycoprofiling Work

We will keep you updated with progress at key stages in the glycoprofiling work. For large projects this will include sending you interim reports (pdf format) on completion of each glycoprofiling module. Throughout the project you are welcome to contact the scientists performing the work on your samples to discuss progress and the results so far. Normally there is no charge for this but we will need to charge consultancy time if the time is significantly higher than we expect for the project (we would advise you before that happened).

9. Final Report

The final report is typically made up by assembling the data from each of the interim reports together with discussion and conclusions for the overall study. A pdf of a draft version of this will be sent you. Get back to us with any comments you have. We will then send you a pdf version of the final report and send you two bound and signed paper copies by courier.

4. Summary of Glycoprofiling Tools at Ludger

The main analysis tools used for our glycoprofiling services at Ludger are as follows:

- Monosaccharide composition analysis using either fluorescence HPLC or HPAE-PAD (high pH anion exchange with pulse amperometric detection) with relative quantitation of monosaccharide species

- Determination of non-human sialylation by quantitative determination of NeuAc:NeuGc ratio using LudgerTag™ DMB sialic acid analysis kit and LudgerSep R1 HPLC with fluorescence detection)

- Release of N- and O-linked glycans from the glycoprotein or glycopeptides by:
  a. Endoglycosidases (for N-linked glycans only)
  b. Hydrazinolysis (in N+O mode, N-mode or O-mode)
  c. Ludger Liberate™ hydrazinolysis system in N-mode, N+O mode or O-mode
  d. Ludger Liberate Orela O-glycan release system

- Fluorescent labeling of released glycans prior to chromatography or mass spectrometry using one of the following LudgerTag labeling systems:
  a. LudgerTag 2AB (2-aminobenzamide) for HPLC and MS
  b. LudgerTag 2-AA (2-aminobenzoic acid) for HPLC and MS
  c. LudgerTag AA-Ac for HPLC and MS

- Glycan mapping of fluorescently labeled glycans by:
  a. LudgerSep™ C3 anion exchange HPLC (for glycan charge analysis)
  b. LudgerSep N1 or N2 amide HPLC (for profiling of charged and neutral glycans by hydrophilic interaction chromatography)
  c. LudgerSep R1 reverse phase HPLC (for profiling of glycans)
  d. Dionex HPAE-FD (fluorescence mode high pH anion exchange used as an orthogonal separation to complement the N1, N2 and R1 profiles)

- Oligosaccharide mapping of non-labeled glycans by HPAE-PAD

- Sequencing of individual glycans and glycan mixtures using a combination of HPLC of fluorescently labeled glycans, exoglycosidase digestion and MS

- Mass spectrometric analysis (including MS^n fragmentation analysis) of derivatized and native glycans and glycopeptides by ion trap MS (Shimadzu Axima QIT)

- 500 MHz ^1H NMR (analyses performed at the University of Oxford)

5. Quality Management Systems (QMS) at Ludger

Jura

Ludger operates a Quality Management System (QMS) known as Jura. Jura covers all aspects of the company's operations, including glycoprofiling, and is based on the ISO 9001:2008 framework. The company gained ISO 9001 accreditation in December 2011.

Jura is comprised of packages of rules, guidelines and standard operating procedures which cover all aspects of the company's activities from administration, interactions with clients, suppliers and business partners, research and development, technology production and analytical services. The Jura packages incorporate principles from a number of industry management systems. These include the following general and laboratory quality management systems (QMSs):

- ISO 9001:2008: General Quality Management System
- ISO 14000: Environmental Management System
- ISO 17025: General requirements for the competence of testing and calibration laboratories
- UKGLPCP: United Kingdom Good Laboratory Practice Compliance Program - 1999 Statutory Implement 3106 of OECD Principles of GLP
- ICH Q2(R1)
- USP <621>: Covers performance qualification for HPLC
- USP <1225>: Validation of compendial methods - principles applied also to Ludger methods

Principles from these QMSs that are relevant to Ludger's operations are distilled into Jura packages.

QMS training is currently delivered via standard operating procedures (SOPs), at weekly meetings and at-the-bench training.

Documentation and IT Systems

Ludger's Jura packages are documented using Microsoft Office software (currently standardized to Office 2010) on a Windows 2003 server with networked Windows XP, Vista and Windows 7 workstations.

Provisions for GMP Glycoprofiling Analyses at Ludger

Glycoprofiling work for biopharmaceutical clients who require GMP level analyses is performed within GMP-compliant modules added to Jura. These GMP-compliant QMS modules implement the GMP protocol requirements for non-GMP external contractors for the particular sponsor which will lead to the following evidence being added to the analytical report:

Operators:

- All operators involved are identified in the protocol. (Name, initials, title)
- Details/cross references of appropriate training are recorded in operators training records.

Equipment:

- All equipment used in the study is identified along with qualification calibration dates as appropriate.

Raw materials/reagents:

- Copies of certificates of analysis are appended for all bought-in reagents & materials.
- For reagents that are produced in-house, full details of their production are given.
- Full details of all weighings and or transfers are given including: material name, batch/lot number, expiry date, balance identification number, date recalibration due, confirmation of satisfactory pre-use performance checks.

Primary raw data:

- This is entered directly onto the appropriate spaces on the working copy of the Standard Operating Procedure (SOP) document.
- All significant steps in the process are signed and dated by the operator(s) performing the operations. Significant steps are countersigned by a second operator.
- Where it has been recorded elsewhere (e.g laboratory notebooks) then verified photocopies of these are appended to the document.
- Printouts obtained from equipment are appended directly to the document.
- All Level 1 and Level 2 glycoprofiling HPLC data is handled and stored by Empower software (Waters Ltd) and is 21 CFP part 11 compliant.
- All data entries and calculations are checked and verified by a second person.

Data and Process Reliability

- The Quality Assurance Department audits the study plan, processes and data entries and provides a signed statement to this affect appended to the glycoprofiling report.

Archiving of samples and data

- All records, data and samples are archived for a period agreed with the Client.

6. Ludger Company Background

Ludger is a bioscience company specialising in technology for the optimisation,measurement and control of biopharmaceutical glycosylation. The company was founded in 1999 by its Chief Executive, Dr Daryl Fernandes, and has glycotechnology production facilities and research laboratories at the Culham Science Centre near Oxford, UK.

Ludger's technology is used in the quality control of FDA and EMEA approved biopharmaceuticals worldwide. These include glycoprotein therapeutics such as erythropoietin (EPO) and monoclonal antibodies (mAbs). Ludger's clients include major pharmaceutical and biotechnology companies throughout the USA, UK, Europe and Asia.

Biopharmaceutical Glycoprofiling Services

Ludger's glycoprofiling services are for companies developing and manufacturing either novel or follow-on biological therapeutics. They are designed to support all stages of drug development including glycoengineering to optimize safety and efficacy profiles, regulatory submissions, comparability studies and intellectual property work.

Our approach to biopharmaceutical glycoprofiling is based on a QbD (Quality by Design) framework as outlined in Fernandes, 2009. This helps us to provide relevant data on biologically important features of drug glycosylation and allows development of a flexible glycoprofiling scheme that later on can be transferred into your own laboratories.

A typical glycorprofiling study employs a range of glycoprofiling modules which allow us to build a forensic profile of the glycosylation of your therapeutic (Royle and Spencer, 2008). These modules include: glycoprofiling pilot study - to optimize analysis methods for your drug samples; N-glycan release by endoglycosidase digestion or hydrazinolysis; O-glycan release by O-mode hydrazinolysis and other non-reductive release methods; oligosaccharide profiling by a range of orthogonal HPLC and MS methods; Level 1 glycoprofiling - to determine the overall glycan profile with classification and relative quantitation of the major glycan species; Level 2 glycoprofiling - which gives detailed sequencing information on the glycan structures; modules for patterns of sialylation, fucosylation and galactosylation; sialic acid profiling - to determine relative levels of human (NeuAc) and non-human sialylation (e.g. NeuGc); neutral monosaccharide composition analysis.

In addition to the standard glycoprofiling methods, we can develop custom glycoprofiling methods optimized for client's particular therapeutics.

Glycoprofiling Method Transfer, Training and Support

On completion of glycoprofiling studies we can transfer both the standard and custom glycoprofiling methods into your own laboratories. These provide you with a set of glycoanalysis tools for biopharmaceutical research, development, process control and product lot release. Methods are supplied as kits and reagents with detailed protocols.

We can provide initial training, troubleshooting, and technical support for your scientists and can help with your in-house validation.

Glycoprofiling Kits and Reagents

The company manufactures a comprehensive range of kits and reagents for ICH-compliant glycoprofiling of biopharmaceuticals throughout the drug development cycle. These include kits for glycan characterisation of EPO and monoclonal antibodies, kits for profiling of N- and O-linked glycans, kits for fluorescent labelling of glycans with 2-AB, 2-AA, AA-Ac and other glycan tags, glycan HPLC columns, glycan analytical standards for glycoprofiling of therapeutics, instrument and process qualification standards and technology for glycan analysis by HPLC, capillary electrophoresis (CE) and mass spectrometry (MS). The kits and reagents are backed up by comprehensive protocols and full technical support.

7. Key People for Glycoprofiling at Ludger

Ludger was founded in 1999 by its Chief Executive **Dr. Daryl Fernandes**. Daryl has 29 years' experience in the development of glycoanalysis methods for glycoproteins of the immune system and biopharmaceuticals. He gained his doctorate at the Glycobiology Institute, University of Oxford, was a consultant to Monsanto and G.D. Searle, and went on to become Process Development Manager and then Head of Analytical Services at Oxford Glycosciences (OGS). Daryl helped develop the hydrazinolysis chemistry used in the OGS GlycoPrep 1000 automated hydrazinolysis instrument which was used for characterisation and QC of biopharmaceutical glycosylation by many biopharmaceutical companies throughout the US and Europe.

Dr Louise Royle is Head of Glycoprofiling at Ludger. Louise has over twelve years experience in analytical glycobiology, particularly in HPLC and LC-MS analysis of O-glycans, N-glycans and glycosphingolipid head groups. She spent eight years working with Prof. Pauline Rudd and Prof. Raymond Dwek at the Oxford Glycobiology Institute before spending a year in Ireland at NIBRT as head of the analytical group. Louise is particularly interested in making glycan analysis robust and rapid. She is an author on over 80 peer-reviewed articles, over 50 of which are on glycobiology.

Dr. Claire Morgan is Business Development Manager at Ludger Ltd. She is responsible for liaising with clients prior to project set up, and setting up mutual CDAs. Claire also coordinates the company's marketing campaigns. Claire has a background in Immunology, having gained her PhD at The Royal London Hospital and Post Doctoral research experience at the Anthony Nolan Bone Marrow Trust (the Royal Free Hospital, London) and St. Bartholomew's Hospital, London.

Dr Daniel Spencer is Head of Development at Ludger. Daniel has over 15 years experience in glycosylation analysis, and many years experience of proteomics and analysis of post-translational modifications by mass spectrometry. Daniel worked for several years as senior proteomics analyst at OGS. Daniel has a background in studying glycosylation effects on protein structure during his PhD at the University of Nottingham, and postdoctoral research on the modification of glycoprotein tumour targeting agents at the Royal Free Hospital, University College London.

Dr Richard Gardner is Senior Chemist at Ludger with expertise in chemical glycan release methods, including both N-glycan and O-glycan release, and glycan derivatisation for MS and HPLC analysis. He is also responsible for biopharmaceutical glycoprofiling projects. Richard has an extensive chemistry background and gained his doctorate in bio-organic chemistry at the University of Leicester with a post-doctoral position in organic

chemistry at the University of Central Florida.

Robert Emery is a Glycoprofiling Technician. He performs the analyses for N-glycans, O-glycans, monosaccharide and sialic acids within the Glycoprofiling group. Robert gained his degree in Forensic Science from the University of Lincoln in 2009 and worked in the Forensic industry for a year before joining Ludger.

Rad Kozak is a chemist at Ludger, working on chemical glycan release methods including O-glycan and N-glycan release and glycan derivatization for HPLC analysis. Rad also works on new product development. Rad has a degree in organic chemistry and is working towards an industrial PhD on O-glycan analysis in conjunction with the University of Leiden, in the Netherlands.

John Froude is the Quality Assurance Manager and Laboratory and Facilities Manager at Ludger. He is responsible for conducting quality audits under ISO 9001 and GMP as well as the maintenance of our Laboratory equipment, the building fabric and running the non scientific services. John has more than 20 years' experience as a Facilities manager for a varied range of organisations from pharmaceutical companies to central government.

Rosa Charlesworth is the Administrator at Ludger. Rosa helps coordinate legal documents and invoicing for glycoprofiling projects.

Magdalena Sharma is works in Accounts at Ludger. She is responsible for credit control for glycoprofiling clients.

8. Nomenclature and Symbols for Glycan Structures

The names and representations of glycan structures in this document follow the conventions given in reference 10 (Mattu *et al*, 2000).

Figure 12 gives a summary of the symbols used for the glycan representations.

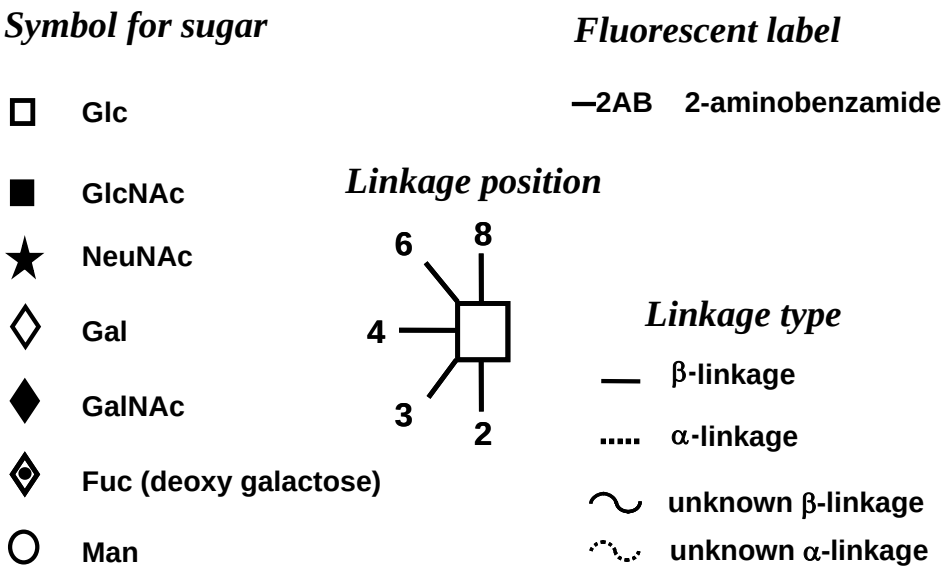


Figure 12: Key to representations of glycan structures

The class of each monosaccharide residue is indicated by the shape and fill in color of the sugar symbols. The glycosidic linkage positions and anomericity are indicated by the direction and shape of the linking lines respectively.

9. References

1. Fernandes, D.L. (2009) 'A QbD approach to biopharmaceutical glycosylation' in Quality for Biologics edited by Nicole Lyscom, epublication published by Biopharm Knowledge Publishing

2. ICH Q8 Guidelines - 'Pharmaceutical Development - Q8' and 'Pharmaceutical Development Annex to Q8'

3. ICH Q9 Guidelines – 'Quality Risk Management - Q9'

4. ICH Q10 Guidelines – 'Pharmaceutical Quality System'

5. ICH Q2(R1) Guidelines – Validation of Analytical Procedures: Text and Methodology – Q2(R1).

6. Fernandes, D.L. (2007) 'Future trends in the Measurement of Biopharmaceutical Glycosylation' 11th WCBP CMC Strategy Forum, Washington DC, January 28, 2007

7. Hermentin, P; Witzel, R.; Kanzy, E-J; Diderrich, G; Hoffmann, D; Metzner, H; Vorlop, J;Haupt, H. (1996) The hypothetical N-glycan charge: a number that characterizes protein glycosylation. Glycobiology **6**: 217-230

8. Loumaye, E., Giartosio, C.E. (2003) Compositions of FSH with high sialylation degree and their use for the preparation of medicaments. Wipo Patent WO/2003/035686, PCT/EP2002/011501

9. Royle, L. and Spencer, D.I. (2008). Introduction to analytical tools for glycan analysis. Society for Experimental Biology Meeting - Comparative Biochemistry and Physiology, Marseilles, July 2008.

10. Mattu, TS; Royle, L; Langridge, J; Wormald, MR; Van den Steen; PE, Van Damme, J; Opdenakker, G; Harvey, DJ; Dwek, RA; Rudd, PM (2000) *O*-Glycan analysis of natural human neutrophil gelatinase B using a combination of normal phase-HPLC and online tandem mass spectrometry: Implications for the domain organization of the enzyme. Biochemistry 39: 15695–15704.