

A Unique Workflow for Glycoprotein Characterization – From Sample Preparation to MS/MS Spectral Interpretation

Using the AB SCIEX TOF/TOF™ 5800 System

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Protein glycosylation is a complex dynamic post-translational modification, which is used by an organism to regulate a number of important protein functions. The structure of attached glycans control protein folding and subsequent delivery to the cell surface, protein interactions with binding partners, and finally protein degradation and turnover. Because glycosylation of a glycoprotein may influence the properties of a protein in the body, the biopharmaceutical industry must characterize the glycosylation patterns on therapeutic protein drugs. The glycan can influence the efficacy and stability of the drug or cause undesirable anti-glycan immune responses^{1,2}.

However, the primary structure elucidation of glycans or glycoproteins remains challenging due to variable composition, linkage, branching and anomericity of constituent monosaccharides in combination with the general heterogeneity. An indirect, non-template control biosynthesis defines the basis of the high structural heterogeneity of glycoprotein glycans³. In general, the structural information obtained from MS/MS (Figure 1) is the greatest when the glycans are released from the peptide or protein backbone and separated by LC^{4,5}. Nonetheless, structural MS analysis of glycan is not yet routine and often requires time consuming manual spectral interpretation.

This technical note presents a complete workflow for glycoprotein characterization comprising sample preparation for

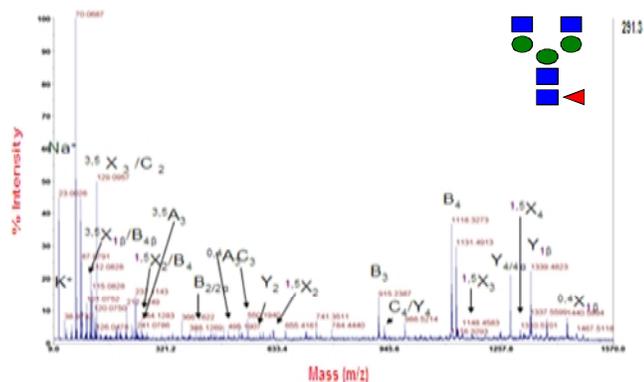
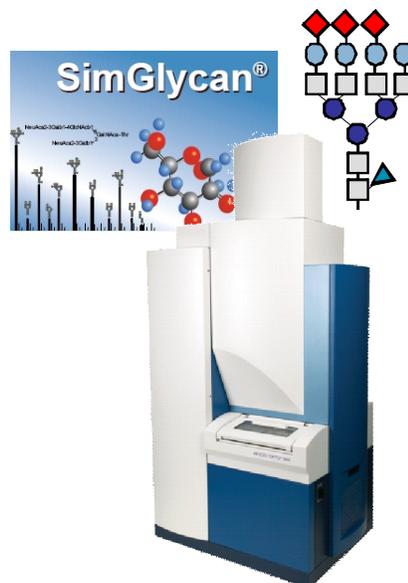


Figure 1. MS/MS of Released Glycans for Structural Characterization. The spectra of $[M+Na]^+$ of a neutral carbohydrate shows specific ring cleavage X- and A-type ions and glycosidic B- and Y-type ions.



glycan release, glycan separation using graphitized carbon separation coupled to MALDI spotting, automated MS and MS/MS analysis and spectral interpretation with SimGlycan® Software (Figure 2).

Key Features of AB SCIEX TOF/TOF™ 5800 System for Automated Glycan Analysis

- AB SCIEX TOF/TOF™ 5800 system, with adjustable collision energy provides wider range of glycan fragments than electrospray MS/MS and MALDI post-source spectra allowing a more complete glycan characterization
- High energy CID MS/MS results in extensive cross-ring fragmentation ions (A-type and X-type ions) allowing to determine branching patterns and provide linkages information of the monosaccharide residues (Figure 1).
- Low energy CID MALDI MS/MS provides strong glycosidic B- and Y-type ions to determine the glycan composition.
- Simultaneous determination of the molar ratios of isomeric glycans is possible from abundances of identified ions.
- Automated data acquisition for LC MALDI separated glycan samples supports a complete workflow, which allows a fast and robust glycan analysis.

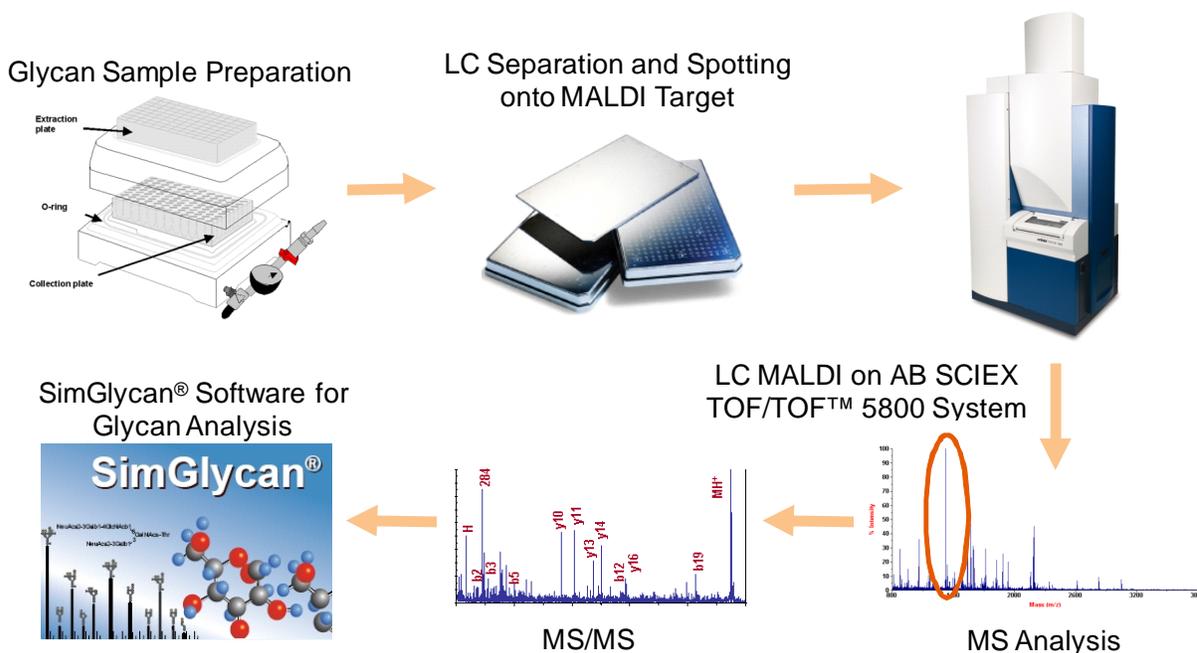


Figure 2. Scheme of Glycoprotein Workflow. The figure illustrates the steps of the workflow, from glycan release and labeling, through MALDI analysis, to final data interpretation.

Robust, Reproducible Glycan Release and Labeling

Existing glycan release methods require extended periods of enzymatic or chemical deglycosylation and laborious sample clean up prior to mass spectrometry analysis⁴. A complete glycan release method in a 96-well plate format that enables deglycosylation, labeling and sample clean up in less than 2 hours using a new Rapid Glycoanalysis Sample Preparation System (RGSPS) with InstantAB™ Labeling kit (ProZyme, Inc.) was investigated. All tests were performed with commercially

available human IgG and a glycan library of defined bisecting and non-bisecting glycan structures (ProZyme, Inc., Part #:GKLB-005, contains both samples).

The human IgG glycoprotein contained predominantly IgG1, IgG2 and IgG3 and was the source for the glycan library sample. Therefore, the glycan library could be used as a control sample for the IgG sample.

First, the completeness of the deglycosylation method of the RGSPS kit was evaluated using 100 µg of human IgG (1 mg/mL). The deglycosylation step was performed according to

the manufacturer protocol with slight changes. After a one hour incubation time with PNGaseF at 45 °C, the glycans were directly eluted into a collection plate. The deglycosylation plate was placed on top of a collection plate and the stacked plates were centrifuged for 10 min at 1200 RCF. Released glycans were spotted directly onto the MALDI target and its MS spectrum compared with control glycan library MS spectrum (Figure 3). Each glycan mass of MS spectra was confirmed by MS/MS analysis. The released glycan spectrum matched the control glycan library very well, indicating complete glycan release.

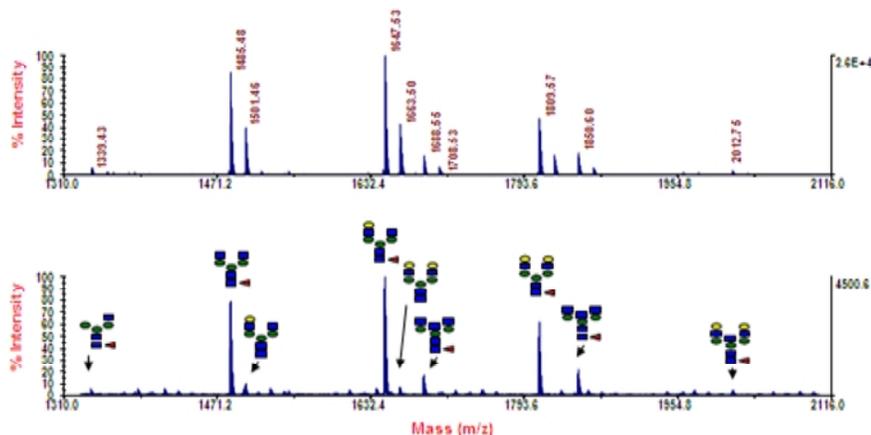


Figure 3. MS Spectra of Glycan, Control and Released. (Top) MS spectrum of the control glycan library sample, glycans in $[M+Na]^+$. (Bottom) MS Spectrum of the glycans released from IgG glycoprotein using kit, glycans in $[M+Na]^+$.

Table 1. Reproducibility Data of the RGSPS kit from ProZyme. The reproducibility of RGSPS kit with InstantAB labeling was tested on 3 consecutive days using 100 µg of de-sialyated human polyclonal antibody. Comparison was done using the HPLC profiles of 2-AB labeled glycans.

Glycan	Experiment 1			Experiment 2			Experiment 3		
	Mean	Std Dev	% CV	Mean	Std Dev	% CV	Mean	Std Dev	% CV
G0	0.9	0	2.4	0.9	0	1.1	0.9	0	3.9
G0F	20.7	0.2	0.9	20.6	0.1	0.4	20.5	0.2	0.9
G1 / Man-5	5.3	0	0.4	5.3	0	0.5	5.3	0	0.5
G1F	33.4	0.1	0.3	33.3	0.1	0.3	33.3	0.1	0.3
GaFB	4.8	0.1	2.6	4.9	0	0.8	4.9	0.1	1.9
G2	2.4	0.1	2.5	2.4	0	0.7	2.4	0	1.9
G2B	0.5	0	2.2	0.5	0	2.5	0.5	0	2
G2F	27.4	0.2	0.6	27.4	0.1	0.2	27.4	0.2	0.6
G2FB	4.1	0.1	2	4.3	0.1	1.6	4.4	0.1	2.2
G2FS1	0.6	0	2.4	0.6	0	5.6	0.6	0	0.8

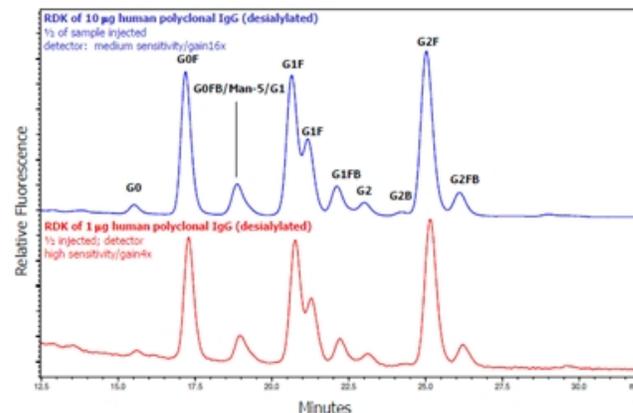


Figure 4. Sensitivity Data of the RGSPS kit from ProZyme. HPLC UV traces after separation of 2-AB labeled glycans with a GlycoSep™ N column illustrate the sensitivity for 10 µg and 1 µg load of human polyclonal antibody.

Next, we applied the entire deglycosylation, labeling and clean up procedure using RGSPS kit following the manufacturer's protocol. The MALDI sample preparation for neutral and labeled N-glycans were carried out with 2,5-dihydroxybenzoic acid matrix (DHB, 10 mg/mL). After mixing the glycan sample 1:1 (v/v) with matrix solution, 0.8 µL was spotted on the MALDI target and dried under vacuum to promote a homogenous co-crystallization for automated measurements. This procedure avoided the typical DHB crystallization of long white needles.

The reproducibility of the process was evaluated at a variety of protein amounts using human polyclonal IgG. Preliminary results of the deglycosylation and 2-AB labeling method showed good sensitivity, UV traces for 1 µg to 10 µg of glycoprotein showed similar results (Figure 4). At the typical sample load of 100µg, excellent reproducibility was obtained in a multiple day test (Table 1).

N-Glycan Separation by Graphitized Carbon LC and Automated Spotting

A very popular and promising method for analysis of glycans is graphitized carbon HPLC–ESI MS. Several publications established this in the early nineties for HPLC of oligosaccharides and glycopeptides with small peptide moieties⁷. The advantage over HILIC type columns is that graphitized carbon HPLC is often able to resolve many glycan isomers structures and can separate even isomeric oligosaccharides.

For the first time, we combined a LC MALDI MS analysis with a nano Hypercarb column for glycan analysis (150 µm ID column, Michrom BioResources, Inc. CN9/99971/00). The combination of the nanoflow rate separation with the LC MALDI spotting system provided femtomole level sensitivity, as determined by serial dilution of glycan standard (data not shown). The hypercarb column allows elution of native glycans or reduced form, after labeling with a fluorescent tag, or in the permethylated form at low acetonitrile concentrations without the use of high salt concentration disturbing the co-crystallization on the MALDI target.

The prepared neutral or labeled glycan sample was diluted and loaded on column and a gradient of 5 – 35 % solvent B in 30 minutes at a flow rate of 600 nL/min (Solvent B: 98 % acetonitrile in 0.1% trifluoroacetic acid) was used for chromatographic fractionation of the glycopeptides (Figure 5).

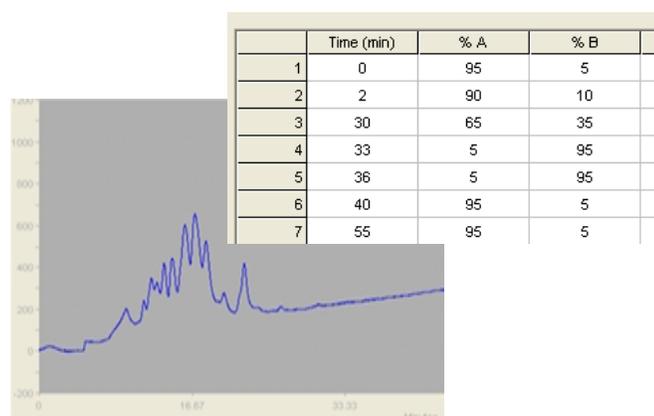


Figure 5. Glycan Separation. The separation of the glycans on the hypercarb column is visualized from the UV trace (214 nm). The eluant from the column was spotted using a nanoLC MALDI Spotting System.

All electrostatic depositions were done using a nano LC MALDI spotting system equipped with a 10 μL sample loop. As neutral or labeled glycans eluted off the column (flow rate of 600 nL/min), DHB matrix (10 mg/mL) was added post-column at 1:1 volume ratio and spots were deposited every 30 seconds from 5-80 minutes (total volume/spot is 600 μL). The DHB crystallization was optimized by re-spotting on top of the entire gradient with DHB matrix (10 mg/mL) using only the syringe at 50 $\mu\text{L}/\text{min}$ with a 1000 msec spotting interval. After a 4 min wait time, the spotted LC run was dried under vacuum resulting in a milky, homogenous layer of analyte and matrix crystals.

MS Analysis of N-Glycans using the AB SCIEX TOF/TOF™ 5800 System

The 5800 system is ideally suited for glycan characterization due to the ability to run both high and low energy MS/MS for detailed fragmentation information. High energy CID provides additional cross-ring fragmentation information on glycosidic linkages and branching. In addition, its superior sensitivity, mass resolution and mass accuracy in both MS and MS/MS modes, as well as its high repetition-rate laser (Nd:YLF laser 1000Hz, 345 nm), allows for the acquisition of high-quality data with high speed.

Survey scans were performed using an MS Reflector Positive mode scan surveying a mass range of 800-3000 amu, firing at a laser repetition rate of 400 Hz. High energy collision-induced dissociation (CID) MS/MS acquisitions were done using an MS/MS 2kV positive method and a collision gas pressure of 1×10^{-6} Torr using a laser repetition rate of 1000 Hz. Argon was used as heavy gas, although no significant differences were observed when argon was replaced by air.

Every glycoform released from the human IgG glycoprotein was detected and confirmed by high CID MS/MS. The glycan library was primarily used as control sample to show the completeness of the deglycosylation method by providing MS and MS/MS spectra for comparison of released and labeled glycans. Figure 6 shows MS spectra of the control glycan library sample in the top panel, then a MS spectrum of the released neutral glycans in MALDI target spot G13 after LC MALDI analysis. The LC-MALDI spectrum of spot G13 shows the concentration effect in an intensity increase of glycan m/z 1647.6 from $1.6 \text{ E}+4$ to $2.3 \text{ E}+4$. All glycans were detected in their sodiated form.

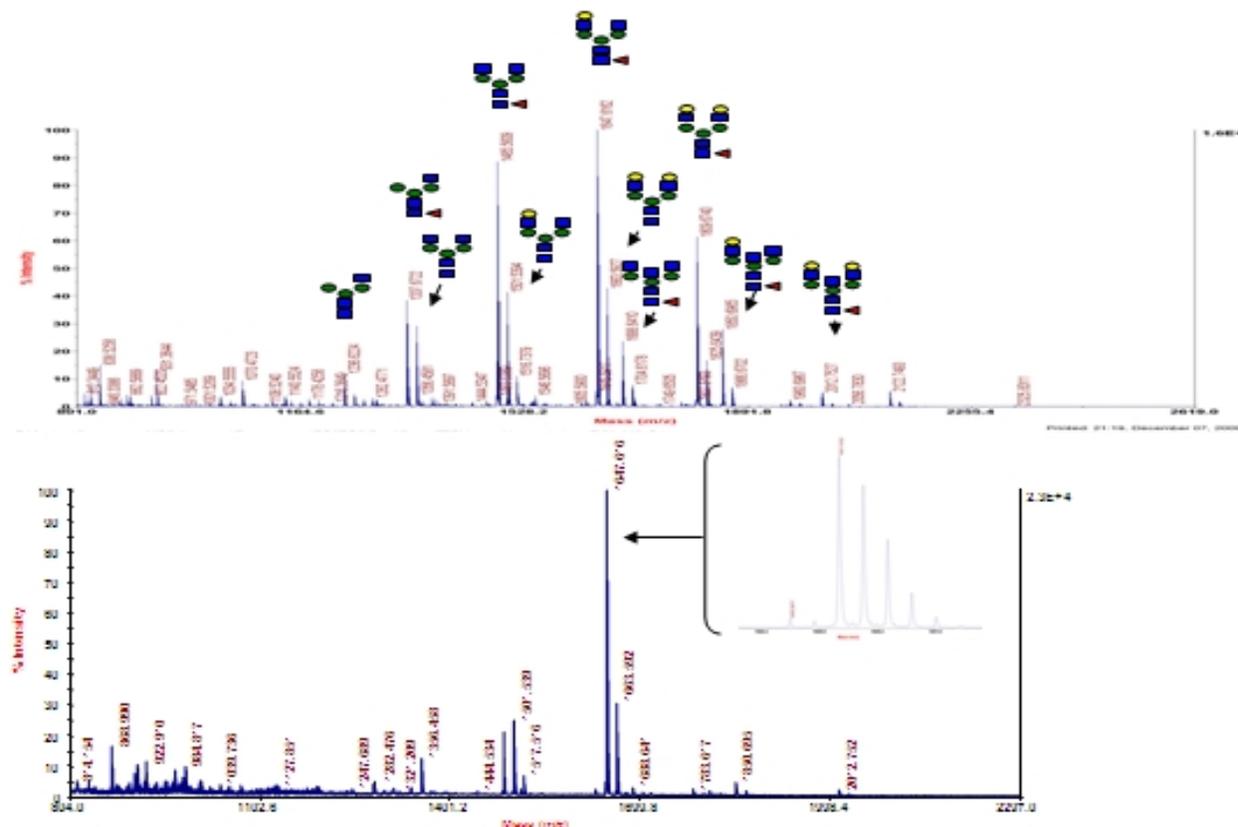


Figure 6. Detection of Glycans by LC MALDI Separation. (Top) Sodiated MS spectrum of the control glycan library obtained by single spot analysis. (Bottom) Released IgG glycans separated on a hypercarb column shows predominantly sodiated glycan at m/z 1647.5 in MALDI spot G13. All glycans were detected by LC MALDI.

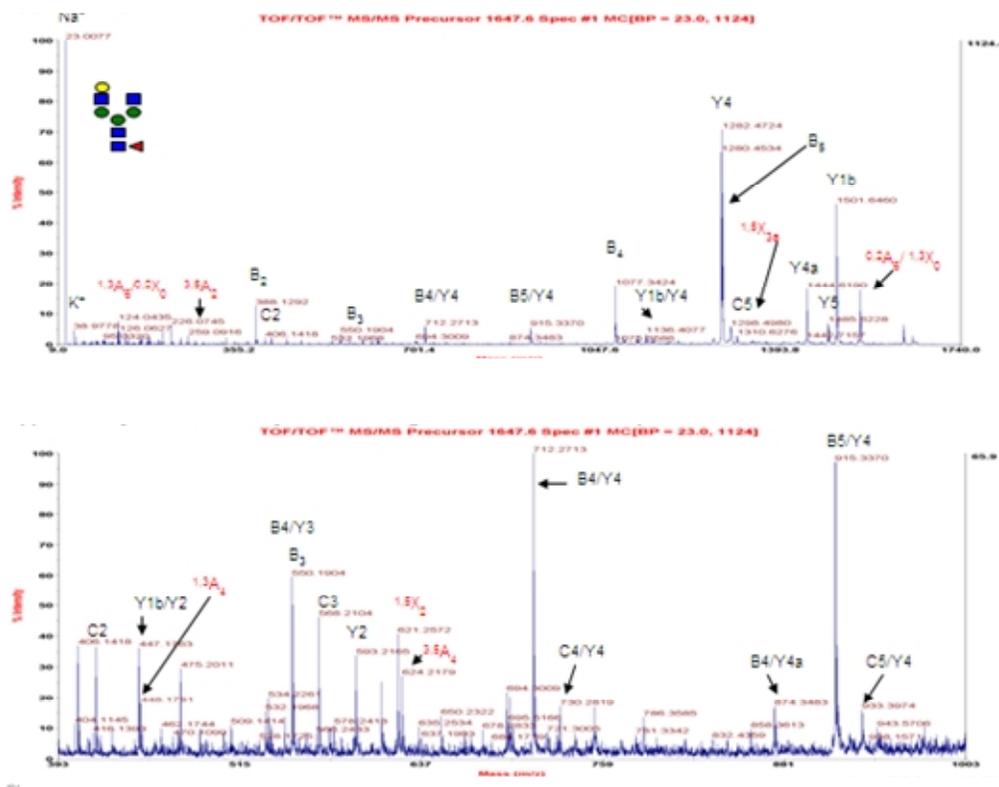


Figure 7. MS/MS Spectra of Released Glycan G1F. (Top) Full mass range of the sodiated MS /MS spectrum of precursor ion 1647.5. (Bottom) Displays a zoomed mass range from m/z 393 to m/z 1003 to show the fragmentation of glycosidic and specific cross ring cleavage ions (red).

Spectral MS/MS Analysis of Released N-Glycans using SimGlycan Software

The high CID spectrum from the 5800 system shows the typical glycosidic cleavage ions (Y and B-ion) and some cross ring fragment ions (A and X-ions). Sometimes C ion scan be observed, but rarely Z-ions in MALDI mass spectrometry. This additional information is very informative for complete characterization of released glycans.

To prepare spectra for SimGlycan Software analysis, all MS/MS data were saved as t2d files and opened in the Data Explorer software, then the SimGlycanPeaklist v1.bas" macro was run to export the spectra in a reduced peak list format for analysis. SimGlycan Software is a comprehensive glycan and glycopeptide drawing and structure analysis software containing a carbohydrate search engine that uses mass spectrometry data to predict glycan structures from primary carbohydrate sequence databases¹¹.

All glycan data, from both the control glycan library and the processed glycoprotein were processed using SimGlycan software. All six of the known glycan structures were detected and characterized when this complete and automated workflow

was applied to 100 µg of the human IgG sample. All glycan MALDI spectra were acquired as sodium adducts [M+Na]⁺.

Table 2. Identified Released Glycans. This table summarizes released and labeled N-glycans deduced from this workflow. It shows the observed and theoretical calculated masses for each assigned glycan structure. Each glycan structure was confirmed by high CID MS/MS analysis.

Glycan Name	Mass of Released Neutral Glycans		Mass of 4AB-Labeled Glycans		Assigned Glycan Structure
	Measured	Calculated	Measured	Calculated	
GOF	1485.56	1485.52	1647.46	1647.57	
G1	1501.53	1501.51	1663.56	1663.45	
G1F	1647.61	1647.57	1809.62	1809.51	
GOFB	1688.64	1688.65	1850.65	1850.53	
G2F	1809.67	1809.67	1971.60	1970.55	
G1FB	1850.69	1850.67	2012.70	2011.57	
G2FB*	2012.75	2012.70	---	2174.75	

* Not stated to be in the glycan library, but was observed

Conclusions

The AB SCIEX TOF/TOF™ 5800 System is the instrument of choice for glycan analysis.

- More structural information is obtained from tandem mass spectrometric studies when glycans are released from the peptide backbone⁵.
- High energy CID capability of the 5800 system provides a greater number of MS/MS fragments than electrospray CID MS/MS spectra or MALDI post source decay (PSD) spectra¹⁰ for more detailed information.
- The RGSPS kit with and without InstantAB labeling provides a fast and robust glycan release and labeling method including sample clean up in less than 2 hours prior to mass spectrometric analysis.
- The combination of the LC MALDI Workflow with the nanoflow hypercarb column enables better glycan separation and therefore more specificity for isomeric glycans structures characterization.
- Final MS/MS spectral comparison of the released and/or labeled glycans with the control samples demonstrates the robustness, speed and sensitivity of this entire workflow.

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