

# A Complete Workflow Solution for Monoclonal Antibody Glycoform Characterization Combining a Novel Glycan Column Technology and Bench-Top Orbitrap LC-MS/MS

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## Overview

**Purpose:** To develop a complete workflow solution for monoclonal antibody (mAb) glycoform characterization using a unique glycan column technology and a Thermo Scientific™ bench-top Orbitrap™ LC-MS/MS.

**Methods:** Glycans are separated using a recently developed high-performance HPLC/UHPLC column, a Thermo Scientific™ GlycanPac™ AXH-1 column. A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans.

**Results:** The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity. A complete workflow solution was developed for glycan profiling combining the unique column technology and a bench-top Orbitrap LC-MS/MS (Figure 1). This workflow was applied to antibody glycoform characterization. Confident identification and structural confirmation were achieved for released glycans from a standard glycoprotein and a monoclonal antibody.

## Introduction

Because glycosylation is critical to the efficacy of antibody therapeutics, the FDA requires that a consistent human-type glycosylation be maintained for recombinant monoclonal antibodies (mAb), irrespective of the system in which they are produced. The complex branching and isomeric nature of glycans pose significant analytical challenges for their identification and characterization. Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans.

The recently developed GlycanPac AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural, qualitative and quantitative analysis of glycans. It has a unique selectivity for biologically relevant glycans including glycans from antibodies, either labeled or native and is designed for high-resolution, high-throughput analysis by LC-fluorescence or LC-MS methods. Because glycans are very hydrophilic and polar, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine or zwitterionic packing materials are often used for their analysis. HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. Identification of the glycan charge state is not possible by HILIC. The GlycanPac AXH-1 column overcomes these limitations and can separate glycans based on charge, size and polarity configuration. It provides both greater selectivity and higher resolution. In this study, we characterized N-linked glycans released from a glycoprotein standard and a monoclonal antibody by LC-MS/MS methods using the new column technology and high-resolution Orbitrap mass spectrometry.

## Methods

### Sample preparation

Native glycans are released from glycoproteins or mAb with PNGase F enzyme. The released glycans are conjugated with 2-amino benzamide (2-AB) label group with reported procedure of Bigge *et. al.*<sup>1</sup>

### Liquid chromatography

All the glycans are separated using a recently developed high-performance HPLC/UHPLC column, GlycanPac AXH-1, on a Thermo Scientific™ Dionex™ Ultimate 3000 UHPLC with either fluorescence or MS detector.

For intact antibody, a Thermo Scientific™ ProSwift RP-10R monolithic column (1 x 50 mm) was used for desalting. LC solvents are 0.1% formic acid in H<sub>2</sub>O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80 °C during analysis. Flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0 min, 20%B; 1.0 min, 35%B; 3.0 min, 55%B; 4.0 min, 98%B; 7.0 min, 98% B; 7.1 min, 20%B; 15.0 min, 20%B).

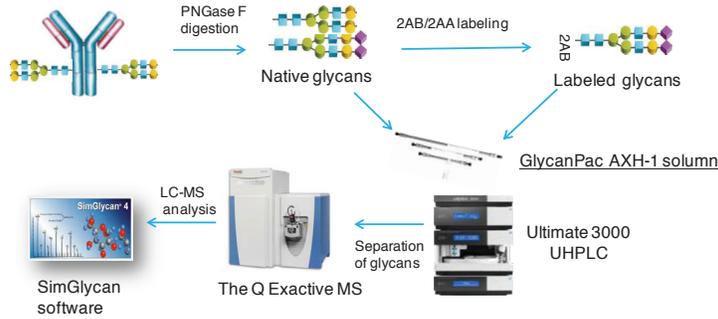
### Mass spectrometry

A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans. The following MS and MS/MS settings were used: MS scan range 380-2000 *m/z*. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of 1x10<sup>6</sup> and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of 2x10<sup>5</sup>. Intact mAbs were analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 °C. S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb. The AGC target was set at 3x10<sup>6</sup>. Maximum IT was set at 250 ms.

## Data analysis

SimGlycan® software from PREMIER Biosoft was used for glycan identification and structural elucidation<sup>2</sup>. SimGlycan software accepts raw data files from Thermo Scientific mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques. Full MS spectra of mAb were analyzed using Thermo Scientific™ Protein Deconvolution™ 2.0 software. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input *m/z* spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses of various combinations of commonly found glycoforms

**Figure 1. A complete LC-MS/MS workflow solution for monoclonal antibody glycan profiling**

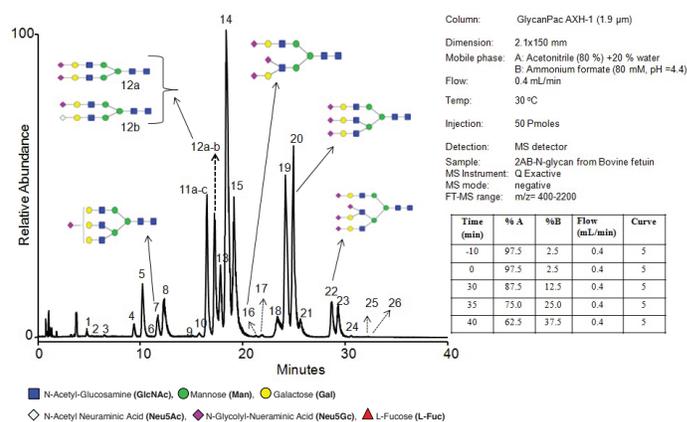


## Results

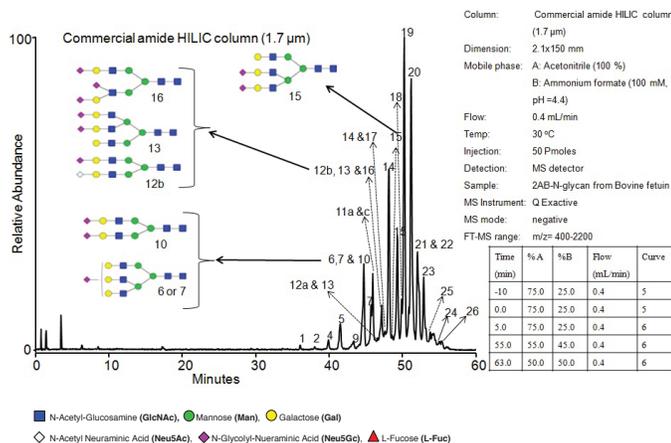
### Separation of Glycans Based on Charge, Size and Polarity

The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis and characterization of uncharged (neutral) and charged glycans present in proteins. The separation and elution of glycans are based on charge; the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. In this study, the structure of glycans present in each peak was determined using high resolution LC-MS/MS. As shown in Figure 2, the detailed structural information obtained from the MS/MS data validated the ability of GlycanPac AXH-1 column to separate labeled N-glycans based on charge, size and polarity. However, co-elution of different charge state glycans is common with other commercially available HILIC column as shown in Figure 3.

**Figure 2. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 μm) column with MS detection.**



**Figure 3. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by a commercial amide HILIC column (1.7  $\mu\text{m}$ ) with MS detection.**

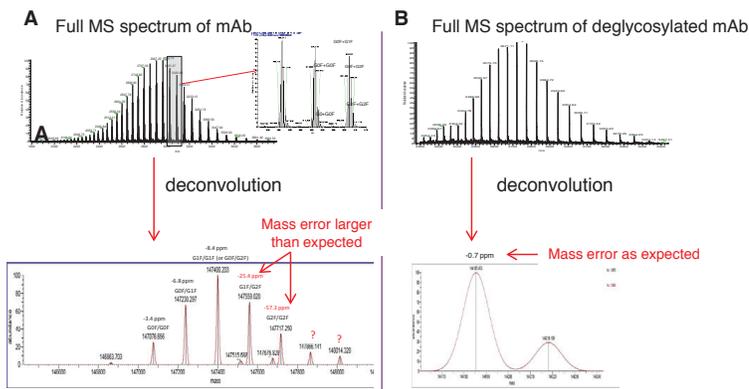


The GlycanPac AXH-1 column is also well suited for high performance LC/MS separation and analysis of native glycans from proteins (data not shown). Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

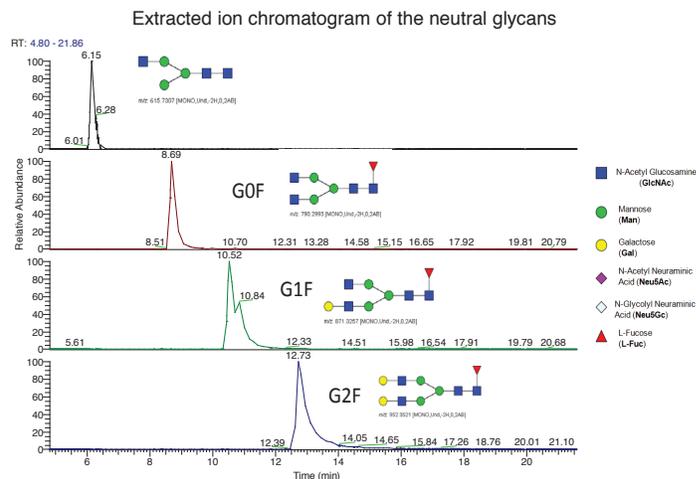
**Monoclonal antibody (mAb) glycan profiling using GlycanPac AXH-1 column and high resolution LC-MS/MS**

Intact mass measurement of a monoclonal antibody identified glycoforms derived from the combination of any two of the three N-glycans, G0F, G1F and G2F. However, the mass errors for some of the intact glycoforms of this antibody ranged from 20-60 ppm (Figure 4A) which is larger than the <10 ppm observed for other samples (data not shown). Furthermore, the intact mass error for the deglycosylated form of this antibody was within 10 ppm (Figure 4B), suggesting that some minor glycosylation forms of this molecule that were not detected at the intact level had interfered with the observed intact mass of the major glycoforms. To further characterize this antibody, released glycans from this protein were separated using the GlycanPac AXH-1 column. The separation and elution of glycans from GlycanPac AXH-1 column are based on charge with neutral glycans eluting first, followed by the acidic sialylated species. Glycans of each charge state are further separated based on their size and polarity (Figure 5).

**Figure 4. Observed molecular mass of glycosylated (A) and deglycosylated (B) forms of a intact monoclonal antibody. Some of the intact antibody major glycoforms have an observed mass error larger than expected. There are also two potentially double fucosylated peaks that need to be confirmed.**

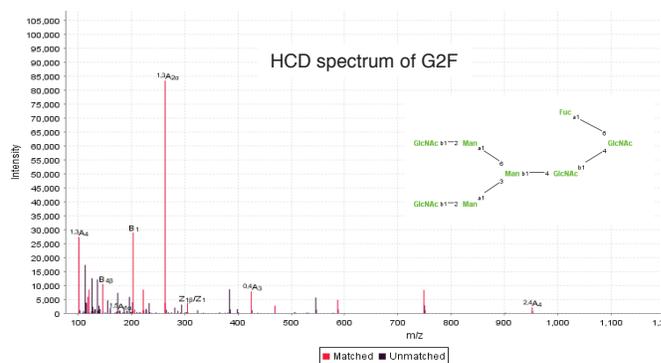


**Figure 5. Separation of the major, neutral N-glycans on GlycanPac AXH-1 column**



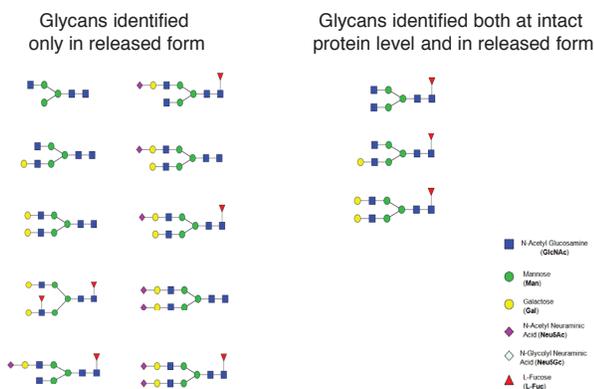
Characterization of glycans in each peak was performed by Full MS and data dependent MS/MS using HCD. The information-rich HCD spectra contain fragment ions that were generated from both cross-ring and glycosidic bond fragmentations (Figure 6). Three different types of glycans were found from this monoclonal antibody, the majority of glycans identified were neutral, including G0F, G1F and G2F which were also the major glycoforms identified at the intact protein level for this antibody (Figure 4A). Also identified were less abundant, non-fucosylated forms of G1 and G2, minor amounts of mono-sialylated and di-sialylated species with and without fucosylation, as well as double fucosylated species that were not identified at the intact protein level (Figure 7).

**Figure 6. Identification and structural confirmation of released glycan using high resolution HCD MS/MS**



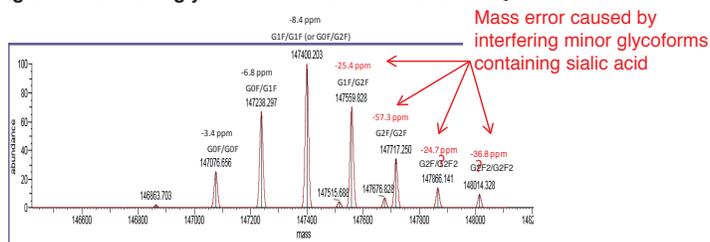
Fragment ion type	Percentage match (%) of theoretical fragments
Single glycosidic	32.14
Glycosidic/glycosidic	30.95
Single cross ring	20.21
Cross ring/glycosidic	14.95

**Figure 7. Identified glycans from monoclonal antibody**



These results explain that the unexpected mass error observed previously is due to the interfering minor glycoforms that have a molecular mass close to the major ones. In the deconvoluted MS spectrum, the base of the antibody major glycoform peaks covers a mass range of about 40 Da due to the distribution of the unresolved isotopic peaks of a large protein of this size. Therefore any interfering species within 20 Da of mass difference would cause a mass shift of the major glycoform peaks, rather than forming a separate peak. For example, in this case, the replacement of a Fuc and a Gal by Neu5Ac, which would have a mass difference of -17Da, could cause the negative mass shift observed in this study, especially when the interfering species is relatively low in abundance (Figure 8). Results in this study indicate that rapid and sensitive antibody glycan profiling can be achieved using GlycanPac AXH-1 column and HR/AM Orbitrap LC-MS/MS.

**Figure 8 Annotated glycoforms of a monoclonal antibody**



## Conclusion

- GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity.
- The GlycanPac AXH-1 columns are compatible with MS instruments. LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- Confident identification and structural confirmation of glycans can be achieved using high-resolution HCD MS/MS which produces an informative spectrum containing glycosidic and cross ring fragment ions.
- A complete workflow solution was developed for glycan profiling combining the unique GlycanPac AXH-1 column technology and a bench-top Orbitrap LC-MS/MS.
- This workflow was applied to characterize a monoclonal antibody glycoforms. Confident identification and structural confirmation was achieved for released glycans from the monoclonal antibody.

## References

1. Bigge, J. C. et al., Non-selective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Analytical Biochemistry*, **1995**, *230*, 229-238.
2. Apte, A and Meitei, N.S., Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan, **2010**, *600*, 269-81.

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