



# Analyze mAb Glycans Using TSKgel® FcR-III A-NPR HPLC Column by Top Down Mass Spectrometry

## Introduction

Monoclonal antibodies (mAbs) comprise the largest class of glycosylated protein therapeutics currently on the market and glycosylation is known to be a major source of mAb heterogeneity. N-glycosylation of IgG-Fc of mAbs is known to impact drug therapeutic mechanism of action (MOA), thus monitoring glycan critical quality attributes (CQAs) is an essential part of biopharmaceutical development. Glycosylation is a critical factor in drug product solubility, kinetics, stability, efficacy, and immunogenicity. Analytical methods utilize a suite of chromatographic modes using high performance liquid chromatography (HPLC) to analyze glycosylation of both intact and digested protein molecules.

The TSKgel FcR-III A-NPR (non-porous resin) column is a high performance affinity chromatography column for the analysis of IgG glycoforms. The stationary phase utilizes a recombinant FcR-III A protein bound to a non-porous polymethacrylate polymer. The retention mechanism is based on the interaction between the FcR ligand and the sugar moieties attached to the ASN amino acid in the conserved region of the monoclonal antibody. The resulting elution profile of the glycoprotein mimics ADCC activity, which is correlated to the composition of the N-glycans.

The purpose of this study is to demonstrate the use of mass spectrometry to characterize the elution profile of a typical IgG<sub>1</sub> molecule separation on a TSKgel FcR-III A-NPR column and verify the observations that certain glycan structures impart higher activity to the monoclonal antibody, especially as it relates to the presence of terminal galactose sugars.

## Experimental HPLC Conditions

### TSKgel FcR-III A-NPR Separation

Column: TSKgel FcR-III A-NPR, 5 µm, 4.6 mm ID × 7.5 cm  
 Instrument: Agilent 1200  
 Mobile phase: A: 50 mmol/L Na citrate, pH 6.5  
 B: 50 mmol/L Na citrate, pH 4.5  
 Gradient: 0 min: 0% B, 20 min: 100% B, 30 min, 100% B  
 Flow rate: 0.85 mL/min  
 Detection: UV @ 280 nm, 25 Hz  
 Temperature: 15 °C  
 Injection vol.: 5 µL  
 Sample: NIST mAb fractions; 5 mg/mL in mobile phase A

### Top Down MS Characterization

Column: TSKgel Protein C<sub>4</sub>-300, 3 µm, 2.0 mm ID × 15 cm  
 Instrument: Shimadzu Nexera® XR  
 Mobile phase: A: 0.1% formic acid in water  
 B: 0.1% formic acid in acetonitrile  
 Gradient: 0 min: 10% B, 40 min: 95% B, 50 min: 95% B  
 Flow rate: 0.2 mL/min  
 Detection: Sciex X500B Q-TOF, ESI positive, m/z 900-4000  
 Temperature: 50 °C  
 Injection vol.: 5 µL  
 Sample: NIST mAb fractions; 100 µg/mL in LC/MS water

MS Conditions:

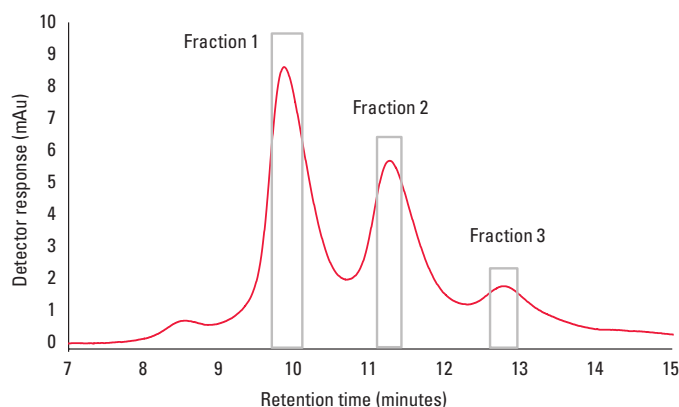
Source gas 1	50 psi	Spray voltage	5000 eV
Source gas 2	50 psi	Declustering potential	250 eV
Curtain gas	50 psi	DP spread	0 eV
CAD gas	7 psi	Collision energy	10 eV
Source temp	400 °C	CE spread	0 eV
Accumulation time	1 sec	Bins to sum	80

## Results and Discussion

Figure 1 demonstrates the separation of NIST mAb on the TSKgel FcR-III A-NPR column. IgG<sub>1</sub> molecules yield this typical type of elution profile based on glycoform composition that is consistent with ADCC activity. This offers a fast orthogonal chromatographic method for determination of antibody activity and comparisons of antibody heterogeneity.

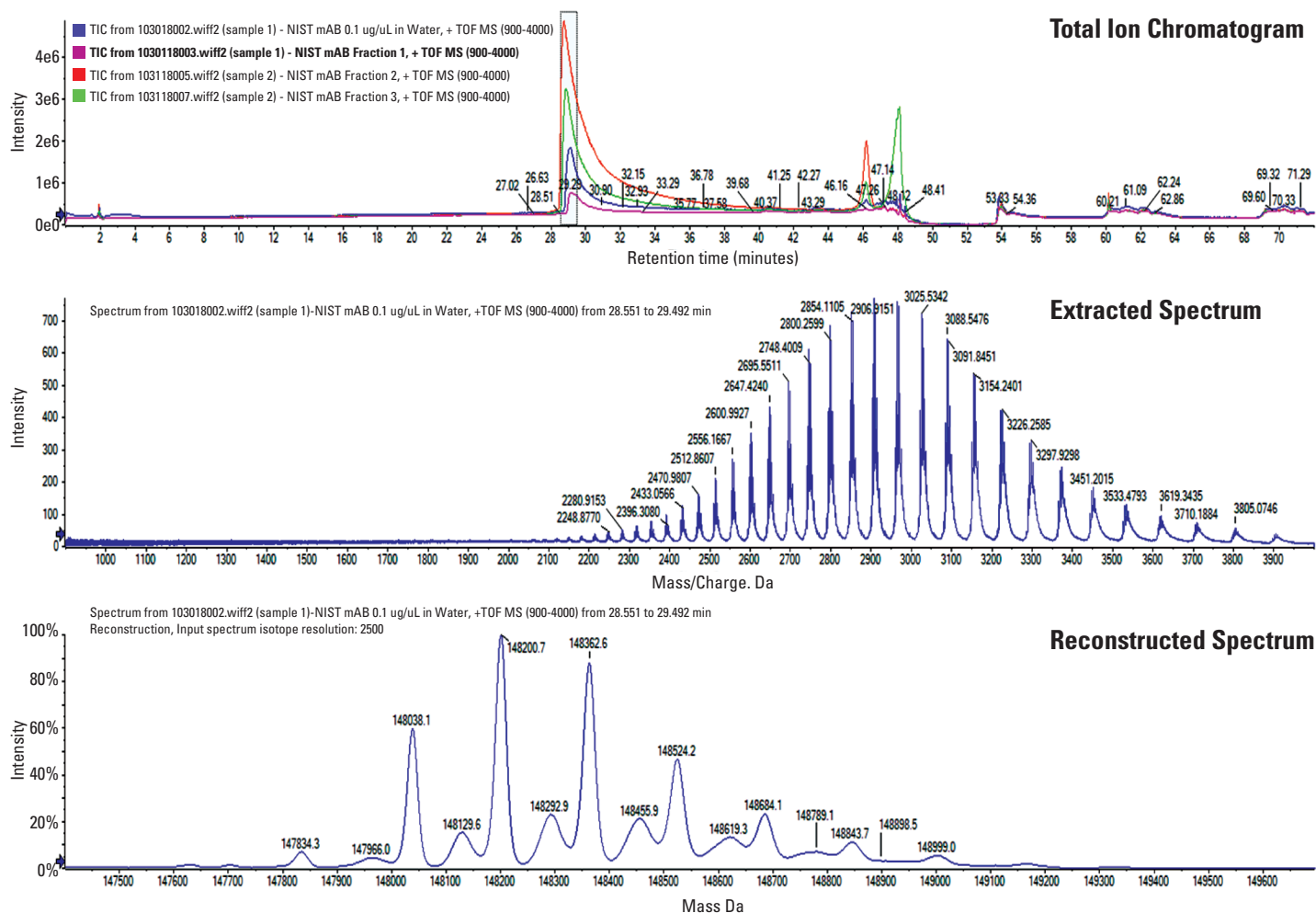
The three largest eluting peaks were collected and analyzed by offline mass spectrometry. Peak fractions were pooled from successive 25 µg on column injections, concentrated, and buffer exchanged to LC/MS grade water.

Figure 1. Zoomed view of the elution profile of NIST mAb on TSKgel FcR-III A-NPR. The boxes highlighting each peak represent fractions that were collected.

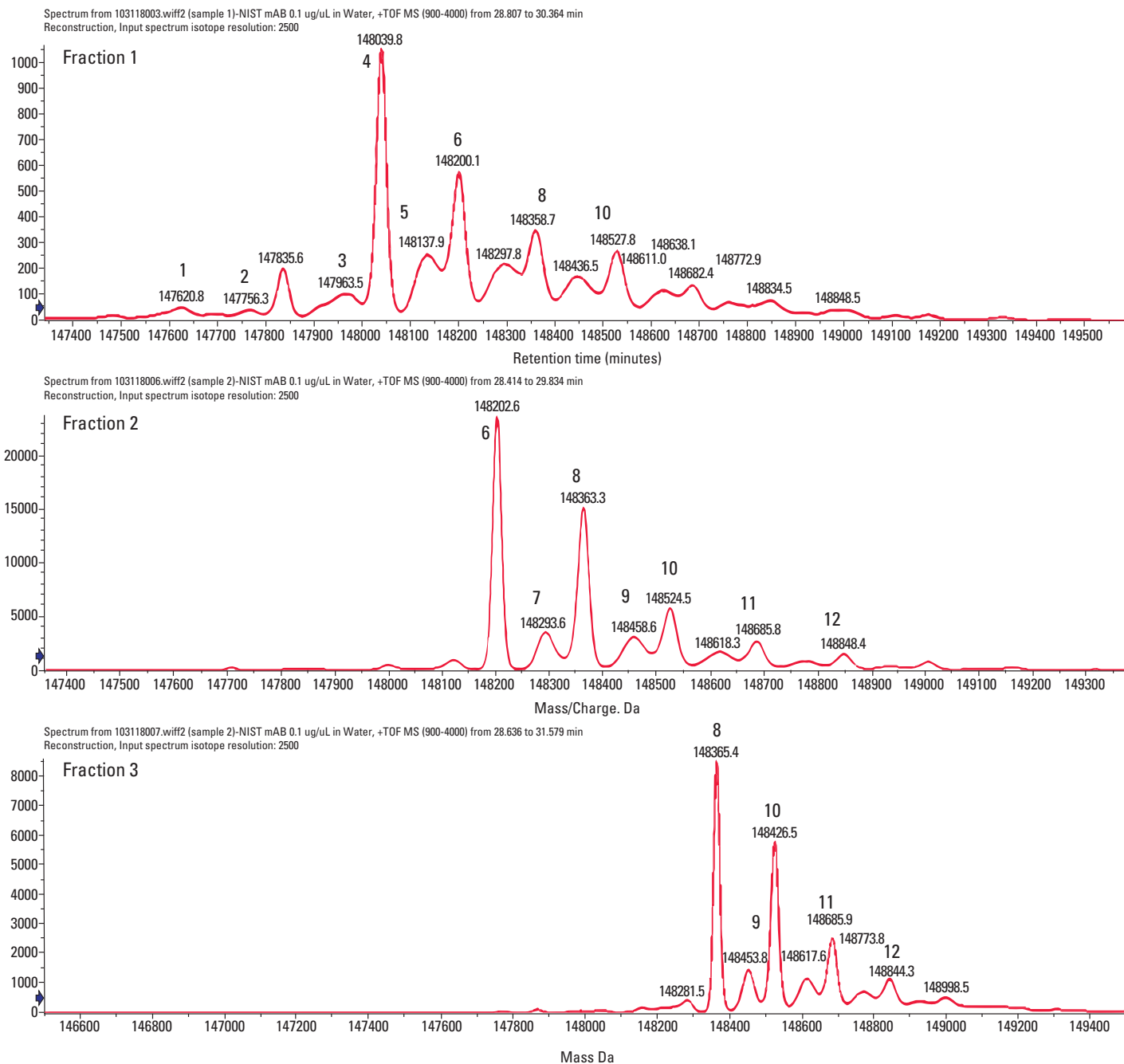


Figures 2 and 3 illustrate analysis of the NIST mAb standard compared against the collected peak fractions. It is observed that each peak has a unique composition of intact mAb glycoforms and that the selectivity of the stationary phase is based on the amount of terminal galactose units on the glycan moiety. This conclusion agrees with studies that show antibodies with higher amounts of G1- and G2-containing sugars show greater ADCC activity. Because of some peak overlap in the initial separation, there is some overlap of different galactose-containing species in the MS profile, though the general trend between galactose and activity has been confirmed.

Figure 2. The TIC, extracted spectrum and reconstructed spectrum for a NIST mAb control sample. The glycoforms observed for this sample are in agreement with accepted literature on characterization of this molecule.



**Figure 3.** Reconstructed spectra for each of the isolated peak fractions, indicating that later eluting fractions have a greater proportion of terminal galactose glycan sugars, consistent with observations of antibody activity and percentage of galactose.



Peak	Mass	Glycoform	Peak	Mass	Glycoform
1	147620	G0F/G0F (-2GlcNAc)	7	148292	G0F/G1F (Adduct)
2	147756	G0F/G0F (-GlcNAc)	8	148362	G1F/G1F
3	147966	G0F/G1F (-GlcNAc)	9	148455	G1F/G1F (Adduct)
4	148038	G0F/G0F	10	148524	G1F/G2F (+Hex)
5	148129	G0F/G0F (Adduct)	11	148684	G2F/G2F (+Hex)
6	148200	G0F/G1F	12	148843	G2F/G2F

### Conclusions

The separation of an IgG<sub>1</sub> molecule was demonstrated using the TSKgel FcR-IIIa-NPR column and peaks from that separation were characterized by high resolution mass spectrometry. The results support that the stationary phase selectivity is based on the same Fc-glycan/Fc receptor interaction as ADCC activity. The glycoform composition of each peak is consistent with previous published observations on the activity of N-glycan sugars with higher amounts of terminal galactose.

This application demonstrates the efficacy of this approach and characterization data that demonstrate the proof of concept of this chromatographic technique for a fast orthogonal analysis to evaluate mAb ADCC activity, potentially for early cell line development, bioreactor modeling and lot-to-lot comparability of therapeutic antibodies.

TSKgel and Tosoh Bioscience are registered trademarks of Tosoh Corporation  
NPR is a registered trademark of Tosoh Corporation in Japan, EU, China and India.  
Nexera is a registered trademark of Shimadzu Corporation