

# Separation of Intact Monoclonal Antibody Sialylation Isoforms by pH Gradient Ion-Exchange Chromatography

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## Key Words

Protein Heterogeneity, Cation Exchange, Glycosylation, ProPac SCX-10 Column

## Goal

To develop a higher-resolution separation of intact protein sialylation isoforms using the Thermo Scientific™ ProPac™ SCX-10 column on a Thermo Scientific™ Dionex™ UltiMate™ 3000 Dual BioRS LC System with either a salt or pH gradient

## Introduction

Glycosylated proteins—including erythropoietins, monoclonal antibodies (MAbs), and various hormones—constitute a large portion of major approved therapeutic biological drugs. Sialic acid, usually attached at terminal positions of glycan molecules, is important to many biological processes such as cell recognition and migration. Sialic acids also have significant effects on the properties of therapeutic proteins, especially on the circulation half-life of the protein. For example, the circulation half-life of sialylated rhEPO is 5.6 h, whereas that of nonsialylated rhEPO is 1.4 min.<sup>1</sup> Thus, monitoring protein glycosylation, including sialylation, is important for both glycoprotein characterization and quality control purposes.

Common approaches for monitoring glycosylated proteins include separating intact protein glycoforms and profiling oligosaccharides. Oligosaccharide profiling (both fluorescently labeled or unlabeled) requires releasing the glycan from the protein, whereas intact protein glycoforms monitoring is convenient and straightforward, and isolated protein glycoforms can be subjected to further characterization. A high-performance liquid chromatography (HPLC) method with higher resolution to separate protein glycoforms with different states of sialylation is needed. Because of the extra negative charge provided by sialic acid, anion-exchange chromatography is the ideal technique for resolving differently sialylated proteins. A recent report shows that pH-gradient-based ion-exchange chromatography (IEC) provides better resolution than routine salt-gradient-based IEC, especially for separation of protein isoforms.<sup>2</sup>



## Equipment

- UltiMate 3000 Dual BioRS LC system, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump (P/N 5042-0066)
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection (P/N 5825-0020)
  - TCC-3000SD Thermostatted Column Compartment (P/N 5730-0010)
  - DAD-3000 Diode Array Detector (P/N 5730-0010) with 13  $\mu$ L flow cell
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2

## Reagents and Standards

- Deionized (DI) water, 18.2 M<sup>-1</sup>cm resistivity
- 2-Propanol (Optima™), ≥99.9% (Fisher Scientific P/N A464-4)
- Sodium Phosphate Monobasic Dihydrate (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O), ≥99.0% (Fisher Scientific P/N S381-500)
- Ammonium Sulfate ([NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>), ≥99.0% (Fisher Scientific P/N A702-500)
- Tris(Hydroxymethyl)Aminomethane, Crystalline (Fisher Scientific P/N T370-500)
- Imidazole, ≥99.0% (Fisher Scientific P/N O3196-500)
- Sodium Chloride (NaCl), Crystalline/Biological, Certified (Fisher Scientific P/N S671-500)
- Piperazine, 99%, Extra Pure (Fisher Scientific P/N AC13129-5000)
- Triethanolamine, ≥99.0% (Fisher Scientific P/N T407-500)
- BIS-TRIS Propane, ≥99.0% (Fisher BioReagents P/N BP2929-25)
- 1-Methylpiperazine, ≥99.0% (Fisher Scientific P/N AC15691-1000)
- Hydrochloric Acid (HCl), 6N (Fisher Scientific P/N 23-011-111)
- Neuraminidase (Fisher Scientific P/N P0720S)
- PNGase F (Fisher Scientific P/N P0704S)

### Conditions

Columns: ProPac SCX-10 Analytical, 4 × 250 mm (P/N 054995)  
 ProPac SAX-10 Analytical, 4 × 250 mm (P/N 054997)  
 Thermo Scientific™ MAbPac™ SEC-1 Analytical, 4.0 × 300 mm (P/N 074696)  
 ProPac HIC-10 Analytical, 4.6 × 250 mm (P/N 074197)

Mobile Phase: **Formula 1** (for ProPac SCX-10 column, salt-gradient-based IEC),  
 A. 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 6.0  
 B. 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, pH 6.0

**Formula 2** (for ProPac SCX-10 column, pH-gradient-based IEC),  
 A. 2.4 mM Tris, 1.5 mM imidazole, 11.6 mM piperazine, pH 6.0  
 B. 2.4 mM Tris, 1.5 mM imidazole, 11.6 mM piperazine, pH 9.5

**Formula 3** (for ProPac SAX-10 column, salt-gradient-based IEC),  
 A. 10 mM Tris, 15 mM NaCl, pH 9.0  
 B. 10 mM Tris, 500 mM NaCl, pH 9.0

**Formula 4** (for ProPac SAX-10 column, pH-gradient-based IEC),  
 A. 20 mM piperazine, 20 mM triethanolamine, 20 mM BIS-TRIS propane, 20 mM 1-methylpiperazine, 5 mM NaCl, pH 10.5  
 B. 20 mM piperazine, 20 mM triethanolamine, 20 mM BIS-TRIS propane, 20 mM 1-methylpiperazine, 5 mM NaCl, pH 4

**Formula 5** (for MAbPac SEC-1 column), 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 M NaCl, pH 6.8

**Formula 6** (for ProPac HIC-10 column),  
 A. 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 7% 2-propanol, pH 7.0  
 B. 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 7% 2-propanol, pH 7.0

Gradient:	For all IEC		
Time (min)	% A	% B	
0	100	0	
30	0	100	
35	0	100	
35.5	100	0	
50	100	0	

For ProPac HIC-10 column		
Time (min)	% A	% B
0	100	0
40	0	100
50	0	100
50.5	100	0
6	100	0

Flow Rate: 1.0 mL/min for all IEC  
 0.6 mL/min for ProPac HIC-10 column  
 0.3 mL/min for MAbPac SEC-1 column

Injection Volume: 50 µL

Temperature: 30 °C

Detection: UV, absorbance at 280 nm

## Preparation of Solutions

### Preparation of Mobile Phases

#### Formula 1

For Mobile Phase A, dissolve 3.12 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O in 950 mL DI water, adjust the pH to 6.0 with 50% NaOH solution, and bring the volume to 1000 mL with DI water.

For Mobile Phase B, dissolve 3.12 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O and 29.22 g NaCl in 950 mL DI water, adjust the pH to 6.0 with 50% NaOH solution, and bring the volume to 1000 mL with DI water.

#### Formula 2

Dissolve 0.581 g Tris, 0.204 g imidazole, and 1.998 g piperazine in 1900 mL DI water. Once dissolved, split the solution into two equal aliquots. Titrate each aliquot to the appropriate pH (6.0 for Mobile Phase A and 9.5 for Mobile Phase B) by adding 6 N HCl. Once titrated, bring the volume of each aliquot to 1000 mL with DI water.

*Note: Thermo Scientific CX-1 pH Gradient Buffers A (pH 5.6, P/N 083273) and B (pH 10.2, P/N 083275) can be substituted for Mobile Phases A and B, respectively. The CX-1 pH Gradient Buffers show higher resolution than this formula (data presented in a separate Thermo Scientific application note).*

#### Formula 3

For Mobile Phase A, dissolve 1.211 g Tris and 0.877 g NaCl in 950 mL DI water, adjust the pH to 9.0 with 6 N HCl, and bring the volume to 1000 mL with DI water.

For Mobile Phase B, dissolve 1.211 g Tris and 29.22 g NaCl in 950 mL DI water, adjust the pH to 9.0 with 6 N HCl, and bring the volume to 1000 mL with DI water.

#### Formula 4

Dissolve 3.446 g piperazine, 5.328 mL triethanolamine, 11.294 g BIS-TRIS propane, 4.436 mL 1-methylpiperazine, and 0.584 g NaCl in 1900 mL DI water. Once dissolved, split the solution into two equal aliquots. Titrate each aliquot to the appropriate pH (10.5 for Mobile Phase A and 4.0 for Mobile Phase B) by adding 6 N HCl. Once titrated, bring the volume of each aliquot to 1000 mL with DI water.

#### Formula 5

Dissolve 7.8 g  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and 17.532 g NaCl in 950 mL DI water, adjust the pH to 6.8 with 50% NaOH solution, and bring the volume to 1000 mL with DI water.

#### Formula 6

For Mobile Phase A, dissolve 14.51 g  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and 245.780 g  $(\text{NH}_4)_2\text{SO}_4$  in 750 mL DI water, adjust the pH to 7.0 with 50% NaOH solution, and bring the volume to 930 mL with DI water. Then bring the volume to 1000 mL with 2-propanol.

For Mobile Phase B, dissolve 14.51 g  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  in 900 mL DI water, adjust the pH to 7.0 with 50% NaOH solution, and bring the volume to 930 mL with DI water. Then bring the volume to 1000 mL with 2-propanol.

### Sample Preparation

The MAb sample was kindly provided by Shanghai National Engineering Research Center of Antibody Medicine Co., Ltd.

#### Neuraminidase Digestion Procedure

This procedure follows vendor-recommended reaction conditions with minor modifications.

Combine 4  $\mu\text{L}$  (equal to 20  $\mu\text{g}$ ) MAb and 31  $\mu\text{L}$   $\text{H}_2\text{O}$  to make a 35  $\mu\text{L}$  total reaction volume. Add 5  $\mu\text{L}$  of 10 $\times$  G1 Reaction Buffer (provided with the neuraminidase) to make a 40  $\mu\text{L}$  total reaction volume. Add 10  $\mu\text{L}$  neuraminidase and incubate at 37  $^\circ\text{C}$  for 1 h.

### Results and Discussion

#### Separation of MAbs by Size-Exclusion and Hydrophobic Interaction Chromatography

Size-exclusion chromatography (SEC) and hydrophobic-interaction chromatography (HIC) are commonly used to monitor the purity of MAbs. The MAb separation using a MAbPac SEC-1 column (Figure 1, Chromatogram A) proves that the MAb sample is mainly monomer (Peak 1), with small fractions of dimer aggregates (Peak 2) and multimer aggregates or unknown impurities (Peak 3). As previously reported, HIC can separate MAb deamidation, heavy chain C-terminal heterogeneity, and methionine or tryptophan oxidation.<sup>3</sup> Thus, the MAb separation using the ProPac HIC column (Figure 1, Chromatogram B) shows that the MAb sample had only minor fractions of MAb species with these modifications. This chromatogram also shows that the MAb sample is primarily pure with respect to its hydrophobicity.

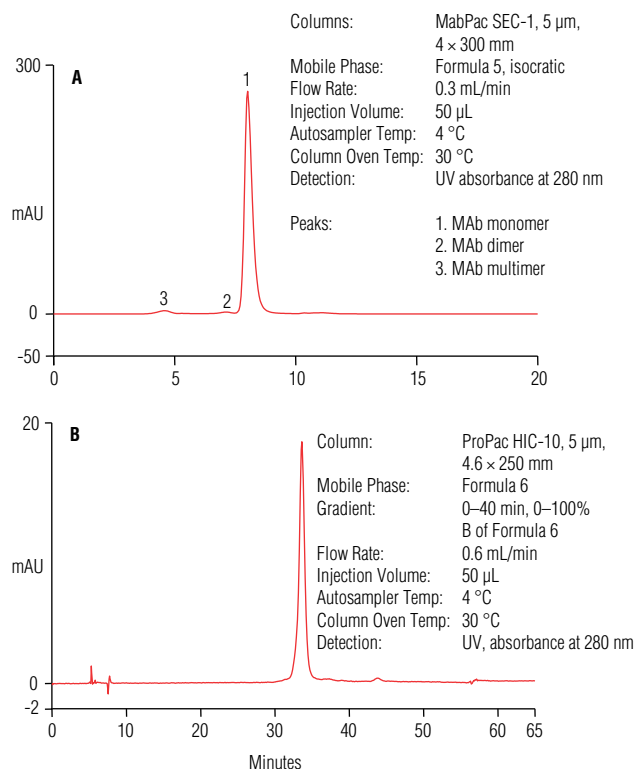


Figure 1. MAb separation using (A) the MabPac SEC-1 column and (B) the ProPac HIC-10 column.

#### Separation of MAbs by Ion-Exchange Columns

To characterize post-translational modifications (PTMs), IEC has proven to be the most powerful method currently available. Salt-gradient-based IEC—the most frequently used IEC—has successfully separated various PTMs such as C-terminal lysine cleavage, amidation, glutamine cyclization, oxidation, deamidation, and others.<sup>3–4</sup> Recently, pH-gradient based IEC has provided higher resolution in the separation of protein isomers.<sup>2</sup>

Sialylation contributes extra negative charges to the glycoprotein; therefore, the ProPac SAX-10 column was used for charge profiling of MAb sialylation in this study. The ProPac WCX-10, ProPac SCX-10, and MAbPac SCX-10 columns—additional products for MAb variant analysis—feature unique selectivity and high resolving power. The ProPac WCX-10 and MAbPac SCX-10 columns have proven especially powerful for MAb variant analysis. The ProPac SCX-10 column was used in this study because it uses the same general column architecture as the ProPac SAX-10 column.

The chromatograms of a strong anion-exchange column (the ProPac SAX-10 column) and a strong cation-exchange column (the ProPac SCX-10 column) were compared for MAb charge variant separation. Both columns were tested using both salt and pH gradients. Figure 2, Chromatogram A shows that using a salt gradient on the ProPac SCX-10 column does not resolve the charge variants of this MAb. In comparison, a pH gradient on the ProPac SAX-10 column and a salt gradient on the same column resolve six MAb isoforms (Figure 2, Chromatograms B and C). The isoform peaks are not baseline separated in either chromatogram. Thus, the resolution cannot be used for precise quantification or purification of individual glycoforms for further characterization.

In comparison, the MAb charge variants are baseline resolved with a pH gradient on the ProPac SCX-10 column. Figure 2, Chromatogram D shows that 10 MAb charge variants can be separated, and the peak resolution is better than the other Figure 2 chromatograms. Table 1 shows the detailed resolution data for the main peaks (Peaks 3–6), as reported by Chromeleon CDS software.

### Characterization of MAb Charge Variants

The pattern of evenly distributed MAb charge variants is similar to sialylation profiling by isoelectric focusing (IEF).<sup>4,5</sup> The authors attempted to show that separation of MAb charge variants on the ProPac SCX-10 column was also due to sialylation using desialylation by neuraminidase and deglycosylation by PNGase F. Figure 3 shows the MAb sample with and without neuraminidase treatment. After neuraminidase treatment, Peaks 1–6 completely disappeared and Peaks 7 and 8 increased. This strongly suggests that desialylation to produce neutral glycans on the MAb diminishes its overall charge and makes it more positive. This results in later elution by cation-exchange chromatography.

Thus, the assumption that the ProPac SAX-10 column would be best to separate sialylated forms of the MAb is incorrect; for this particular MAb, the ProPac SCX-10 column proved better. The PNGase F treatment experiment was unsuccessful because no conditions were found to completely deglycosylate without denaturing the protein.

Table 1. Comparison of MAb charge variant resolution values.

Chromatography Method	Peak 3	Peak 4	Peak 5	Peak 6
Salt-Gradient-Based Strong Cation Exchange	Not Applicable			
pH-Gradient-Based Strong Anion Exchange	1.40	1.41	1.44	1.44
Salt-Gradient-Based Strong Anion Exchange	1.52	1.50	1.47	1.48
pH-Gradient-Based Strong Cation Exchange	3.05	2.73	2.54	2.83

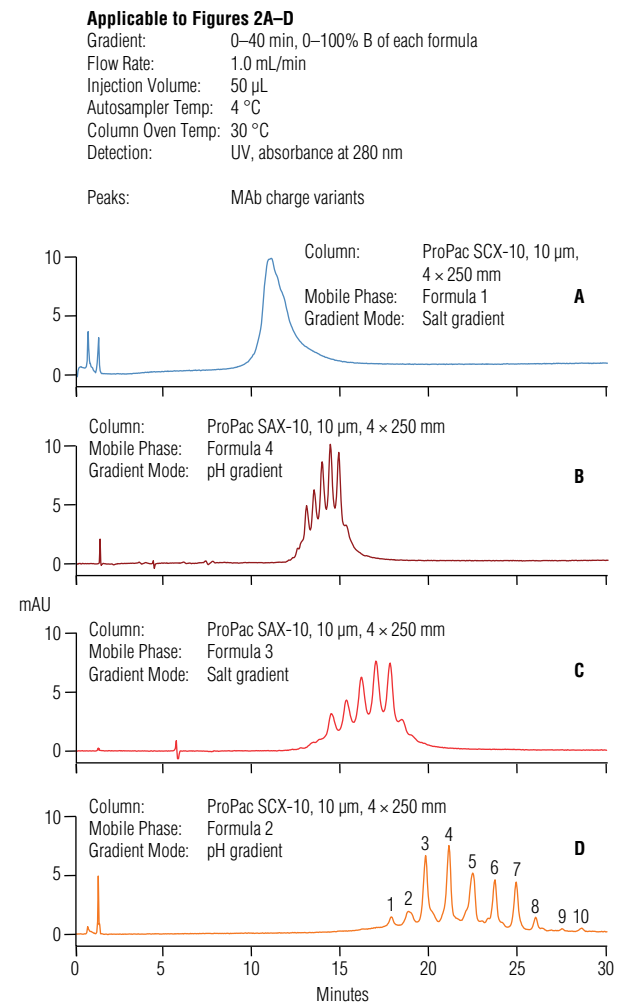


Figure 2. Separation of MAb charge variants.

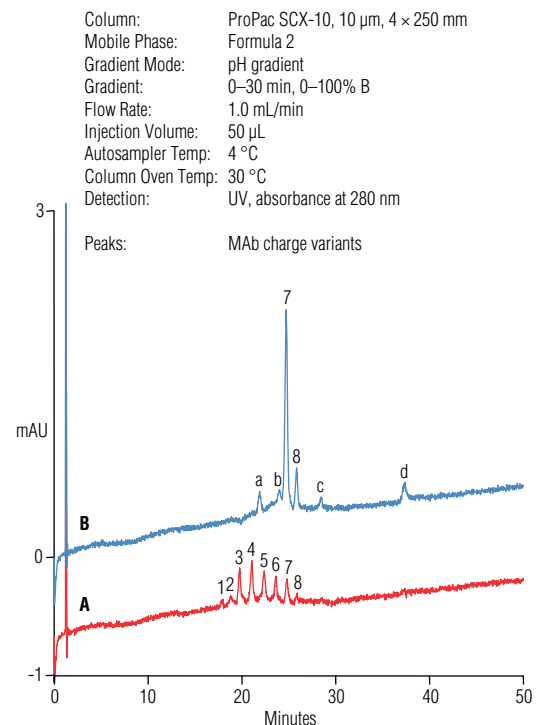


Figure 3. MAb neuraminidase digestion (A) without and (B) with neuraminidase treatment.

## Conclusion

This study shows that pH-gradient-based strong cation-exchange chromatography on the ProPac SCX-10 column can provide excellent resolution for MAb charge variants. The variants were partially identified as sialylation variants. Compared to the routine IEF method, this method is more convenient and straightforward for protein quality control (i.e., protein sialylation profiling). Future work will focus on fast separation using the smaller particle size MAbPac SCX-10 column and the UltiMate-3000 BioRS system.

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