

# Monitoring Monoclonal Antibody Heterogeneity by Cation-Exchange Chromatography

## **INTRODUCTION**

During the development and production of therapeutic proteins, characterization of structural variants is a critical challenge. C-terminal processing of lysine residues on the heavy chain of monoclonal antibodies (MAbs) is a common structural variation that demands careful analysis.<sup>1-7</sup> As a result of this processing, C-terminal lysine or arginine residues are often absent in proteins isolated from mammalian cell cultures, even though their presence may be expected on the basis of the gene sequence. This discrepancy, which is common in plasma-derived proteins, results from the activity of one or more basic carboxypeptidases. Incomplete protein processing results in charge heterogeneity which is readily identified by cation-exchange chromatography on the Dionex ProPac® WCX-10, a weak cation-exchange column. The packing in this column is a unique pellicular resin with a hydrophilic coating and

carboxylate functional groups on grafted linker arms. The physicochemical properties of this support eliminate secondary (nonionic) interactions between the protein analytes and the stationary phase, affording minimal band broadening and high selectivity.<sup>8</sup> The UltiMate® 3000 Titanium System is an HPLC whose flow path ensures that neither solvents nor sample are in contact with stainless steel materials, removing concerns about iron and other transition metals contaminating the column and samples. This application note describes a method for analyzing a humanized IgG<sub>1</sub> MAb for C-terminal lysine variants. These variants are baseline resolved from the native antibody using the ProPac WCX-10 column and a 4-morpholineethanesulfonic acid (MES)-based mobile phase. Other acidic and basic variants are also better resolved than had been observed with traditional phosphate-based mobile phases buffered at pH 7, enabling the detection of additional MAb variants, each with heavy chain C-terminal lysine heterogeneity.

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## **EQUIPMENT**

Dionex UltiMate 3000 Titanium System consisting of:  
SRD-3600 Solvent Rack with 6 Degasser Channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2-L glass bottles for each pump  
LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability  
WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020) with 50  $\mu$ L biocompatible sample loop  
TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK ten-port two-position valves (P/N 5723.0025) for added productivity  
VWD-3400 Variable Wavelength Detector (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)  
Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)  
Chromleon<sup>®</sup> Chromatography Data System  
Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)  
Filter unit, 0.2  $\mu$ m Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent Nylon filter)  
Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)  
0.3 mL polypropylene (Vial Kit, P/N 055428) injection vials with caps  
Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

## **REAGENTS AND STANDARDS**

Deionized water, 18 M $\Omega$ -cm resistance or higher  
4-Morpholineethanesulfonic acid (MES) hydrate, minimum 99.5% titration (Sigma-Aldrich; P/N M8250)  
Sodium chloride, crystal (J.T. Baker; P/N 4058-05)  
Sodium hydroxide solution, 50% W/W (Thermo Fisher Scientific; P/N SS254)  
Carboxypeptidase B, chromatographically purified,  $\geq$  170 U/mg, 5.0 mg/mL (Worthington Biochemical Corporation; P/N LS005305) or equivalent  
Humanized monoclonal IgG<sub>1</sub> antibody (a generous gift from a biotechnology company)

## **CONDITIONS**

### **Method**

Column: ProPac WCX-10 Analytical  
4  $\times$  250 mm (P/N 054993)  
Flow Rate: 1.00 mL/min  
Inj. Volume: 10  $\mu$ L (partial loop)  
Sampler Temp: 5  $^{\circ}$ C  
Column  
Temperature: 30  $^{\circ}$ C  
Detection: Absorbance, 280 nm (absorbance at 214 and 254 nm also collected)  
Data  
Collection Rate: 1.0 Hz  
Noise: 12-24  $\mu$ AU  
Typical System Operating  
Backpressure:  $\sim$  125 bar ( $\sim$ 1830 psi)  
Mobile Phase: A: 20 mM MES,  
60 mM sodium chloride, pH 5.6  
B: 20 mM MES,  
240 mM sodium chloride, pH 5.6  
Gradient: Linear, 20% B for 2 min,  
20–50% B from 2–52 min

## Gradient Method

Time (min)	A(%)	B(%)	Comments
-20.00	80.0	20.0	Equilibration
0.00	80.0	20.0	Sample Injection
2.00	80.0	20.0	Sample Binding
52.00	50.0	50.0	End Gradient
52.10	0.0	100.0	Column Regeneration
57.00	80.0	20.0	Re-equilibration

## PREPARATION OF SOLUTIONS AND REAGENTS

All mobile phases are filtered through a 0.2  $\mu\text{m}$  nylon filter under vacuum to remove particulates and to degas prior to their use. The mobile phases are blanketed under 34–55 kPa (5–8 psi) of helium headspace to reduce the growth of opportunistic microorganisms, and maintain a low concentration of dissolved air.

### 20 mM MES, 60 mM Sodium Chloride, pH 5.6 (Mobile Phase A)

Combine 7.81 g of MES, 7.01 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500  $\mu\text{L}$  needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

### 20 mM MES, 240 mM Sodium Chloride, pH 5.6 (Mobile Phase B)

Combine 7.81 g of MES, 28.04 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500  $\mu\text{L}$  needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

## Sample Preparation

MAB samples were diluted to 10 mg/mL using filtered, degassed water. One 100  $\mu\text{L}$  aliquot of the MAB solution was combined in a microcentrifuge tube with 0.5  $\mu\text{L}$  of thawed carboxypeptidase B solution using a micropipette while another 100  $\mu\text{L}$  aliquot was combined with 0.5  $\mu\text{L}$  of DI water. Both sample and control were incubated at 37  $^{\circ}\text{C}$  for 2 h. After the 2-h incubation period, sample and control were diluted 5-fold with Mobile Phase A and analyzed.

## RESULTS AND DISCUSSION

The ProPac WCX-10 weak cation-exchange column was assessed for its ability to separate humanized IgG<sub>1</sub> variants. As shown in Figure 1 this antibody not only produces three major peaks but also separates a number of other variants, both more acidic as well as more basic variants compared to the three major peaks. The MES-based buffers resolved additional details not seen when using phosphate-based buffers, an observation sometimes reported when separating other protein variants.<sup>9</sup>

To verify that the three major peaks were due to variations in C-terminal lysine content, the IgG<sub>1</sub> sample was treated with carboxypeptidase B, an exopeptidase that specifically cleaves C-terminal lysine residues. A comparison of the two chromatographic traces in Figure 2 shows a disappearance of the second and third major peaks, which are proposed to contain one and two terminal heavy chain lysine residues, respectively. The disappearance of peaks 2 and 3 was accompanied by a corresponding increase in the area under the first major peak, the variant with no terminal lysine. Additionally, the pattern for the minor peaks eluting before the first major peak is maintained, suggesting that these peaks do not contain terminal heavy chain lysine residues. The complex chromatographic pattern of peaks eluting after the three major peaks is also simplified with carboxypeptidase B incubation, showing MAB variants, each with heavy chain C-terminal lysine heterogeneity, are present in the original sample.

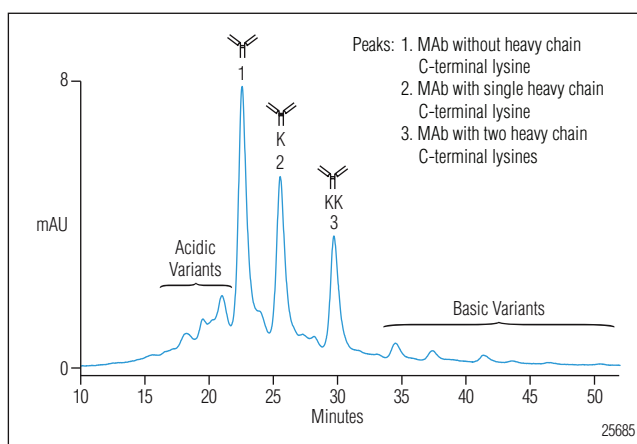


Figure 1. Separation of acidic and basic terminal lysine variants of an IgG<sub>1</sub> monoclonal antibody using the ProPac WCX-10 column and MES-based buffers on the UltiMate 3000 Titanium System.

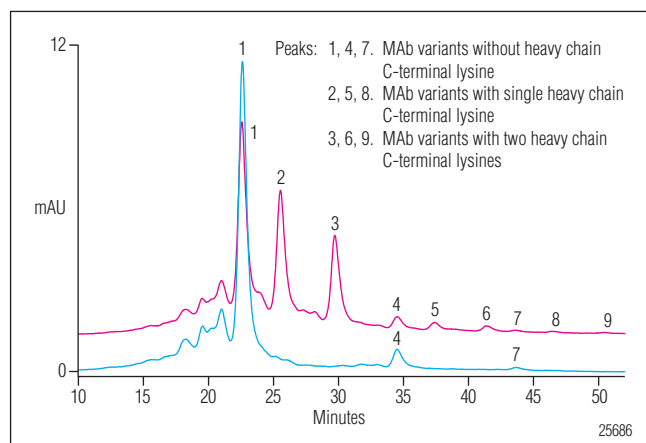


Figure 2: Analysis of IgG<sub>1</sub> monoclonal antibody before (upper trace) and after (lower trace) treatment with carboxypeptidase B for 2 h at 37 °C.

## CONCLUSION

The ProPac WCX-10 weak cation-exchange column, used with MES-based buffers on an inert titanium system, produced detailed chromatograms of variants of an IgG<sub>1</sub> MAb, including baseline separation of C-terminal lysine variants. These results demonstrate that this chromatographic system is well suited for analyses such as quality control monitoring of protein-based therapeutics that require high efficiency, high resolution separation of closely eluting proteins.

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LPN 1047-01 PDF: 02/09  
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