

# Monitoring Monoclonal Antibody Stability by Cation-Exchange Chromatography

## **INTRODUCTION**

Protein microheterogeneity can be attributed to a variety of post-translational modifications including glycosylation, oxidation, phosphorylation, amino-terminal modifications (e.g., to pyroglutamate), asparagine (Asn) deamidation,<sup>1</sup> and incomplete C-terminal processing.<sup>2</sup> Variations in protein composition can impact a protein's activity and stability as a biotherapeutic.<sup>3-7</sup> Monitoring stability of therapeutic proteins and peptides is regarded as essential for demonstrating safety and efficacy of these drugs, and is expected by the FDA and other regulating agencies.

Recombinant monoclonal antibodies (MAb) have been shown to have heterogeneity with either arginine (Arg) or lysine (Lys) at the C-terminus of the heavy chain(s). When a MAb is treated with carboxypeptidase B, an exopeptidase, Arg and Lys are cleaved from the C-terminus, eliminating C-terminal heterogeneity.<sup>8-14</sup> Proteins and peptides containing Asn adjacent to glycine

(Gly) are particularly susceptible to Asn deamidation, converting Asn to aspartic acid (Asp) or isoaspartic acid to varying extents.<sup>15-18</sup> Monitoring the extent of deamidation is of interest to quality control and process development chemists concerned with product quality and stability.

Shelf-life studies of proteins and peptides typically monitor a variety of degradation products. This evaluation commonly uses ion-exchange chromatography. In this application note a sample of humanized MAb was first treated with carboxypeptidase B to remove C-terminal lysine charge heterogeneity. This modified antibody was subsequently subjected to forced deamidation conditions.<sup>19</sup> Changes in MAb heterogeneity were monitored using the Dionex ProPac<sup>®</sup> WCX-10 weak cation-exchange column housed in the UltiMate<sup>®</sup> 3000 Titanium HPLC System. The combination of a bio-inert HPLC system with a high resolution cation-exchange column is well suited for the analysis of protein microheterogeneity.<sup>20</sup>

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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  
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TCC-3000 Column Compartment without Switching  
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Compartment with 2 PEEK ten-port two-position  
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Chromleon<sup>®</sup> Chromatography Data System  
Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)  
Filter unit, 0.2 µm 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  
eluent)  
SpeedVac Evaporator System (Thermo Scientific Savant  
or equivalent) consisting of:  
SpeedVac microcentrifuge, model SVC100  
Refrigerator Vapor Trap, Model RVT400  
Vacuum Gauge, Model VG-5  
Welch Duo-Seal Vacuum Pump, Model 1402 capable  
of pulling 0.2 Torr (200 µm Hg) vacuum  
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 MΩ-cm resistance or higher  
MES hydrate, minimum 99.5% titration  
(Sigma-Aldrich; P/N M8250)  
Sodium chloride, crystal (J.T. Baker; P/N 4058-05)  
Hydrochloric acid, Ultrapure Reagent (J.T. Baker,  
P/N 6900-05)  
Sodium hydroxide solution, 50% W/W  
(Thermo Fisher Scientific; P/N SS254)  
Carboxypeptidase B, chromatographically purified,  
≥ 170 U/mg, 5.0 mg/mL (Worthington Biochemical  
Corporation; P/N LS005305) or equivalent  
Ammonium carbonate, HPLC Reagent grade  
(J.T. Baker; P/N 0651-08)  
Sodium azide (Sigma-Aldrich; P/N S8032)  
Protease inhibitor cocktail, general use, containing  
4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF),  
trans-epoxysuccinyl-L-leucyl-amido (4-guanidino)  
butane (E-64), bestatin, leupeptin, aprotinin, and  
sodium EDTA (Sigma-Aldrich; P/N P2714)  
or equivalent  
Ribonuclease A, Type XII-A from bovine pancreas,  
minimum 90% (Sigma-Aldrich; P/N R5500)  
Humanized monoclonal antibody (IgG<sub>1</sub>,  
50 mg protein/mL) was a generous gift from a  
biotechnology company)

## **CONDITIONS**

### **Method**

Column: ProPac WCX-10 Analytical  
4 × 250 mm (P/N 054993)  
Flow Rate: 1.00 mL/min  
Inj. Volume: 10 µL (partial loop)  
Autosampler Temp: 5 °C  
Column Temp: 30 °C  
Detection: Absorbance, 280 nm (absorbance at  
214 and 254 nm also collected)  
Data  
Collection Rate: 1.0 Hz  
Noise: 12-24 µAU  
Typical System Operating  
Backpressure: ~ 125 bar (~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
0.0	80.0	20.0	Sample Injection
2.0	80.0	20.0	Sample Binding
52.0	50.0	50.0	End Gradient
52.1	0.0	100.0	Column Regeneration*
57.0	80.0	20.0	Start Re-equilibration
77.0	80.0	20.0	End Re-equilibration

\*Need for column regeneration has not been demonstrated

#### PREPARATION OF SOLUTIONS AND REAGENTS

Prepare mobile phases as described below. Slightly different volumes of sodium hydroxide may be required to produce 2 L volumes of mobile phases A and B. Filter mobile phases through a 0.2 µm nylon filter under vacuum to remove particulates and to degas. Blanket the mobile phases 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.

#### Buffers

##### 20 mM MES, 60 mM Sodium Chloride, pH 5.6 (Buffer 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 (~650 µL 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 (Buffer 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 (~650 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

#### Stock Standards

##### 10% Ammonium Carbonate Buffer, pH 8.2

Dissolve 1.5 g ammonium carbonate in 12.0 mL water. Adjust pH to 8.2 with concentrated HCl.

Adjust total volume to 15.0 mL with additional water.

##### 1% Sodium Azide

Dissolve 10 mg sodium azide in 1.0 mL water

*Caution: sodium azide is very toxic. Use necessary precautions to protect against exposure*

##### 10× Protease Inhibitor Cocktail

Reconstitute the protease inhibitor cocktail vial with 10 mL DI water. Cocktail concentrate consists of 20 mM AEBSF, 14 µM E-64, 1.3 mM Bestatin, 10 µM Leupeptin, 3 µM Apotinin, and 10 mM EDTA.

##### Ammonium Carbonate-Azide-Protease Inhibitor Cocktail Buffer (ACAPIC buffer)

Combine:

1.05 mL 10% ammonium carbonate

0.53 mL 1% sodium azide

0.11 mL 10× protease inhibitor cocktail

8.31 mL water

Adjust pH to 8.2, if necessary.

##### Monoclonal Antibody

Dilute with mobile phase A to yield a 20 mg/mL solution. Use immediately after dilution.

##### Ribonuclease A (positive control)

Prepare 100 µL of a 20 mg/mL solution in mobile phase A. Use immediately after preparation.

## SAMPLE PREPARATION

Combine 35  $\mu\text{L}$  of 20 mg/mL MAb with 1.0  $\mu\text{L}$  of carboxypeptidase B, and incubate at 37  $^{\circ}\text{C}$  for 2 h. Remove a 2.0  $\mu\text{L}$  aliquot, dilute to 1.0 mg/mL MAb with mobile phase A, and analyze 10  $\mu\text{L}$  of the solution to ensure the removal of MAb heavy chain C-terminal lysines by carboxypeptidase B. Concurrently incubate positive controls (ribonuclease A) and negative controls (mobile phase A), each with and without carboxypeptidase B. Combine the remaining 34  $\mu\text{L}$  of carboxypeptidase B-treated MAb sample with 627  $\mu\text{L}$  of ACAPIC. Final deamidation conditions consist of 1.0 mg/mL protein (MAb or ribonuclease A positive control), 1% ammonium carbonate, 0.05% sodium azide, 210  $\mu\text{M}$  AEBSF, 150  $\mu\text{M}$  E-64, 14  $\mu\text{M}$  Bestatin, 0.1  $\mu\text{M}$  Leupeptin, 0.03  $\mu\text{M}$  Aprotinin, and 100  $\mu\text{M}$  EDTA. Incubate at 37  $^{\circ}\text{C}$ , remove 100  $\mu\text{L}$  aliquots at 0, 25, and 50 h, add 900  $\mu\text{L}$  of DI water, and freeze these samples. When ready to analyze the samples, remove water and other volatile components by placing frozen samples into a SpeedVac evaporator system. Add 300  $\mu\text{L}$  of water to the dried samples, freeze, and repeat the evaporation process. Dissolve the remaining solid in 100  $\mu\text{L}$  of mobile phase A, transfer to a 0.3 mL autosampler vials, and analyze a 10  $\mu\text{L}$  injection. The final protein concentration is 1.0 mg/mL. Controls include ribonuclease A (positive control) with and without carboxypeptidase B, and reagent blank with and without carboxypeptidase B.

## RESULTS AND DISCUSSION

The MAb solution was treated with carboxypeptidase B to make the results of antibody exposure to deamidation conditions simpler to interpret. Heavy chain C-terminal lysine heterogeneity (the three major peaks between 20-30 min retention time in Figure 1-panel A) is eliminated after carboxypeptidase treatment (Figure 1-panel B).<sup>2</sup> Ribonuclease A, tested in parallel as a positive control, did not show any change upon addition of carboxypeptidase B (data not shown). The carboxypeptidase B-treated antibody and treated controls were then incubated for up to 50 h at 37  $^{\circ}\text{C}$  in 1% ammonium carbonate containing sodium azide to eliminate microbial contamination, and a broad spectrum protease inhibitor cocktail to stop proteolytic cleavage from contaminating proteases that may be present in the carboxypeptidase B preparation.

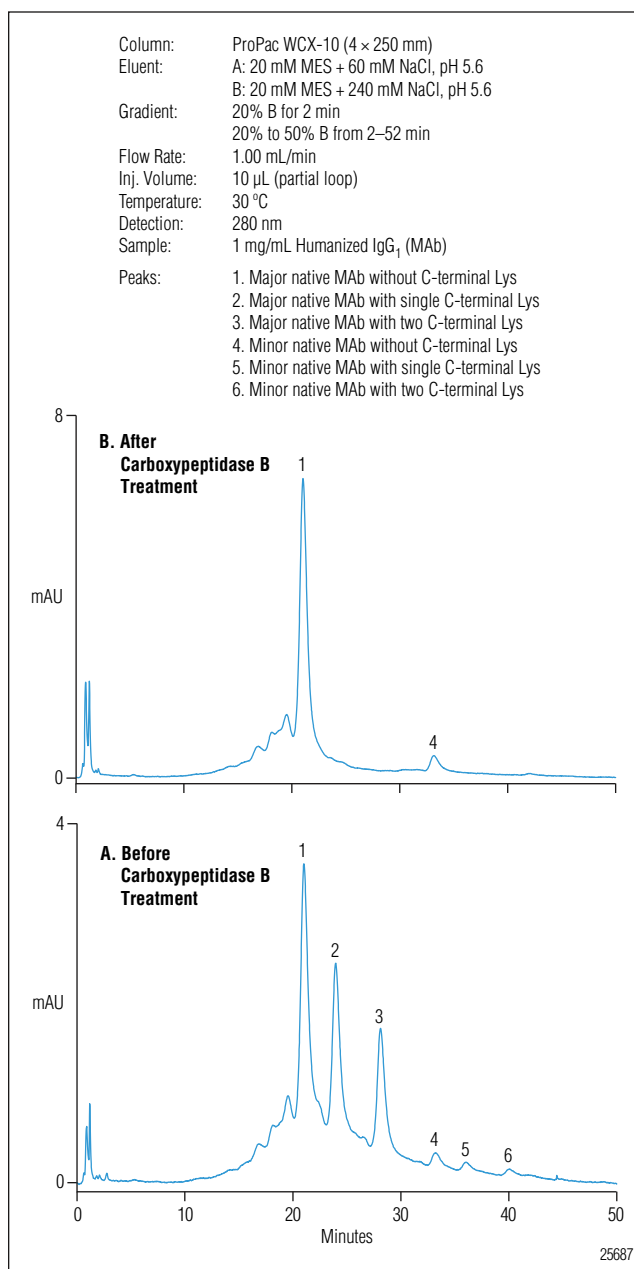


Figure 1. Monoclonal antibody (MAb) separations before (panel A) and after (panel B) carboxypeptidase B treatment (2 h at 37  $^{\circ}\text{C}$ ).

Figure 2 shows chromatograms from aliquots taken 0, 25, and 50 h after adding the ACAPIC mixture and incubating at 37 °C. Several incompletely resolved peaks (#1-4 in Figure 2) eluting before the major native MAb peak increase in abundance at longer incubation times. The appearance of these multiple peaks is expected as a consequence of deamidation.<sup>1,5,19</sup> Having Asn adjacent to Gly makes the Asn susceptible to conversion to either Asp or isoaspartic acid. While the MAb's primary sequence was not disclosed, the complexity of the chromatogram implies the presence of multiple deamidation sites. The ribonuclease A positive control for deamidation showed the predicted formation of new peaks<sup>1</sup>, confirming that this experiment replicated forced deamidation conditions. Peak 6, eluting after the major native MAb, appears immediately at time zero and is not present in the buffer control sample. Its area decreases with increasing incubation time. The source of this peak is unknown but may be the result of a rapid initial degradation. Peaks 1–4, and 6 are broadly defined as decomposition products because there is no additional analysis to confirm deamidation of the monoclonal antibody.

## CONCLUSION

This study highlights the high efficiency of the ProPac WCX-10 column, coupled with an inert, titanium HPLC system, for the analysis of protein charge microheterogeneity. This system is an alternative to reversed-phase or affinity chromatography for characterizing proteins.

*Caution: Sodium azide and some ingredients in the protease inhibitor cocktail are toxic. Consult the relevant MSDS and use necessary precautions to protect against exposure.*

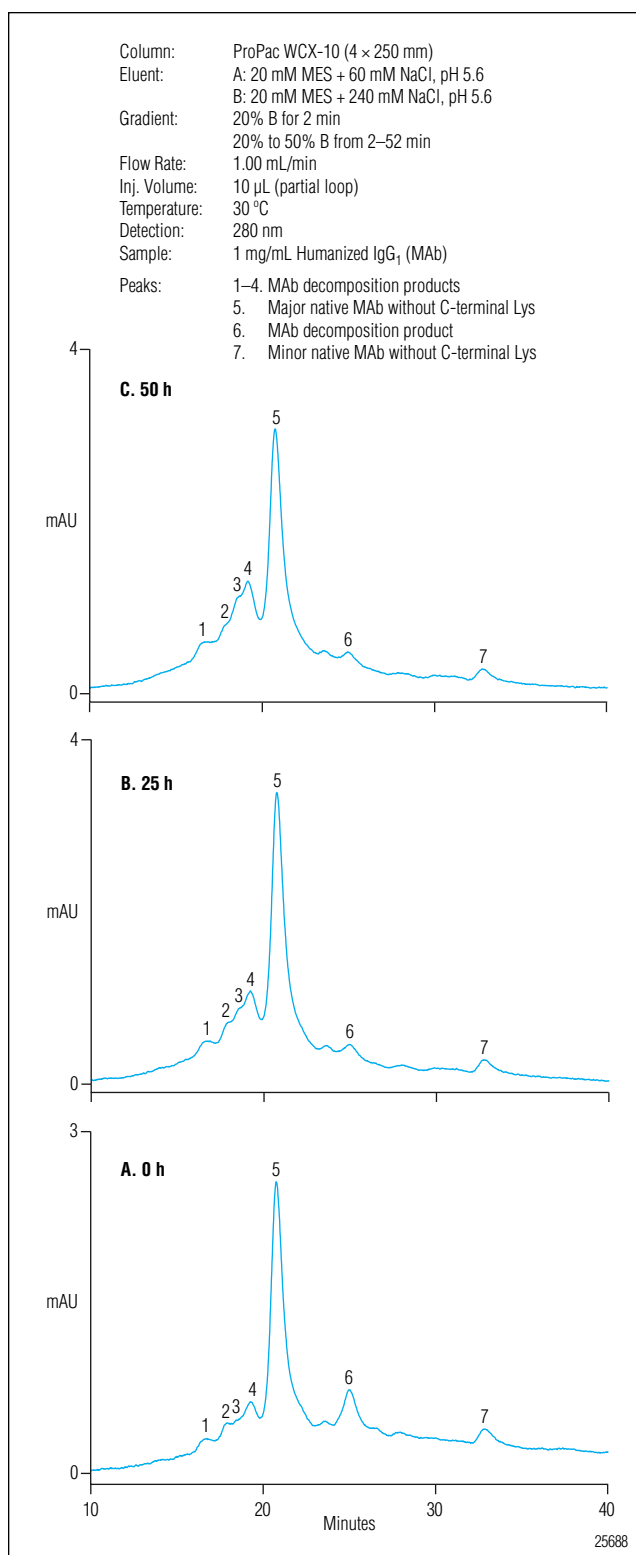


Figure 2. Forced deamidation of carboxypeptidase B-treated MAb (1 mg/mL) incubated at 37 °C with 1% ammonium carbonate – 0.05% sodium azide – 0.1× protease inhibitor cocktail mixture for 0 h (panel A), 25 h (panel B), and 50 h (panel C).

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