

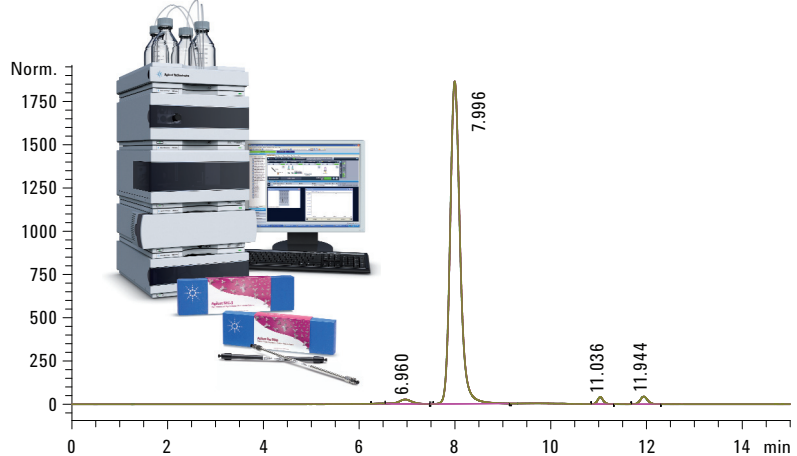
# Characterization of monoclonal antibodies on the Agilent 1260 Infinity Bio-inert Quaternary LC by Size Exclusion Chromatography using the Agilent BioSEC columns

## Application Note

### Biopharmaceuticals

#### Authors

Srividya Kailasam, Jochen Strassner,  
Faizy Ahmed and Martin Vollmer  
Agilent Technologies  
Bangalore, India and  
Waldbronn, Germany



#### Abstract

The Agilent 1260 Bio-inert Quaternary LC System and Agilent BioSEC columns were used to investigate the purity and stability of two monoclonal antibodies (mAb): Anti-FLAG and BL05. The high sensitivity of the Agilent 1260 Infinity Diode Array Detector was used to characterize these antibodies at low concentrations. The bio-inert LC system in combination with BioSEC columns resulted in the development of reliable and rugged methods amenable for routine size exclusion chromatography of therapeutic mAbs and proteins. For both the mAbs, superior separation of variants from the main antibody peak was achieved and compared to the results obtained with a competitive column.



**Agilent Technologies**

## **Introduction**

Sensitive analytical techniques are necessary for monitoring the purity of recombinant proteins and mAbs during formulation, storage, and clinical use. The aggregates that form in these samples due to improper production, formulation, storage, or handling conditions can be separated and detected based on their difference in size from the main drug product. Typically, Size Exclusion Chromatography (SEC) coupled to UV detection is used for analyzing aggregation and conformational variants in order to monitor the integrity of the investigated sample. This Application Note demonstrates the suitability of the Agilent 1260 Bio-inert Quaternary LC System for separating and monitoring the integrity of mAbs by SEC with Agilent BioSEC columns. The instrument, in combination with smaller particle columns, provides a very efficient tool for separating variants from the native conformations while minimizing secondary interaction on both the instrument and the column particle surface.

## **Experimental**

### **System**

The completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC system with a maximum pressure of 600 bar consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)  
Agilent 1260 Infinity Thermostatted Column Compartment containing Bio-inert heating elements (G1316C #19)
- Agilent 1260 Infinity Diode Array Detector with bio-inert Max-Light 60 mm, high sensitivity flow cell (G4212A #33)
- ChemStation B.04.02 software

Due to the innovative capillary technology of the system, the sample flow path is

### **Size Exclusion Chromatography**

#### **Columns**

Column 1:	Agilent BioSEC 3, 3 $\mu\text{m}$ , 300 $\text{\AA}$ , 7.8 mm $\times$ 300 mm (p/n 5190-2511)
Column 2:	Agilent BioSEC 5, 5 $\mu\text{m}$ , 300 $\text{\AA}$ , 7.8 mm $\times$ 300 mm (p/n 5190-2526)
Column 3:	Brand A, 5 $\mu\text{m}$ , 250 $\text{\AA}$ , 7.8 mm $\times$ 300 mm

#### **LC Method**

Injection volume:	1 $\mu\text{L}$
Mobile phase A:	150 mM sodium phosphate (pH = 7.0) and
Mobile phase B:	150 mM sodium phosphate (pH = 7.0) containing 150 mM sodium chloride
Flow rate:	1 mL/min
UV detection:	220 nm/4 nm Reference: Off

completely free of any metal components ensuring that the sample never touches metal surfaces. Solvent delivery was free of any stainless steel components preventing iron release from corrosion.

### **Chemicals and Reagents**

Monoclonal antibodies: 1. Anti-FLAG, 3.8 mg/mL (Sigma-Aldrich, St. Louis, MO) and 2. BL05, 0.76 mg/mL (Stratagene, San Diego CA)

SEC standard mix: (Thyroglobulin, Immunoglobulins (IgA and IgG), Ovalbumin, RNase, Cytidine), di-sodium hydrogen phosphate, monosodium hydrogen phosphate, sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO).

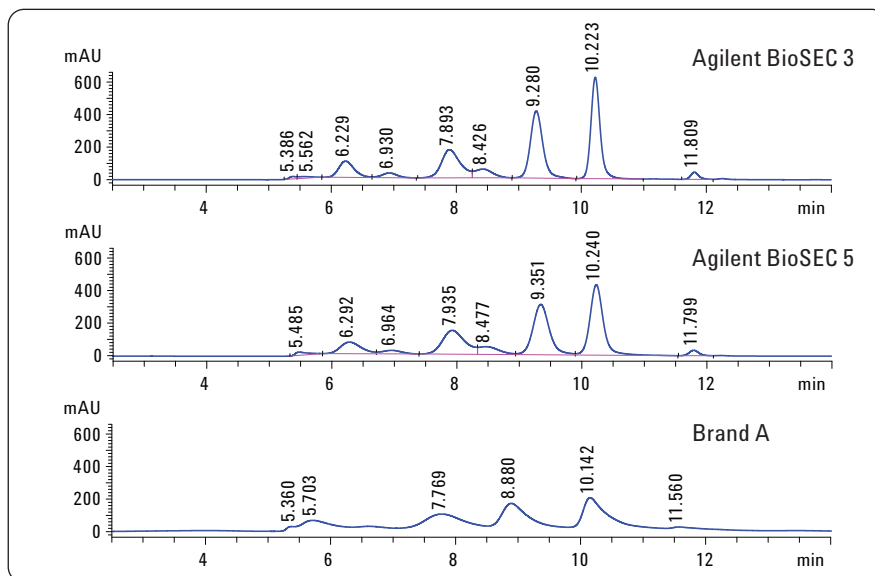
## Results and Discussion

### Analysis of standard proteins under different buffer conditions

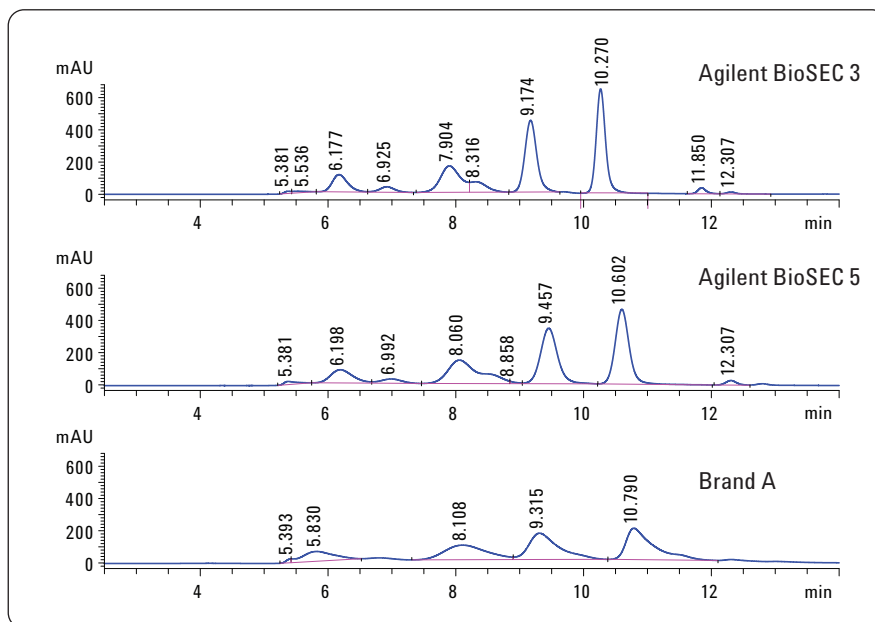
Buffer optimization is an important prerequisite for mAb analysis by SEC to minimize secondary stationary phase interaction and obtain maximum resolution. Frequently, significant amounts of corrosive salts are used to prevent non-specific binding, peak broadening, or peak tailing on many of the commercially available SEC columns.

In the present work, two buffer systems were used: A) 150 mM sodium phosphate buffer (pH 7.0) and B) 150 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride for the SEC separation of the two mAbs.

In order to evaluate column and system performance prior to injecting the mAbs, a standard sizing mix of proteins of known molecular weight was analyzed under both buffer conditions: no NaCl (Figure 1) and 150 mM NaCl (Figure 2). The separation of standard proteins resulted in significantly broader peaks and tailing when using the Brand A column from a different vendor with buffer A (Figure 1). Using the same buffer A, the Agilent BioSEC 3 column showed the highest separation efficiency with increased resolution when compared to the Agilent BioSEC 5 column. These results demonstrated the benefit of smaller particle size, which provides improved mass transfer for protein separations compared to columns using larger diameter particles (Figure 1). The Agilent BioSEC column performance is not affected by the lack of a salt additive, indicating that high concentrations of salt are not needed to prevent secondary interactions.



**Figure 1**  
SEC of 1 µL standard mix on the three columns using mobile phase A.



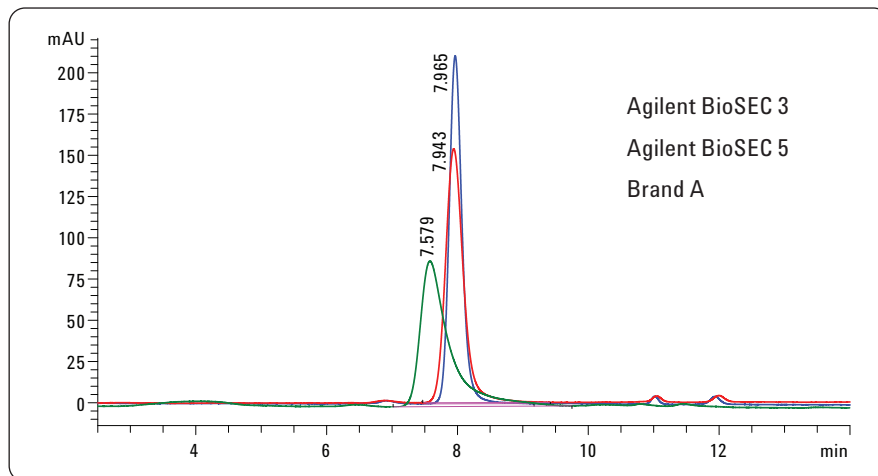
**Figure 2**  
SEC of 1 µL standard mix on the three columns using mobile phase B.

## Separation of monoclonal antibodies

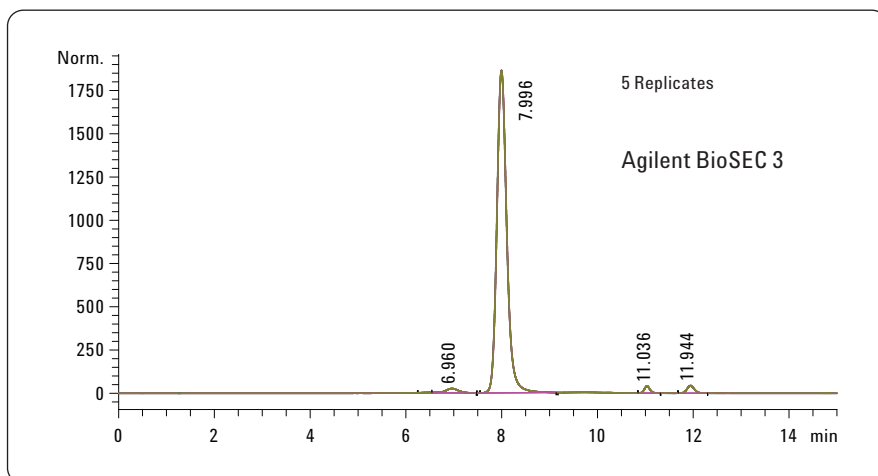
Performance of the Agilent Bio-inert solution was further evaluated using an mAb separation (Anti-FLAG 0.76 µg on-column). Agilent BioSEC 3, and Agilent BioSEC 5, were benchmarked against a commercially available column (Brand A) with 5 µm particle diameter, a 250Å pore size and the same column dimensions. Results clearly confirm the findings obtained during experiments performed on the protein standard mix.

While significant tailing can be observed for the antibody peak using the Brand A column (no salt additive experiment), analyses with the Agilent BioSEC columns result in symmetrical peak shape, superior resolution, and increased sensitivity (Figure 3) without using high concentrations of corrosive salts.

The degree of repeatability of analyses, usually an indicator of method ruggedness, is especially important in a QA/QC type of environment. Figure 4 shows the overlay of five chromatograms generated by replicate analyses of undiluted Anti-FLAG mAb on the Agilent BioSEC 3 µm column. Perfect overlay of chromatograms clearly demonstrates excellent repeatability of the method, and stable instrument and column performance.



**Figure 3**  
SEC of 1 µL Anti-FLAG mAb (five times diluted) on the three columns using mobile phase A.



**Figure 4**  
Undiluted Anti-FLAG mAb (1 µL) on the Agilent SEC 3; mobile phase A.

## Induction of changes to physico-chemical mAb properties by heat stress

Aggregation of therapeutic proteins during drug manufacturing and formulation is considered a critical attribute. Therefore it needs to be monitored tightly in order to prevent loss of drug efficacy or to prevent adverse immunogenic effects. SEC is commonly used to monitor aggregation, since it enables size-based separation and uses particles designed to not interact with bio-molecules.

To promote the alteration of physico-chemical properties (aggregation and denaturation), aliquots of antibody BL05 were subjected to heat stress and exposed at 60 °C for 5, 10, 30 and 60 minutes. The time course of the changes of the BL05 mAb conformation is clearly visible when analyzing the sample at different incubation times. While the height of the monomer peaks at 8 min decrease over time, the height of the peaks at a retention time of 5.5 min clearly increases (Figure 5). Differences in chromatographic performance are visible when comparing the Agilent BioSEC columns to the Brand A column. While chromatograms on Figure 5c (Brand A) show significantly lower separation efficiency and peak tailing, separation on Agilent BioSEC (Figure 5a and 5b) show superior resolution. Separation efficiency is further increased using the Agilent BioSEC 3 (Figure 5b).

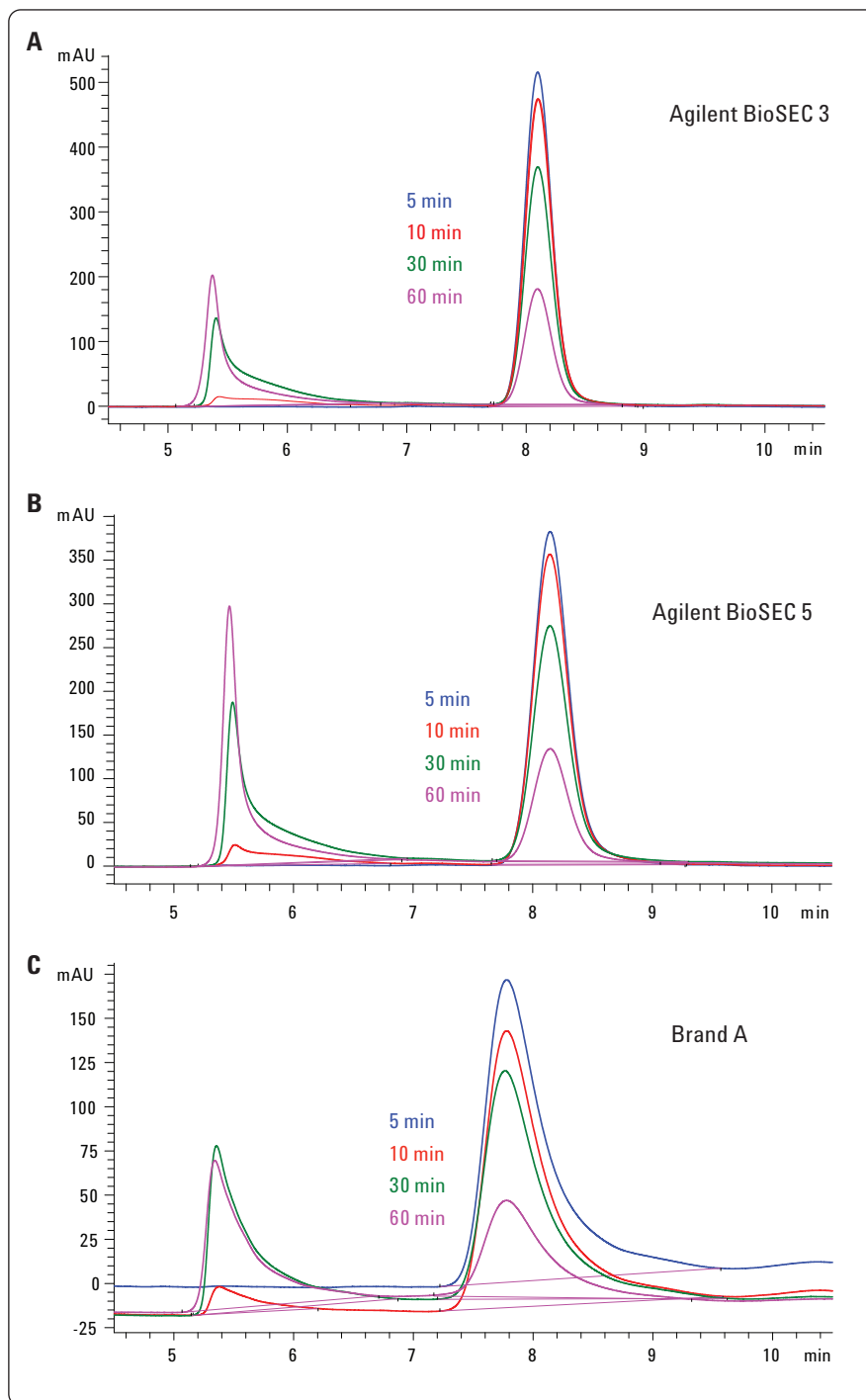
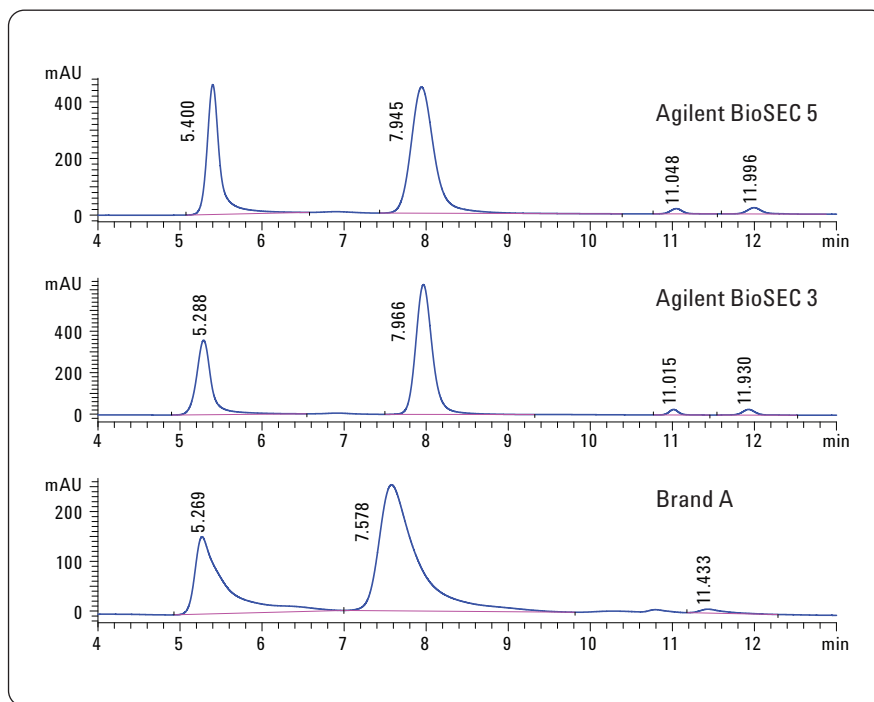


Figure 5a, 5b, and 5c  
Effect of heat stress (60 °C) on BL05 mobile phase A.

This result was confirmed by using a second, different sample. In this example the Anti-Flag mAb was subjected to analysis after 30 min incubation at 60 °C (Figure 6). While both Agilent BioSEC columns demonstrate higher efficiency and symmetry, the separation with Brand A resulted in lower sensitivity and decreased resolution.



**Figure 6**  
Effect of heat stress (60 °C, 30 min) on undiluted Anti-FLAG (1  $\mu$ L, 3.8  $\mu$ g on-column) with mobile phase A.

## **Conclusions**

This study demonstrated the superior performance of the Agilent 1260 Infinity Bio-inert HPLC system in combination with Agilent BioSEC columns for the analysis of physico-chemical properties of mAbs. Higher separation efficiency was achieved with smaller id columns. This solution provides an excellent tool for method development and monitoring the purity and stability of therapeutic monoclonal antibodies.

[www.agilent.com/chem/bio-inert](http://www.agilent.com/chem/bio-inert)

© Agilent Technologies, Inc., 2011  
February 1, 2011  
Publication Number 5990-6414EN



**Agilent Technologies**