# Agilent AdvanceBio SEC Columns for Aggregate Analysis: Instrument Compatibility

## Technical Overview

### Introduction

Agilent AdvanceBio SEC columns are a new family of size exclusion chromatography (SEC) columns packed with 2.7 µm particles of a unique, low binding, polymer coated silica stationary phase. This technology provides minimal nonspecific interactions, and gives improved peak shape for the analysis of biotherapeutic molecules such as monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs).

While it is recommended to use an Agilent 1260 Infinity Bio-Inert LC System with AdvanceBio SEC columns, the highly uniform 2.7 µm particles provide excellent column efficiency providing maximum protein recovery with operating pressures that allow operation on any HPLC system. This includes 400 bar legacy instruments through to 1,200 bar UHPLC instrumentation. AdvanceBio SEC 300Å has been designed specifically for monoclonal antibody aggregate analysis providing optimum performance where it matters, monomer and dimer resolution. AdvanceBio SEC 130Å has a similar high pore volume but covers smaller proteins and polypeptides.

The wide selection of column dimensions covers all application needs: conventional 7.8 mm id to 4.6 mm id for higher sensitivity or where sample availability is restricted. Column lengths are 30 cm for traditional high-resolution separations, and 15 cm for higher speed where increased sample throughput is required. Five-centimeter guard columns are also available.

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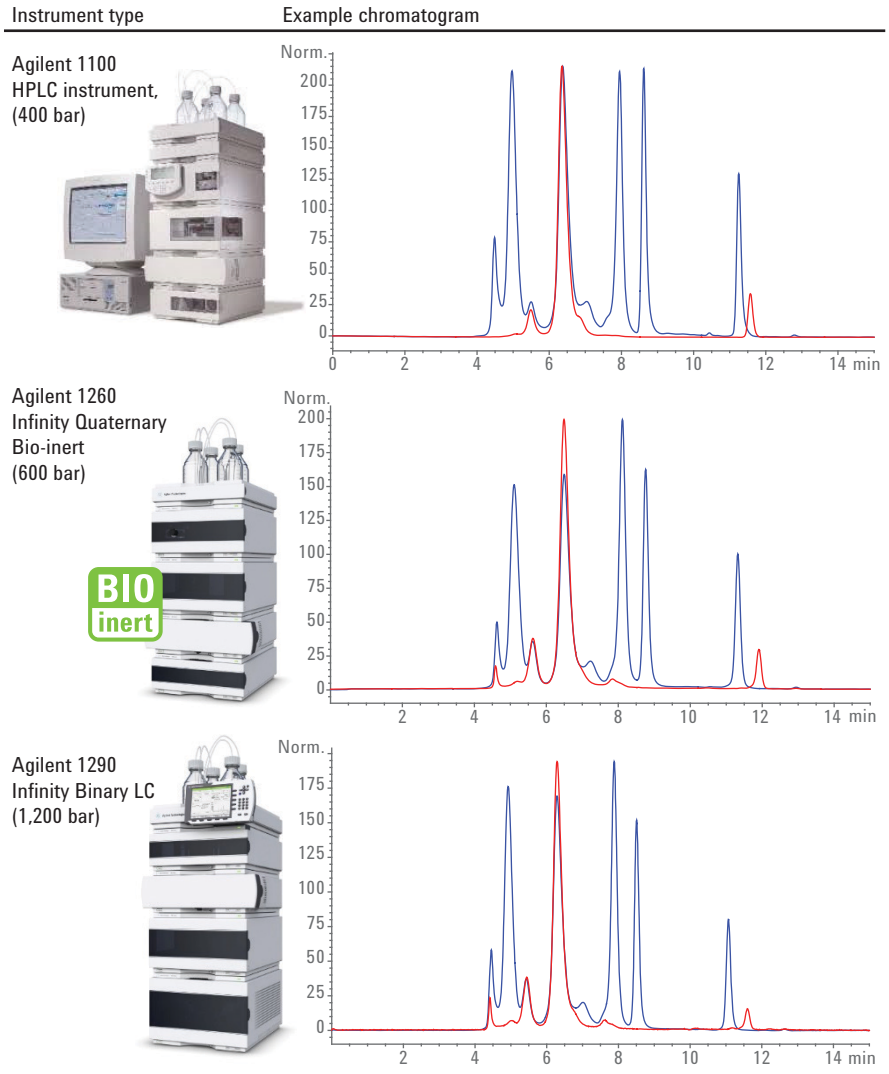



Figure 1. Illustrative examples of protein separations using different HPLC instruments (blue trace: BioRad protein standard mixture; red trace: γ-globulin). Note: These were different batches of proteins used in different laboratories.

## Conditions

| Parameter | Value |
|---|---|
| Eluent | 150 mM Sodium phosphate buffer, pH 7.0 |
| Flow rate | 1.0 mL/min (7.8-mm columns) or 0.35 mL/min (4.6-mm columns) |
| Temperature | Ambient |
| Sample | 1 mg/mL protein concentration |
| Instrument | Agilent 1100 HPLC instrument (legacy, 400 bar)<br>Agilent 1260 Infinity Quaternary Bio-inert (600 bar)<br>Agilent 1290 Infinity Binary LC with G1315D DAD and Bio-inert flow cell (1,200 bar) |
| Wavelength | 220 nm |

## Choosing the Right Column Diameter

Operating columns of different internal diameters requires adjustments in flow rate (to maintain the same linear velocity) and sample volume. The normal operating flow rate for 7.8 mm columns is 1.0 mL/min, therefore, the correct flow rate to use for 4.6 mm columns is 0.35 mL/min. The injection volume should be reduced by a similar amount to obtain comparable detector response, therefore, a 6 µL injection on a 7.8 mm column is reduced to 2 µL for a 4.6 mm column. There may be slight differences in retention time observed due to extra-column volume in the instrument. Similarly, the observed backpressure from an instrument operated at 1.0 mL/min will be somewhat higher than the backpressure observed if the same instrument is operated at 0.35 mL/min, particularly where low dead volume capillaries have been used. Figure 2 shows the same batch of stationary phase packed into 30-cm columns in 7.8 mm and 4.6 mm dimensions, and tested on the same Agilent 1260 Infinity Quaternary Bio-inert instrument.

This clearly illustrates how similar in performance the columns are. The ability to use smaller injection volumes for 4.6 mm columns is ideal when sample availability is limited, and is better suited to modern low dead volume instrumentation. For applications requiring the use of less sensitive detectors, including light scattering detectors, refractive index detectors, or longer UV detector wavelengths (when using mobile phase eluents that have a high background at lower wavelengths for example), the 7.8 mm column offers the capability to inject much larger sample volumes.

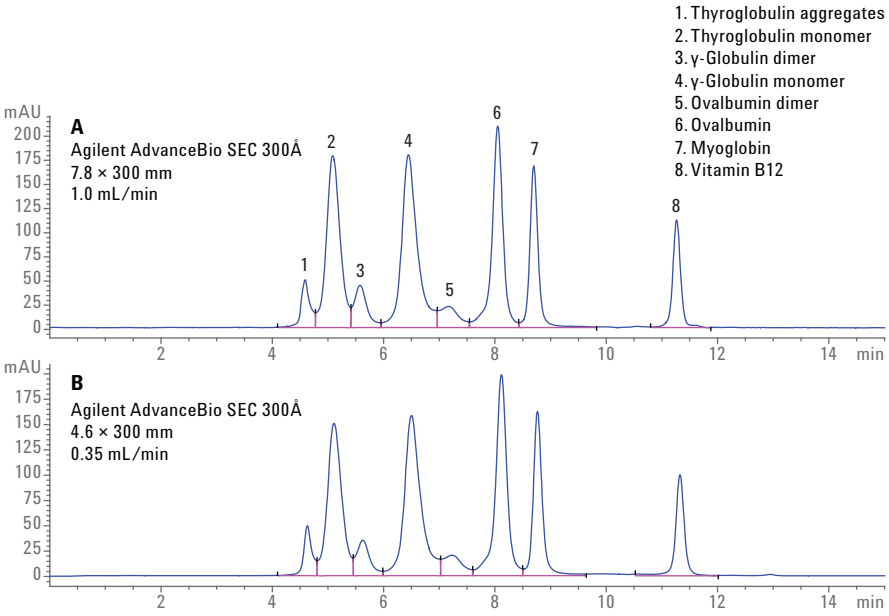



Figure 2. Comparison of protein standard separation on 7.8 mm and 4.6 mm columns.

The strong buffer compositions used for analysis means there is a risk of precipitation or corrosion (if chloride ions are present in the mobile phase). There is also the possibility of bacterial growth in aqueous buffers that do not contain preservative. Regular preventative maintenance and always flushing instruments so that they are not left stored in buffer is essential. Freshly prepared buffer that has been filtered through a 0.2 µm membrane filter is a prerequisite for dependable operation.

For these reasons, the instrument of choice is the Agilent 1260 Infinity Quaternary Bio-inert system.

## Ensuring Ongoing Reliability

Monitoring column performance is an important part of robust size exclusion chromatography. Phosphate buffers are known to cause deterioration of silica-based stationary phases. AdvanceBio SEC columns have a proprietary hydrophilic polymeric layer to help minimize nonspecific interactions. They are routinely tested to demonstrate robust column lifetime even in concentrated sodium phosphate buffers (Figures 3 and 4). Column lifetime may be diminished if operated at higher temperatures or faster flow rates.

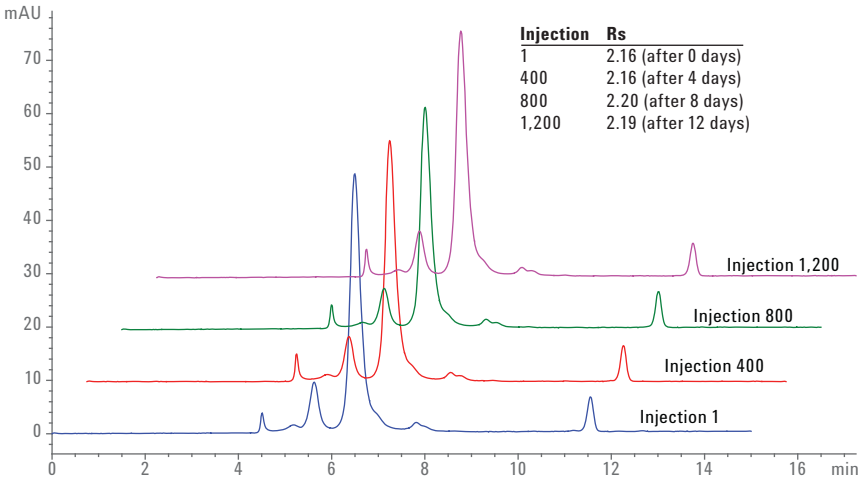



Figure 3. Lifetime experiment on an Agilent AdvanceBio SEC 300Å 7.8 × 300 mm column carried out with 1,200 injections during 12 days operation.

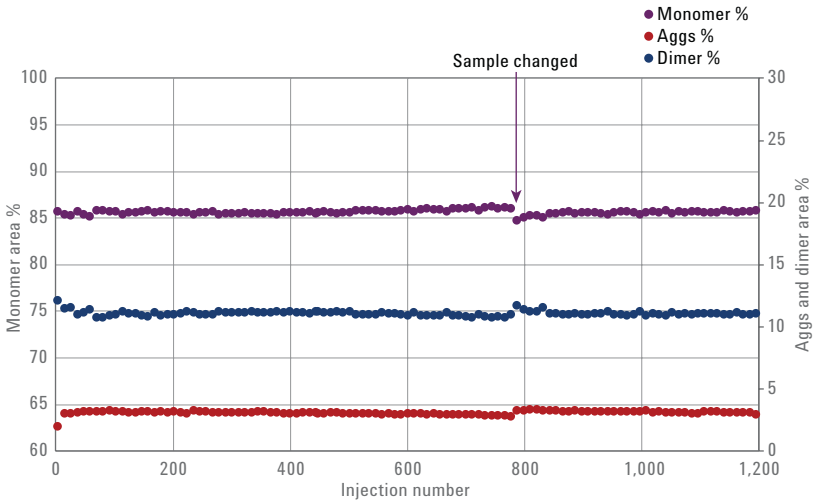



Figure 4. Minimal change in monomer/dimer area% during lifetime experiment on an Agilent AdvanceBio SEC 300Å 7.8 × 300 mm column during 12 days operation.

Ensuring consistent results from instrument to instrument and laboratory to laboratory is essential for effective method transfer. Only by regularly using a protein standard mixture is it possible to determine if factors other than the column need to be optimized. Figure 5 shows the excellent reproducibility shown from closely matched instruments that are operating correctly.

## Agilent 130A AdvanceBio SEC protein standards

A protein mix consisting of five carefully selected proteins (Ovalbumin, Myoglobin, Aprotinin, Neurotensin, and Angiotensin II) designed to calibrate Agilent's 130Å AdvanceBio size exclusion columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

## Agilent 300A AdvanceBio SEC protein standards

A protein mix consisting of five carefully selected proteins (Thyroglobulin, γ-globulin, Ovalbumin, Myoglobin, and Angiotensin II) designed to calibrate Agilent's 300Å AdvanceBio size exclusion columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

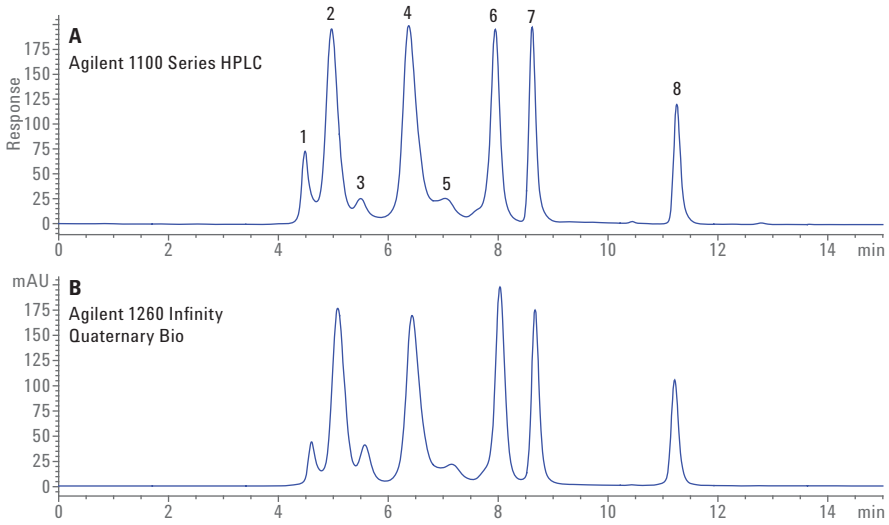
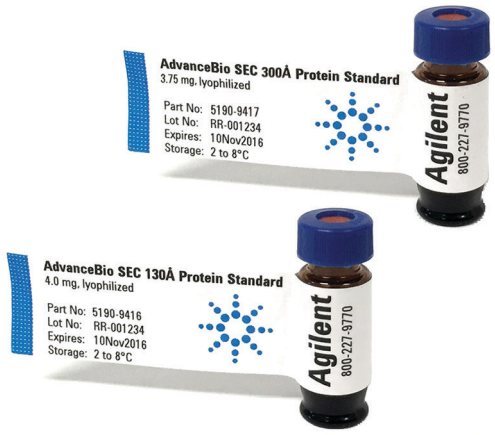



Figure 5. Comparison of a Protein Standard Mixture run on Agilent AdvanceBio SEC 300Å 7.8 × 300 mm columns using an Agilent 1100 Series HPLC instrument and an Agilent 1260 Infinity Quaternary Bio-inert instrument.

## Agilent AdvanceBio SEC protein standards




| Description | Size | Part no. |
|---|---|---|
| 130Å | 1.5 mL vial | 5190-9416 |
| 300Å | 1.5 mL vial | 5190-9417 |

With an isocratic separation mechanism, the most likely explanations for differences in retention time are either flow rate inaccuracy (perhaps resulting from poor pump maintenance or general wear and tear), or differences in extra-column dead volume (changes in capillary length or diameter, or sample loop size). This is particularly important when transferring methods to LC instruments from other vendors (Figures 6 and 7).

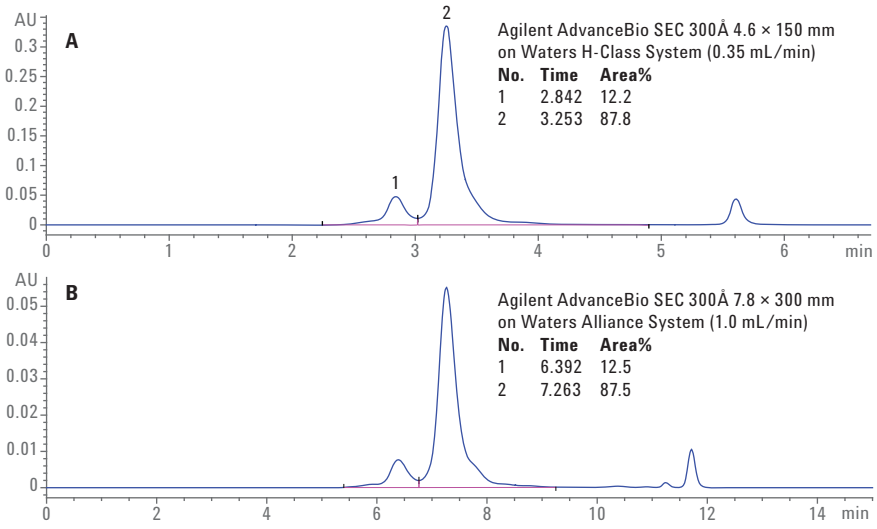



Figure 6. Comparison of γ-globulin separation using different column dimensions and different instruments.

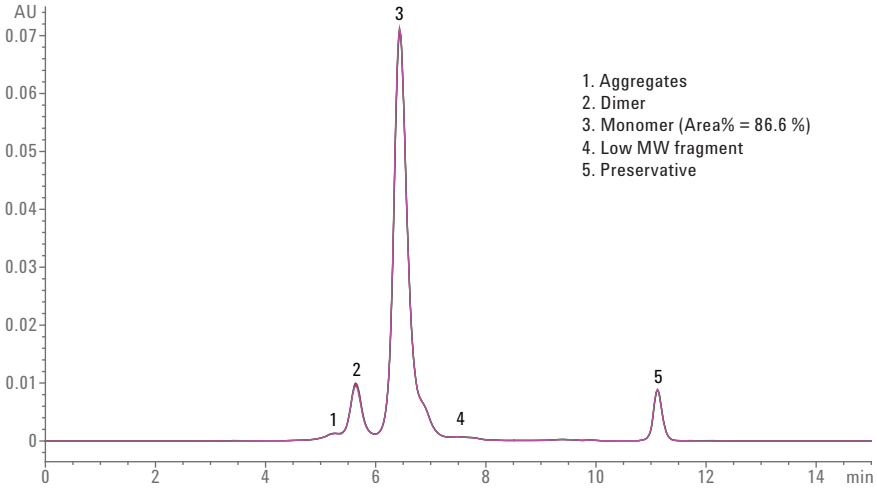



Figure 7. Four replicate injections of γ-globulin on a Waters Alliance HPLC system.

## Control of Temperature

A factor often overlooked when determining method robustness is the separation temperature. Very often separations are performed at ambient temperature; however, the normal temperature in a particular environment may depend on time of day and seasonal variations as well as the influence of heating or air conditioning. With aqueous separations, backpressure will vary noticeably with changing temperature, however some proteins are also affected. An improvement in resolution may be expected with increasing temperature; however, there may also be a change in the level of aggregation too (Figures 8 and 9). For this reason, it is highly recommended that separations are performed with the column contained in a thermostatically-controlled oven, and that the samples are maintained in a temperature-controlled environment.

## Conclusions

Agilent AdvanceBio SEC columns have been developed to provide optimum performance regardless of the instrument used. This Technical Overview highlights some of the areas that need consideration to obtain comparable results on older 400 bar instruments through to modern UHPLC-capable instrumentation regardless of location or operator.

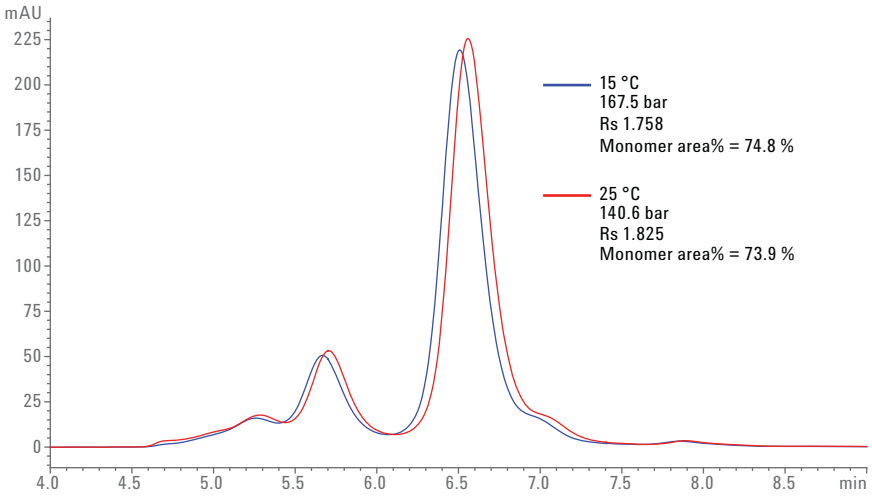



Figure 8. Effect of analysis temperature of γ-globulin.

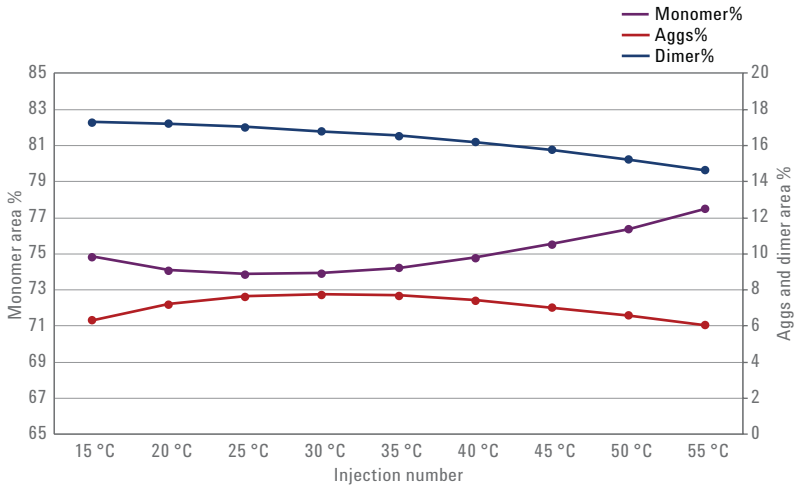



Figure 9. Effect of analysis temperature of on monomer/dimer/aggregate content of γ-globulin.

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