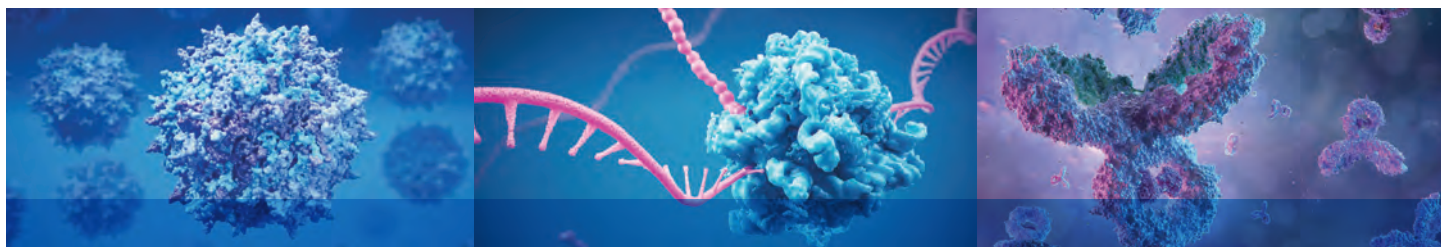


Aggregate Analysis of Biotherapeutics

Agilent size exclusion columns



Accurately determine biomolecule aggregation and fragmentation

Size exclusion chromatography (SEC) is a technique for separating proteins, oligonucleotides, and other complex biopolymers by size, using aqueous eluents. It is an essential tool for quantification of aggregates present in biotherapeutics. Because the size of aggregates, including dimers, is sufficiently different from the protein monomer, you can separate the various forms using SEC. In fact, SEC with UV or light scattering is a standard technique for quantifying biotherapeutic aggregation.

Manufacture of a biopharmaceutical, such as a monoclonal antibody, is a complex process, and aggregation of the protein is an issue that can arise during cell culture, isolation, purification, and formulation. Aggregate formation may be covalent via disulfide bonds, or noncovalent. The presence of dimers and higher aggregates can affect both efficacy and safety of the final product. Quantification of aggregate content must be carried out during process development to establish the product's critical quality attributes (CQA), as well as during final product characterization to ensure the extent of aggregation is minimized and controlled at safe levels.

As a leading manufacturer of SEC columns and instruments for over 30 years, Agilent is continually developing new SEC products that will provide even higher resolution and quicker separations. Agilent ensures reliability in every product by adhering to strict quality standards. A broad SEC portfolio is highlighted here, spanning the robust workhorse, advanced sub-2 μm columns, to next-generation columns for an evolving biotherapeutic landscape.

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Which SEC column is right for your application?

Pore size is arguably the most critical aspect of SEC column selection, as it dictates what analytes can be separated. Agilent AdvanceBio SEC columns are available in pore sizes ranging from 120 to 1000 Å, providing high-resolution separations for samples, including peptides, monoclonal antibodies (mAbs), and larger biotherapeutics such as adeno-associated viruses (AAVs) and mRNA. Molecular weight ranges for SEC columns are based on correlations established for globular proteins. SEC ultimately separates based on size in solution, which does not strictly correlate with molecular weight for different types of biomolecules. AAVs are more compact than their molecular weight suggests, and work best with a smaller-than-predicted pore size. Oligonucleotides tend to be much larger in solution, requiring a larger pore. For samples that are too large for the 1000 Å AdvanceBio SEC, the 2000 Å Agilent Bio SEC-5 column is recommended.

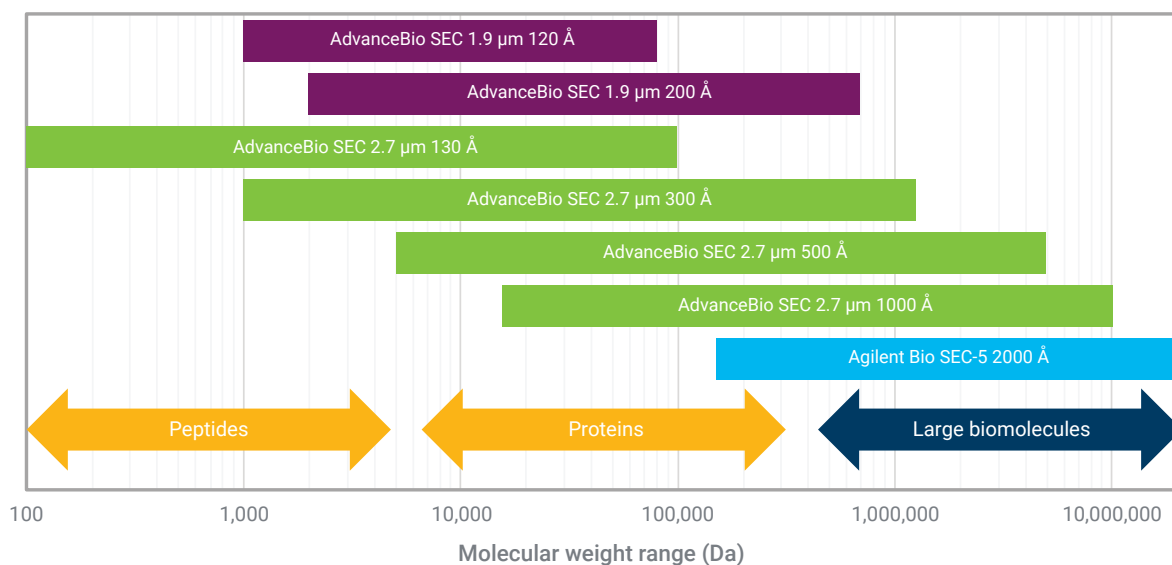


Figure 1. Agilent AdvanceBio SEC column recommendations based on your application. Molecular weight ranges are based on proteins.

Application	Particle Size	Pore Size	Sample Size Range*	Maximum Pressure
AdvanceBio SEC				
Peptides and small proteins	2.7 µm	130 Å	100 Da to 120 kDa	400 bar
	1.9 µm	120 Å	1 to 80 kDa	620 bar
mAbs and other proteins	2.7 µm	300 Å	1 kDa to 1.25 MDa	400 bar
	1.9 µm	200 Å	2 to 700 kDa	620 bar
AAVs, small VLPs	2.7 µm	500 Å	5 kDa to 5 MDa or Rh 3-25 nm	400 bar
VLPs, large oligonucleotides, LNPs	2.7 µm	1000 Å	15 kDa to 10 MDa or Rh 5-40 nm	400 bar
Agilent Bio SEC-5				
VLPs, large oligonucleotides	5 µm	2000 Å	150 kDa to 10 MDa or Rh 5-75 nm	240 bar
PROTEEMA				
Natural and synthetic polymers and peptides	3 µm	100 Å	100 Da to 150 kDa	200 bar
Natural and synthetic polymers and proteins	3 µm	300 Å	1 kDa to 1.2 MDa	200 bar
	5 µm	300 Å	1 kDa to 1.2 MDa	150 bar
	5 µm	1000 Å	1 kDa to 7.5 MDa	150 bar

*Molecular weight ranges are based on proteins.

AdvanceBio SEC

Premier solution for aggregate analysis



Size exclusion columns for routine and advanced applications

AdvanceBio SEC columns deliver accurate, precise quantitation for aggregate and fragment analysis of mAbs, other proteins, and peptides. These innovative SEC columns were designed and engineered by Agilent to improve lab productivity by providing robust, reliable methods that eliminate sample reanalysis. Consistent results are achieved from column to column, batch to batch, and lab to lab, ensuring that methods can be transferred across departments and locations to put an end to uncertainty.

AdvanceBio SEC 2.7 μm columns are robust columns for routine SEC needs, with a unique hydrophilic coating to minimize secondary interactions between the sample and stationary phase without adding extra salt or organic solvent to the mobile phase. Even for sticky samples such as antibody-drug conjugates, this column has minimized secondary interactions for accurate answers. Larger 500 and 1000 $\text{\AA}$ pore sizes are available for AAVs, mRNA, and other large biomolecules.

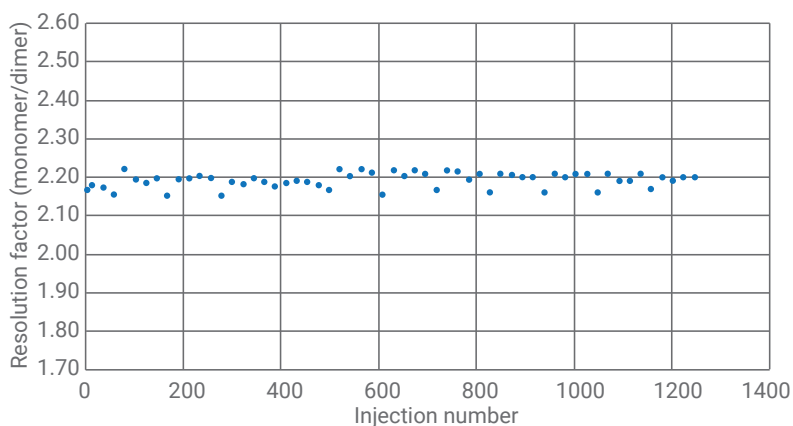
AdvanceBio SEC 1.9 μm columns bring your SEC separations to the next level. The smaller particle and hydrophilic chemistry results in high-efficiency, high-resolution separations. Stability up to 620 bar enables faster flow rates for high-throughput needs. The 200 $\text{\AA}$ pore size was specifically optimized for simultaneous separation of dimer, monomer, and fragment for thorough mAb characterization.

- Increased analytical speed to help you meet vital deadlines
- Increased resolution for more accurate quantitation
- Increased sensitivity for quantitate aggregates, even at low levels
- Increased reproducibility to eliminate rework



Long column lifetime with robust AdvanceBio SEC columns

The stability and low secondary interactions of AdvanceBio SEC chemistry, along with mechanically strong particles and column packing contribute to robust, long-lasting columns that ensure you get the most value from your SEC column.



Column: AdvanceBio SEC 2.7 μm 300 $\text{\AA}$,
7.8 x 300 mm
Mobile phase: 150 mM sodium phosphate, pH 7.0
Sample: IgG
Flow rate: 1.0 mL/min

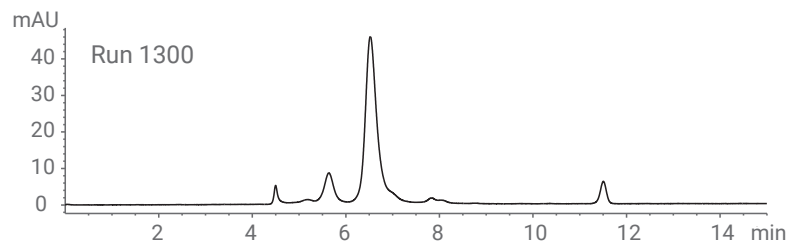
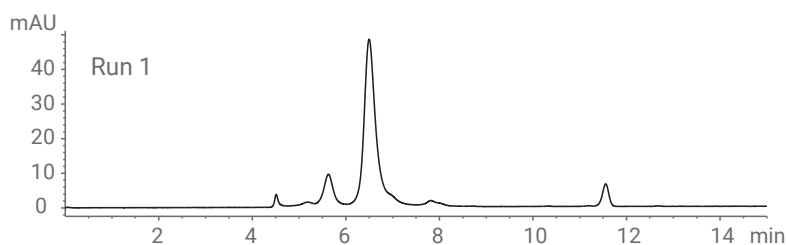
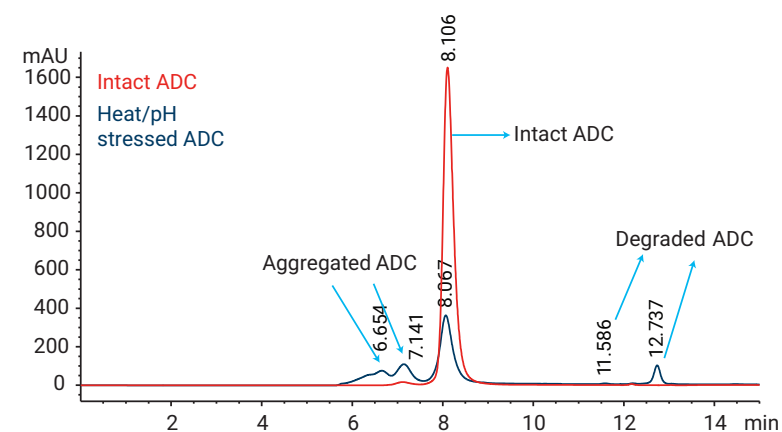


Figure 2. The profile of an IgG sample did not change—even after 1300 injections (bottom). Resolution factors and quantitation of the IgG monomer and dimer also remained within working range throughout the column lifetime (top).

Low secondary interactions with hydrophilic SEC media, ideal for complex biotherapeutics

The unique hydrophilic coating on AdvanceBio SEC columns minimizes secondary interactions, improving recovery and preserving information about your sample.



Column: AdvanceBio SEC 2.7 μm 300 $\text{\AA}$, 7.8 x 300 mm
Instrument: Agilent 1260 Infinity bio-inert quaternary LC system
Mobile phase: PBS, 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4
Column temperature: Ambient
Injection volume: 10 μL
Flow rate: 0.8 mL/min
Detector: UV, 220 nm

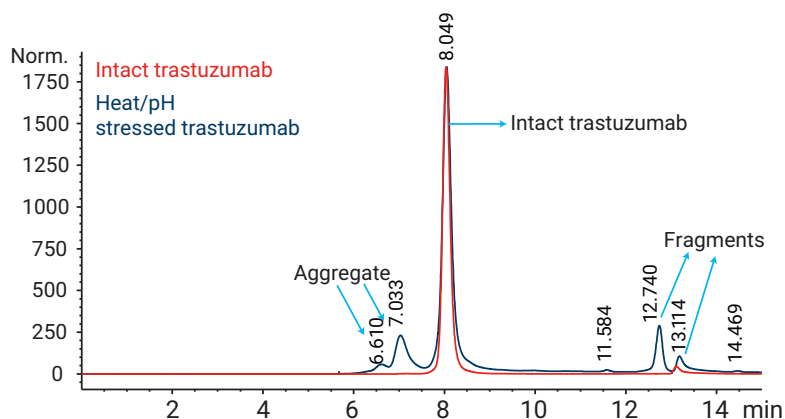
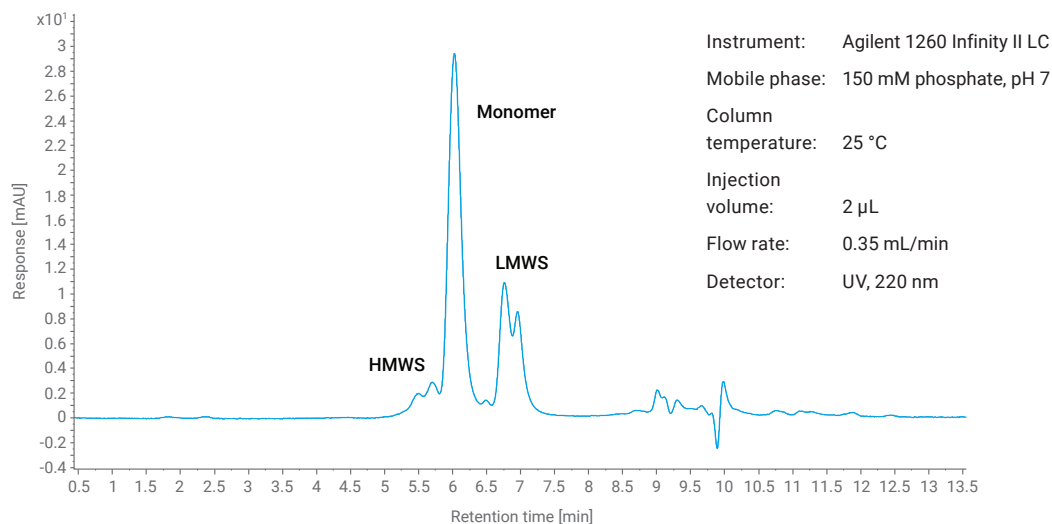


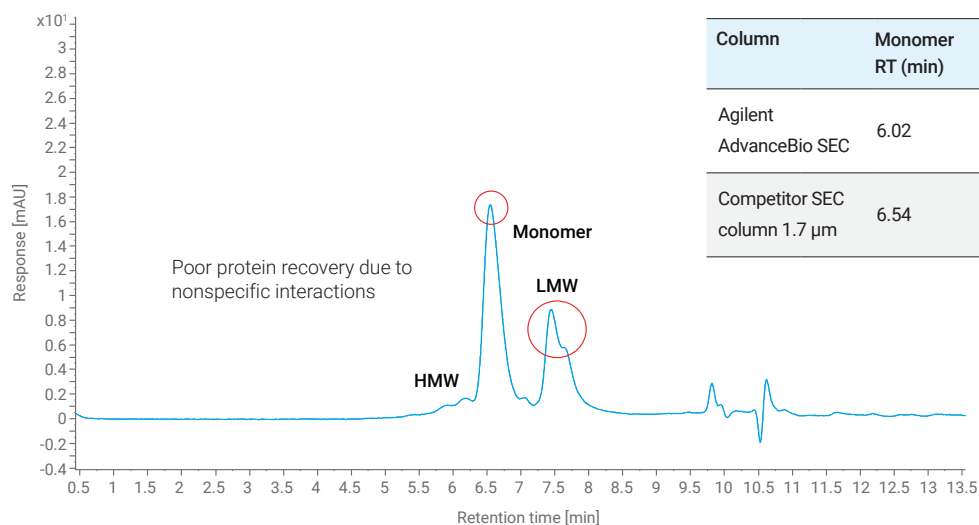
Figure 3. The inert coating on AdvanceBio SEC particles result in sharp peaks with low tailing for ADCs (top), which are often more hydrophobic than the related mAb (bottom).

AdvanceBio SEC columns have fewer secondary interactions than competitor columns, leading to improved recovery and higher resolution.

A: Agilent AdvanceBio SEC 1.9 μm , 120 Å, 4.6 x 300 mm



B: Competitor column 1.7 μm , 125 Å, 4.6 x 300 mm



Column	Monomer RT (min)	Total Peak Area (Average $n = 2$)	Monomer Peak Tailing	Monomer Peak Width
Agilent AdvanceBio SEC	6.02	691.81	1.22	0.21
Competitor SEC column 1.7 μm	6.54	581.10	1.33	0.28

Figure 4. Separation of high molecular weight species (HMWS), monomer and low molecular weight species (LMWS) on an Agilent AdvanceBio column versus a competitor column. The competitor column shows significant secondary interactions, leading to lower sample recovery and lost resolution between two LMWS.

High-resolution, high-throughput separations save time

Smaller particles with large pore volume enable users to switch from a traditional 300 mm column to a 150 mm column. This cuts the run time in half, increasing throughput and freeing up more time—so you can run more samples or move onto your next project.

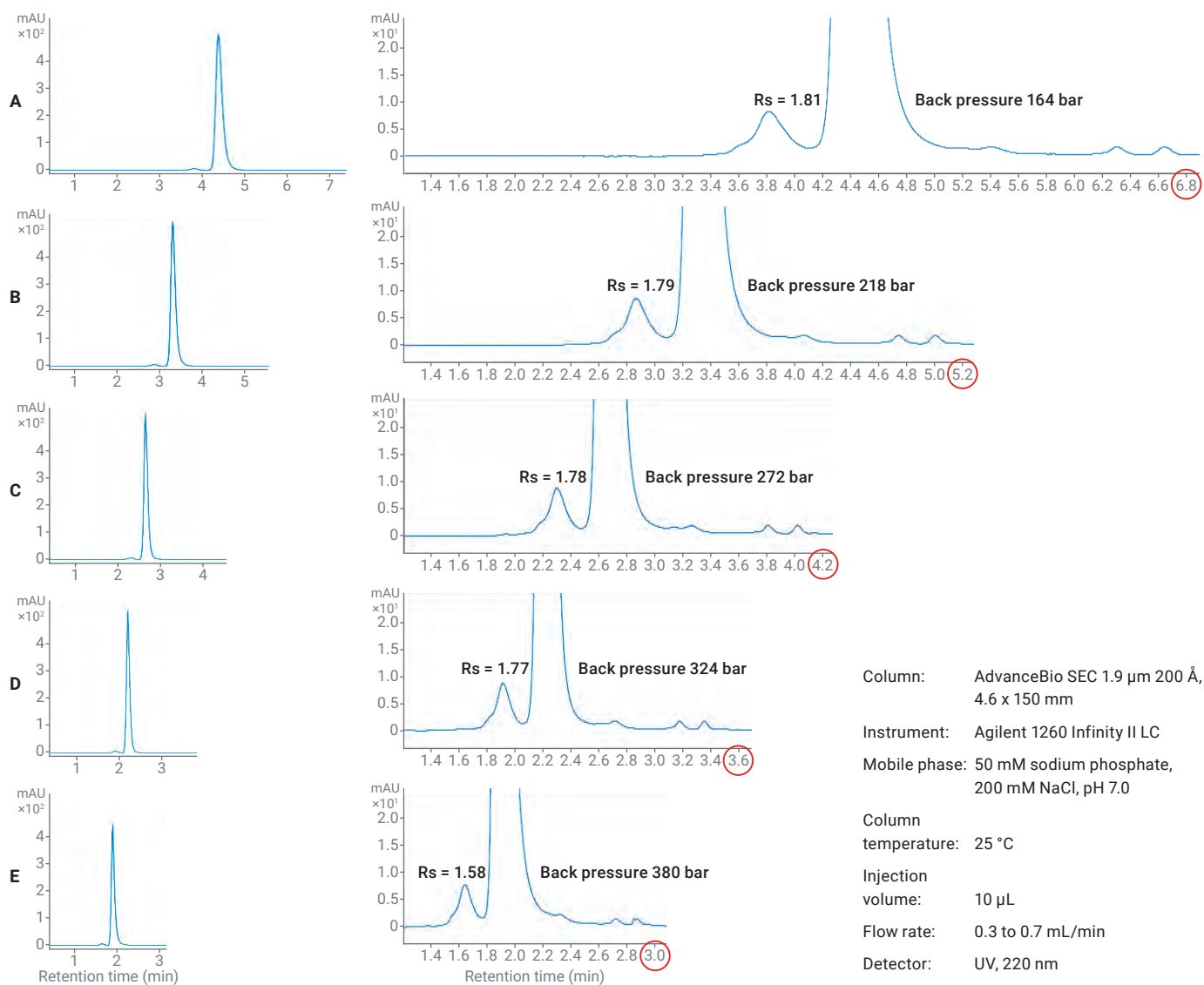


Figure 5. Size exclusion chromatograms of SigmaMAb using a 4.6 × 150 mm SEC column running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.3 mL/min; B) 0.4 mL/min; C) 0.5 mL/min; D) 0.6 mL/min; E) 0.7 mL/min. At 0.7 mL/min, as many as 20 samples/hour, or 480 samples in a 24-hour day can be run.

Optimal pore sizes for your ultralarge biotherapeutics analyses

AAVs, virus-like particles (VLP), oligonucleotides, and lipid nanoparticles (LNP) represent the next generation of biotherapeutics for cell and gene therapy, as well as vaccines. Characterizing these novel and challenging biotherapeutics requires advanced and capable technology.

While maintaining all the best attributes of earlier Agilent AdvanceBio SEC columns, the new 500 and 1000 Å pore sizes now provide suitability for samples of larger diameter, making them ideal for your aggregation analyses of ultralarge biomolecules (> 1 MDa) such as AAVs, VLPs, oligonucleotides, and LNPs. Compared to competitor columns, AdvanceBio SEC columns demonstrate excellent resolution and tailing (Figure 4). This state-of-the-art resolution enables more robust data analysis and reliable results.

AdvanceBio SEC columns

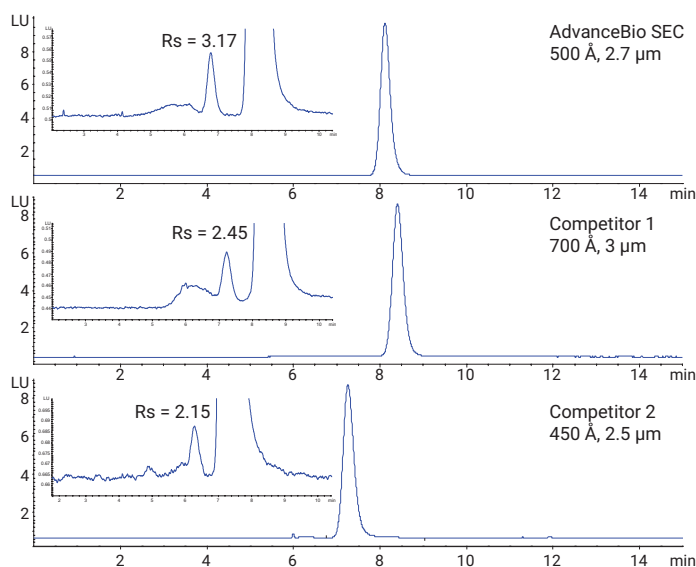
Column: AdvanceBio SEC 500 Å, 2.7 µm, 4.6 x 300 mm (p/n PL1580-5325)
AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 x 300 mm (p/n PL1580-5302)
Competitor 1 700 Å, 3 µm, 4.6 x 300 mm
Competitor 2 450 Å, 2.5 µm, 4.6 x 300 mm
Competitor 2 1000 Å, 3 µm, 4.6 x 300 mm)

Mobile phase: 50 mM sodium phosphate + 400 mM NaCl, pH 7.2

Flow rate: 0.35 mL/min

Detector: Fluorescence λ_{ex} 280 nm, λ_{em} 348 nm

A) AAV-9



B) HPV-16

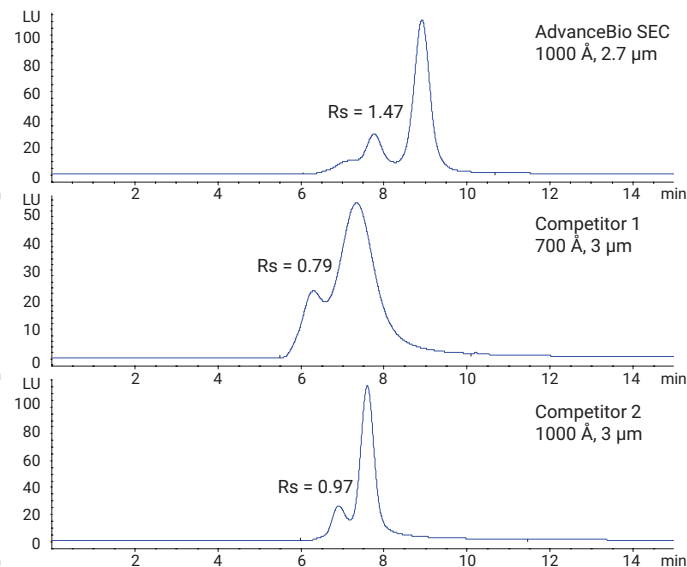


Figure 6. Agilent AdvanceBio SEC columns show enhanced resolution and tailing versus competitor columns for protein-based next-generation therapeutics.

AdvanceBio SEC 500 and 1000 Å columns show good resolution and a wide resolving range for oligonucleotide standards.

AdvanceBio SEC 500 and 1000 Å columns

Column: AdvanceBio SEC 500 Å, 2.7 µm, 4.6 x 300 mm

AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 x 300 mm

Mobile phase: 150 mM phosphate buffer, pH 7.0

Flow rate: 0.175 mL/min

Column

temperature: 30 °C

Detector: UV, 260 nm

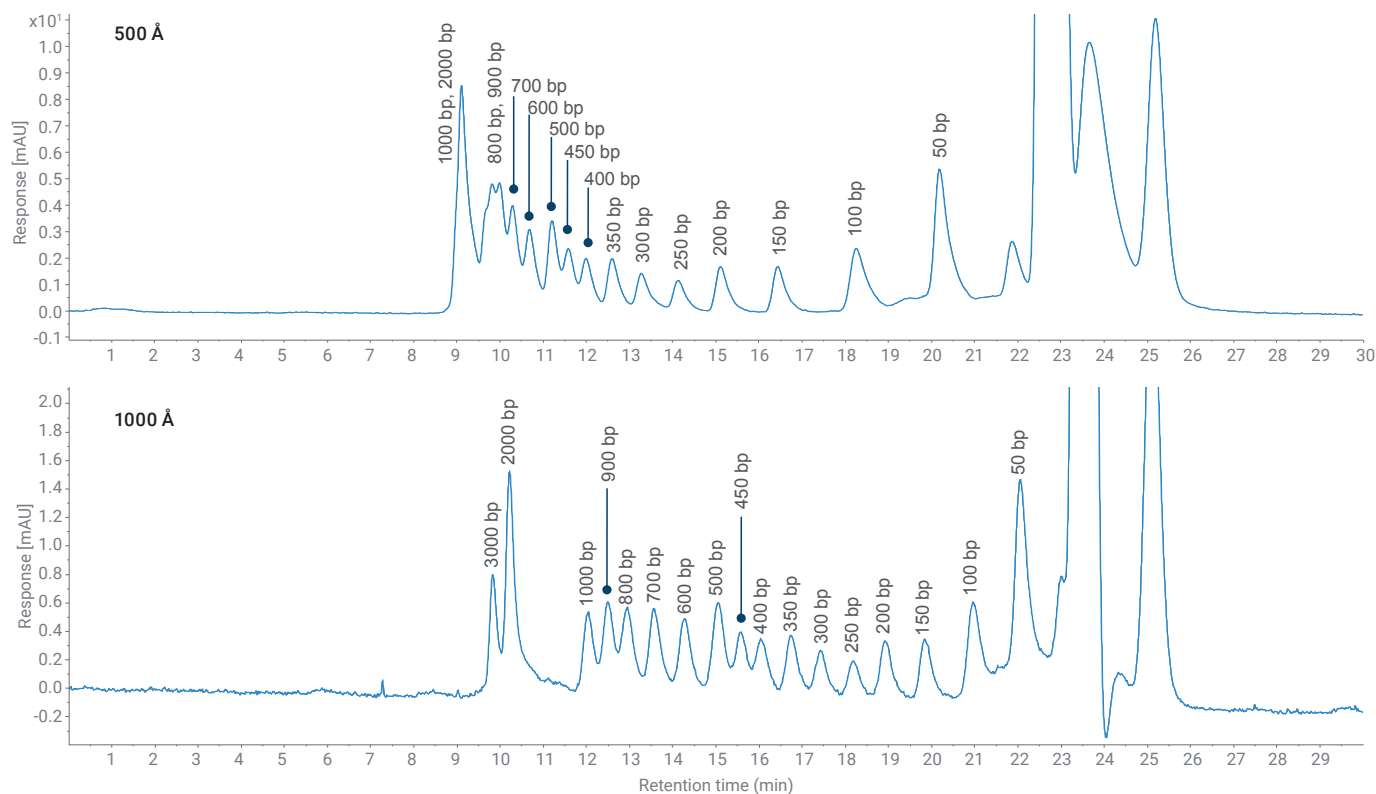


Figure 7. 50 bp DNA ladder ranging from 50 bp to 3,000 bp, showing excellent resolution particularly for larger oligonucleotides.



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Agilent Bio SEC-5

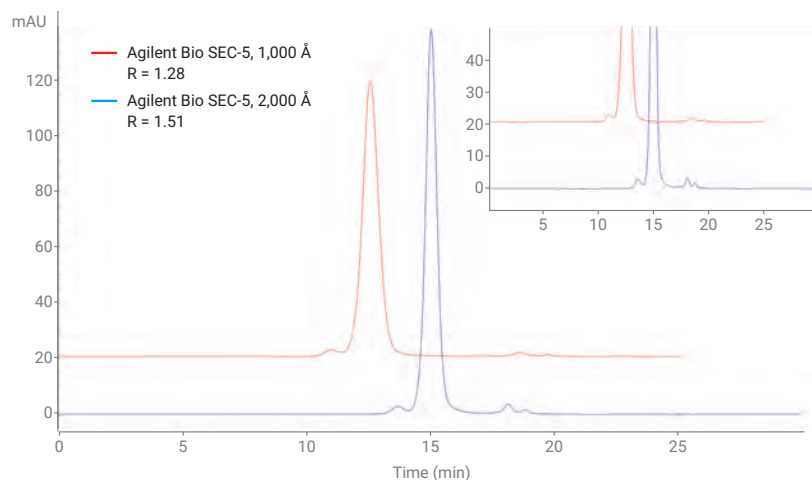
2000 Å pore size



Ultralarge pores for ultralarge biotherapeutics

When sample sizes exceed the largest pore size of the AdvanceBio SEC columns, Agilent Bio SEC-5 2000 Å columns provide the necessary larger pore size. Bio SEC-5 2000 Å columns are part of a larger Bio SEC portfolio, with pore sizes ranging from 100 to 2000 Å, and dimensions up to 21.2 mm id.

Agilent Bio SEC-5 columns



Column: Bio SEC-5, 7.8 × 300 mm, 5 µm, 2000 Å
Bio SEC-5, 7.8 × 300 mm, 5 µm, 1000 Å

Instrument: Agilent 1260 Infinity II LC with quaternary pump

Mobile phase: 50 mM phosphate buffer (pH 7.4) with 400 mM sodium chloride

Sample: 5 µL

Column temperature: Ambient

Flow rate: 0.6 mL/min

Detector: UV, 220 nm

Figure 8. The same VLP was analyzed using Agilent Bio SEC-5 2000 Å and Bio SEC-5 1000 Å columns.

PROTEEMA SEC Columns

Diol chemistry



Versatile columns with USP L20 classification

PROTEEMA columns are suited for aqueous gel permeation chromatography/size exclusion chromatography (GPC/SEC) analysis of natural and synthetic proteins, peptides, enzymes, and gelatins/collagens with molecular weights ranging from 100 to 7,500,000 Da. Multiple column configurations are available, including two particle sizes, three pore sizes, and both stainless steel and bio-inert hardware options. PROTEEMA Lux columns arrive preconditioned for light scattering detection. This diol SEC phase is suitable for separations requiring USP designation L20.

Column: PROTEEMA 100 Å, 300 Å, 1000 Å, 8 x 300 mm
Mobile phase: Phosphate buffer pH 6.6 (34 mmol) + 0.5M NaCl
Column temperature: 23 °C
Flow rate: 1 mL/min
Detector: RI

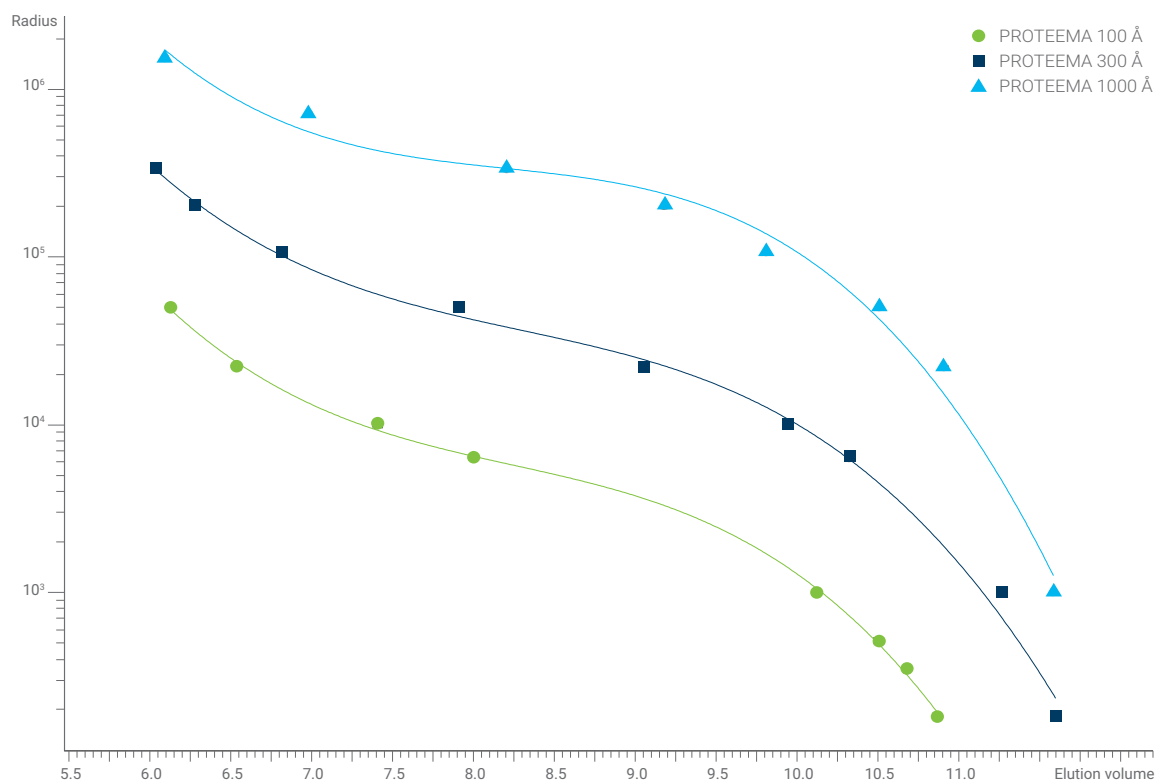


Figure 9. Pullulan calibration curves marking the separation ranges of 100, 300, and 1000 Å pore size PROTEEMA columns.

Protein standards for SEC

Ensure column performance
and system suitability



AdvanceBio SEC 130 and 300 Å protein standards

Each standard is a protein mix consisting of five carefully selected proteins (ovalbumin, myoglobin, aprotinin, neurotensin, angiotensin II) designed to calibrate Agilent's 130 Å or 300 Å 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.

Column: AdvanceBio SEC 2.7 µm 7.8 x 300 mm

Mobile phase: 150 mM sodium phosphate, pH 7.0

Flow rate: 1.0 mL/min

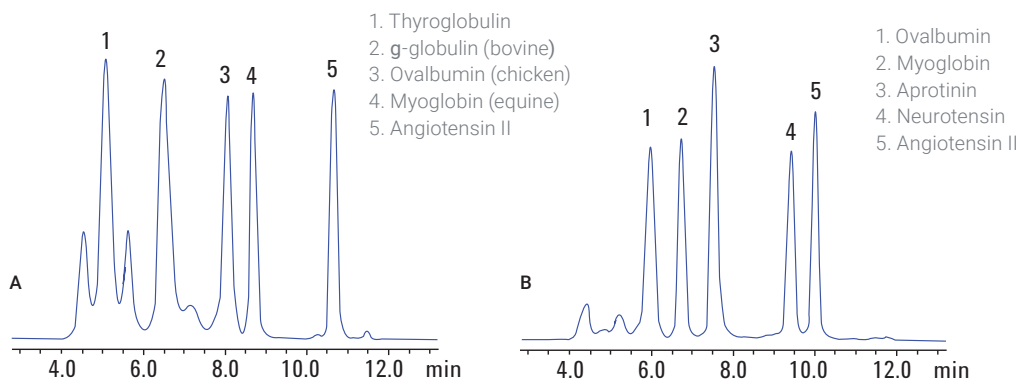


Figure 10. A) AdvanceBio SEC 300 Å protein standard separated on a 300 Å AdvanceBio SEC column.
B) AdvanceBio SEC 130 Å protein standard separated on a 130 Å AdvanceBio SEC column.

Protein standards kits offer greater flexibility

Kitted protein standards enable users to choose the best protein standards for a given analysis or troubleshooting circumstance. PSS-PROKIT contains 10 individually bottled, lyophilized standards ranging in molecular weight from 243 Da (cytidine) to 670 kDa (thyroglobulin, Rh 9.1). PSS-PROKITR1 contains five each of three different vials, containing three to four lyophilized standards that are already mixed. These kits give users the flexibility to design their own mixture, troubleshoot with individual proteins, or use ready-mixed combinations of fully resolved standards.

Agilent ReadyCal Kit Components

Vial	Components
Green cap	Thyroglobulin, albumin, cytochrome C, cytidine
Red cap	Gamma-globulins, β-lactoglobulin, aprotinin
White cap	Albumin (bovine), myoglobin, vitamin B12

Ordering Information

One-click ordering



You can always count on Agilent to support your entire workflow—including sample preparation, columns, supplies, standards, and instruments. To add items to your shopping cart at the Agilent online store, simply click the part number links. Then, enter the quantities for the products you need.

AdvanceBio SEC columns, 2.7 μm

Dimension (mm)	Particle Size (μm)	130 Å	300 Å	500 Å	1000 Å
4.6 x 300	2.7	PL1580-5350	PL1580-5301	PL1580-5325	PL1580-5302
4.6 x 150	2.7	PL1580-3350	PL1580-3301	PL1580-3325	PL1580-3302
4.6 x 50, guard	2.7	PL1580-1350	PL1580-1301	PL1580-1325	PL1580-1302
7.8 x 300	2.7	PL1180-5350	PL1180-5301	PL1180-5325	PL1180-5302
7.8 x 150	2.7	PL1180-3350	PL1180-3301		
7.8 x 50, guard	2.7	PL1180-1350	PL1180-1301	PL1180-1325	PL1180-1302

AdvanceBio SEC columns, 1.9 μm

Dimension (mm)	Particle Size (μm)	120 Å	200 Å
4.6 x 300	1.9	PL1580-5250	PL1580-5201
4.6 x 150	1.9	PL1580-3250	PL1580-3201
4.6 x 30, guard	1.9	PL1580-1250	PL1580-1201
2.1 x 150, PEEK-lined SS	1.9	PL1980-3250PK	PL1980-3201PK
2.1 x 50, PEEK-lined SS, guard	1.9	PL1980-1250PK	PL1980-1201PK

Bio SEC-5 columns

Dimensions (mm)	Particle Size (μm)	Bio SEC-5 2000 Å
4.6 x 300	5	5190-2543
4.6 x 150	5	5190-2544
4.6 x 50, guard	5	5190-6862
7.8 x 300	5	5190-2541
7.8 x 150	5	5190-2542
7.8 x 50, guard	5	5190-2545
21.2 x 300	5	5190-6868
21.2 x 50, guard	5	5190-6874

PROTEEMA columns

Dimensions (mm)	Particle Size (µm)	Pore Size (Å)	Stainless Steel	Bio-Inert	PROTEEMA Lux Light Scattering Ready
4.6 x 30	3	Guard column	PRM050303	PRM050303BI	
4.6 x 250	3	100	PRM0525031E2	PRM0525031E2BI	
4.6 x 250	3	300	PRM0525033E2	PRM0525033E2BI	
4.6 x 250	5	300	PRM0525053E2	PRM0525053E2BI	
8 x 50	5	Guard column	PRA080505	PRA080505BI	PRA080505LS
8 x 300	5	100	PRA0830051E2	PRA0830051E2BI	PRA0830051E2LS
8 x 150	5	300	PRA0815053E2	PRA0815053E2BI	
8 x 300	5	300	PRA0830053E2	PRA0830053E2BI	PRA0830053E2LS
8 x 300	5	1000	PRA0830051E3	PRA0830051E3BI	PRA0830051E3LS

AdvanceBio SEC standards

Description	Form	Part No.
AdvanceBio SEC 130 Å Protein Standard	Lyophilized solid	5190-9416
AdvanceBio SEC 300 Å Protein Standard	Lyophilized solid	5190-9417

PSS Protein standards

Description	Form	Part No.
GPC/SEC-calibration kit, 10 individual standards	Lyophilized solid	PSS-PROKIT
ReadyCal Kit Protein, 5 mixes of 3-4 proteins each	Lyophilized solid	PSS-PROKITR1

Agilent NIST mAb

Description	Form	Part No.
Agilent-NISTmAb, 25 µL (10 mg/mL, 0.25 mg total)	Frozen liquid	5191-5744
Agilent-NISTmAb, 4 x 25 µL (10 mg/mL, 1 mg total, 0.25 mg per aliquot)	Frozen liquid	5181-5745

Visit: www.agilent.com/chem/advancebio-aggregate for other SEC columns, including Bio SEC-3 and other pore sizes of Bio SEC.

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