Agilent BioHPLC Size Exclusion Chromatography Columns

RESOLVE PROTEIN AGGREGATES AND DEGRADANTS WITH SPEED AND CONFIDENCE

The Measure of Confidence

Agilent Technologies

AGILENT BioHPLC SIZE EXCLUSION CHROMATOGRAPHY COLUMNS

ACHIEVE FAST, HIGH-RESOLUTION SEPARATIONS FOR PROTEIN AGGREGATION AND DEGRADATION

Size exclusion chromatography (SEC) is a critical tool for quantitating monomers, dimers, aggregates, and potential degradants. These types of protein separations demand the highest levels of accuracy and speed.

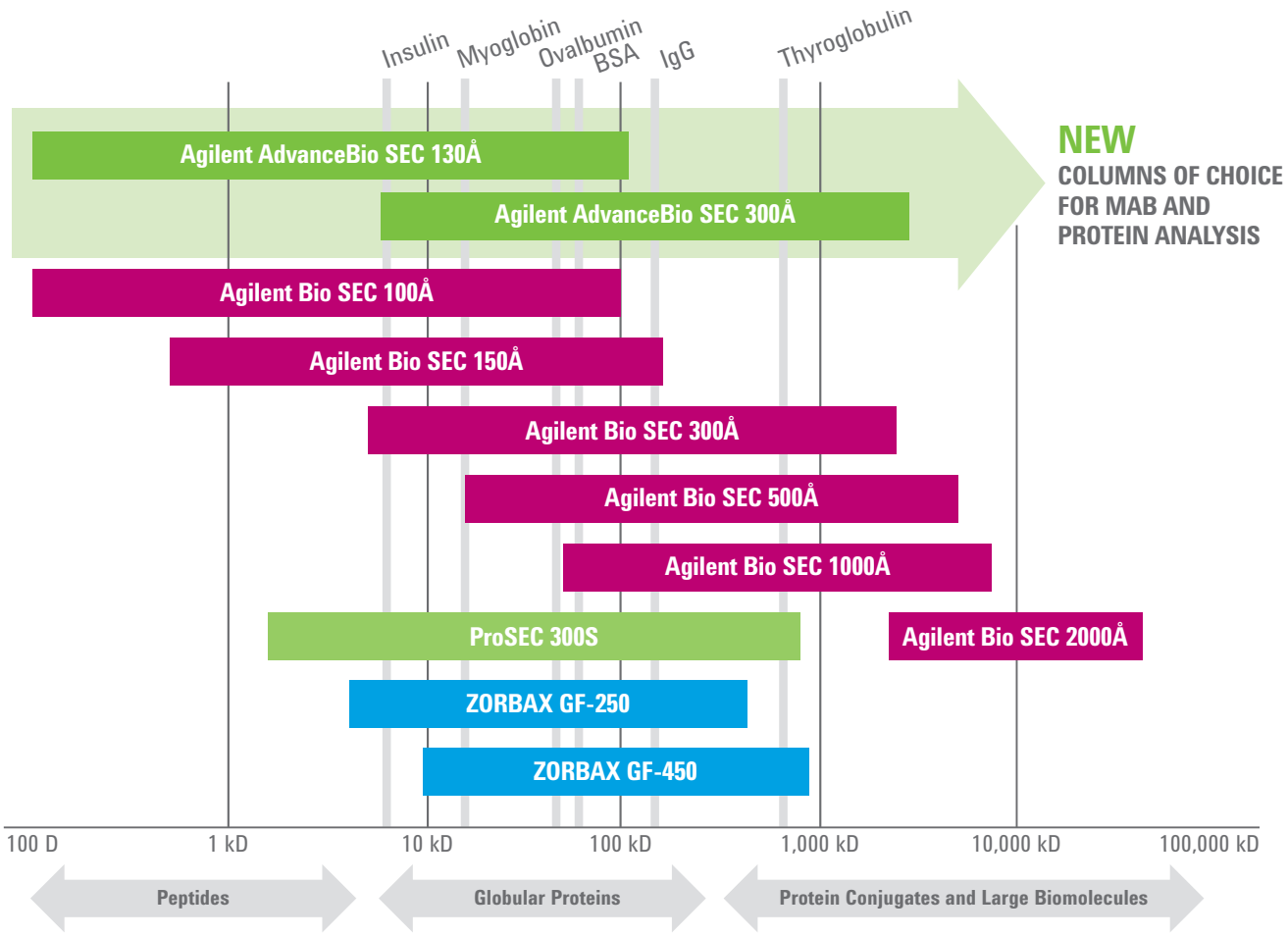
Agilent BioHPLC columns for size exclusion chromatography (SEC) offer fast, reliable, accurate performance for your biopharmaceutical analysis. They are easy to integrate into your workflow, and are available in a full range of pore sizes and dimensions to ensure the perfect separation every time.

As a leading manufacturer of SEC columns and instruments for more than 30 years, Agilent is continually developing new products that provide the highest resolution and fastest separations. So you can quickly—*and cost-effectively*—get life-changing products into the hands of those who need them.

The latest addition to the Agilent SEC column family is AdvanceBio SEC—a new technology, particle, and chemistry designed and engineered for precise, accurate quantitation of mAb aggregates and proteins.

Which SEC column is right for your application?

Agilent's wide selection of SEC columns gives you the choices you need to perfect separations based on your analytes and method parameters. This chart gives you an overview of the pore size ranges that yield the best results for common molecule types.



INSIDE: Complete Agilent portfolio of BioHPLC SEC columns covering the very latest biomolecule applications

New technology designed for monoclonal antibody separations
Agilent AdvanceBio SEC Columns 4

Protein analysis using mass spec
Agilent Bio SEC-3 HPLC Columns 9

Large biomolecules
Agilent Bio SEC-5 HPLC Columns 11

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To learn more about performing fast, high-resolution separations for protein aggregation and degradation, visit **www.agilent.com/chem/BioHPLC**

AGILENT ADVANCEBIO SEC COLUMNS

AdvanceBio SEC columns deliver accurate, precise quantitation for mAb aggregation and protein SEC analysis. This new SEC technology is designed and engineered for:

- High resolution for more accurate quantitation
- Faster analysis speeds for delivery to deadlines
- No change to sample integrity
- Sensitive aggregate quantitation even at low levels

In addition, AdvanceBio SEC columns improve lab productivity by providing robust, reliable methods that eliminate sample re-analyses. You can also easily transfer methods to other locations, including QA/QC, reducing the risk of late-stage candidate and batch failures.

Optimal resolution for accurate quantitation

The chromatograms at right compare the separation of the BioRad Gel Filtration achieved with AdvanceBio SEC 300Å and 130Å columns.

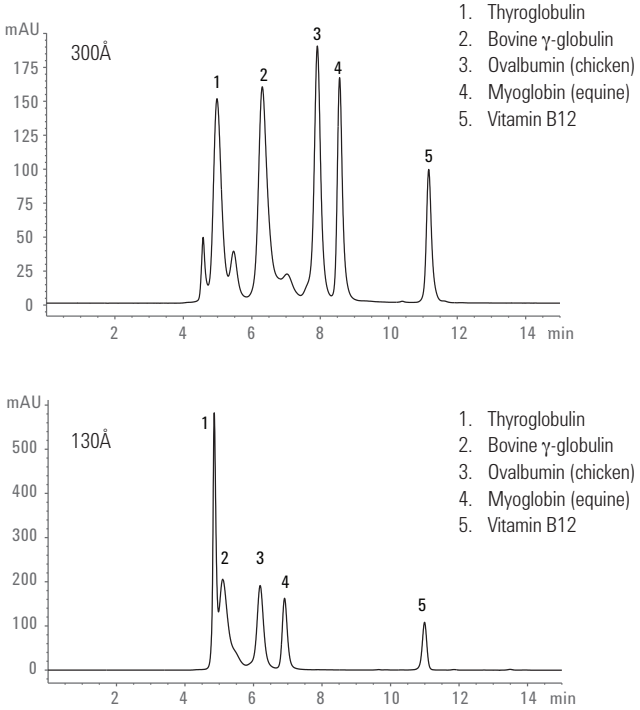
Top chromatogram: The AdvanceBio SEC 300Å column provides resolution for quantitating large proteins, including mAb.

Bottom chromatograms: The AdvanceBio SEC 130Å column provides resolution for quantitating protein fragments, small proteins, and peptides.

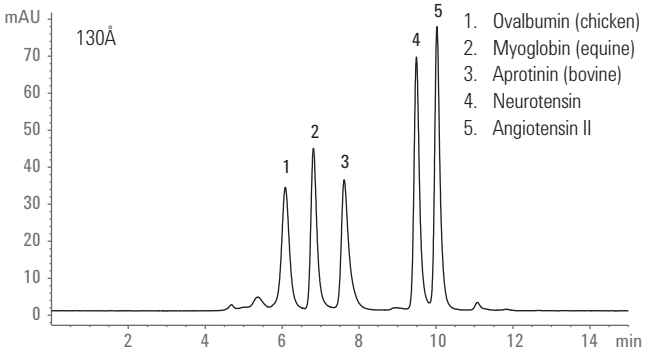
BioRad Gel Filtration Standard #1511901

Column: AdvanceBio SEC, 7.8 x 300 mm

Mobile phase: 150 mM sodium phosphate, pH 7.0



AdvanceBio SEC 300Å and 130Å separation of the BioRad Gel Filtration mix. The 300Å provides increase resolution.

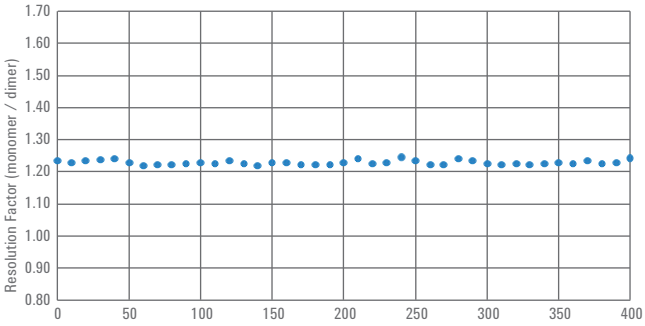


AdvanceBio SEC 130Å separation of protein and peptide mix showing the resolution of small peptides and proteins.

Delivery to deadlines—Increasing sample throughput

By decreasing the AdvanceBio SEC column length from 300 mm to 150 mm, and increasing the flow rate from 1.0 mL/min to 2.0 mL/min, analysis time was reduced from 12 to 3 minutes. In addition, the resolution of the IgG monomer and dimer was sufficient for quantitation—and was consistent for more than 400 injections.

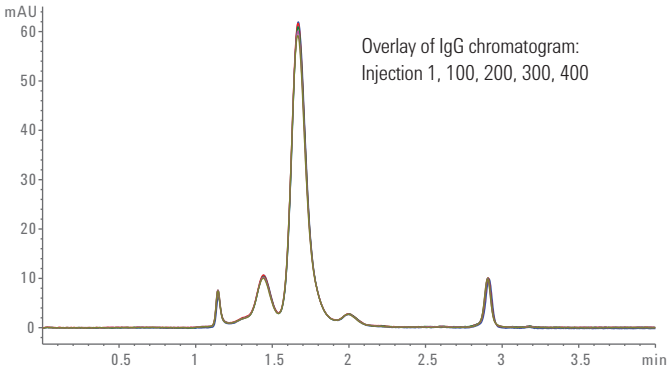
Resolution over 400 injections

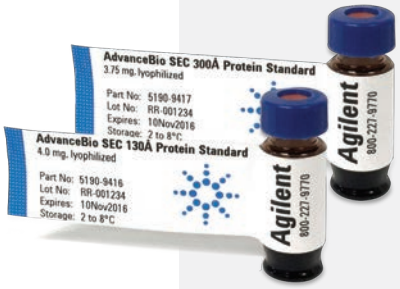


Column: Agilent AdvanceBio SEC 300Å, 7.8 x 150 mm

Mobile phase: 150 mM sodium phosphate, pH 7.0

Overlays of chromatograms 1 and 400





Agilent 130Å AdvanceBio SEC protein standards

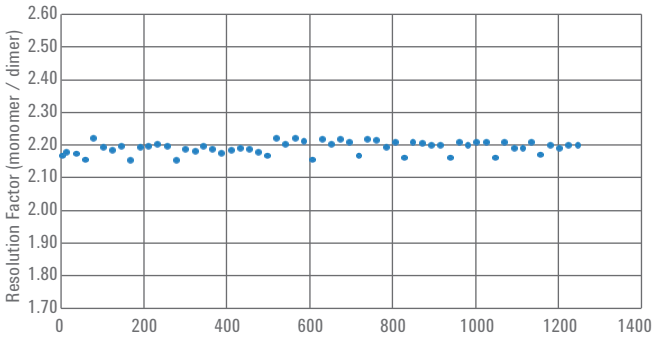
A protein mix consisting of 5 carefully selected proteins (Ovalbumin, Myoglobin, Aprotinin, Neurotensin, Angiotensin II) designed to calibrate Agilent's 130Å AdvanceBio size exclusion columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

Agilent 300Å AdvanceBio SEC protein standards

A protein mix consisting of 5 carefully selected proteins (Thyroglobulin, γ-globulin, Ovalbumin, Myoglobin, 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.

No change to sample integrity

As part of the installation protocol, older generations of protein SEC columns had to be conditioned with BSA to block the sites responsible for non-specific interactions. The new bonding chemistry used in Agilent AdvanceBio SEC columns eliminates this problem by providing an inert surface that reduces non-specific interactions and maintains sample integrity.



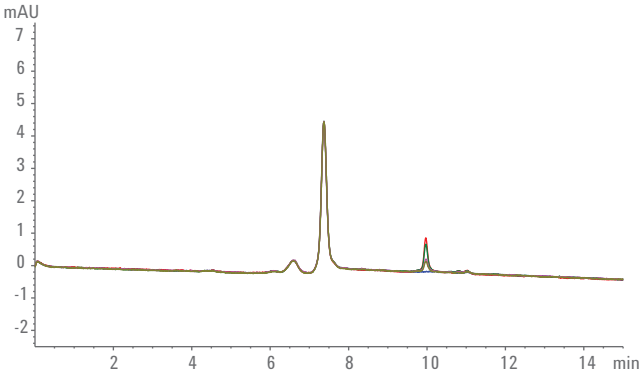
Column: AdvanceBio SEC, 300Å, 7.8 x 300 mm

Mobile phase: 150 mM sodium phosphate, pH 7.0

Sample: IgG

Plot showing the resolution between IgG monomer and dimer over a 1300 injection sequence.

No protein recovery issues observed—Sample integrity maintained

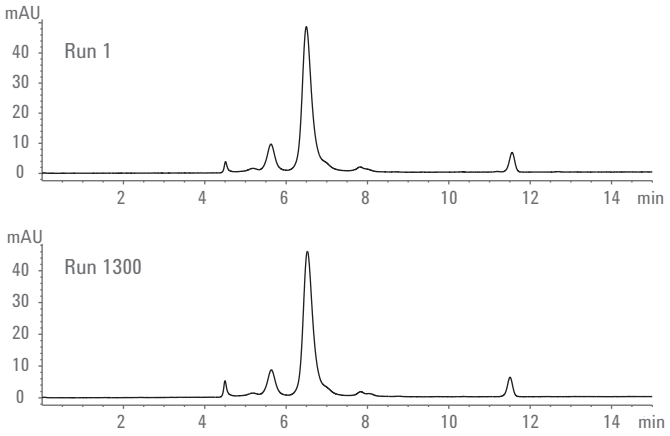


Column: AdvanceBio SEC, 300Å, 7.8 x 300 mm

Mobile phase: 150 mM sodium phosphate, pH 7.0

Five replicate injections of BSA 0.1 mg/mL, 1 µL equivalent to 0.1 µg of BSA on-column. BSA peak area is also consistent, even with 0.1 µg on-column loads.

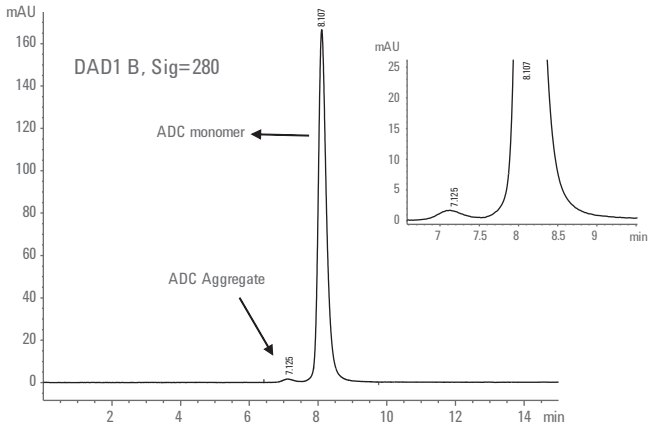
Injections	Monomer Peak Area
1	50.5
2	49.6
3	50.6
4	50.0
5	50.2



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

Perform aggregate quantitation—even at low levels

Resolution and baseline stability were achieved with an AdvanceBio SEC-3, 300Å, 7.8 x 300 mm, 2.7 µm column. This enabled low-level quantitation of dimers and aggregates in mAb and Antibody-Drug Conjugate (ADC) sample.

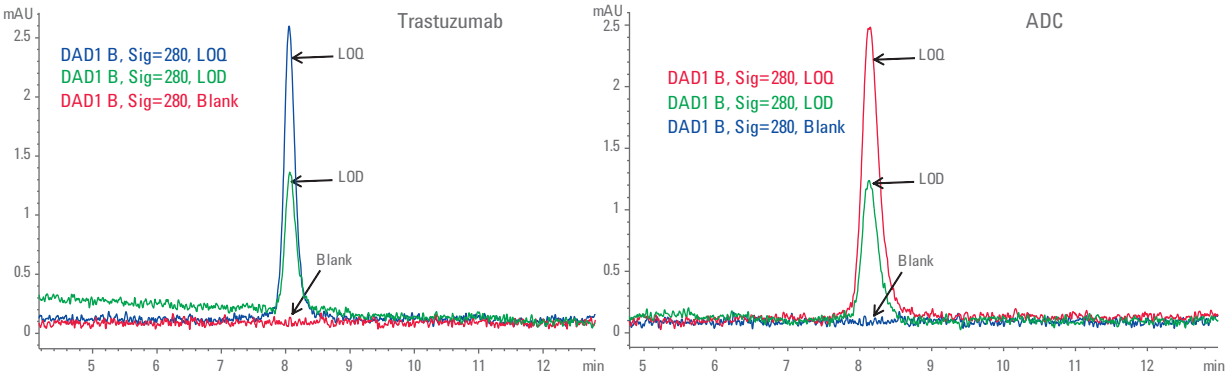


Column:	AdvanceBio SEC-3, 300Å, 7.8 x 300 mm
Mobile phase:	(PBS) 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow rate:	0.8 mL/min
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Sample:	Trastuzumab and Antibody Drug Conjugate (T-DM1)

SEC profile of intact T-DM1 (ADC).

Lower limits of detection (LOD) and limits of quantitation (LOQ)

The LOD and LOQ were found to be 15 µg/mL and 31 µg/mL, respectively, for trastuzumab and ADC—proving method sensitivity.



LOD and LOQ chromatograms of trastuzumab and ADC overlaid with blank.

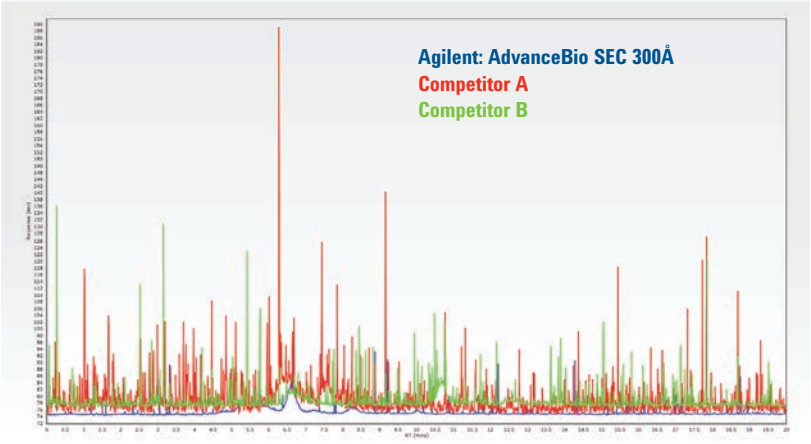
INFINITELY BETTER BIOMOLECULE ANALYSIS

SEC has been a workhorse in the analysis of biopolymers for decades, especially in detecting and quantifying protein aggregation. The use of advanced detectors can provide increased sensitivity to these critical parameters and provide further information for the analyst, especially important with increasing regulations. The Agilent 1260 Infinity Bio-SEC MDS is a dedicated solution for large biomolecule analysis.

Advanced detectors determine accurate molecular weights, size and shape of the biomolecule. The design of metal-free components in the sample flow path and the absence of iron and steel in solvent delivery ensure the integrity of the biomolecule, minimizing unwanted surface interactions and providing confidence in quality of data. Combined with intuitive and easy-to-use software, this advanced information is easily and reproducibly obtained from this high performance system.

- **Accurate molecular weights**—Determine actual molar mass rather than relative
- **Sensitive aggregation**—Discover presence of aggregation at much lower quantities
- **Study molecular size**—Determine radius of hydration of the biomolecule
- **Access to all**—Get the results you need, no matter your level of experience
- **Reproducible**—Increasing the reproducibility of advanced detection
- **Maintain sample integrity**—Complete metal-free sample contacting surfaces
- **Simplicity**—Advanced information through a simple user-interface

Advanced detection techniques



The AdvanceBio SEC column has the lowest particle shedding and hence the least baseline noise.

MASS SPEC COMPATIBILITY FOR PEAK IDENTIFICATION

The Bio SEC-3 particle technology is stable toward MS-friendly eluents, making **Agilent Bio SEC-3 columns** fully mass spec compatible. Robust particles also ensure baseline stability and low MS signal suppression. Other advantages include:

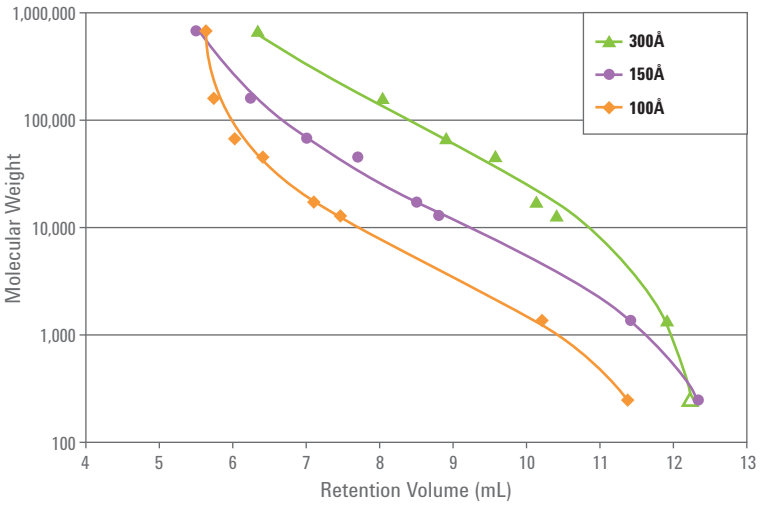
- Compatibility with denaturing organic/aqueous mobile phases used for SEC-MS
- Excellent stability with both high- and low-salt conditions
- Scalability from analytical to laboratory prep

The Agilent Bio SEC-3 columns use the same particle technology as the Agilent SEC-5 columns and so provide a higher efficiency option when more resolution is needed for the smaller biomolecules.

Bio SEC-3 column specifications

Bonded phase	Pore size (Å)	Particle size (µm)	Protein MW range (Da)	pH range	Flow rate (mL/min)	Typical operating pressure (bar)
Bio SEC-3	100	3	100 to 100,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-3	150	3	500 to 150,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-3	300	3	5,000 to 1,250,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)

Bio SEC-3 protein calibration curves



Column: Bio SEC-3, 7.8 x 300 mm, 3 µm

Mobile phase: Sodium phosphate 150 mM, pH 7.0

Flow rate: 1.0 mL/min

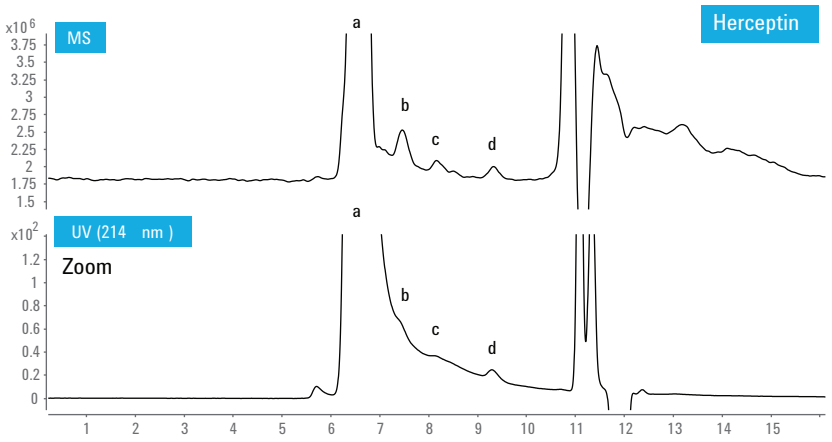
Detector: UV

		Retention volume		
Proteins	MW/Dalton	300Å	150Å	100Å
Thyroglobulin	670,000	6.34	5.50	5.63
γ-Globulin	150,000	8.03	6.24	5.74
BSA	67,000	8.90	7.00	6.03
Ovalbumin	45,000	9.57	7.70	6.41
Myoglobin	17,000	10.12	8.50	7.10
Ribonuclease A	12,700	10.40	8.80	7.46
Vitamin B12	1,350	11.90	11.40	10.20

Mass spectrometry is an excellent technique for determining peak structure and identity.

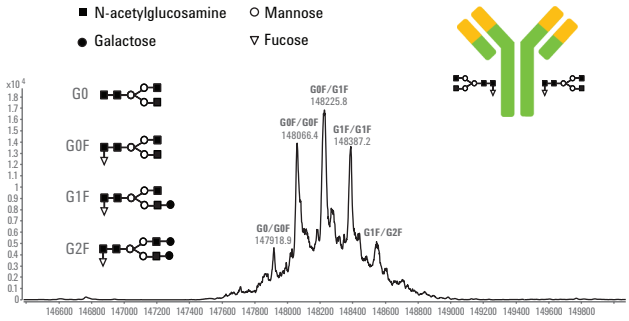
Interfacing SEC with MS can provide additional information not available when DAD or UV is used. However, typical mobile phases used for SEC contain buffers and salts, making them incompatible with MS. By changing to a denaturing mobile phase (such as acetonitrile with water mixed with formic and trifluoroacetic acid) the SEC column can be interfaced directly to the MS.

SEC-MS Analysis (Intact mAb)



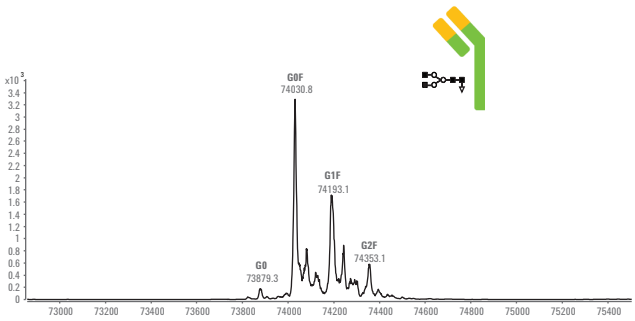
Column:	Bio SEC-3 7.8 x 300 mm, 3 µm	Mobile phase:	20% ACN, 0.1% formic acid, 0.1% TFA
LC:	Agilent 1290 Infinity Bio-inert Quaternary LC	Flow rate:	1 mL/min
MS:	Agilent 6540 Q-TOF	Temperature:	24 °C

Peak a



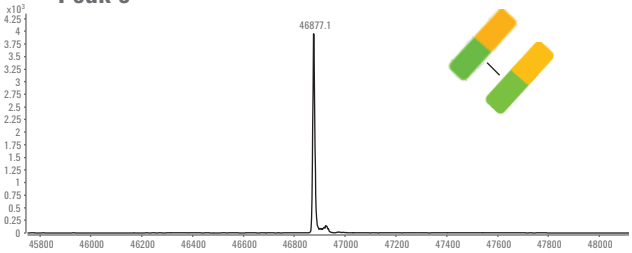
Counts vs. Deconvoluted Mass (amu)

Peak b



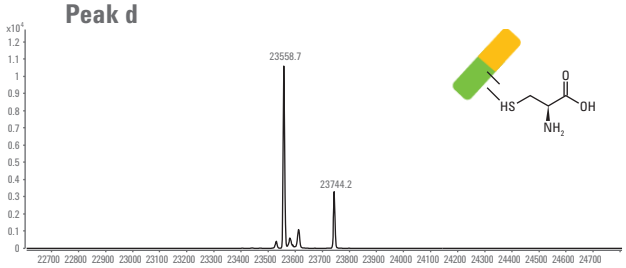
Counts vs. Deconvoluted Mass (amu)

Peak c



Counts vs. Deconvoluted Mass (amu)

Peak d



Counts vs. Deconvoluted Mass (amu)

Peak a represents the intact mAb monomer peak with different glyco forms. Peaks b, c, and d are fragments.

EXCELLENT PERFORMANCE FOR LARGER BIOMOLECULES

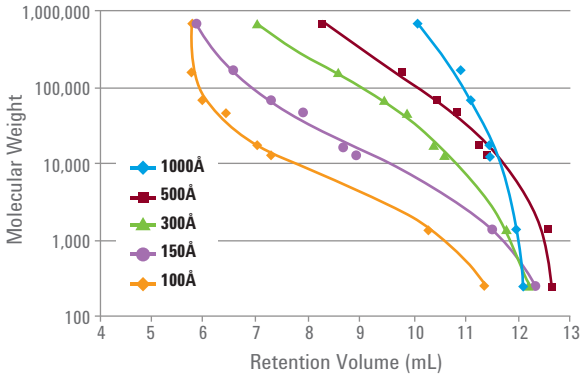
For large biomolecules and samples with components of multiple molecular weights, **Agilent Bio SEC-5 columns** are an ideal choice. They are packed with 5 µm silica particles coated with a proprietary, neutral, hydrophilic layer for maximum efficiency and stability, with 6 different pore sizes to provide optimum resolution over the molecular weight range.

- **Exceptional resolution** for large molecules
- **High stability and efficiency** due to a proprietary neutral hydrophilic layer
- **Improved peak capacity and resolution** due to specially designed packing that increases pore volume
- **Rugged performance:** Outstanding reproducibility and column lifetime
- **Excellent stability**, even under high-pH, high-salt, and low-salt conditions
- **Flexible method development:** Compatible with most aqueous buffers
- **Broad applicability:** Up to 2000Å pore size for vaccines and high molecular weight biomolecules

Bio SEC-5 column specifications

Bonded phase	Pore size (Å)	Particle size (µm)	Protein MW range (Da)	pH range	Flow rate (mL/min)	Typical operating pressure (bar)
Bio SEC-5	100	5	100 to 100,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-5	150	5	500 to 150,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-5	300	5	5,000 to 1,250,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-5	500	5	15,000 to 2,000,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-5	1000	5	50,000 to 7,500,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)
Bio SEC-5	2000	5	>10,000,000	2 to 8.5	0.1 to 1.25 (7.8 mm id); 0.1 to 0.4 (4.6 mm id)	103 (1500 psi)

Bio SEC-5 protein calibration curves



Column: Bio SEC-5, 7.8 x 300 mm, 5 µm
Mobile phase: 150 mM Na phosphate, pH 7.0
Flow rate: 1.0 mL/min
Detector UV

		Retention volume				
Proteins	MW/Dalton	1000Å	500Å	300Å	150Å	100Å
Thyroglobulin	670,000	10.07	8.23	7.03	5.82	5.77
γ-Globulin	158,000	10.88	9.80	8.57	6.55	5.79
BSA	67,000	11.13	10.44	9.44	7.29	6.00
Ovalbumin	45,000	11.28	10.83	9.89	7.90	6.40
Myoglobin	17,000	11.44	11.28	10.42	8.66	7.05
Ribonuclease A	12,700	11.52	11.41	10.58	8.93	7.32
Vitamin B-12	1,350	12.00	12.59	11.78	11.49	10.30
Uracil (total permeation marker)	112	12.08	12.68	12.21	12.13	11.41

Multiple pore sizes available for optimum resolution over molecular weight range.

ANALYZE GLOBULAR PROTEINS WITH A SINGLE COLUMN

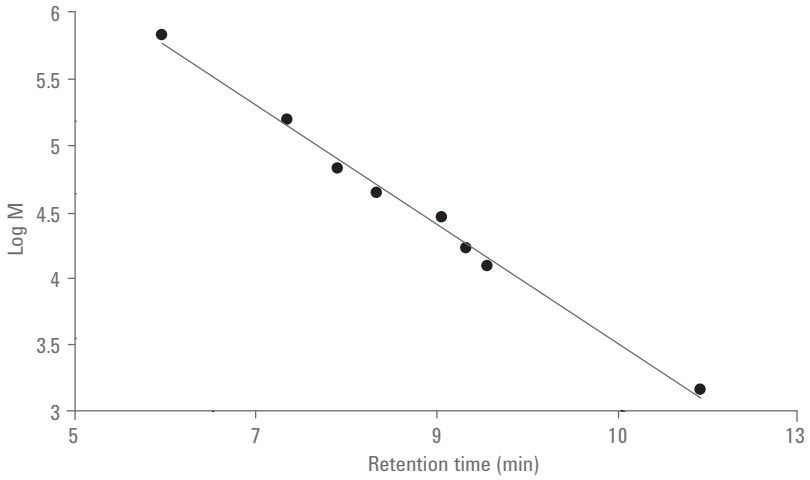
ProSEC 300S columns are designed as a single column solution for globular protein analysis. The pore size selection and optimization provides an extended linear resolving range so that this single column can be used for analysis across the full range of globular proteins. Their robust particle does not fragment during use, which prevents particulate leaching and gives you an exceptionally stable baseline.

- **Stable performance:** Mechanically robust silica particles that do not bleed during use
- **Easy method development:** Extended linear resolving range eliminates the need for pore size selection—a *single column* to analyze most globular proteins
- **Choices to help you perfect your separation:** Two column ids to suit multi-detector SEC
- **Increased sensitivity** when used with light-scattering detectors, to identify dimers, trimers, and aggregates

ProSEC 300S column specifications

Bonded phase	Pore size (Å)	Particle size (µm)	Protein MW range (Da)	pH range	Flow rate (mL/min)	Typical operating pressure (bar)
ProSEC 300S	300	5	1,500 to 800,000	2 to 7.5	<1.5 (7.5 mm id); <0.5 (4.6 mm id)	250 (3700 psi)

Calibration of the ProSEC 300S column with globular proteins



Column:	ProSEC 300S 7.5 x 300 mm, 5 µm
Mobile phase:	50 mM KH_2PO_4-K_2HPO_4 (at pH 6.8) containing 0.3 M NaCl
Flow rate:	1.0 mL/min
Detection:	UV 280 nm
Sample:	Protein samples

Molecular weights of the proteins

MW/Dalton	Protein
670,000	Thyroglobulin
155,000	g-Globulin
66,430	Bovine serum albumin
44,287	Ovalbumin
29,000	Carbonic anhydrase
16,700	Myoglobulin
12,384	Cytochrome c
1,423	Bacitracin

This calibration curve shows a linear relationship between retention time and the log of the molecular weight—demonstrating that a pure size exclusion separation is taking place.

FOR SEC METHODS THAT REQUIRE USP DESIGNATION L35

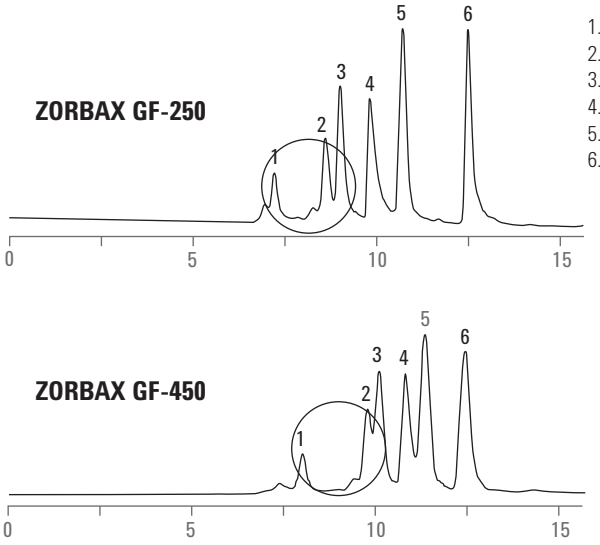
ZORBAX GF-250 and ZORBAX GF-450 size exclusion (gel filtration) columns feature a hydrophilic diol bonded phase for high protein recovery (typically >90%)—plus a unique zirconia silica modification for a wider pH operating range.

- **End-to-end analysis:** A choice of semi-prep and prep column dimensions, usable at flow rates of up to 3 mL/min
- **High recovery:** Hydrophilic diol bonded base typically >90% recovery
- **Rugged:** Precisely-sized porous silica microspheres of narrow pore size and particle size distribution
- **Compatible with organic modifiers** (<25%) and denaturants to minimize non-specific interactions
- **Broad applicability:** A wide usable pH range: 3 to 8

ZORBAX GF-250 and ZORBAX GF-450 column specifications

Bonded phase	Pore size (Å)	Particle size (µm)	Protein MW range (Da)	pH range	Flow rate (mL/min)	Typical operating pressure (bar)
ZORBAX GF-250	150	4	4,000 to 400,000	3.0 to 8.0	<3.0	350 (5,104 psi)
ZORBAX GF-450	300	6	10,000 to 900,000	3.0 to 8.0	<3.0	350 (5,104 psi)

Separations of proteins on semi-preparative columns



1. Thyroglobulin, 5.43 min
2. BSA dimer, 6.19 min
3. BSA monomer, 6.93 min
4. Ribonuclease A, 8.74 min
5. Poly-DL-alanine (1-5 kDa), 9.90 min
6. Uracil, 12.13 min

Column:	ZORBAX GF-250, 9.4 x 250 mm, 4 µm
Column:	Agilent ZORBAX GF-450, 9.4 x 250 mm, 6 µm
Mobile phase:	0.2 M Na_2HPO_4, pH 7.0
Flow rate:	5.0 mL/min
Detection:	UV 280 nm
Sample:	200 µL

This protein separation shows the complementary relationship between GF-450 and GF-250 preparative columns. The GF-450 column reliably separated the high molecular weight biomolecules, which were excluded from the linear separation range of the GF-250 column (with a smaller pore size).

SAMPLE FLEXIBILITY FROM A SINGLE ADVANCEBIO SEC COLUMN

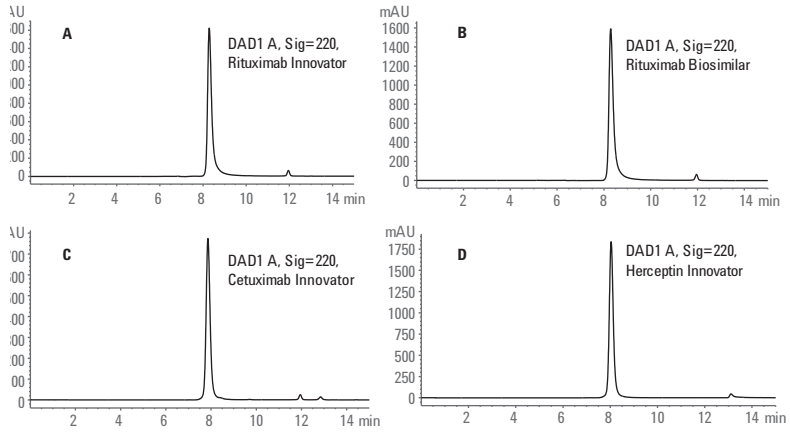
A single AdvanceBio SEC column and method used for analysis of multiple biotherapeutics

Multiple sample types, biotherapeutic mAbs, innovators, biosimilars and ADC using the same aqueous mobile phase.

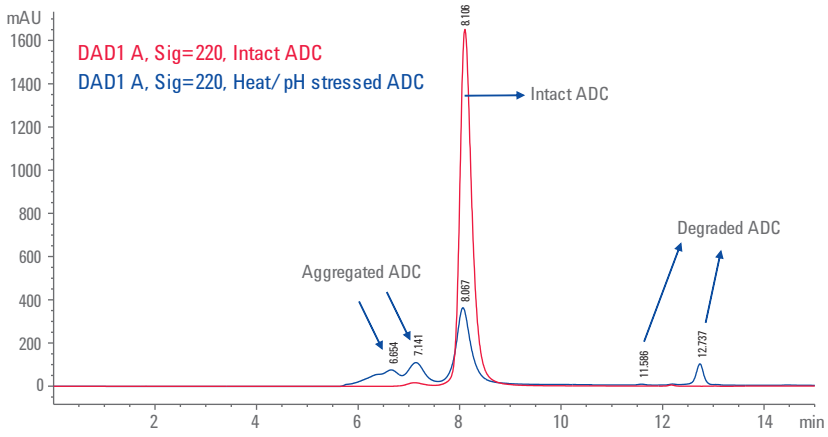
Column:	AdvanceBio SEC-3, 300Å, 7.8 x 300 mm
Mobile phase:	(PBS) 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow rate:	0.8 ml/min Agilent 1260 Infinity Bio-inert Quaternary LC
Sample:	Rituximab Innovator, Rituximab Biosimilar, Cetuximab Innovator, Herceptin Innovator, ADC

Retention time and peak area precision (n=6)

	Retention Time		Peak Area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Rituximab Innovator	8.28	0.04	99.33	1.21
Rituximab Biosimilar	8.29	0	100	0
Cetuximab Innovator	7.86	0	99.60	0.96
Herceptin Innovator	8.034	0	100	0
ADC	8.106	0.005	98.91	0.33



SEC profile of intact therapeutic mAbs, A- Rituximab Innovator, B- Rituximab Biosimilar, C-Cetuximab Innovator and D- Herceptin Innovator.



AdvanceBio SEC chromatogram of native (control; red trace) ADC overlaid with 2 mg/mL ADC pH/heat stressed.

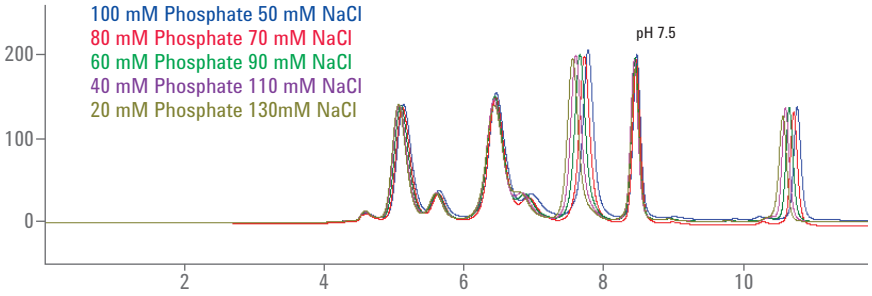
PERFORM EFFICIENT SIZE SEPARATIONS WITH A WIDE RANGE OF MOBILE PHASES

Rugged performance and resolution in varying salt conditions

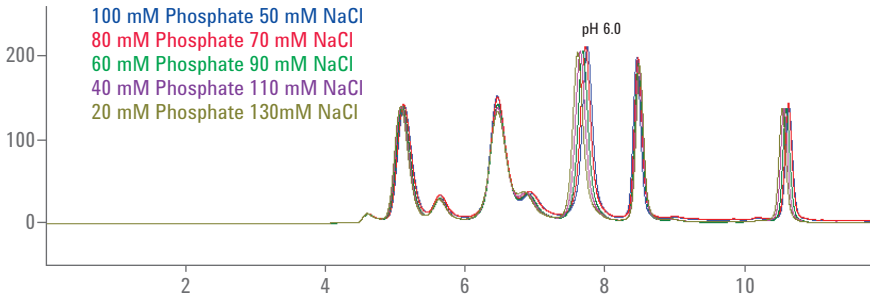
Agilent AdvanceBio SEC columns provide rugged performance across varying salt conditions, and over time.

Your SEC separation mechanism must be based on *size*, with no secondary interactions with the stationary phase. The innovative particle technology employed in AdvanceBio SEC columns eliminates the need to use high salt concentrations to achieve size separations—reducing non-specific ionic interactions.

Column: AdvanceBio SEC-3, 300Å, 7.8 x 300 mm
BioRad Gel Filtration Standard #1511901



Here, peak shapes are consistent and do not show the characteristic distortion of non-specific interactions.



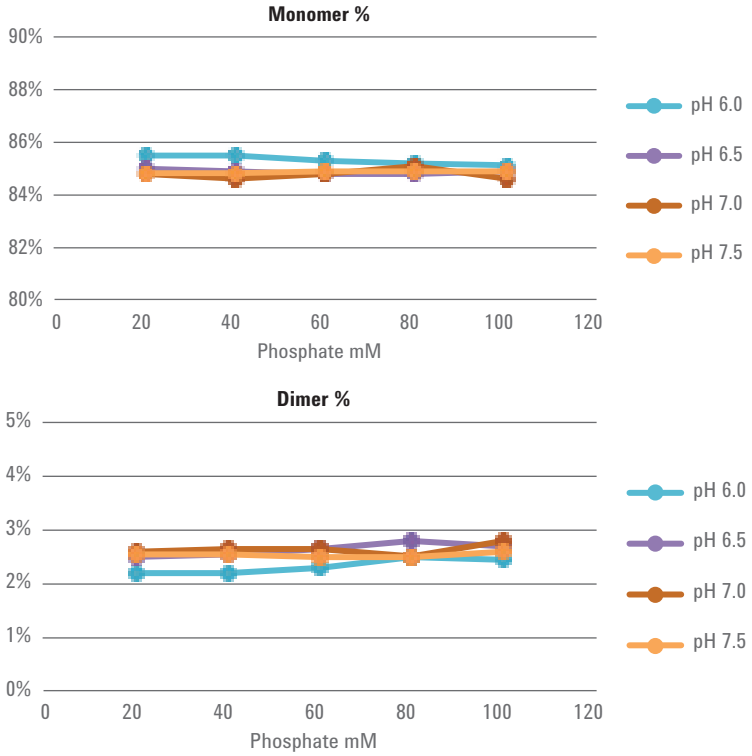
No salt required: SEC retention is related to molecular size in solution. Slight changes in elution times are likely due to the impact of pH and salt concentration on the hydrodynamic radius of the proteins.

SOFTWARE ENABLES METHOD OPTIMIZATION IN A FRACTION OF THE TIME

Regulatory bodies require that experimental designs establish the optimal analytical method, along with method robustness and reproducibility. Doing this manually involves preparing a vast number of mobile phases and equilibrating the LC as each mobile phase is changed—a time-consuming process.

Agilent Buffer Advisor software simplifies the protocol by allowing you to prepare buffers on-system from just four components (two buffers, plus a water and salt solution).

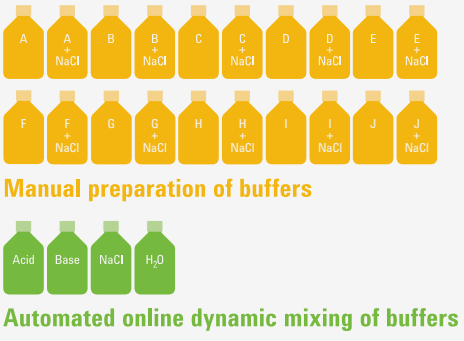
In the analysis at right, BSA monomer and dimer quantitation was investigated for robustness using 20 mobile phases (pH 6.0 to 7.5) and buffer concentrations ranging from 20 mM to 100 mM.



Column: AdvanceBio SEC, 300Å, 7.8 x 300 mm

Sample: BSA

The impact of phosphate buffer concentration and pH on monomer and dimer quantitiation.



Save time and money on buffer preparation

Agilent Buffer Advisor software helps to automate the production of buffers. Dynamic mixing of only 4 stock solutions eliminates the need to prepare and titrate multiple buffer solutions.

Boost Performance: Rapid method development through automated buffer preparation

Save Time: Quaternary mixing enables easy blending of multiple buffers of different pH or salt concentration, for faster buffer preparation

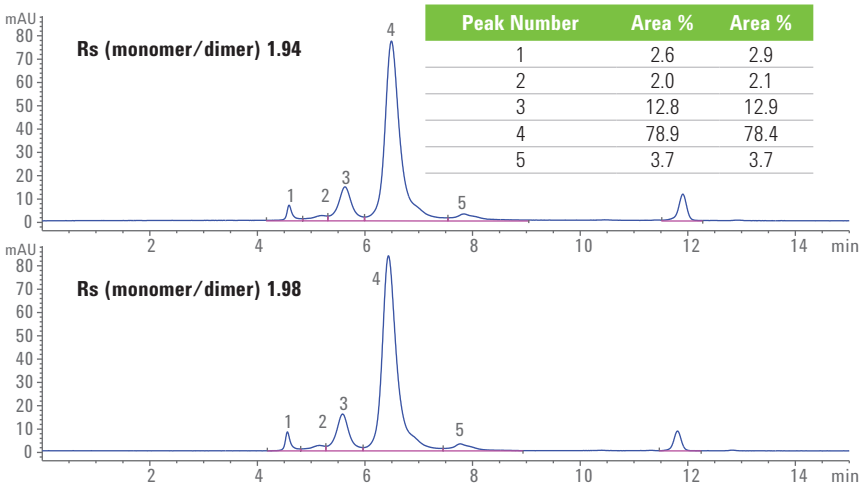
Reduce Costs: Evaluate conditions for optimum eluent before running samples, for less sample waste and shorter analysis time

RELY ON ADVANCEBIO SEC TO END UNCERTAINTY

Batch to batch reproducibility

When working in regulated laboratories and needing to deliver data to deadlines, having robust methods and columns is a necessity.

The AdvanceBio SEC columns deliver on performance and reproducibility across the lifetime of the column. See pages 5 and 6 for data and from column to column and batch to batch.



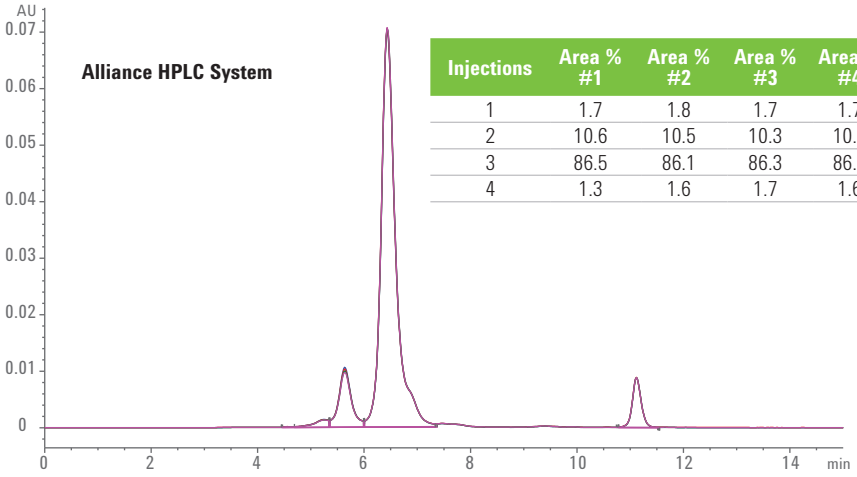
Peak Number	Area %	Area %
1	2.6	2.9
2	2.0	2.1
3	12.8	12.9
4	78.9	78.4
5	3.7	3.7

Analysis of γ-globulin (IgG) using AdvanceBio SEC 300Å 4.6 x 300 mm, 2.7 µm columns from two different batches of media. The resolution between peaks 3 and 4, the dimer and monomer, and the percentage peak areas show excellent batch to batch reproducibility

Instrument to instrument

Transferring methods to other LC instruments within the same department or in different departments, locations or companies is required as candidate biotherapeutics progress through development to approval. The 2.7 µm particle size of the AdvanceBio SEC columns deliver resolution at pressures below 200 bar. Analysis can be done using HPLC systems—no need to consider instrument availability when developing methods.

Overlay of four consecutive injections on Waters Alliance HPLC System



Injections	Area % #1	Area % #2	Area % #3	Area % #4
1	1.7	1.8	1.7	1.7
2	10.6	10.5	10.3	10.2
3	86.5	86.1	86.3	86.6
4	1.3	1.6	1.7	1.6

Overlay of four consecutive chromatograms of a polyclonal IgG on AdvanceBio SEC 300Å, 7.8 x 300mm, 2.7µm performed on Waters' Alliance HPLC System.

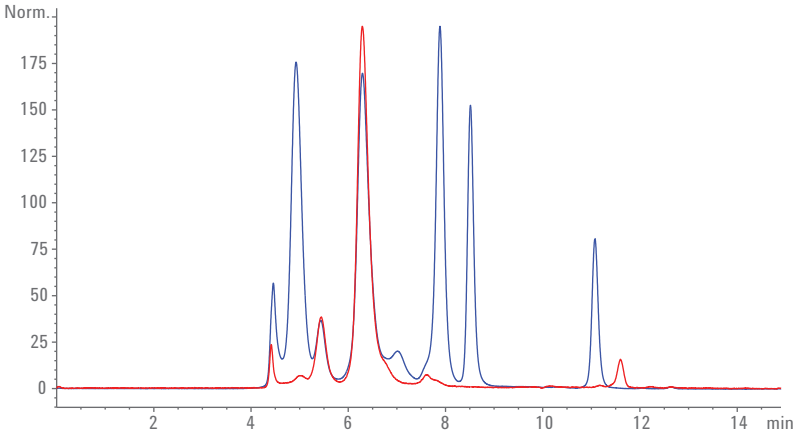
MULTIPLE COLUMN DIMENSIONS— SAME PERFORMANCE

UHPLC methods for increased sensitivity

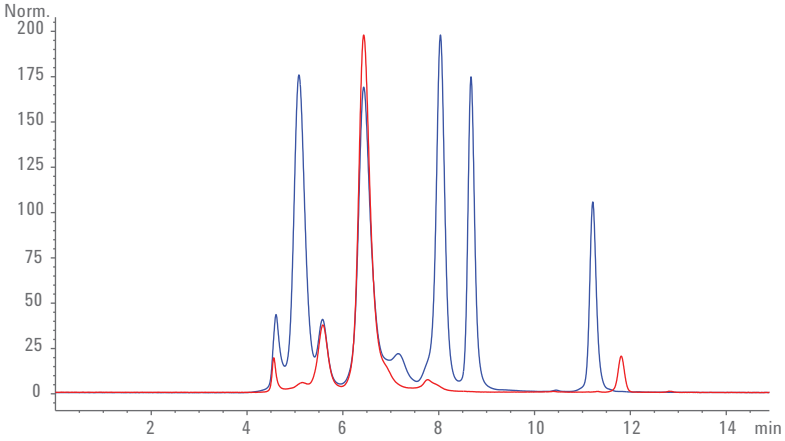
UHPLC instruments provide the lowest dispersion and high precision and are the LCs of choice for early development. For applications that require sensitivity and/or small amounts of sample, a smaller ID of column is used. For SEC, this would be 4.6 mm ID. Typically flow rates used with these columns would be 0.3 to 0.7 mL/min .

To compare the column performance on different instruments the BioRad Gel Filtration Standard #1511901 (blue peaks) and a commercially available polyclonal IgG were used. Data was generated in different laboratories, using different columns and different lots of test samples.

Agilent 1290 Infinity Binary LC with Agilent 1260 Infinity DAD (G1315D) and Bio-inert flow cell (1200 bar).
AdvanceBio SEC 4.6 x 300 mm column and a flow rate of 0.35 mL/min.



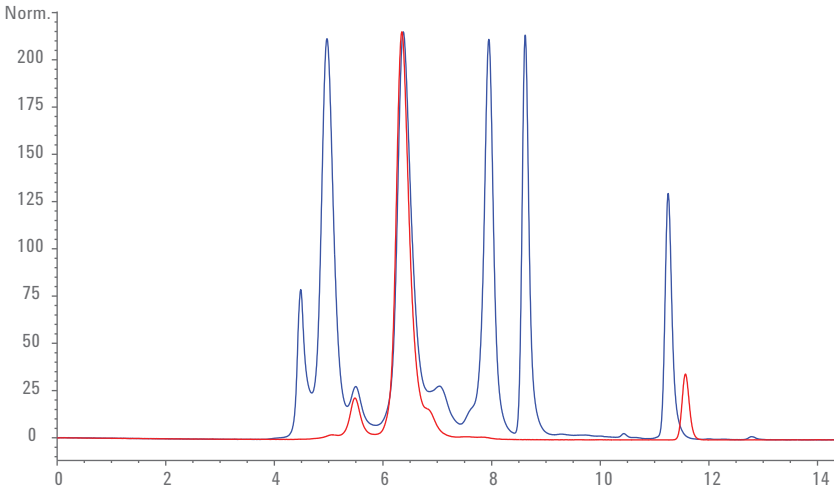
Agilent 1260 Infinity Quaternary Bio-inert LC (600 bar).
AdvanceBio SEC 4.6 x 300 mm column and a flow rate of 0.35 mL/min.



HPLC methods for robustness

For robust methods able to deliver the accuracy and reproducibility needed for QC methods, HPLC instruments are often the systems of choice. For robust HPLC methods, the larger 7.8 mm id columns are routinely used with HPLC systems. The ideal flow rates for this type of LC system are 1.0 mL/min with the 300 mm long columns for resolution and up to 2 mL/min with the 150 mm long columns for sample throughput.

Agilent 1100 HPLC instrument (400 bar).
AdvanceBio SEC 7.8 x 300 mm column and a flow rate of 1.0 mL/min.



With the AdvanceBio SEC columns there is no need to change the column or validate a new method when a candidate moves from discovery to development and into manufacture just simply change the dimensions of the AdvanceBio SEC column.

Agilent's AdvanceBio SEC columns with the optimized 2.7µm particle size provide outstanding performance with typical pressures of less than 200 bar.

Methods can be transfered from instrument to instrument and lab to lab.

The UHPLC data was generated in Europe in an R&D laboratory and the HPLC data in a technical support laboratory in the US–by different scientists using different samples and different columns.

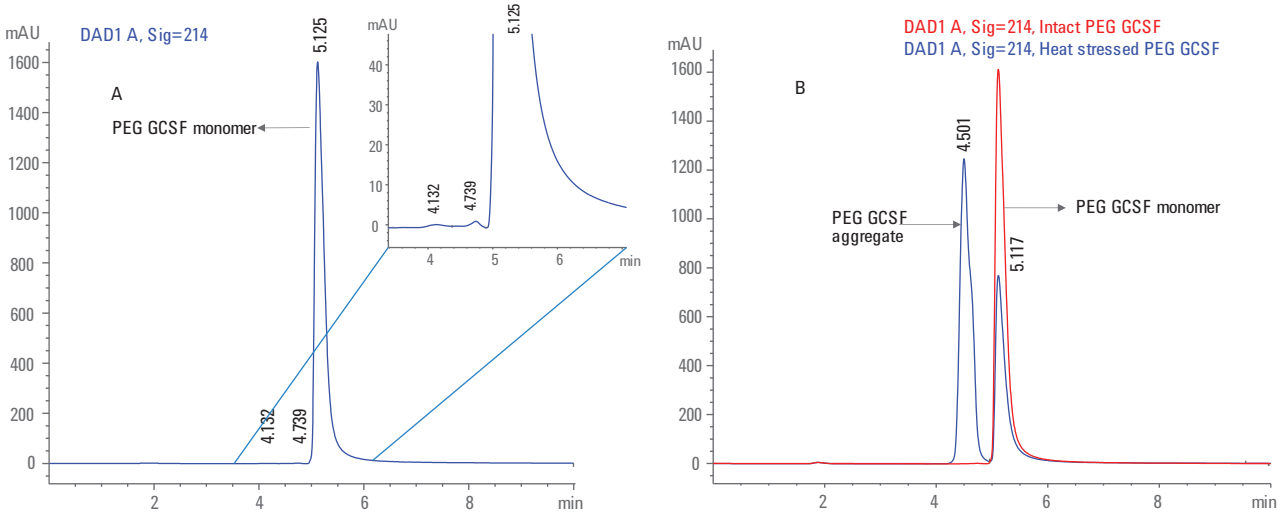
SEC MONOGRAPH METHODS

Analysis of PEGylated proteins

PEGylation is the covalent attachment of polyethylene glycol (PEG) polymers to proteins or drugs. Advantages of PEGylating a molecule include increased bioavailability, increased serum half-life, and decreased immunogenicity.

Size exclusion chromatography (SEC) is the method of choice for identifying high molecular mass impurities of PEGylated proteins. Below, an SEC method was used to identify pegfilgrastim (PEG GCSF) per a draft monograph using an Agilent AdvanceBio SEC 130 Å, 7.8 x 300 mm, 2.7 µm column.

The results confirm that the area percent of monomer *and* the retention time standard deviation were within acceptable criteria set by the monograph. Additionally, Agilent SEC columns successfully separated, detected, and quantified aggregates of PEG GCSF produced by forced stress studies.



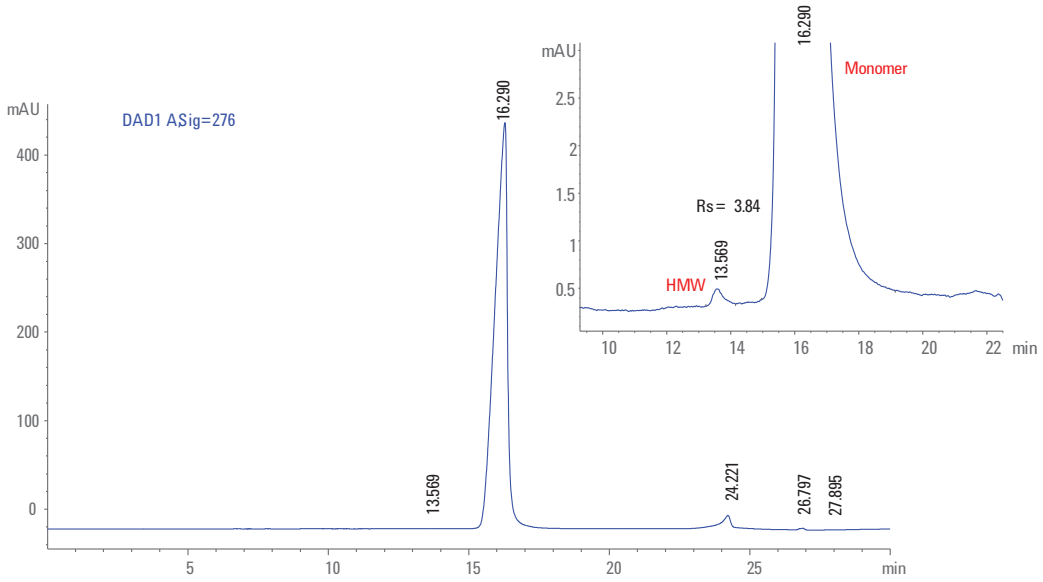
Column	AdvanceBio SEC 130Å, 7.8 x 300 mm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase (as per monograph):	a mixture of 6.8 ml of 85% v/v solution of orthophosphoric acid in 800 volumes of water, adjust pH to 2.5 with 10 N sodium hydroxide, 50 volumes of ethanol, and 150 volumes of water.
Flow rate:	1 mL/min
Temperature:	25 °C
Thermostat autosampler:	4 °C
Detector:	214 nm (1260 DAD VL+, G5615-60022 Bio-inert flow cell)
Injection volume:	3 µL

Size exclusion chromatography of PEG GCSF on AdvanceBio SEC 130Å, 7.8 x 300 mm, 2.7 µm column.
A. Intact PEG GCSF, zoom in view showing aggregates. B. Intact PEG GCSF, overlay with heat stressed sample showing aggregate.

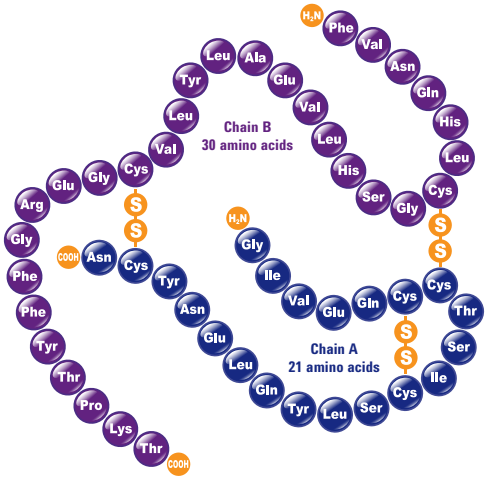
Analysis of insulin

Insulin is a small polypeptide hormone that controls blood glucose homeostasis. Biopharmaceutical companies use genetic engineering techniques to develop diverse, long-acting insulin analogues.

The data below were produced by analyzing an insulin analogue and its aggregates using a modified draft monograph SEC method. The smaller pore size Agilent AdvanceBio SEC 130Å, 7.8 x 300 mm, 2.7 µm column provided superior resolution between monomer and aggregate.



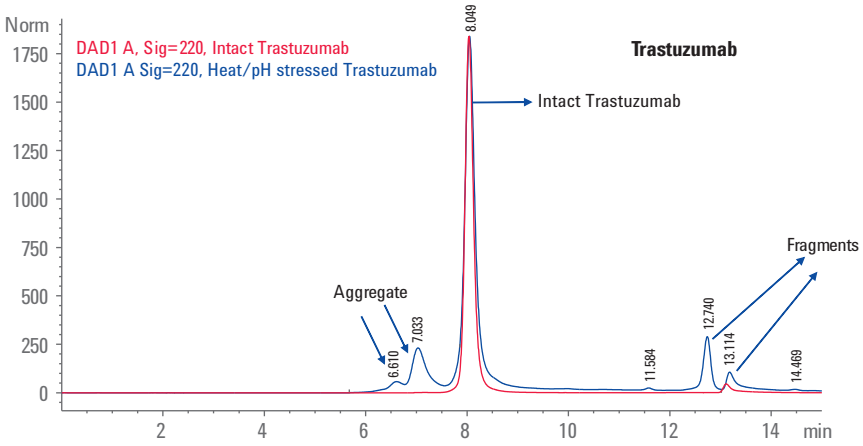
Column:	AdvanceBio SEC 130Å, 7.8 x 300 mm
Instrument:	Agilent 1290 Infinity Binary LC System
Mobile phase	L-arginine (1.0 g/L)/acetic acid (99%)/acetonitrile; 65/15/20 (v/v/v)
Flow rate:	0.4 mL/min
Temperature:	Ambient
Thermostat autosampler:	4 °C
Detector:	276 nm (1260 DAD VL+, G5615-60022 Bio-inert flow cell)
Injection volume:	10 µL



MONITOR PROTEIN STABILITY

The AdvanceBio SEC column resolved the aggregates—plus the degraded Trastuzumab and ADC. In both cases, aggregation increased as the samples were stressed.

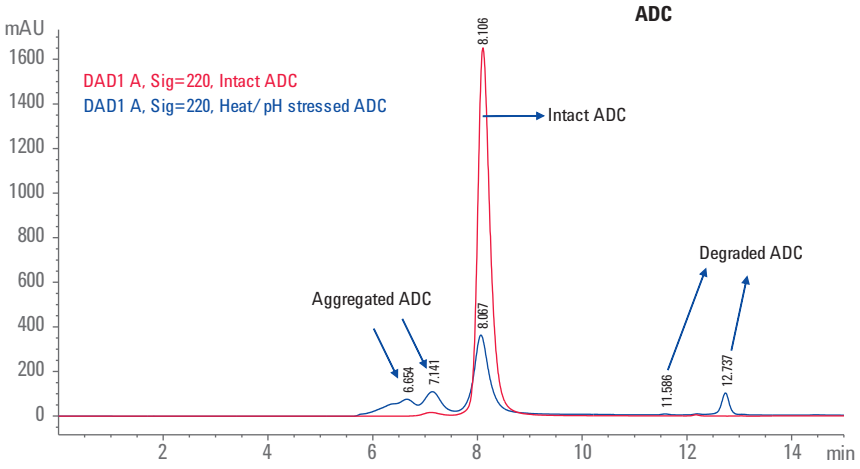
The same aqueous mobile phase was used for both the mAb and the more hydrophobic ADC.



Chromatogram of native (red trace) Trastuzumab overlaid with 2 mg/mL Herceptin pH/heat stressed.

Column:	AdvanceBio SEC 300Å, 7.8 x 300 mm
Mobile phase:	50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4
Flow rate:	0.8 mL/min
System:	Agilent 1260 Infinity Bio-inert Quaternary LC
Sample:	Trastuzumab and Antibody Drug Conjugate (T-DM1)

Stressed samples were prepared by adjusting pH to 1.0, 10, and 6.0, then incubated at 60 °C for 60 min.



Chromatogram of native (red trace) ADC overlaid with 2 mg/mL ADC pH/heat stressed.

IMPROVE YOUR ACCURACY FOR BIO-PURIFICATION AND SEMI-PREPARATIVE WORK

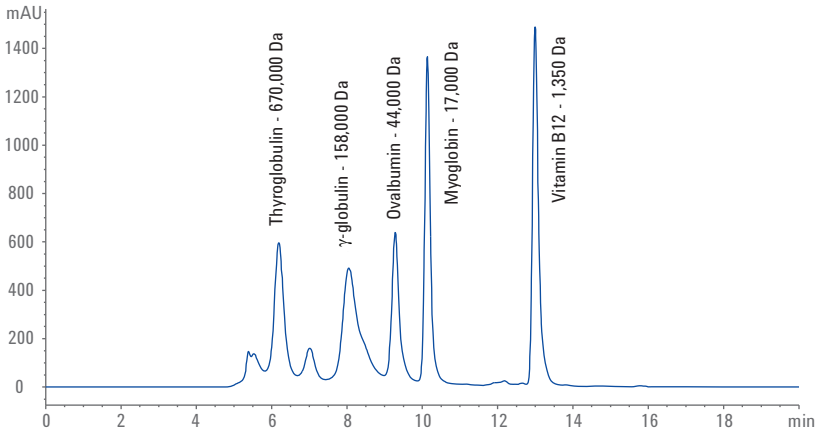
Agilent BioHPLC columns are available in sizes that support reliable bio-purification and semi-preparative applications. Together with the Agilent 1260 Infinity Bio-inert LC System and the 1260 Infinity Bio-inert Fraction Collector, they can increase the accuracy of your peak-based fraction collection.

SEC and fraction collection

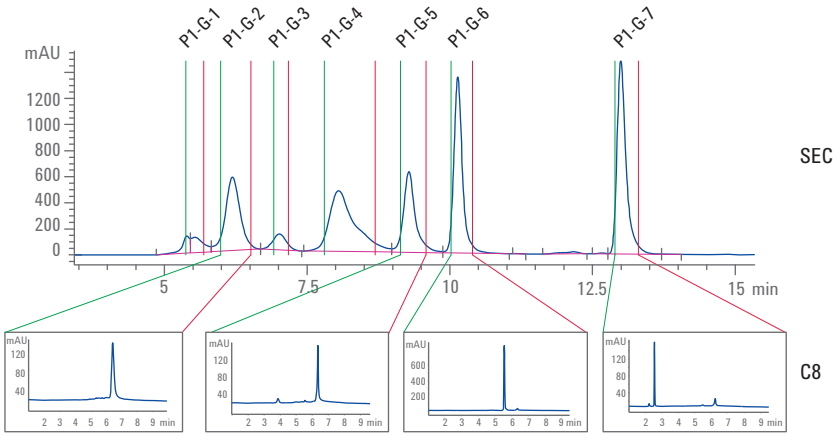
Column:	Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm
Mobile phase:	Buffer A: 50 mM sodium phosphate buffer + 150 mM NaCl, pH 6.8
Flow rate:	1 mL/min
Gradient:	Isocratic
Injection volume:	30 µL
Thermostat autosampler and FC:	8 °C
Temperature TCC:	RT
DAD:	280 nm/4 nm, Ref.: OFF
Peak width:	>0.05 minute (1.0 second response time) (5 Hz)

Re-analysis—reversed-phase C8

Column:	ZORBAX 300SB-C8, 4.6 x 50 mm, 5 µm
Mobile Phases:	Buffer B: ACN + 0.09% TFA Buffer C: H_2O_{dd} + 0.1% TFA
Flow rate:	1 mL/min
Gradient:	0 min 5 % B, 95% C 10 min 95% B, 5% C Runtime: 10 min Stoptime: 10 min
Injection volume:	100, 50 and 10 µL
Thermostat autosampler and FC:	8 °C
Temperature TCC:	70 °C
DAD:	280 nm/4 nm Ref.: OFF
Peak width:	>0.05 minute (1.0 second response time) (5 Hz)



This gel filtration standard containing thyroglobulin, γ-globulin, ovalbumin, myoglobin, and Vitamin B12 was separated using SEC. Afterwards, we automatically collected the fractions (using peak-based fraction trigger mode) into ascertained wells of a deep-well plate. The fractions were then re-analyzed using a reversed-phase C8 column.



A re-analysis of the fractions confirms the exact fractionation procedure using peak-based fraction-trigger mode. The 2-position/6-port valve in the column compartment enabled automated column switching.

INCREASE SENSITIVITY WITH LIGHT SCATTERING DETECTION

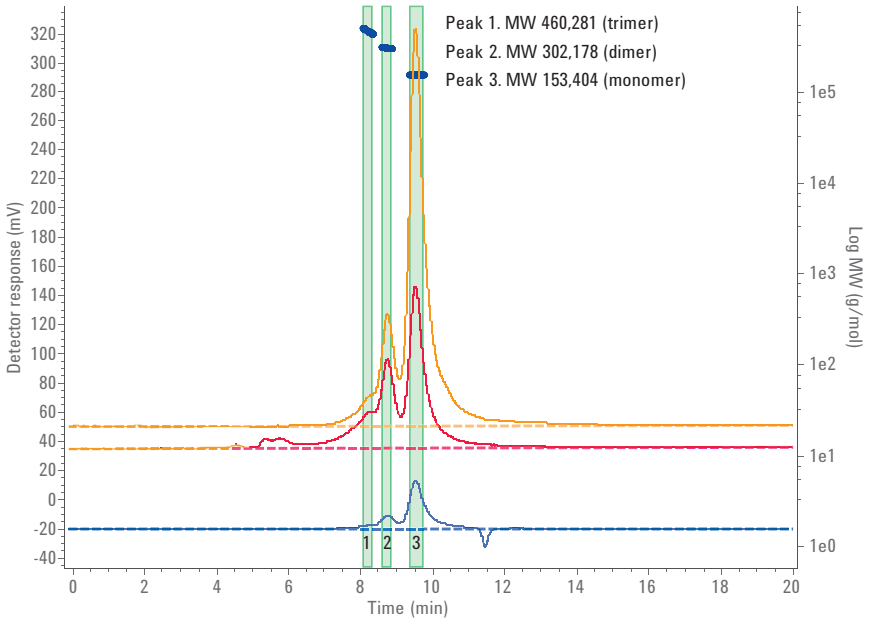
Combining a light scattering detector with a concentration detector (such as UV or RI) can provide absolute molecular weight data. Light scattering provides higher sensitivity of aggregates compared with concentration detectors such as UV and RI.

Here we show three regions of the chromatogram—monomer, dimer and trimer—along with the software-derived molecular weight information. As expected, the signal from the light scattering detector was noticeably more responsive to higher molecular weight material, which improved detection limits and accuracy for aggregate quantitation.

When studying the higher aggregates of a large protein, you may need to use a larger pore size column to maximize the information obtained.

Here, an Agilent Bio SEC-5 500Å column was used to achieve separation across the range of higher aggregates.

Improved detection limits through light scattering



Orange = UV 280 nm
Red = light scattering 90°
Blue = refractive index

Columns:	Bio SEC-5, 500Å, 7.8 × 300 mm, stainless steel
Mobile phase:	50 mM sodium phosphate, 250 mM NaCl, pH 7.0
Injection volume:	50 µL
Flow rate:	1.0 mL/min
Temperature:	30 °C
Sample:	Bovine γ-globulin
Sample concentration:	1.0, 2.0 and 4.0 mg/mL
Detection:	UV 280 nm; LS 15° and 90°; RI
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity GPC/SEC Multi Detector Suite

Detector signals from monomer (region 3), dimer (region 2), and trimer (region 1) of bovine IgG. The run length was 20 minutes.

AGILENT 1260 INFINITY BIO-INERT QUATERNARY LC: INFINITELY BETTER BIOMOLECULE ANALYSIS

From solvent delivery that is free from iron and steel... to metal-free sample-flow-path components... the Agilent 1260 Infinity Bio-inert Quaternary LC sets new standards in performance and reliability.

This robust system stands up to the challenging solvent conditions commonly used for analyzing proteins and biotherapeutics and it also minimizes problems associated with nonspecific binding. Paired with Agilent ion-exchange BioHPLC columns, you can achieve the highest resolution time.

100% bio-inert sample flow path

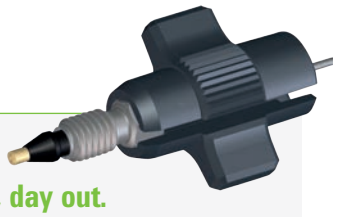
All capillaries and fittings throughout the autosampler, column compartment, and detectors are completely metal-free, so biomolecules only come into contact with ceramics or PEEK. This helps you avoid the pitfalls of peak tailing, low recovery, and decreased column life by minimizing secondary interaction of proteins and peptides with metallic surfaces.

True UHPLC performance

Power range of up to 600 bar, capable of handling the higher pressures demanded by emerging column technologies with smaller particles. It's the perfect match for all SEC and IEX columns with particle sizes down to 1.7 µm.

Agilent Buffer Advisor software provides a fast and simple way to create salt and pH gradients.

To learn more about the Agilent 1260 Infinity Bio-inert LC, visit **www.agilent.com/chem/1200BioLC**



Capillary and fitting technology for robust and secure operation—day in, day out.

With the 1260 Infinity Bio-inert LC, Agilent uses capillary and fitting technology that facilitates the unique combination of metal-free bio-inertness and high pressure operation. Three different types of capillaries are deployed:

- Highly corrosion resistant titanium capillaries for the solvent delivery lines
- Metal-clad PEEK capillaries in the autosampler and column compartment
- PEEK capillaries in the low pressure parts of the system downstream of the separation column

The metal-clad PEEK capillaries feature a unique connection system for complete bio-inertness at every connection. The mechanically interlocked PEEK tip is highly resistant to lateral or rotational tension, eliminating torque at the capillary while tightening the fitting.

To learn more visit **www.agilent.com/chem/LCcapillaries**

AGILENT INSTRUMENTS FOR PROTEIN IDENTIFICATION AND IMPURITY PROFILING

The new Agilent 1290 Infinity II LC:
The next generation in UHPLC, raises the efficiency in three dimensions

- Maximize analytical efficiency: Unmatched separation and detection performance deliver analysis data of the highest quality
- Maximize instrument efficiency: Highest sample capacity and fastest injection cycles combine with new levels of usability
- Maximize laboratory efficiency: Seamless integration in current infrastructure and smooth method transfer from legacy equipment

The 1290 Infinity II LC is available with high-speed pump or with flexible pump

Agilent 1260 Infinity Binary LC System:
Raising the standard in analytical HPLC with 600 bar, high-speed 80 Hz detector, and up to 10x higher sensitivity

100% HPLC compatibility, UHPLC capability, plus:

- Corrosion-resistant and biologically-inert flow path
- Widest pH range
- For bio-analysis and bio-purification

Use for any standard UHPLC application

Agilent 1260 Infinity Multi-Detector Bio-SEC System:
A dedicated solution for reproducible advanced analysis of protein-based pharmaceuticals.

If size-exclusion chromatography (SEC) is combined with advanced light scattering detectors, it enables biochemists to determine accurate molecular weights and size in solution, while providing more sensitive aggregation detection for analysis of large bio-molecules.

- Reproducible and accurate molecular weights and size information
- Sensitive detection of aggregates with market-leading low dead volume light scattering detection
- Accuracy for size and molecular weight due to advanced detection

Use for applications that need more sensitivity for large biomolecules

For a closer look at these advanced systems, visit **www.agilent.com/chem/BioHPLC**

ORDERING INFORMATION

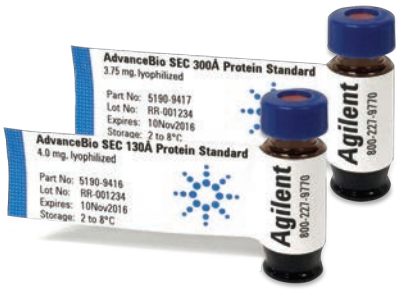
Agilent AdvanceBio SEC HPLC columns

The latest technology for SEC analysis of monoclonal antibodies, proteins, and peptides

Description	130Å	300Å
Analytical columns		
4.6 x 300 mm, 2.7 µm	PL1580-5350	PL1580-5301
4.6 x 150 mm, 2.7 µm	PL1580-3350	PL1580-3301
7.8 x 300 mm, 2.7 µm	PL1180-5350	PL1180-5301
7.8 x 150 mm, 2.7 µm	PL1180-3350	PL1180-3301
Analytical guards		
4.6 x 50 mm, 2.7 µm	PL1580-1350	PL1580-1301
7.8 x 50 mm, 2.7 µm	PL1180-1350	PL1180-1301

Agilent AdvanceBio SEC protein standards

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



Agilent Bio SEC-3 HPLC columns for protein analysis with MS detection

Description	100Å	150Å	300Å
Analytical columns			
4.6 x 300 mm, 3 µm	5190-2503	5190-2508	5190-2513
4.6 x 150 mm, 3 µm	5190-2504	5190-2509	5190-2514
7.8 x 300 mm, 3 µm	5190-2501	5190-2506	5190-2511
7.8 x 150 mm, 3 µm	5190-2502	5190-2507	5190-2512
Analytical guards			
4.6 x 50 mm, 3 µm	5190-6846	5190-6847	5190-6848
7.8 x 50 mm, 3 µm	5190-2505	5190-2510	5190-2515
Prep columns			
21.2 x 300 mm, 3 µm	5190-6850	5190-6851	5190-6852
Prep guards			
21.2 x 50 mm, 3 µm	5190-6854	5190-6855	5190-6856

Agilent Bio SEC-5 HPLC columns for large biomolecules and samples with multiple molecular weight components

Description	100Å	150Å	300Å	500Å	1000Å	2000Å
Analytical columns						
4.6 x 300 mm, 5 µm	5190-2518	5190-2523	5190-2528	5190-2533	5190-2538	5190-2543
4.6 x 150 mm, 5 µm	5190-2519	5190-2524	5190-2529	5190-2534	5190-2539	5190-2544
7.8 x 300 mm, 5 µm	5190-2516	5190-2521	5190-2526	5190-2531	5190-2536	5190-2541
7.8 x 150 mm, 5 µm	5190-2517	5190-2522	5190-2527	5190-2532	5190-2537	5190-2542
Analytical guards						
4.6 x 50 mm, 5 µm	5190-6857	5190-6858	5190-6859	5190-6860	5190-6861	5190-6862
7.8 x 50 mm, 5 µm	5190-2520	5190-2525	5190-2530	5190-2535	5190-2540	5190-2545
Prep columns						
21.2 x 300 mm, 5 µm	5190-6863	5190-6864	5190-6865	5190-6866	5190-6867	5190-6868
Prep guards						
21.2 x 50 mm, 5 µm	5190-6869	5190-6870	5190-6871	5190-6872	5190-6873	5190-6874

Agilent ProSEC 300S columns for globular proteins (USP L33)

Description	100Å
Analytical columns	
4.6 x 250 mm, 5 µm	PL1547-5501
7.5 x 300 mm, 5 µm	PL1147-6501
7.5 x 600 mm, 5 µm	PL1147-8501
Analytical guards	
4.6 x 50 mm, 5 µm	PL1547-1501
7.5 x 50 mm, 5 µm	PL1147-1501

ZORBAX GF-250 and GF-450 Gel Filtration columns for analysis protocol that requires the use of USP designation L35

Description	Size	Part No.
Analytical columns		
GF-250, 150Å	4.6 x 250 mm, 4 µm	884973-701
GF-250, 150Å	9.4 x 250 mm, 4 µm	884973-901
GF-450, 300Å	9.4 x 250 mm, 6 µm	884973-902
Analytical guards and kits		
ZORBAX Diol Guard Cartridge, 4/pk	4.6 x 12.5 mm, 6 µm	820950-911
ZORBAX Diol Guard Cartridge, 2/pk	9.4 x 15 mm, 6 µm	820675-111
Guard Hardware Kit for 4.6 mm id		820999-901
Guard Hardware Kit for 9.4 mm id		840140-901
PrepHT columns		
PrepHT GF-250, 150Å	21.2 x 250 mm, 6 µm	877974-901
PrepHT GF-450, 300Å	21.2 x 250 mm, 6 µm	877974-910
PrepHT guards and kits		
ZORBAX Diol PrepHT Guard Cartridge, 2/pk	17 x 7.5 mm, 5 µm	820212-911
Guard Cartridge Hardware Kit for 21.2 mm id		820444-901
PrepHT endfittings, 2/pk		820400-901

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Agilent continues to innovate with the AdvanceBio family of columns to address the requirements for protein and mAb characterization. Agilent AdvanceBio columns are designed to advance accuracy and speed for your characterization, aggregation with SEC, charge variants with IEX, intact mass, primary structure and impurities profiles by reversed-phase, cleaved glycans by hydrophilic interaction, and mAb titer determination by affinity chromatography.

Agilent BioHPLC columns

Affinity Titer determination and purification	**Reversed Phase** Protein identification and impurity profiling	**HILIC** Glycan analysis	**Size Exclusion** Aggregation analysis	**Ion Exchange** Charge variant analysis
Bio-Monolith Protein A	AdvanceBio RP-mAb	AdvanceBio Glycan Mapping	AdvanceBio SEC, 2.7 µm	Bio-Monolith (QA, DEAE, SO_3)
Bio-Monolith Protein G	AdvanceBio Peptide Mapping	ZORBAX RRHD 300Å, 1.8 µm	Bio SEC-3	Bio mAb
	ZORBAX RRHD 300Å, 1.8 µm		Bio SEC-5	Bio IEX (SAX, SCX, WAX, WCX)
	Poroshell 300		ProSEC 300S	PL-SAX
	PLRP-S		ZORBAX GF-450 ZORBAX GF-250	PL-SCX
	ZORBAX 300SB			
	ZORBAX Amino Acid Analysis			

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