Agilent BioHPLC Size Exclusion Chromatography Columns

RESOLVE PROTEIN AGGREGATES AND DEGRADANTS WITH SPEED AND CONFIDENCE

The Measure of Confidence

Agilent Technologies

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

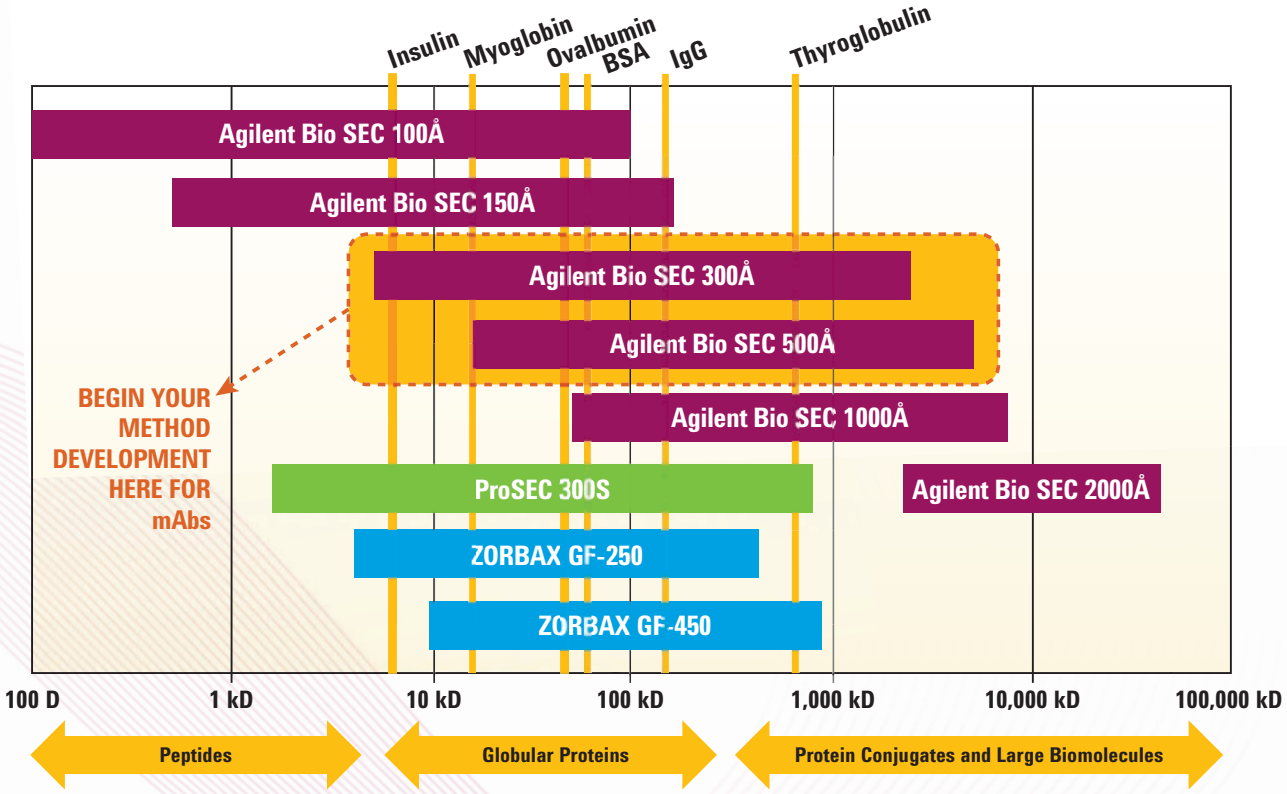
Size exclusion chromatography (SEC) is a critical tool for quantitating monomers, dimers, aggregates, and potential degradants. These types of protein separations demand the highest levels of accuracy and speed.

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.

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. We recommend that you begin your method development with Agilent Bio SEC-3 and 5 columns.



INSIDE: our complete portfolio of BioHPLC SEC columns covering the very latest biomolecule applications

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

Agilent Bio SEC-3 HPLC columns

HIGHER RESOLUTION FOR FASTER PEPTIDE AND PROTEIN SEPARATIONS

Agilent Bio SEC-3 columns offer speed and resolution advantages over other SEC columns, thanks to their small, efficient particles.

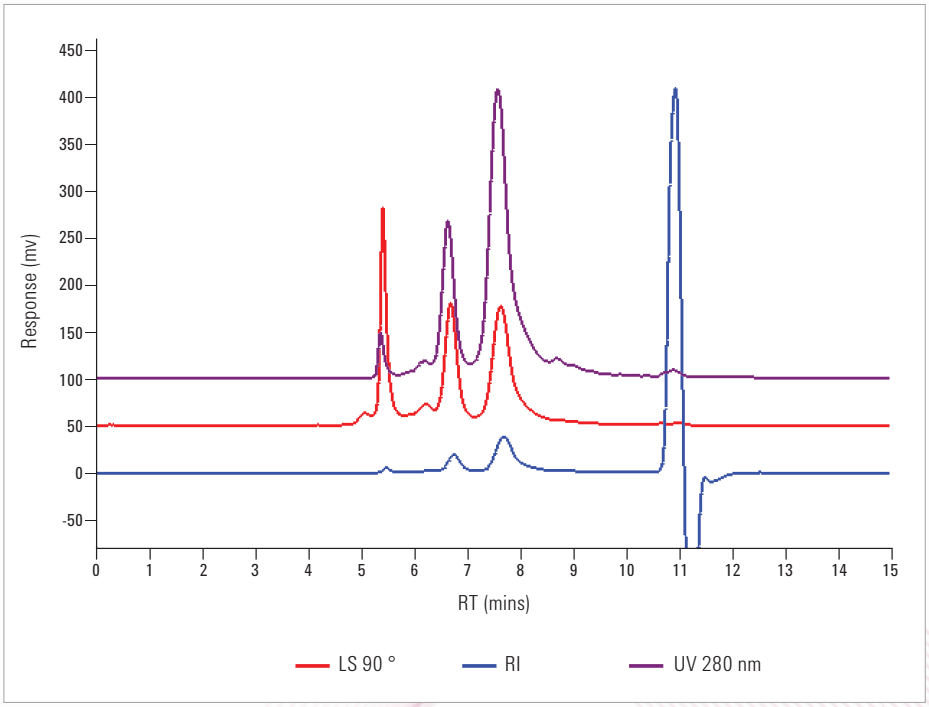
- **Faster separations** than large-particle SEC columns
- **High resolution:** Sharper peaks and better protein recovery
- **Exceptional loading capacity and recovery** due to proprietary hydrophilic layer
- **Flexible method development:** Compatible with most aqueous buffers
- **Excellent stability** under both high- and low-salt conditions
- **Reliable, consistent performance:** Narrowly dispersed particles; proprietary hydrophilic layer provides for minimal secondary interactions
- **Robust particles** compatible with multi-detectors including light scattering

Bio SEC-3 columns help you achieve more consistent SEC separations. Each column is packed with spherical, narrowly dispersed 3 µm silica particles coated with a proprietary hydrophilic layer for high recovery and minimal secondary interactions, which provides more consistent separations. This thin polymeric layer is chemically bonded to pure, mechanically stable silica under controlled conditions, ensuring a highly efficient and stable size exclusion particle.

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)

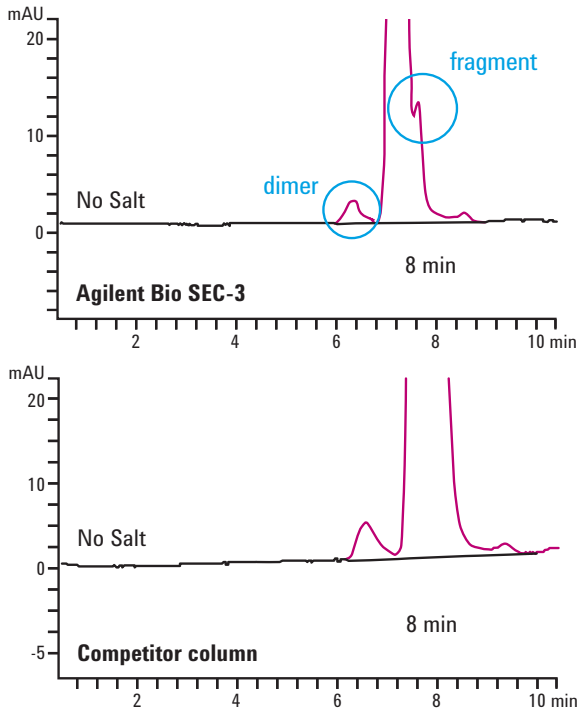
Advanced detection techniques



Results of using different detectors on MDS protein separation.

In a column comparison test, the improvement in data quality produced by the Agilent Bio SEC-3 column is evident in the table. The chromatogram demonstrates the value of the column by revealing the presence of monoclonal antibody fragment that is missed by the competitor column.

Fast, high resolution SEC characterizations



Conditions

Column:	Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm (p/n 5190-2511)
Column:	Competitor 7.8 × 300 mm
Sample:	mAb (2 mg/mL)
Injection:	5 µL
Flow rate:	1.0 mL/min
Eluent:	150 mM sodium phosphate
Detection:	220 nm

Eluent	Column	Resolution ratio monomer:dimer	Monomer efficiency	Percentage dimer
No Salt	Agilent	2.08	7,942	0.60
No Salt	Competitor	1.92	4,164	0.57

Monoclonal antibody monomer and dimer analysis using Agilent Bio SEC-3 and a competitor column.

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

Agilent Bio SEC-5 HPLC columns

EXCELLENT PERFORMANCE FOR LARGER BIOMOLECULES

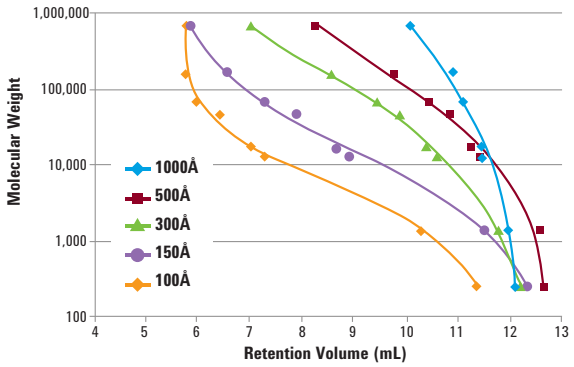
For large biomolecules and samples with components of multiple molecular weights, **Agilent Bio SEC-5 columns** are an ideal choice. They are packed with 5 µm silica particles coated with a proprietary, neutral, hydrophilic layer for maximum efficiency and stability, with 6 different pore sizes to provide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



Conditions

Column: Agilent 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

Proteins	MW	Retention volume				
		1000Å	500Å	300Å	150Å	100Å
Thyroglobulin	670000	10.07	8.23	7.03	5.82	5.77
Gamma globulin	158000	10.88	9.80	8.57	6.55	5.79
BSA	67000	11.13	10.44	9.44	7.29	6.00
Ovalbumin	45000	11.28	10.83	9.89	7.90	6.40
Myoglobin	17000	11.44	11.28	10.42	8.66	7.05
Ribonuclease A	12700	11.52	11.41	10.58	8.93	7.32
Vitamin B-12	1350	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.

Agilent 1260 Infinity Bio-SEC Multi Detector Suite (MDS)

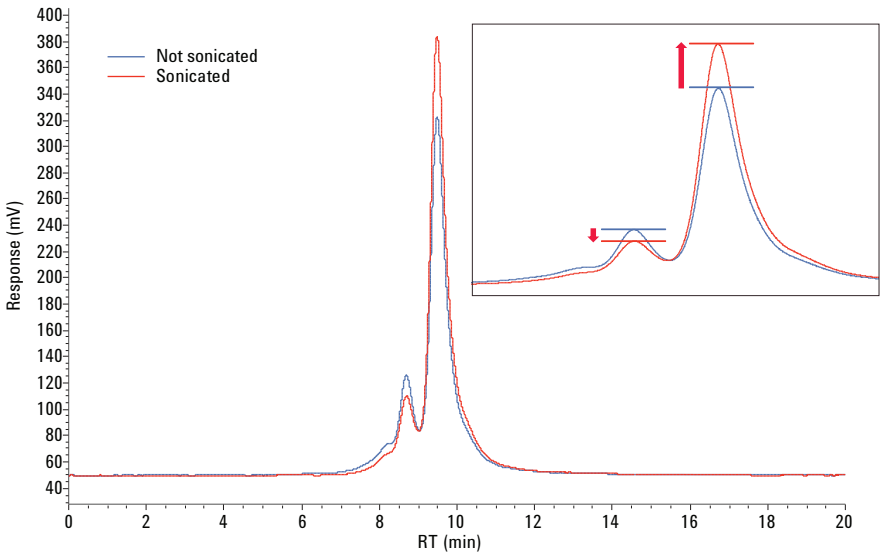
INFINITELY BETTER BIOMOLECULE ANALYSIS

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

Effect of sonication on level of aggregation in protein analysis



Conditions

Columns:	Agilent Bio SEC-5 500Å, 7.8 × 300 mm, 5 µm stainless steel (p/n 5190-2531)
Mobile phase:	50 mM sodium phosphate, 250 mM NaCl, pH 7.0
Injection volume:	50 µL
Flow rate:	1.0 mL/min
Gradient:	Isocratic
Temperature:	30 °C
Sample:	Bovine γ-globulin
Sample concentration:	1.0, 2.0 and 4.0 mg/mL
Detection:	UV 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity GPC/SEC Multi Detector Suite

Effect of sonication on sample aggregation in bovine IgG; 4 mg/mL, 7 °C, sonicated (red) and not sonicated (blue).

	Monomer (%)	Dimer (%)	Higher aggregates (%)
Before sonication	76.12	16.45	7.44
After sonication	83.80	12.25	3.95

Quantification of protein aggregation – effect of sonication.

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

Agilent ProSEC 300S columns

ANALYZE GLOBULAR PROTEINS WITH A SINGLE COLUMN

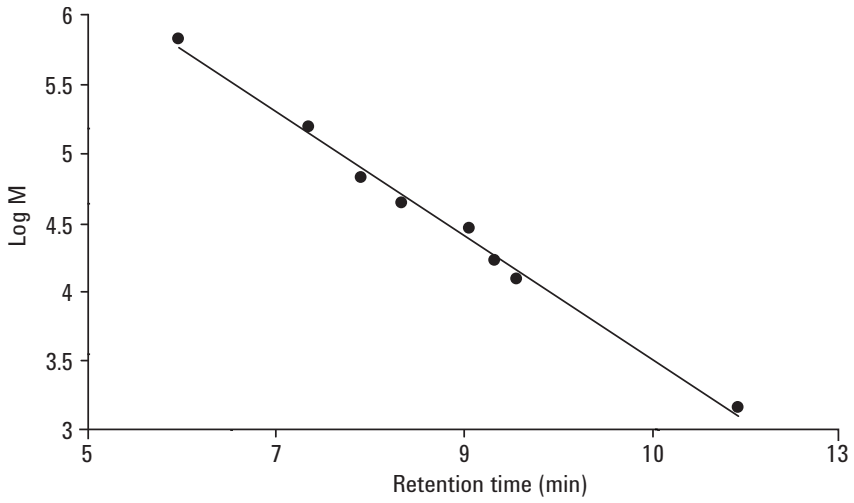
ProSEC 300S columns are designed as a single column solution for globular protein analysis. The pore size selection and optimization provides an extended linear resolving range so that this single column can be used for analysis across the full range of globular proteins. Their robust particle does not 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



Conditions

Column:	Agilent ProSEC 300S 7.5 x 300 mm, 5 µm (p/n PL1147-6501)
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/Daltons	Protein
670,000	Thyroglobulin
155,000	γ-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.

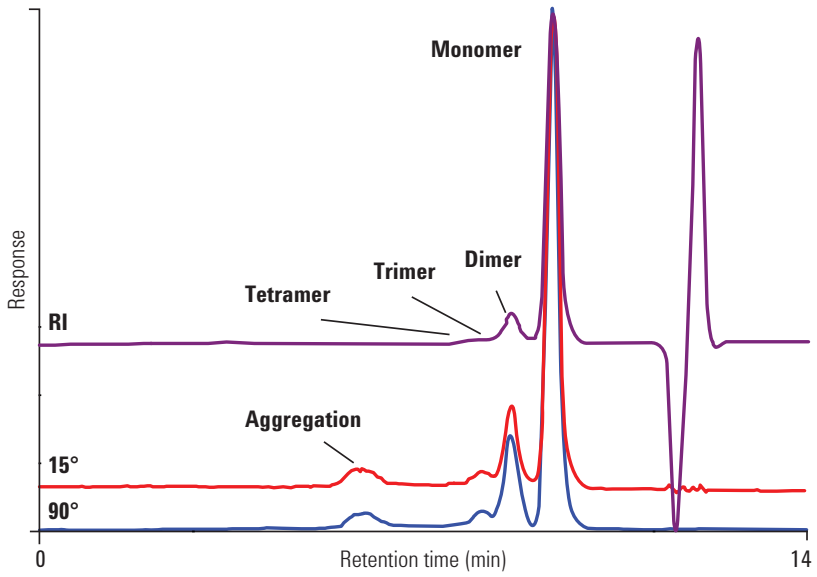
Analysis of bovine serum albumin by light scattering using ProSEC 300S columns

Conditions

Column:	Agilent ProSEC 300S, 7.5 x 300 mm, 5 µm (p/n PL1147-6501)
Mobile phase:	Water + 120 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4
Flow rate:	1.0 mL/min
Detection:	Agilent 1260 Infinity Multi-Detector GPC/SEC system with refractive index and dual angle light scattering
Sample:	Bovine serum albumin

Molecular weights of the proteins

Monomer	**66,900 Daltons, 88.5%**
670,000	Thyroglobulin
155,000	γ-Globulin
66,430	Bovine serum albumin
44,287	Ovalbumin
29,000	Carbonic anhydrase
16,700	Myoglobulin
12,384	Cytochrome c
1,423	Bacitracin



Overlay of the differential refractive index and dual-angle light scattering data showing monomer, dimer, trimer, and aggregation peaks. These results illustrate the power of light scattering, coupled with SEC, in protein characterization.

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

Agilent Gel Filtration (SEC) columns

ANALYTICAL TO PREP WITH FLOW RATES UP TO 3 mL/min

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.

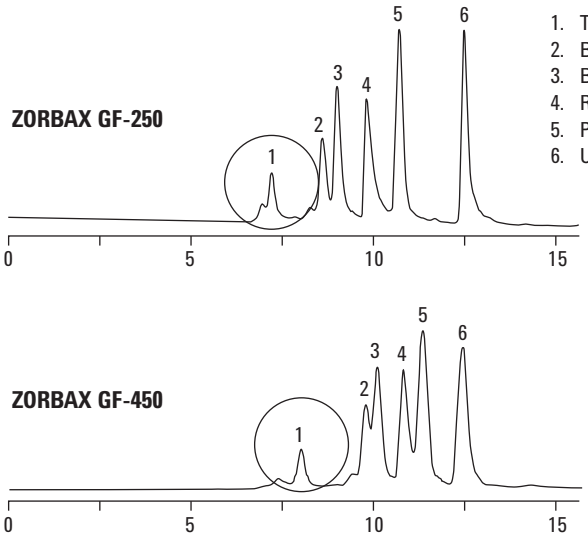
- **End-to-end analysis:** A choice of semi-prep and prep column dimensions, usable at flow rates of up to 3 mL/min
- **High recovery:** Hydrophilic diol bonded base typically >90% recovery
- **High efficiency and reproducibility** with short analysis time
- **Rugged and reproducible:** Precisely-sized 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

Agilent Gel Filtration columns are suitable for desalting, estimating protein molecular weight, and separating modified proteins.

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 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

Conditions

Column:	Agilent ZORBAX GF-250, 9.4 x 250 mm, 4 µm (p/n 884973-901)
Column:	Agilent ZORBAX GF-450, 9.4 x 250 mm, 6 µm (p/n 884973-902)
Mobile phase:	0.2 M Na_2HPO_4, pH 7.0
Flow rate:	5.0 mL/min
Detection:	UV 280 nm
Sample:	200 µL

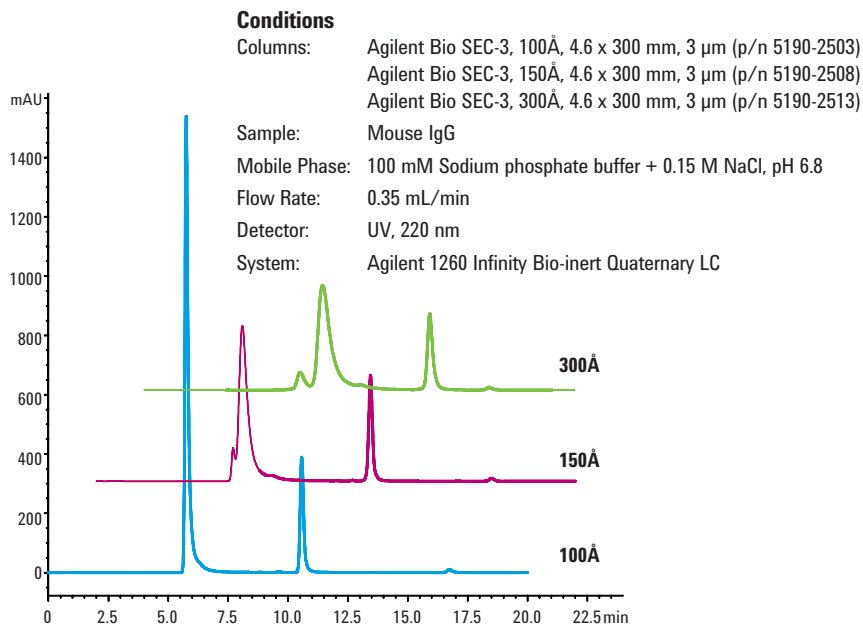
This protein separation shows the complementary relationship between GF-450 and GF-250 preparative columns. The GF-450 column reliably separated the high molecular weight biomolecules, which were excluded from the linear separation range of the GF-250 column (with a smaller pore size).

INCREASE RESOLUTION AND FLEXIBILITY WITH A WIDE SELECTION OF PORE SIZES AND CONFIGURATIONS

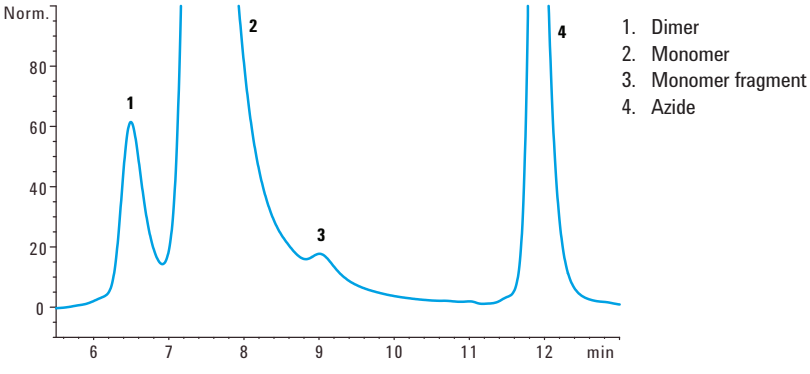
Aggregates are formed through a variety of mechanisms, including disulfide bonding and non-covalent interactions. The size, type, and content of aggregates present in protein biopharmaceuticals can affect both efficacy and formulation – or worse, induce an immunogenic response. Excessive aggregation will also reduce the process yield, hampering productivity.

The separations that follow compare the performance of varying pore sizes when separating a mouse IgG sample. Because media pore size influences the resolution obtained with SEC, it is worthwhile to test a range of pore sizes for each analyte. This approach lets you identify the best column choice *before* conducting in-depth investigations, reducing your risk of missing valuable data.

Higher pore size, higher resolution



Separation of mouse IgG on Agilent Bio SEC-3 columns with three different pore sizes. Here, resolution increased with pore size: The 100Å pore size column excluded the IgG, so no definition was achieved; partial exclusion with the 150Å, and 300Å columns successfully resolved the monomer and dimer.



Magnification of the mouse IgG separation using an Agilent Bio SEC-3 300Å column – the best choice for this analysis.

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

Discovery, QA/QC, and Manufacturing

PERFORM EFFICIENT SIZE SEPARATIONS WITH MINIMAL SECONDARY INTERACTIONS

Your SEC separation mechanism must be based on *size*, with no secondary interactions with the stationary phase. The materials used to manufacture Agilent Bio SEC columns are chemically modified to provide an inert hydrophilic surface, eliminating any underlying ion-exchange character.

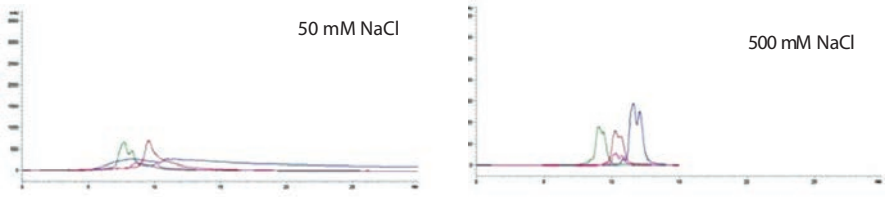
Rugged performance and resolution in varying salt conditions

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

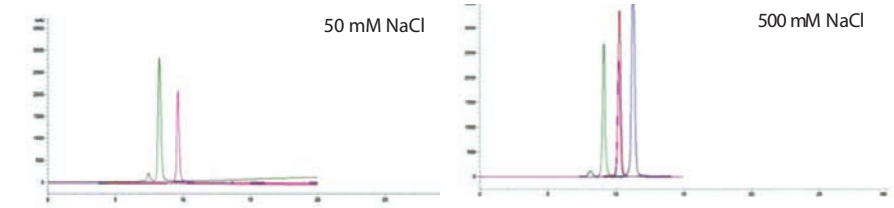
Column performance is evaluated first by looking at low salt conditions and high salt conditions. At low salt conditions, both columns exhibit ionic interactions, as demonstrated by the retention of lysosome, in blue. With the higher salt concentration (500 mM) the Agilent Bio SEC-3 exhibits minimal non-specific interactions due to hydrophobicity. Peaks are sharp and symmetrical. The Agilent Bio SEC-3 column provides size separations over a wider range of salt concentrations.

Size separations over a range of salt concentrations

Vendor A



Agilent Bio SEC-3



Blue: Lysozyme
Red: α-chymotrypsinogen A
Green: Ovalbumin
Pink: Myoglobin

Conditions

Columns:	Agilent Bio SEC-3, 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513) Vendor A, 4.6 x 300 mm, 300Å, 1.7 µm
Instruments:	Agilent 1290 Infinity or Agilent 1260 Infinity UHPLC
Mobile phase:	A: 150 mM phosphate buffer, pH 7 B: 20 mM phosphate buffer + x mM NaCl, pH 7, blended using Agilent Buffer Advisor software
Flow rate:	0.35 mL/min
Detection:	UV 220 nm
Samples:	Lysozyme, α-chymotrypsinogen A, ovalbumin, myoglobin

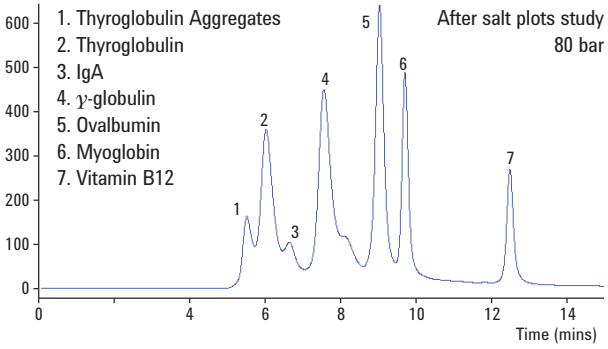
Six runs were completed over a range of salt concentrations, to evaluate the robustness of the columns and impact on peak shape, efficiency, and resolution. The benefit of the hydrophilic coating is seen, which delivered robust performance, maintaining retention times, and peak shapes for the four proteins. The column from Vendor A demonstrated a significant reduction in performance, suggesting column fouling due to poor recovery or packed bed instability. There was also a reduction in column efficiency, which was partially recovered by performing the recommended wash procedure.

Better robustness than competitor

Before salt plots study

Plates/m (Vitamin B12)	Rs IgA/IgG	Rs Ovalb/Myo
76,612	1.37	1.82

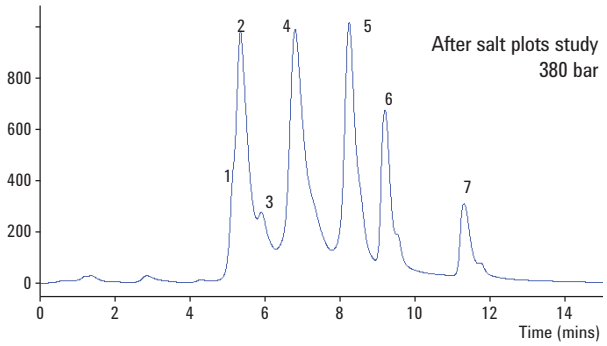
Agilent Bio SEC-3



Before salt plots study

Plates/m (Vitamin B12)	Rs IgA/IgG	Rs Ovalb/Myo
26,820	1.23	1.83

Vendor A



Conditions

Columns:	Agilent Bio SEC-3, 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513) Vendor A, 4.6 x 300 mm, 300Å, 1.7 µm
Instruments:	Agilent 1290 Infinity or Agilent 1260 Infinity UHPLC
Mobile phase:	A: 150 mM phosphate buffer, pH 7 B: 20 mM phosphate buffer + x mM NaCl, pH 7, blended using Agilent Buffer Advisor software
Flow rate:	0.35 mL/min
Detection:	UV 220 nm
Samples:	Bio-Rad Gel Filtration Standard (1 vial diluted with 5 mL of eluent), thyroglobulin, y-globulin, ovalbumin, myoglobin, vitamin B12

The change in efficiency and resolution of the protein standard separations after completing the evaluations of non-specific interactions using different salt concentrations is evident. The Agilent Bio SEC-3 column is more robust.

Save time and money on buffer preparation

Manual preparation of buffers

Automated online dynamic mixing of buffers

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*

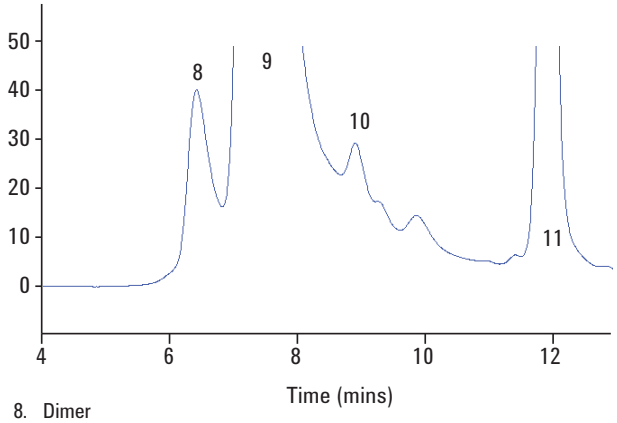
To learn more about performing fast, high-resolution separations for protein aggregation and degradation, visit **agilent.com/chem/BioHPLC**

Discovery, QA/QC, and Manufacturing

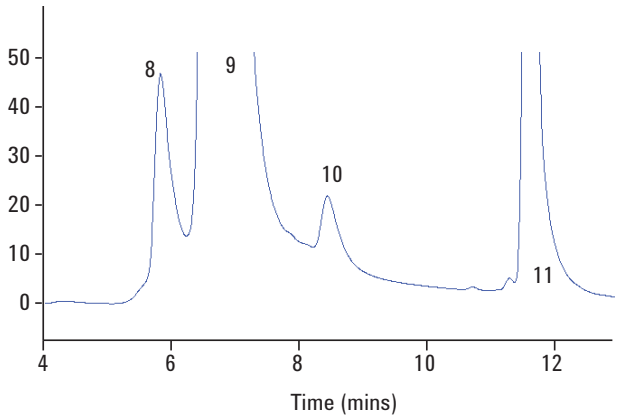
PERFORM EFFICIENT SIZE SEPARATIONS WITH MINIMAL SECONDARY INTERACTIONS

Best fragmentation pattern definition in polyclonal antibody mouse IgG

Agilent Bio SEC-3



Vendor A



8. Dimer
9. Monomer
10. Monomer fragment
11. Azide

Conditions

Columns:	Agilent Bio SEC-3, 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513) Vendor A, 4.6 x 300 mm, 300Å, 1.7 µm
Instruments:	Agilent 1290 Infinity or Agilent 1260 Infinity UHPLC
Mobile phase:	A: 150 mM phosphate buffer, pH 7 B: 20 mM phosphate buffer + x mM NaCl, pH 7, blended using Agilent Buffer Advisor software
Flow rate:	0.35 mL/min
Detection:	UV 220 nm
Sample:	Mouse IgG

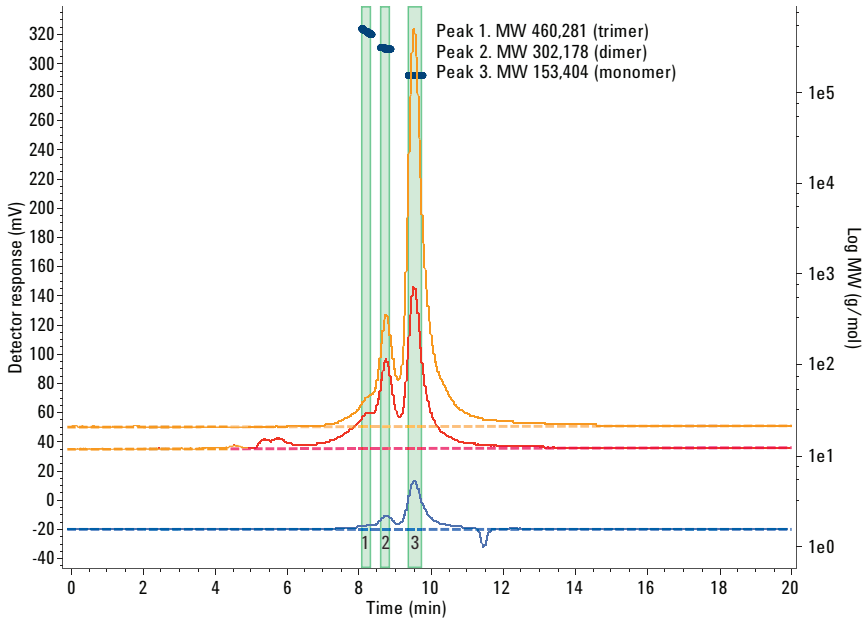
We further evaluated the effect of chemistry and particle size on separation with a polyclonal mouse IgG test mix. The Agilent Bio SEC-3 had the best definition of the fragmentation pattern for the mouse IgG, due to the proprietary hydrophilic coating of the Agilent Bio SEC-3 column, which reduces secondary interactions and improves resolution.

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 & 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.

Improved detection limits through light scattering



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

Conditions

Columns:	Agilent Bio SEC-5, 500Å, 7.8 × 300 mm, stainless steel (p/n 5190-2531)
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.

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Discovery, QA/QC, and Manufacturing

RELY ON BIO SEC-3 FOR REPRODUCIBILITY AND ROBUSTNESS OF THE ANALYSIS

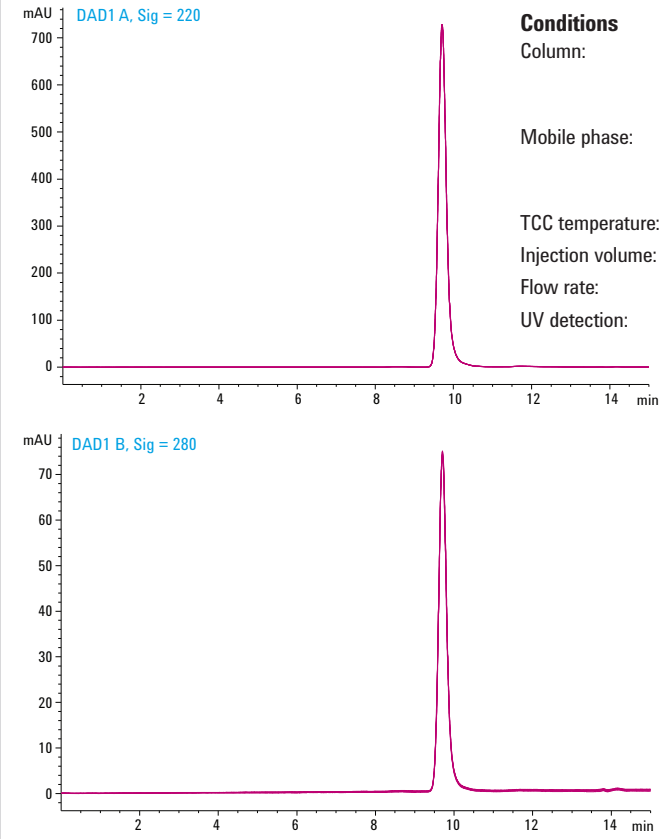
Quality control applications demand robust methods that can be transferred from lab to lab, and user to user. That is why Agilent BioHPLC columns are built to deliver robust performance over time.

In the following IgG1 study, we varied four critical parameters to evaluate method robustness:

- Injection volume (± 10%)
- Buffer pH (± 10%)
- Flow rate (± 5%)
- Buffer composition (± 150 mM NaCl)

Allowed deviations for retention time and area RSD were set to ± 3.0% and ± 5% respectively.

Overlay of 6 chromatograms



Profile of intact IgG1 on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm column (overlay of six replicants).

Retention time		Peak area	
Mean (min)	**RSD**	**Mean (mAU/min)**	**RSD**
9.708	0.008	10815	0.693

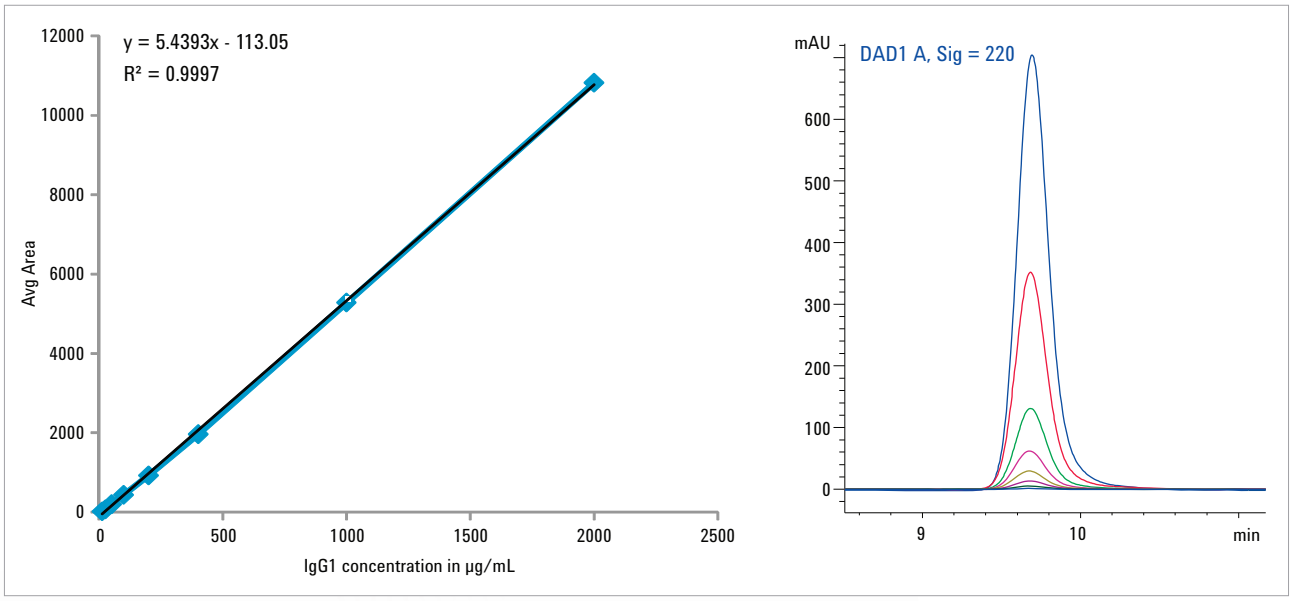
Retention time and peak area precision (n=6).

Linearity

Linearity curves for IgG1 were constructed from the LOQ level to a highest concentration level in the study using area response and concentration of IgG1. The accuracy results are shown in this table. The observed accuracy values of 82-112%, representing the range of 100-2,000 µg/mL, indicate that SEC method is quantitative and accurate. The linearity curve for IgG1 in the concentration range 12.5 to 2,000 µg is shown in the figure below.

Concentration of IgG1 (µg/mL)	Avg area	Standard deviation area	Back calculation using the standard line equation	% Accuracy	% CV
12.5	21	2	-4.87	-39	7.27
25	71	3	5.57	22	4.27
50	197	3	31.74	63	1.46
100	438	1	81.58	82	0.26
200	921	1	181.75	91	0.06
400	1,959	3	397.01	99	0.16
1,000	5,275	10	1,084.76	108	0.18
2,000	10,815	0	2,233.66	112	0

Summary of linearity range (n = 6).



These IgG1 linearity curves were constructed from the LOQ level to the highest concentration of the study. Observed accuracy values of 82 to 112%, representing the range of 100 to 2,000 µg/mL, indicate that the SEC method is quantitative and accurate.

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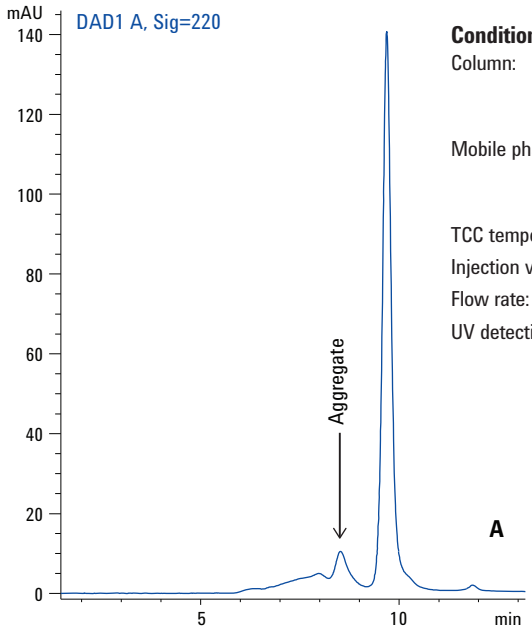
Discovery, QA/QC, and Manufacturing

RELY ON BIO SEC-3 FOR REPRODUCIBILITY AND ROBUSTNESS OF THE ANALYSIS

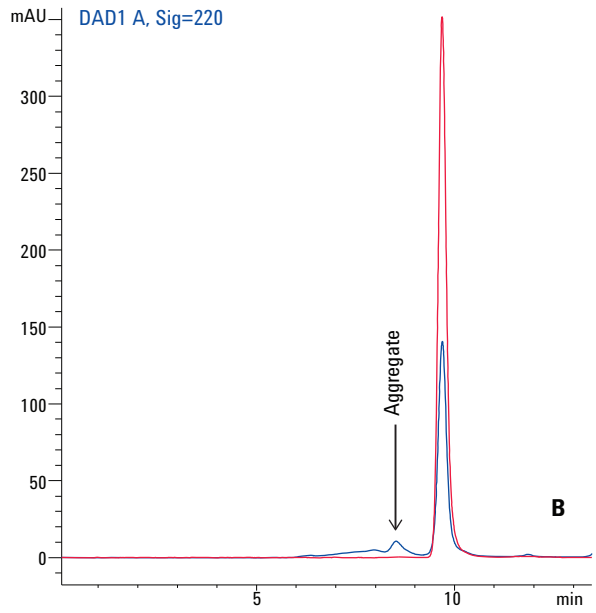
pH stress was created by adding 1 M HCl slowly by pipette to change the pH from 6.0 to 1.0, then adding 1 M NaOH to adjust pH to 10 and another 1 M HCl to adjust back to 6.0. This chromatogram of pH-induced aggregates, demonstrates how the method distinctly separated and detected both aggregates and intact IgG1. The intact IgG1 and aggregates eluted at 9.69 minutes and 8.5 minutes, respectively.

Baseline resolution for accuracy of IgG1 and aggregate quantitation



Conditions

Column:	Agilent Bio SEC-3, 7.8 × 300 mm, 3 µm (p/n 5190-2511)
Mobile phase:	150 mM sodium phosphate (pH = 7.0) containing 150 mM sodium chloride
TCC temperature:	Ambient
Injection volume:	5 µL
Flow rate:	0.8 mL/min
UV detection:	220 and 280 nm



Agilent Bio SEC-3 chromatogram of IgG1 with pH-stress induced aggregates (A) and overlay with intact IgG1 (B).

The impact of injection volume, buffer pH, and buffer composition on RT was within acceptable limits. A variation in flow rate (± 10% compared to the actual method) caused the RT RSD to deviate significantly; however, this deviation was expected due to the isocratic elution on a SEC column. The area RSD of the IgG1 peak was also within the acceptable limit.

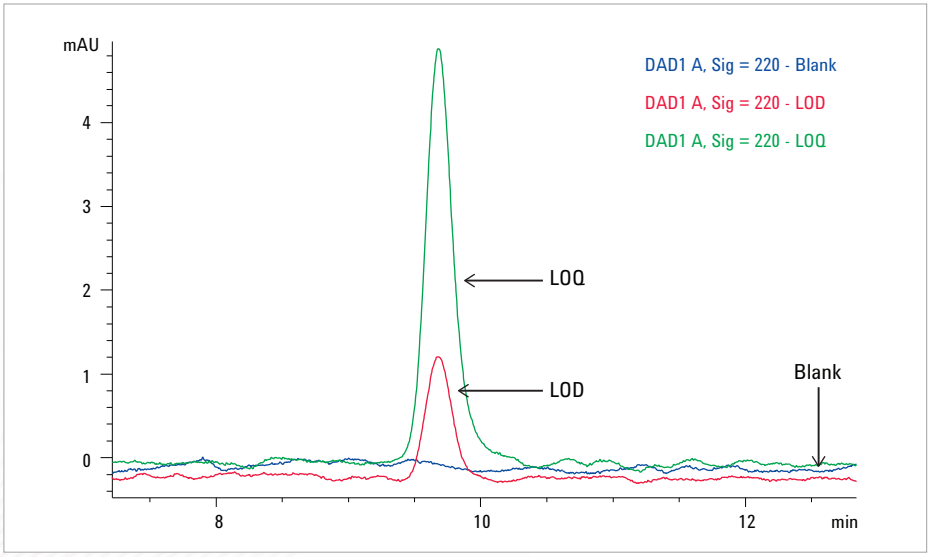
When the mobile phase pH was varied by ± 10%, the area RSD deviated more than the allowed limits. There were no further significant changes in the chromatographic pattern when deliberate variations were made in experimental conditions – proving that the method is robust.

Parameters	Variations	RT deviation (limit ± 3.0%)	Area deviation (limit ± 5.0%)
Variation in injection volume (5 µL ± 10%)	- 10%	0.015	0.295
	+10%	0.056	1.229
Variation in buffer pH (7.0 ± 10%)	- 10%	0.169	-2.71
	+10%	0.159	**6.870**
Variation in flow rate (0.8 ± 5%)	-5%	**-5.227**	3.10
	+5%	**4.763**	3.42
Variation in buffer composition (± 150 mM NaCl)	-150 mM NaCl	0.169	3.10
	+150 mM NaCl	0.169	3.42

Robustness (RT and Area RSD %) n = 6.

Concentration of IgG1 (µg/mL)	S/N = Signal-to-noise ratio	Average area
6.25	1.8	2.7
12.5 (LOD)	6.8	21
25 (LOQ)	19.6	71
50	76.7	197
100	185	454

LOD, LOQ and S/N results (n = 6).



LOD (12.5 µg/mL) and LOQ (25 µg/mL) chromatograms of IgG1 overlaid with blank.

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MONITOR PROTEIN STABILITY

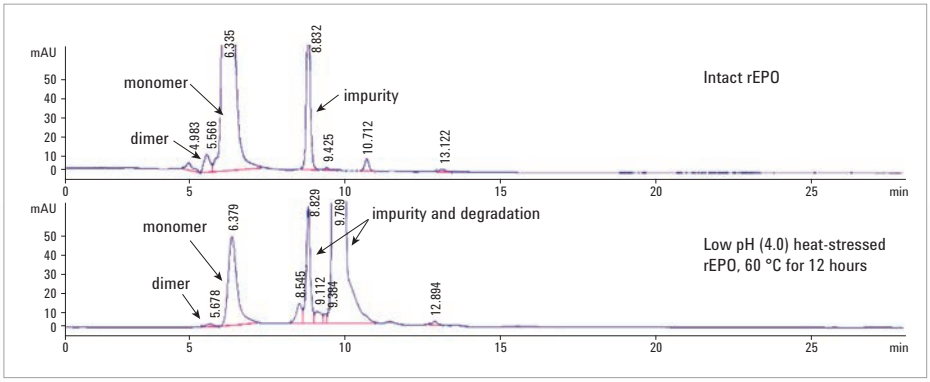
Degradation of therapeutic proteins during drug manufacturing and formulation is considered a critical attribute; therefore, it must be closely monitored to prevent adverse immunogenic effects and loss of drug efficacy.

To test the separation ability of small-pore-sized particles, rEPO was heat-stressed at low pH (4.0) with acetic acid for 12 hours at 60 °C, then separated on an Agilent Bio SEC-3, 100Å column. As you can see, the separation showed superior peak resolution of heat-degraded rEPO – and revealed how much its separation profile differed from intact rEPO.

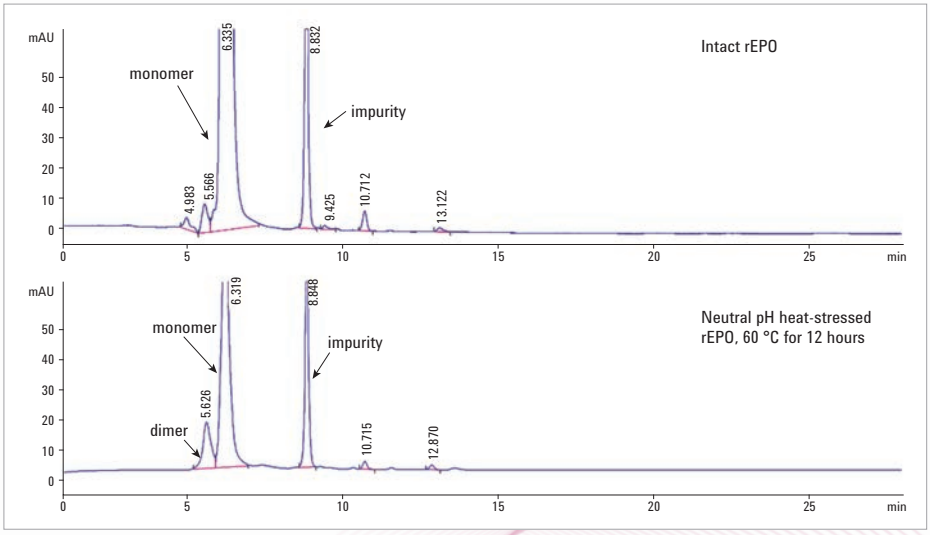
The significant reduction of monomers, dimers, and aggregates demonstrates that the rEPO was degraded by the low pH buffer at high temperature, and effectively separated by the Bio SEC-3 100Å column.

Conditions

Column:	Agilent Bio SEC-3, 100Å, 4.6 × 300 mm, 3 µm (p/n 5190-2513)
Sample:	Recombinant human EPO protein (rEPO)
Sample concentration:	1.0 mg/mL
Injection volume:	5 µL
Flow rate:	0.35 mL/min
Mobile phase:	150 mM sodium phosphate buffer, pH 7.0
Detector:	UV 225 nm
System:	Agilent 1260 Infinity Bio-inert Quaternary LC



rEPO has limited stability at low pH and high temperature – the monomer peak is reduced in size and peaks due to smaller fragments resolved.



The dimer peak increased to 11%, whereas the monomer peak decreased to 62.5%. Although the reduction was not as severe as with low-pH, heat-stressed rEPO, the data clearly demonstrate that the Bio SEC-3 column successfully resolved different sizes of rEPO impurities and degradation products.

Conditions of rEPO	% aggregates	% dimer	% monomer	% impurities
Intact	0.6	1.6	85.6	10.3
Heat-stressed at low pH	not detected	0.2	6.1	92.2

Calculation of percentage heterogeneity of intact rEPO and heat-stressed rEPO (by low pH at high temperature).

Conditions of rEPO	% aggregates	% dimer	% monomer	% impurities
Intact	0.6	1.6	85.6	10.3
Heat-stressed at neutral pH	not detected	11.7	62.5	24

Calculation of percentage heterogeneity of intact rEPO and heat-stressed rEPO (by neutral pH at high temperature).

Discovery, QA/QC, and Manufacturing

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

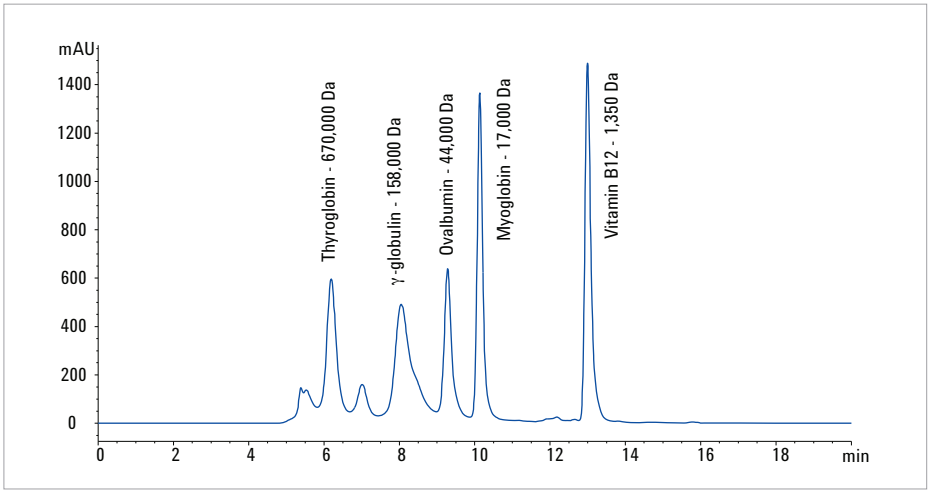
Conditions

Column:	Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 µm (p/n 5190-2511)
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)

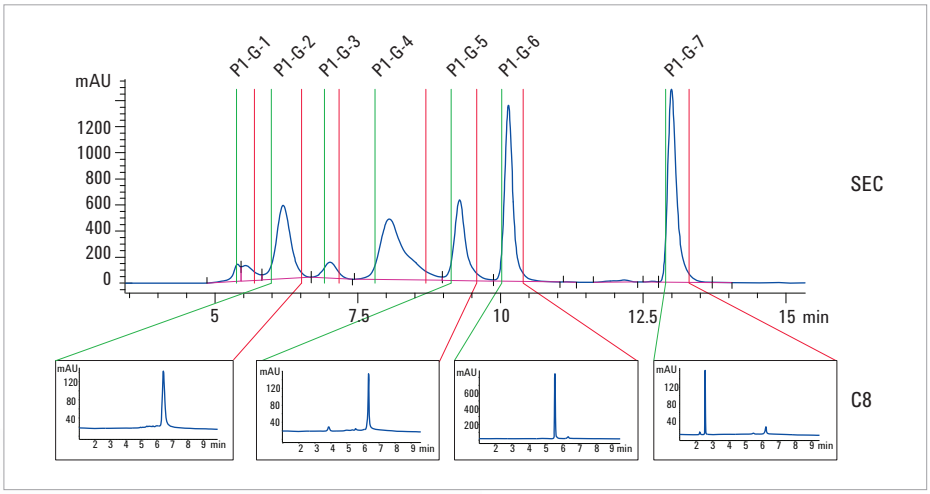
Re-analysis – reversed-phase C8

Conditions

Column:	Agilent ZORBAX 300SB-C8, 4.6 x 50 mm, 5 µm (p/n 860950-906)
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.

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AGILENT INSTRUMENTS FOR PROTEIN IDENTIFICATION AND IMPURITY PROFILING

Agilent 1260 Infinity Bio-inert Quaternary LC System:
Your best choice for protein separations

The only UHPLC that provides a metal-free sample flow path. Other advantages include:

- **100% Bio-inertness**
  - No stainless steel: sample does not touch metal surfaces
  - pH 1 to pH 13 (pH 14 short-term)
  - Handles 2 M salt and 8 M urea
  - New capillary technology
- **UHPLC capability:** 600 bar
- **Robust and easy to use** with low surface activity, corrosion resistance, active seal wash, and quaternary buffer mixing

Ideal for protein identification

For best results, use with AdvanceBio Peptide Mapping columns, Bio SEC-3 and Bio IEX 1.7 µm

Agilent 1290 Infinity Binary LC System:
Our most adaptive UHPLC system with the widest application range

Best-in-class resolution per time, dispersion, sensitivity, accuracy, and precision in LC/UV and LC/MS. Combines innovative active damping, microfluidic mixing, and optofluidic waveguides detection technology to achieve:

- UHPLC power range with up to 1200 bar and 5 mL/min
- The fastest and easiest method transfer using ISET, Agilent's unique Intelligent System Emulation Technology
- UHPLC productivity with HPLC ownership costs

Use for impurity profiling, peptide mapping or ultra-fast gradients

For best results, use with ZORBAX RRHD 300Å 1.8 µm columns

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:

- UHPLC performance with HPLC ownership costs
- Supports LC and LC/MS applications, with any narrow and standard bore analytical column (2.1 to 4.6 mm id)
- Superior gradient accuracy by high-pressure mixing

Use for any standard UHPLC application

Agilent 1290 Infinity Quaternary LC System:
Combining performance with flexibility

The only quaternary UHPLC system with binary-like accuracy and precision.
Other advantages include:

- UHPLC power range with up to 1200 bar and 5 mL/min
- BlendAssist, the easiest tool for accurate buffer and additive blending
- UHPLC productivity with HPLC ownership costs

Use for method development or walk-up systems with accurate buffer blending

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

ORDERING INFORMATION

Agilent Bio SEC-3 HPLC columns for faster peptide and protein separations

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*

**See website for availability date*

Agilent Bio SEC-5 HPLC columns for size-based biomolecules

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*

**See website for availability date*

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

ORDERING INFORMATION

Agilent ProSEC 300S columns for globular proteins

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

ZORBAX GF-250 (USP L33) and GF-450 (USP L35) Gel Filtration columns for analytical to prep with flow rates up to 3 mL/min

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

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.

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

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

For more information

To learn more about
Agilent BioSEC HPLC columns, visit
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Agilent BioHPLC columns:
Reliably identify proteins and profile impurities

- **Superior choice and flexibility** for reversed-phase, size exclusion, ion exchange, HILIC, and affinity chromatography.
- **Cutting-edge fast LC for reversed-phase:** Advances such as superficially porous Poroshell 300 columns give you higher resolution on any HPLC or UHPLC.
- **UHPLC reversed-phase method refinements:** Agilent ZORBAX RRHD 1.8 µm columns deliver UHPLC performance for reversed-phase separations with 1200-bar stability.

Confirm protein identity and identify post-translational modifications

Agilent AdvanceBio Peptide Mapping BioHPLC columns let you quickly resolve and identify amino acid modifications in primary structure. With their 2.7 µm particles and C18 functionality, AdvanceBio Peptide Mapping columns deliver excellent retention, resolution, and peak shape for basic hydrophobic peptides.

Printed in the USA, December 4, 2013
5991-2898EN

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