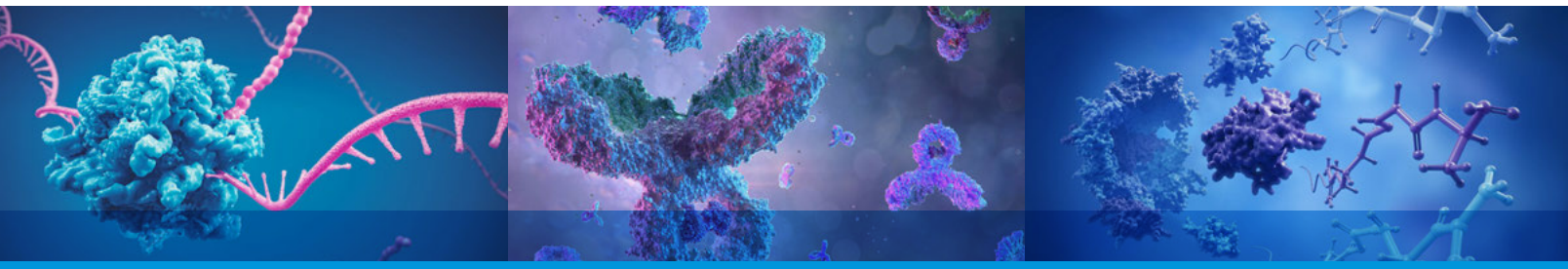
Agilent CrossLab
From Insight to Outcome

Agilent BioHPLC Columns and Consumables

Your essential resource for biomolecule analysis



Agilent

Agilent CrossLab
From Insight to Outcome

Who We Are. What We Do.

Across the lab, around the world, with you every step of the way.

CrossLab is an Agilent capability that integrates services, consumables and lab-wide resource management to help laboratories improve efficiency, optimize operations, increase instrument uptime, develop user skill, and more.

Agilent CrossLab supports Agilent and select non-Agilent instruments and provides consultative support for workflow enablement, lab analytics, compliance, inventory management and asset management, including relocation services.

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Look out for CrossLab stories from the lab to find out how we can help.

Real stories from the lab

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www.agilent.com/chem/crosslabstories

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Biocolumn Selection Guidelines

Biotherapeutics characterization is an ever-evolving challenge

Biotherapeutics have enormous potential to improve human health. The number of approved protein and antibody therapeutics continues to grow around the world as this important therapeutic class addresses unmet medical needs. But discovery and development of biopharmaceuticals is difficult. Scientists face many challenges and must not only stay abreast of advances in knowledge and improvements in technology, but also navigate the maze of shifting government regulations. Making good decisions fast is critical. At every stage in the process, from disease research to QA/QC and manufacturing, Agilent can help you make the right choices for moving therapeutics successfully to market. And it's not just because we build reliable instruments and consumables that provide accurate, reproducible results. We understand the biopharmaceutical workflow and provide families of products that work together seamlessly—as engines of research, discovery, and development—to move candidate biopharmaceuticals forward.

Given that protein biopharmaceuticals are very heterogeneous, they will require a number of chromatographic methods to accurately characterize the active pharmaceutical ingredient (API). Methods include size exclusion chromatography for the quantitation of dimers and aggregates, and ion-exchange chromatography for charge variant analysis. As part of the full characterization, it will be necessary to look at primary amino acid sequence and any post-translational modification to the sequence that may occur during purification or formulation steps. To enable complete, reproducible, and high-quality analysis for key characterization workflows, Agilent provides a broad range of columns and supplies.

Agilent solutions simplify biologics characterization

This comprehensive guide will help you find the right column for your characterization workflow. We have also included advice and tips on method development, solvent choice, mobile phase modification, optimization, and many example separations, all to assist you in column selection and method development.

Agilent has complete solutions for your needs. These include the Agilent 1260 Infinity II bio-inert LC with a metal-free sample path and the Agilent 1290 Infinity II LC, designed to provide highest speed, resolution, and ultrasensitivity for UHPLC applications. Biomolecules may be complex in structure, but their analysis is simplified by using Agilent HPLC columns, systems, and supplies.

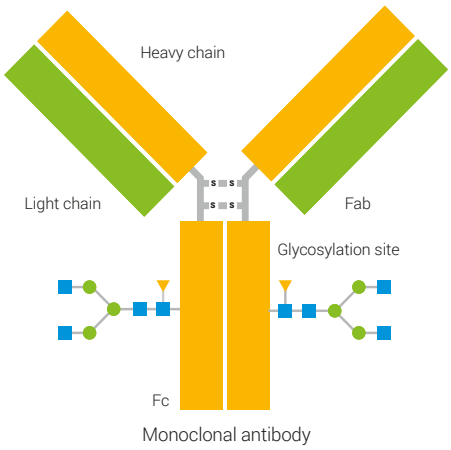
Tips and tools

For applications examples spanning the biocolumn portfolio, please see the Critical Quality Attributes Application Compendium at **www.agilent.com/chem/cqa-applications**

What is a biomolecule?

Biomolecules are compounds made by living organisms. They can range in size from amino acids and small lipids to large polynucleotides, such as DNA or RNA.

In this section, we deal with the separation of:



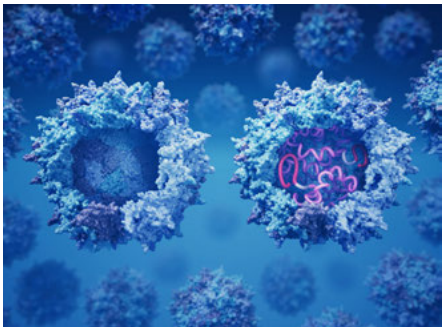
Proteins
Separation based on size with size exclusion chromatography, charge with ion-exchange chromatography, and hydrophobicity with reversed-phase or hydrophobic interaction chromatography.

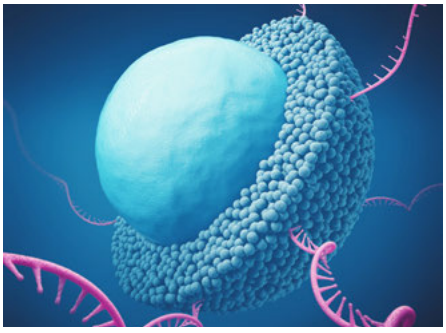
Peptides
Biocolumns for the analysis and purification of the full range of peptides, including hydrophobic, hydrophilic, basic, and acidic peptides across the full-size range. Also, columns for peptide mapping by HPLC and UHPLC.

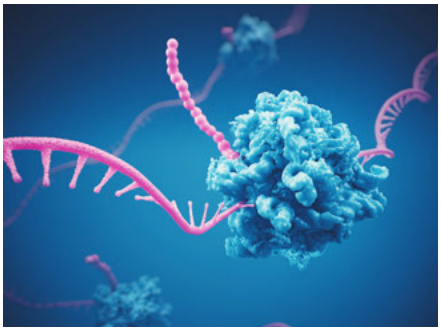
Amino acids
The AdvanceBio Amino Acid Analysis columns provide a high-efficiency solution for analysis of 24 amino acids. Typical analysis times range from 14 minutes, with a 75 mm column, to 24 minutes with a 150 mm column.

DNA/RNA oligonucleotides
Reversed-phase and ion-exchange options for DNA and RNA oligos, and with particle pore sizes to cover the full range of oligonucleotide sizes, from small synthetic oligos to large plasmids.

Adeno-associated virus (AAVs)
AAVs are large molecular complexes consisting of an icosahedral protein shell encapsulating a single stranded DNA genome. The protein capsid and encapsulated DNA each require dedicated analytical techniques to ensure the quality and safety of the overall product.







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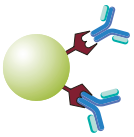
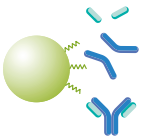
What is a biocolumn?

Biochromatography columns, or biocolumns, are liquid chromatography columns used for the separation of biological compounds, such as peptides and proteins, oligonucleotides and polynucleotides, and other biomolecules and complexes. Biocolumns are specifically designed for biomolecule analysis with larger pore sizes to accommodate the larger molecule sizes. Media are designed to minimize nonspecific binding of analytes for improved recovery. Separation mechanisms are chosen to either retain biological function so bioactivity is not lost during analysis, or to deliberately denature for primary structure characterization.

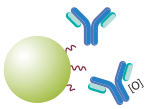
Agilent's biocolumn offering provides solutions for all the major characterization techniques required for your biomolecule analysis. These include:



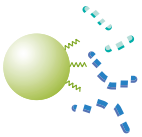
Titer determination
Use unique technology, such as Agilent Bio-Monolith Protein A, to perform titer determination and cell line optimization.



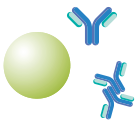
Intact and subunit purity using reversed-phase
Use key technologies such as AdvanceBio RP-mAb, ZORBAX RRHD 300 Å, and PLRP-S, for confidence in results from primary structural characterization through analysis of intact or fragmented proteins.



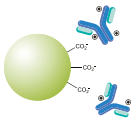
Intact analysis using hydrophobic interaction
AdvanceBio HIC columns will resolve various protein variants (PTMs) including oxidation in mAbs and drug-antibody species observed in ADCs.



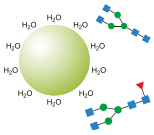
Peptide mapping
Detect and identify key post-translational modifications in digested protein samples using AdvanceBio Peptide Mapping.



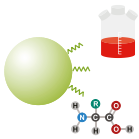
Aggregate and fragment analysis
AdvanceBio SEC accurately measures aggregates (such as dimers, trimers, and tetramers, and so on) and separates low molecular excipients and impurities from larger molecular weight proteins.



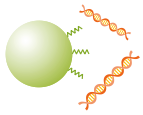
Charge variant analysis
Agilent ion-exchange columns include optimized chemistries for monoclonal antibody analysis, such as Bio MAb and Bio IEX for accurate isoform analysis.



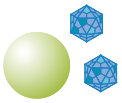
Glycan analysis
Agilent hydrophilic interaction chromatography (HILIC) columns deliver accurate and reproducible glycan and glycopeptide analysis.



Amino acid and cell culture analysis
Analyze critical cell culture media components with either an LC/UV-based workflow with AdvanceBio AAA or an LC/MS-based workflow with AdvanceBio MS Spent Media.



Oligonucleotide analysis
Robust, high-efficiency solutions for DNA/RNA analysis.



Adeno-associated virus (AAVs)
Optimized reversed-phase solutions for intact capsid proteins and post translational modifications (PTMs). Strong anion exchange analysis of full and empty capsids, and SEC for aggregate and fragment analysis.

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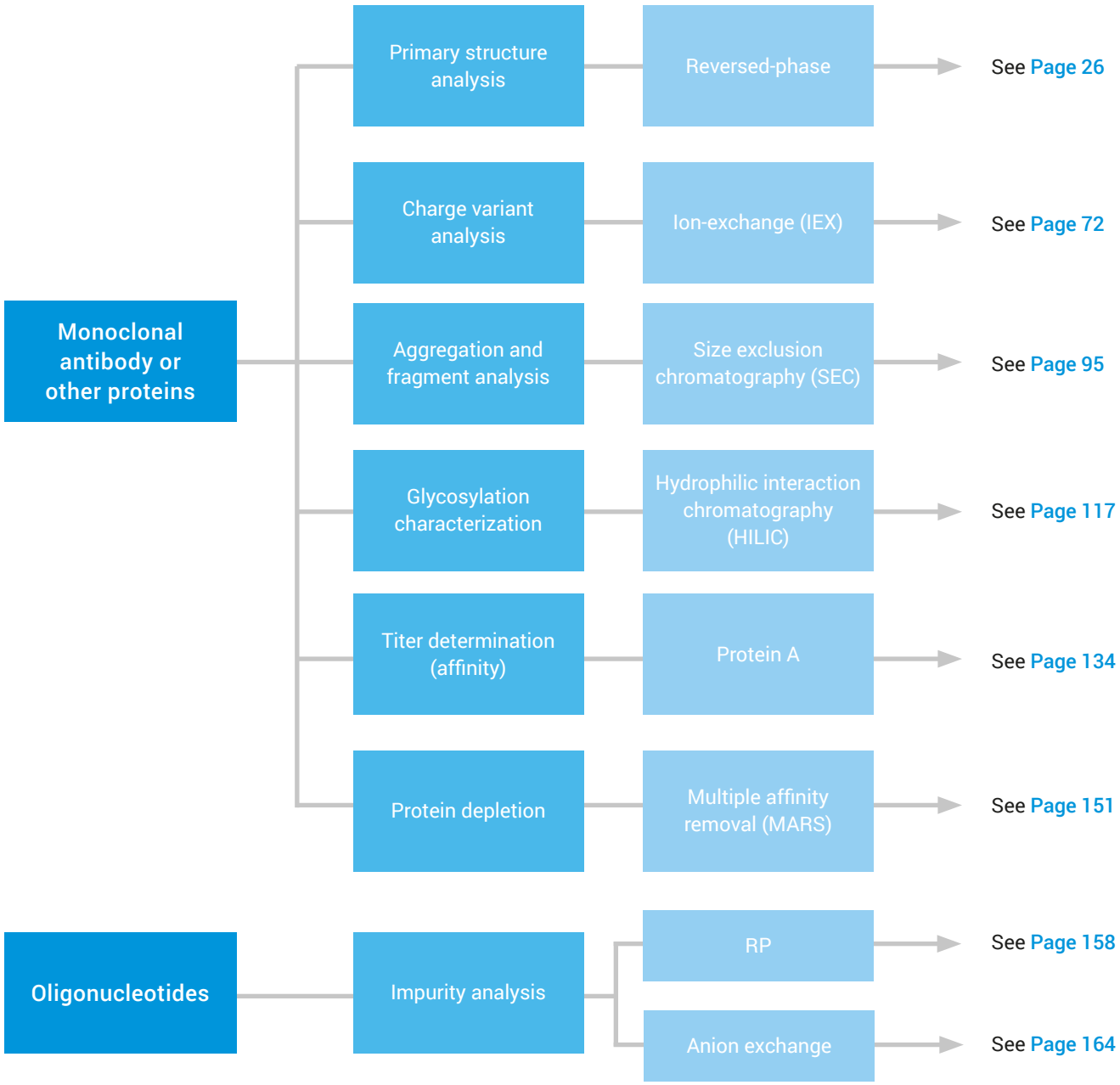
Column selection flowchart

The flowchart below indicates the page numbers that will take you to the selection guides in the individual chapters to help you choose the best column for your biomolecule application.

There are various guidelines that can be followed to help with the selection of the optimum column for a biomolecule separation. The starting point is the size of the molecule, as this determines the pore size of the HPLC method used for the separation. Secondly, consider the solubility of the molecule. Thirdly, note the separation mechanism, size, hydrophobicity, and charge.



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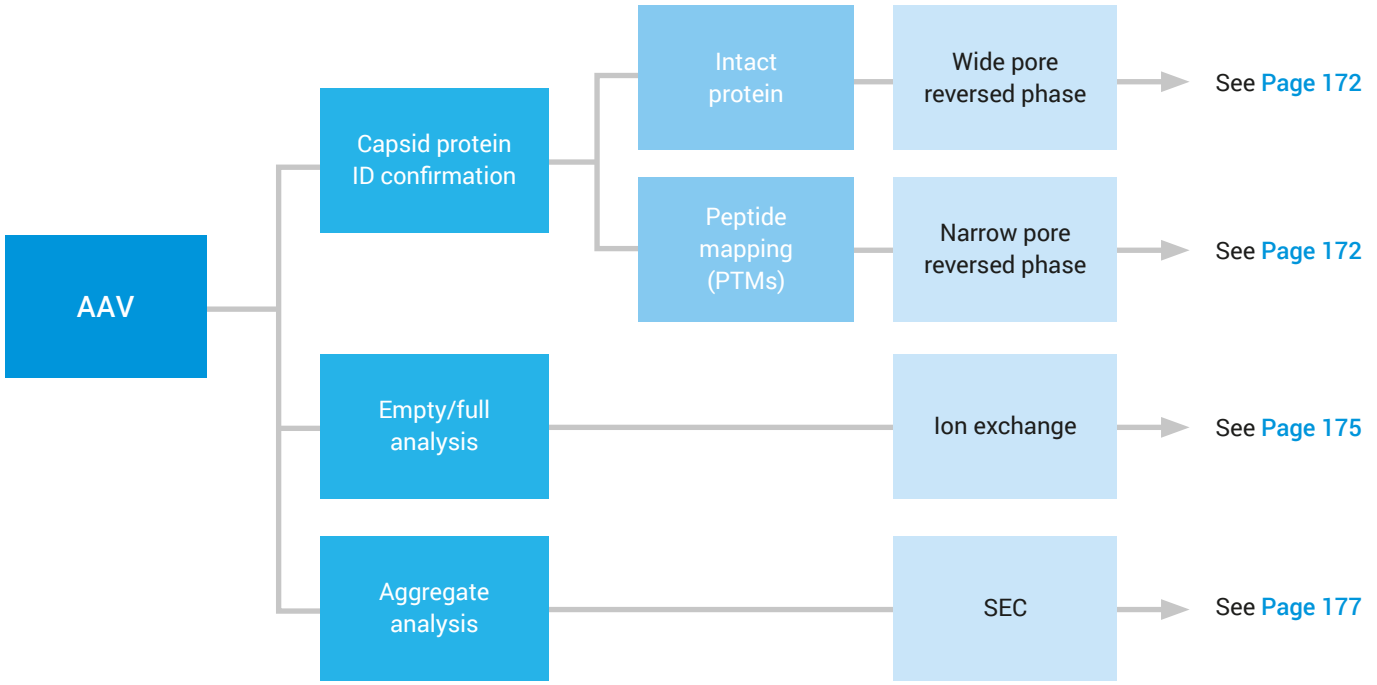
Adeno-associated virus (AAV) analysis



Tips and tools

The **Agilent Community** is a valuable online resource for questions and practical advice from Agilent experts and other users. Search "AdvanceBio Blog" in the Community to find best practices and troubleshooting tips for biomolecule separations.

Agilent AdvanceBio columns

Agilent AdvanceBio columns are designed to advance accuracy and speed for your characterization of monoclonal antibodies and other intact proteins, aggregation with SEC, charge variants with IEX, intact mass, primary structure, and post-translational modifications (PTMs) by reversed-phase, and cleaved glycan analysis by hydrophilic interaction chromatography.

This guide provides more details on the complete Agilent biocolumn portfolio, along with information on choices within the AdvanceBio family to accurately characterize biotherapeutics.

Tips and tools

To learn more about our AdvanceBio family of columns and various tools to advance your characterization needs, visit: **www.agilent.com/chem/advancebio**

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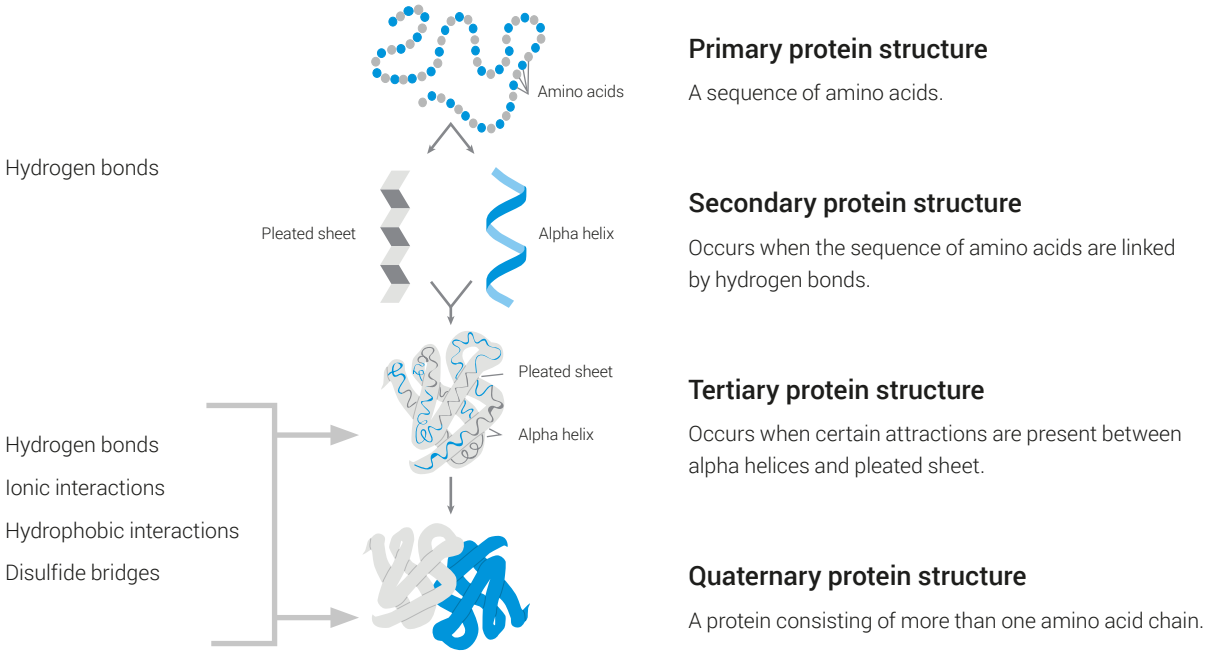
Biomolecule Separations

Protein separations

Proteins are complex molecules that require multiple techniques to provide full characterization. They exist as three-dimensional structures and these structures confer their biological activity.

The sequence of the amino acid chains defines the primary structure of the protein. Hydrogen bonding between amino acids of the primary structure then confers a secondary structure, typically in the form of alpha helices and pleated sheets. A further series of interactions, hydrogen bonding, ionic, hydrophobic, and disulfide bridges, between regions of the secondary structure, then provides the tertiary protein structure, or three-dimensional conformation. If the protein is composed of a number of amino acid chains, the interaction between these chains gives the quaternary structure.

When looking at methods for protein characterization, it is therefore clear from Figure 1 that techniques will be required that characterize the protein in its native state, without disrupting the tertiary and quaternary structures. We also need techniques for assessing the primary amino acid sequence, in the fully denatured state, with the three-dimensional structure stripped away.



Schematic showing the various levels of protein structure.

Separation considerations for proteins

The environment of the protein can influence, stabilize, or disrupt its structure. Factors to consider include pH, temperature, salt concentrations, aqueous or organic solvent content, and for some proteins, the presence of a stabilizing small molecule or metal ions. Protein structure can also be disrupted by the use of sulfhydryl reducing agents to break -S-S- bonds or chaotropic agents, such as urea or guanidine HCI. With the complexity of proteins and the intramolecular interactions that determine the three-dimensional structure, you can also expect that there will be intermolecular associations between protein molecules and other molecular entities and the surfaces with which they come into contact. This can result in protein complexes, aggregation (with possible precipitation), and deposition on surfaces, including those of the HPLC column and system. Therefore, you should consider the handling and environment in which the protein is maintained.

Protein Column Selection Guide

Application	Technique	Agilent Columns	Notes
Primary structure analysis	UHPLC/HPLC reversed-phase separations	AdvanceBio RP-mAb PLRP-S ZORBAX RRHD 300 Å Poroshell 300 Å ZORBAX 300 Å AdvanceBio Peptide Mapping	Reversed-phase separations require (or cause) denaturing of the protein to obtain detailed information about the amino acid sequence and amino acid modifications (including post-translational modifications).
Charge variant analysis	Ion-exchange separations	Agilent Bio IEX Agilent Bio MAb PL-SAX PL-SCX	The ratio of individual amino acids determines the net charge of the protein molecule. The pH at which the net charge is zero is called the isoelectric point (pI). When the solution pH is less than the pI, the protein will be positively charged (acidic), and when the solution pH is greater than the pI, the protein is negatively charged (basic). For ion-exchange analysis, we recommend the eluent pH be at least one pH unit away from its pI. Protein analysis using ionexchange columns requires buffered mobile phase and either salt gradients or pH gradients for elution.
Aggregation and fragment analysis	Size exclusion separations	AdvanceBio SEC Bio SEC-3 Bio SEC-5	Aggregates in protein biopharmaceuticals are of major concern, as they can induce an immunogenic response and can influence the composition of the final formulation.
Glycosylation characterization	Hydrophilic interaction chromatography	AdvanceBio Glycan Mapping ZORBAX RRHD 300 HILIC	Understanding glycosylation and glycan structures of proteins and mAbs is growing in importance due to the effect of immunogenicity and safety of the biotherapeutic. HILIC chromatography provides orthogonal information to reversed-phase columns as it retains the hydrophilic portion of the sample.
Titer determination	Affinity separation	Bio-Monolith Protein A Bio-Monolith Protein G	To monitor monoclonal antibody titer and yield from cell-culture supernatants before expensive preparative and large amounts of protein A are employed, a small (analytical) scale procedure is necessary to determine the titer of monoclonal antibody for the optimal time for harvest of the monoclonal antibody products.
Protein depletion	Affinity purification	MARS Human-14 MARS Human-7 MARS Human-6 MARS Human-6 High Capacity MARS Human-2 MARS Human-1 MARS Mouse-3	Remove the high-abundance proteins from biological samples. Removal of these abundant proteins improves the subsequent LC/MS and electrophoretic analysis of the sample by effectively expanding the dynamic range.

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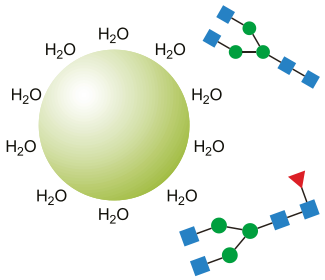
Glycan separations

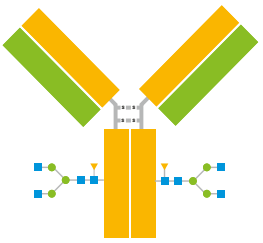


Glycan profiling

Glycan analysis is required for the characterization of biotherapeutics as the glycosylation pattern can affect the safety and efficacy of the final product.

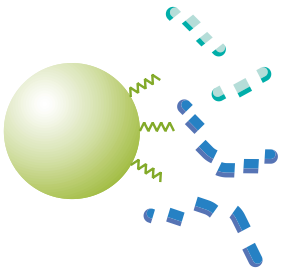
The intact glycoprotein is treated with an enzyme such as PNGase F to cleave the glycans from the protein. The glycans are then labeled with a fluorescent dye, as they are not inherently visible by UV or fluorescence. Following labeling, a cleanup step is performed to remove excess reagent and deglycosylated protein from the sample mixture. The purified, released glycan sample is then most commonly analyzed with hydrophilic interaction chromatography (HILIC) with either fluorescence or mass spectrometry detection.

The chromatographic profile is characteristic of the starting glycoprotein samples, and can vary widely in complexity. The AdvanceBio Glycan Mapping columns are well suited to delivering high resolution separation in a short amount of time.



Peptide separations

Peptide mapping



Peptide mapping is required for the characterization of proteins. It is used to confirm the identity of a protein and to identify and quantify post-translational modifications.

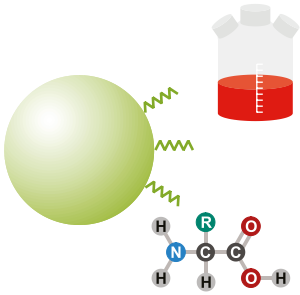
The purified protein is first digested using an enzyme, such as trypsin, yielding a range of peptide fragments. The specificity of the enzyme cleavage produces a fingerprint of peptides which is characteristic of that protein. Identification of the peptide fragments confirms the identity of the protein, and changes in the profile of the peptide digest can be used to identify post-translational modifications to the protein that may have occurred during the manufacturing or purification processes.

Reversed-phase UHPLC/HPLC is the preferred technique for the analysis of peptide digests with either MS or UV detection. LC/MS is used for the identification of the peptide fragments and determination of sequence coverage whereas LC/UV is more commonly used for peptide map comparisons in the monitoring/QC segments.

Peptide digests are complex mixtures, and for complete coverage, that is, resolution of the individual peptides, a high efficiency/high resolution column is required. AdvanceBio Peptide Mapping columns are designed to provide high-resolution peptide maps for protein identification and determination of post-translation modifications. These columns let you quickly resolve and identify amino acid substitutions/modifications in a protein primary sequence.

Amino acid and spent media analysis

During the production of biotherapeutic proteins, the cell culture media is monitored to ensure that the correct nutrient balance and levels are maintained for the expression of the product protein. Amino acids serve as the building blocks of proteins and thus are critical components of the feedstock and must be monitored and adjusted during the production process. Amino acids represent a diverse group of polar compounds that all share a similar structural backbone with an amine and carboxylic acid functional group. They differ in the structure of their side chain which can be basic, nonpolar (hydrophobic), polar uncharged, and acidic. Their differing physiochemical properties make chromatographic analysis challenging, but Agilent offers an AdvanceBio solution to meet these challenges in different ways. The AdvanceBio Amnio Acid Analysis column and derivatization reagent kit yields a highly reliable and reproducible reversed-phase chromatographic solution, while the AdvanceBio MS Spent Media column utilizes a HILIC separation paired with mass spectrometry detection for analysis of underivatized amino acids.



Amino acids

His — Histidine
H | 155.16 | 137.14 | $C_6H_9N_3O_2$

Asp — Aspartic Acid
D | 133.10 | 115.09 | $C_4H_7NO_4$

Arg — Arginine
R | 174.20 | 156.19 | $C_6H_{14}N_4O_2$

Phe — Phenylalanine
F | 165.19 | 147.18 | $C_9H_{11}NO_2$

Ala — Alanine
A | 89.09 | 71.08 | $C_3H_7NO_2$

Cys — Cysteine
C | 121.16 | 103.14 | $C_3H_7NO_2S$

Gly — Glycine
G | 75.07 | 57.05 | $C_2H_5NO_2$

Gln — Glutamine
Q | 146.15 | 128.13 | $C_5H_{10}N_2O_3$

Glu — Glutamic Acid
E | 147.13 | 129.11 | $C_5H_9NO_4$

Lys — Lysine
K | 146.19 | 128.17 | $C_6H_{14}N_2O_2$

Leu — Leucine
L | 131.17 | 113.16 | $C_6H_{13}NO_2$

Met — Methionine
M | 149.21 | 131.20 | $C_5H_{11}NO_2S$

Asn — Asparagine
N | 132.12 | 114.10 | $C_4H_8N_2O_3$

Ser — Serine
S | 105.09 | 87.08 | $C_3H_7NO_3$

Tyr — Tyrosine
Y | 181.19 | 163.17 | $C_9H_{11}NO_3$

Thr — Threonine
T | 119.12 | 101.10 | $C_4H_9NO_3$

Ile — Isoleucine
I | 131.18 | 113.16 | $C_6H_{13}NO_2$

Trp — Tryptophan
W | 204.23 | 186.21 | $C_{11}H_{12}N_2O_2$

Pro — Proline
P | 115.13 | 97.12 | $C_5H_9NO_2$

Val — Valine
V | 117.15 | 99.13 | $C_5H_{11}NO_2$

- Basic
- Nonpolar (hydrophobic)
- Polar, uncharged
- Acidic

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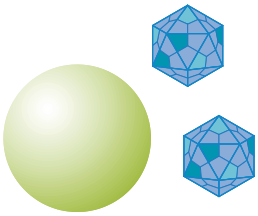
Adeno-associated viruses (AAVs)



Adeno-associated viruses (AAVs) are an emerging therapeutic delivery platform and the most widely used vectors for gene therapy that have been successful in treating inherited retinal diseases and spinal muscular atrophy. AAVs are composed of an icosahedral protein shell containing a linear single-stranded DNA genome of approximately 4.7 kb. The intact AAVs act as vehicles to protect and deliver oligonucleotide therapeutics. As AAVs continue to be explored as therapeutic delivery platforms, monitoring all the critical quality attributes of the therapeutic product is essential. The protein capsid and encapsulated DNA each require dedicated analytical techniques to ensure quality and safety of the overall product.

AAVs Column Selection

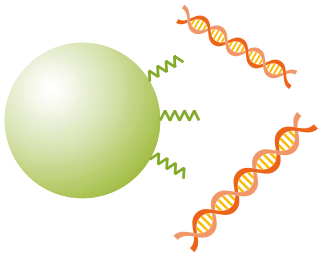
Application	Technique	Agilent Columns	Notes
Capsid Protein ID Confirmation: Intact Protein	Wide Pore Reversed-phase	ZORBAX RRHD Wide Pore Columns	1.8 µm 300 Å ZORBAX RRHD Stablebond C18 and Diphenyl column options for high efficiency reversed-phase separation and characterization of intact capsid proteins.
Capside Protein ID: Peptide Mapping (PTMs)	Narrow Pore Reversed-phase	AdvanceBio Peptide Mapping	2.7 µm Poroshell particle for optimal efficiency and resolution.
Empty/Full Capsid Analysis	Strong Anion Exchange	Agilent Bio SAX	Agilent Bio SAX strong anion exchange columns ideal for separating full and empty AAV capsids.
Aggregate Analysis	Size Exclusion Chromatography	Agilent Bio SEC-5	Agilent Bio SEC-5 with 1,000 Å pore size ideally suited for AAV aggregate and fragment analysis and purification.

Tips and tools

Agilent bio-inert supplies provide a metal-free sample flow path, which minimizes interactions with your biomolecules.

Visit: **www.agilent.com/chem/bio-inert-uhplc**

DNA and RNA oligonucleotide separations



There is growing interest in oligonucleotides (oligos) in the biopharmaceutical market as research has shown their promise as therapeutic agents. These oligos can range in size, sequence complexity, and overall modifications. Smaller oligonucleotides may be prepared synthetically and can span a range of sizes from a few nucleotides to 40 or 50 bases, with varying DNA of RNA modifications. The chemical synthesis process will produce a variety of impurities with closely related sequences that contain omissions, insertions, or chemical modifications. Larger oligos – including guide RNA or NGS primers and probes – can span from 50 to 200 bases with varying modifications and tags for their particular use. RNA therapeutics can have sequences that are thousands of bases in length requiring their own unique purification considerations. As a result of this diversity, the analysis and purification of oligonucleotides by liquid chromatography is complex and utilizes a range of techniques, column chemistries, and particle supports.

There are several UHPLC/HPLC techniques used for oligonucleotide separations.

Trityl-on – This procedure separates the full-length target oligo, which still has the dimethoxytrityl (DMT) group attached, from the deprotected failure sequences. The analytical information obtained is limited and this is considered to be a purification method.

Ion-exchange separations of the trityl-off, deprotected oligos – This method uses the negative charge on the backbone of the oligo to facilitate the separation. Resolution is good for the shorter oligos, but decreases with increasing chain length. Aqueous eluents are used, but oligos are highly charged and high concentrations of salt are needed to achieve elution from the column.

Ion-pair reversed-phase separation of the trityl-off, deprotected oligos – This technique uses organic solvents and volatile ion-pairing agents, and is suitable for LC/MS. The technique is best performed with high-efficiency particles. Conditions that fully denature the oligos and prevent association with complementary sequences are required. Thus, the separation is best performed at elevated temperatures.

DNA and RNA Oligonucleotide Column Selection

Application	Technique	Agilent Columns	Notes
Trityl off oligonucleotide characterization/ purfication	Ion-pairing reversed-phase separation	AdvanceBio Oligonucleotide	2.7 µm and 4 µm superficially porous particles for high resolution analysis and purification of oligonucleotides and their impurities
		PLRP-S	Macroporous polymeric particles in a wide range of particle sizes and pore sizes, offering the greatest pH and temperature stability.
Trityl off Aggregation and Fragment Analysis	Ion-exchange separation	Agilent Bio IEX	Non-porous polymeric particles with hydrophilic coating eliminating mass transfer issues, providing exceptional performance. Available in a range of particle sizes for analytical and lab scale preparative capabilities.
		PL-SAX	Fully porous polymeric particles with strong anion exchange functionality, available from analytical to prep scale, including bulk media.

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Instrumentation for Biotherapeutics Analysis

Agilent offers total workflow solutions

While the LC column is a critical piece of many purification or analytical characterization workflow, it is ultimately only one component. Agilent offers end-to-end solutions and expert support for many biomolecule workflows, including sample preparation, vials and other supplies, instrumentation, and data analysis and management tools.

1260 Infinity II bio-inert LC system

For completely (bio-)inert applications

The 1260 Infinity II bio-inert LC is fully bio-inert, eliminating metal interactions and facilitating analysis of your most challenging compounds. The corrosion-resistant titanium solvent delivery system and metal-free sample flow path ensure integrity of biomolecules in your routine bio applications. The system also serves as an ideal front end for ICP-MS analysis.

- The metal-free sample flow path at 600 bar offers a reliable analysis of biological samples which means that none of your precious sample touches metal surfaces, minimizing unwanted surface interactions while increasing column lifetime
- Inert flow cells for UV and fluorescence detection and inert solvent and column selection valves for multimethod/multi-attribute analysis offer instrument variety for highest adaptability
- High salt tolerance (2 M) and wide pH range (1–13, short term 14) with active seal wash and quaternary solvent blending offer increased flexibility
- Novel bio-inert capillary and connection design and InfinityLab Quick Connect/Quick Turn column fittings offer ease of use

1260 Infinity II Prime bio LC system

For everyday analysis with high flexibility

The biocompatible 1260 Infinity II Prime bio LC System is a versatile HPLC for bio separations and offers outstanding functionality and operational convenience for bioanalytical HPLC and entry-level quaternary bio UHPLC.

- Biocompatible solvent and sample flow path ensure integrity of biomolecules and minimize unwanted surface interaction
- High salt tolerance and wide pH range offer enhanced flexibility and robustness for increased instrument uptime
- Power range combines high pressure up to 800 bar and high analytical flow rates up to 5 mL/min for maximum UHPLC performance
- Shallow microplate drawers take a maximum load of 6,144 samples for unmatched sample capacity

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1290 Infinity II bio LC system

For ultra-high performance in complex analyses

The 1290 Infinity II bio LC system is available with either the 1290 Infinity II bio high-speed pump or flexible pump. The system is designed for the most demanding applications, providing very shallow gradients and enabling high performance, accuracy, and reliability. The biocompatibility of the system ensures robustness and biomolecule integrity.

- Flexibility for all applications - through wide power, temperature, an automatically scalable injection range, and gradient options (binary and quaternary solvent mixing)
- Lowest delay volume throughout the system enables highest chromatographic resolution and lowest dispersion
- Different bio accessories include a range of bio heat exchangers, bio capillary kits, bio loops, and analytical heads to cover all the different application needs for instrument versatility and efficiency
- Power range combines ultrahigh pressure up to 1300 bar and high analytical flow rates up to 5 mL/min for maximum UHPLC performance
- Your choice for performance and sustainability: The 1260/1290 Infinity II LC system family has received the My Green Lab ACT (Accountability, Consistency, Transparency) label after an independent audit to verify its environmental impact throughout the product life cycle.

1290 Infinity II bio 2D-LC system

Ultimate separation power with a bio-compatible 2D-LC system

The 1290 Infniity II bio 2D-LC system combines highest separation power and peak capacity of a 2D-LC system with the biocompatibility of a bio LC system.

- Whether samples require superior resolution for co-eluting compounds or samples containing a high number of compounds that cannot be resolved efficiently with conventional (U)HPLC, 2D-LC is the method of choice.
- Agilent InfinityLab 2D-LC solutions are based on dedicated 1290 Infinity II hardware and special software products and offer highest performance, quality and ease of use.
- Biopharma and applications from other industries use demanding conditions with respect to samples, solvents and reagents. The biocompatible 2D-LC system provides superior separation performance and robustness for such applications. MP35N is used in the bio 2D-LC flow path with other noble materials like ceramic, PEEK, or gold. These materials reduce surface binding and interaction with biomolecules, and they are compatible with demanding conditions such as solvents, samples, and reagents like a wide pH range or high salt concentrations.
- All biocompatible and bio-inert LC modules and parts can be used with the 1290 Infinity II bio 2D-LC system.

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1260 Infinity II Bio-SEC system

Reliable analysis of biological polymers with metal-free flow path and optional advanced detectors

The 1260 Infinity II Bio-SEC system is a size exclusion chromatography (SEC) system designed to meet the challenges of analyzing biological polymers. It is the perfect solution for protein molar mass, purity, and aggregation analysis with advanced detection.

- Metal-free sample flow path allows robust characterization of biological polymers
- High salt tolerance and wide pH range with active seal wash and quaternary mobile phase blending offer increased flexibility
- Dedicated SEC column thermostat provides high capacity and cooling capabilities
- Flexible addition of multi-angle light scattering (MALS) detection enables the characterization of aggregation, size, and shape of biological polymers
- Compliant software support by WinGPC software provides a dedicated SEC software platform

1260 Infinity II multidetector GPC/SEC system

Accurate and reproducible characterization of proteins and biomolecules with light scattering detection

Select from any combination of detection by refractive index, light scattering, and viscometry. The system delivers accurate, absolute molecular weights and sizes, and can be used to analyze a wide range of polymers, regardless of molecular weight range or solvent.

- **Accurate molecular weights** – light scattering detection does not require column calibration and when combined with PLgel columns delivers perfect results about the true polymer molecular weight, size, shape, and long chain branching
- **Accurate size and structure** – viscometry can be used to measure Rg (sample radius) and sample structure over a wide molecular weight range
- **Low molecular weight sample analysis** – the viscometer can measure molecular weights down to as low as 100 s thanks to the latest advances in silica and pressure transducer technology
- **Triple detection delivers the ultimate in polymer analysis** – absolute molecular weights are determined by light scattering detection and molecular size and solution behavior comes from the light scattering and viscometry detectors

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1290 Infinity II bio analytical-scale LC purification system

Ultra high-performance biocompatible analytical LC purificiation system to protect your biomolecules

The 1290 Infinity II bio analytical-scale LC purification system is a UHPLC, binary or quaternary, consisting of biocompatible material for use in biopharma. It is a most flexible and versatile solution for compound isolation utilizing high salt and extreme pH conditions.

- Biocompatible solvent and sample flow path ensure integrity of biomolecules and minimize unwanted surface interaction
- High salt tolerance and wide pH range offer enhanced flexibility and robustness for increased instrument uptime
- Collection of up to 4 x 96 fractions in microtiter plates, or up to 216 fractions in glass tubes with 4 outer diameters of tubes available collector
- Integrated and automated fraction delay sensor technology for increased purity and recovery of collected fractions
- Upgradeable fraction capacity based on your demand: improved lab efficiency through orthogonal analytical information only available from isolated compounds

1260 Infinity II Prime bio analytical-scale LC purification system

Biocompatible analytical LC purification system with a pressure range up to 800 bar

The 1260 Infinity II Prime bio analytical-scale LC purification system is a versatile HPLC for bioseparations. It offers outstanding functionality and operational convenience for bioanalytical HPLC and entry-level quaternary bio UHPLC at pressures up to 800 bar and flow rates up to 5 mL/min.

- Biocompatible solvent and sample flow path ensure integrity of biomolecules and minimize unwanted surface interaction
- Power range combines high pressure up to 800 bar and high analytical flow rates up to 5 mL/min for maximum UHPLC performance
- A wide range of sensitive optical detection capabilities with various flow cells for VWD, DAD, FLD, or LC/MS detection for exceptional adaptability
- Integrated and automated fraction delay sensor technology for increased purity and recovery of collected fractions
- Upgradeable fraction capacity based on your demand: improved lab efficiency through orthogonal analytical information only available from isolated compounds

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Load & Lock preparative HPLC columns

Highest bed stability and enhanced flow distribution for routine purification of large quantities of material

Agilent offers a complete range of laboratory scale Load & Lock columns and the Mobile Packing Station. Designed to let you easily and quickly pack your own preparative high- efficiency columns, this is the right solution for development applications of pharmaceutical compounds, peptides, and natural products. Our Load & Lock columns have a unique fluid/sample distribution system to maximize productivity. This improves column performance by diffusing the sample more efficiently over the complete bed surface.

- Highest performance: achieve superior results with a unique flow distribution system
- Maximum flexibility: all the 1 inch, 2 inch, and 3 inch Load & Lock columns conveniently use the same mobile packing station and perform both Dynamic Axial Compression (DAC) or Static Axial Compression (SAC)
- Greater convenience: pack or unpack your column in a few minutes
- Maximum mobility: column and packing station are combined in one easy-to-move skid, wherever it's needed

Dual mode packing formats ensure easy operation and the delivery of consistent, high-performance results by using the DAC or SAC mode.

Agilent laboratory scale Load & Lock columns combine excellent packed-bed stability with enhanced flow distribution to deliver the highest quality purification possible with maximum speed, flexibility, and ease of operation.

Requiring only compressed air, the Load & Lock packing station uses no power supply, making it safe to use with any type of solvent and the solution of choice for hazardous environments. The quick-release single-bolt clamp offers speedy and easy packing and unpacking within minutes.

Load & Lock Preparative HPLC Columns

Description	Water Jacket	Inner Diameter x Length (mm)	Part No.
Load & Lock column	Yes	27.0 x 500	PCG93LL500X25WJ
	Spare parts kit		PCG931AAKIT
Mobile packing station (air-driven hydraulic)			PCG93LLSTAND123

Tips and tools

Compare the features, physical specifications, and performance characteristics of InfinityLab LC purification systems, modules, columns, and media in our selection guide **5991-9153EN**.

www.agilent.com/chem/lc-purification-selection-guide

6545XT AdvanceBio LC/Q-TOF system

Designed for large biomolecule analysis

The Agilent 6545XT AdvanceBio LC/Q-TOF system is designed to handle multiple workflows in biopharmaceutical characterization. When you need to access the most information at the intact protein level, automatically confirm a sequence through peptide mapping, and confidently understand PTMs, Agilent provides a UHPLC Q-TOF that delivers.

Accurately profile major and minor proteoforms with time-of-flight technology, offering new levels of sensitivity and clarity to intact protein mass spectrometry. Easily dig deeper into peptide digests with the Iterative MS/MS mode of operation. The 6545XT AdvanceBio Q-TOF is the analytical cornerstone in a comprehensive set of tools designed for biopharma.

- Excellent protein spectral clarity from ultra-high TOF vacuum (10E-8)
- One-click optimization for large molecules with SWARM autotune
- Capable of analyzing very large molecules, with a variable mass range up to 30k m/z
- 50k resolution from improved beam optics
- Ease of maintenance with vent-free capillary removal

6230B time-of-flight (TOF) LC/MS

Accurate mass TOF LC/MS for biomolecules

The Agilent 6230B time-of-flight LC/MS (TOF LC/MS) system is a high-resolution accurate mass system for enhanced analytical capabilities. Simultaneous measurement of the full spectrum of mass-to-charge ratios up to 20,000 improves speed and sensitivity. The full mass spectrum scan of a TOF LC/MS ensures that all necessary information is captured for biomolecule characterization. The 6230B TOF LC/MS is compatible with Agilent Bio-inert HPLC systems for optimized intact protein characterization, peptide, glycan, and oligonucleotide analysis. The high resolution and accurate mass enable detection and identification of extractables and leachables, and other impurities. Intuitive software tools enable anyone to routinely achieve reliable results. The 6230B TOF LC/MS MassHunter software suite is compliance ready.

- Analyze monoclonal antibodies (mAb) with high mass accuracy and resolving power for glycoforms and truncation
- Characterize large biologic compounds with a broad mass range of up to 20,000 m/z
- Reduce administrative tasks and risks while eliminating the need for LC/MS expertise with intuitive MassHunter WalkUp software
- Go beyond UHPLC separation with support for capillary electrophoresis, SFC, and 2D-LC
- Find impurities at extremely low concentrations, using Agilent Jet Stream technology with low picogram on-column sensitivity

MassHunter BioConfirm software

Take a giant step forward in biomolecule characterization

Agilent MassHunter BioConfirm software enables routine characterization of biomolecules through easy-to-use workflows for oligonucleotide analysis, intact protein analysis, peptide mapping, and routine glycan profiling. This biopharma software for LC/Q-TOF is part of a comprehensive solution for mass spectrometry analysis of biopharmaceutical products such as monoclonal antibodies and oligonucleotides. From automated sample preparation to separation and detection through to data analysis and reporting, the Agilent biopharma workflow provides highly sophisticated analyses. Tools like the drug-to-antibody ratio (DAR) calculator, flexible report templates, and integrated operation with MassHunter WalkUp bring expert LC/MS capabilities to nonexperts.

Meets data integrity standards with technical controls to securely acquire, process, report, and store data in laboratories that must follow the compliance guidelines of FDA 21 CFR Part 11, EU Annex 11, GAMP 5, ISO/IEC 17025, and EPA 40 CFR Part 160

Complete workflows for:

- Intact proteins (including DAR)
- Peptide mapping
- Released glycans
- Oligonucleotides

Tips and tools

Achieve optimal performance and efficiency using Agilent LC supplies

Agilent InfinityLab supplies work seamlessly with InfinityLab LC series instruments to achieve the highest performance, operational efficiency, and lab safety. Smart consumables track usage for added confidence in your analysis.

Visit: **www.agilent.com/chem/infinitylab** or discover the InfinityLab LC Supplies catalog at **www.agilent.com/chem/catalog**

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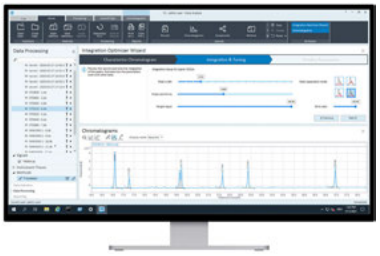
OpenLab software suite

The Agilent OpenLab Software portfolio is an integrated suite of products that includes sample management, data acquisition, data analysis, data management, lab workflow management.

OpenLab CDS – Streamline your large molecule analytical workflows

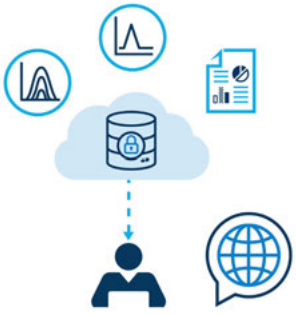


OpenLab CDS is a chromatography data system that combines productivity, usability, and data integrity. With software tools like Custom Calculator and Integration Optimizer, the process of calculating oligonucleotide purity or determining DAR can be automated, eliminating the need for manual entry of peak data into spreadsheets. This enables fast interpretation of data, resulting in accurate and consistent results.

Application-specific tools to support biopharma workflows

- **MatchCompare for OpenLab** for objectively comparing unknown samples to a known standard based on user defined constraints.
- **2D-LC software** for running two-dimensional measurements and analyzing multidimensional data.
- **WalkUp software** an intuitive sample submission and method selection software allows open access to LC and LC/MS.

OpenLab ECM XT – A single point of access for all your analytical data



OpenLab ECM XT is a scientific data management system (SDMS) software that provides centralized cloud and on-premises storage of data. By pairing MassHunter with OpenLab Server/ECM XT for secure storage, your lab will benefit from extensive technical controls that enhance your readiness for regulatory audits:

- **Secure on-premises or cloud storage** of active records including data files, methods, worklists, report templates, and results
- **Audit trails** that clearly show the file history with details of who did what, when, and why
- Assign **access privileges** based on the function of the user and the data each can access

Break down data silos with networked systems

Whether for an mRNA therapeutic or bispecific monoclonal antibody, analytical data to support a large molecule modality is critical. However, having standalone workstations make instrument data difficult to access and manage. The move to a networked configuration can improve your overall lab operations.

InfinityLab LC supplies

A perfect fit for your biomolecule analysis

Agilent InfinityLab supplies are innovative consumables designed to work seamlessly together with Agilent LC instruments and columns for maximum efficiency and performance for your bio HPLC analysis.

Protect your column against particles

Particulates can lead to column clogging, poor chromatographic results and increased downtime. To safeguard against these issues, employing effective filtration techniques is vital. LC filtration assemblies can be used to filter mobile phases, especially when using water-based buffer solutions, to remove residue of undissolved salt crystals and microbes.

Inline filters can be installed to capture any particles in the flow path that come from solvents, samples or worn system parts. InfinityLab Quick Change inline filter offer tool-free replacement of filter discs and "click and seal" feedback to ensure ultimate ease of use.

Perfect connection for your bio application

Within the InfinityLab family, Agilent offers HPLC capillaries in a variety of materials to meet your needs. Capillaries made of MP35N, PEEK-lined stainless steel and titanium are inert and corrosion resistant and are particularly suitable for bio-applications. In combination with InfinityLab Quick Connect fittings, you can create a perfect finger-tight connection up to 1300 bar. For the best analytical performance.

Reduce chemical vapor in the lab

Acetonitrile and methanol are just two of the many toxic compounds you may be exposed to every day. The InfinityLab Stay Safe caps stop solvents from leaching into the air. Combined with the innovative InfinityLab Stay Safe purging bottle the purging of an HPLC with up to four solvent lines becomes a safe task.

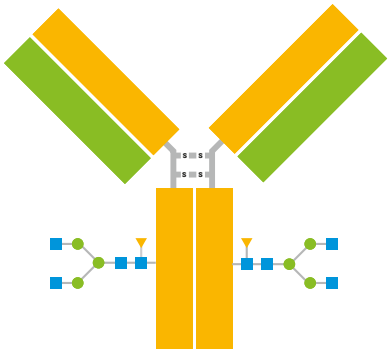
Your sample's journey starts in a vial – make it the right one!

Agilent offers a comprehensive line of vials, caps and inserts; whether a standard borosilicate glass vial will suffice, a surface-deactivated glass vial or polypropylene vial we have the containment solution for you. Beyond vial composition we offer vials in various designs reflecting the nature and sample volume available, from 3-5 µL ,when sample is limited to 1.75 mL when an abundance of sample is available.

Tips and tools

Download our vials catalog (**5994-4803EN**) to help guide you in making the final decision on vials, supported by our **vial selection tool**.

Intact Protein Analysis using Reversed-Phase and Hydrophobic Interaction Chromatography



Get the selectivity and resolution necessary to separate impurities at the intact protein or subunit level

Proteins are commonly analyzed at the intact or subunit level to monitor impurities or to measure accurate molecular weights using LC/MS. Intact protein measurements often require far less sample preparation than peptide-level analyses, while offering a relatively quick assessment of product quality and purity.

Agilent offers the most comprehensive range of wide-pore, 300 Å, 450 Å, and larger, reversed-phase BioHPLC columns, all backed by technical support experts and application chemists around the globe. The family includes 1.8, 3.5, and 5 µm porous particles for pressures from 400 to 1200 bar, three different superficially porous particles for UHPLC separations at lower pressure, and polymeric columns for analysis under a wide variety of conditions, including formic acid mobile phases for MS analysis.

Additionally, Agilent offers both hydrophobic interaction chromatography (HIC) and hydrophilic interaction chromaography (HILIC) columns for alternative and complementary approaches to intact protein or subunit separations.

Reversed-phase

- **AdvanceBio RP-mAb columns** are the only reversed-phase columns designed especially for mAb characterizations. 450 Å pore size Poroshell technology and the right bonded phase selectivity provide fast, high resolution characterization of intact mAbs and mAb fragments.
- **PLRP-S columns** include macroporous polymer particles that deliver HPLC separations over the widest pH range. With three wide-pore sizes and eight particle sizes, the PLRP-S columns provide optimum solutions for analytical prep separations of peptides, proteins, and protein complexes.
- **ZORBAX RRHD 300 Å 1.8 µm columns** deliver UHPLC performance for reversed-phase separations of intact proteins, protein fragments, and digests with 1200 bar stability.
- **ZORBAX 300 Å 3.5, and 5 µm columns** are made from fully porous materials for HPLC and prep separations; many of the bonded phases scalable from the 1.8 µm particle.
- **Poroshell 300 columns** are the industry's first superficially porous small particle columns for fast polypeptide and protein separations.

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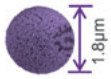
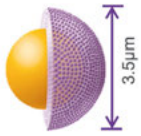
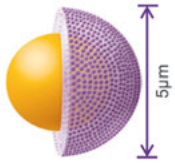
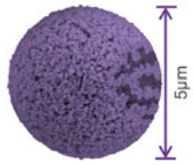
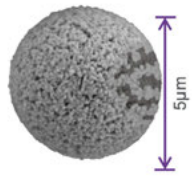
Column choices for reversed-phase separations

	ZORBAX RRHD SB 300	AdvanceBio RP-mAb	Poroshell 300	ZORBAX 300SB	PLRP-S
					
Phase Options	SB-C3, SB-C8, Diphenyl	C4, SB-C8, Diphenyl	SB-C3, SB-C8	SB-C3, SB-C8, Diphenyl	Polymeric
Pore Size	300 Å	450 Å	300 Å	300 Å	1000 Å
HPLC		●	●	●	●
UHPLC	●				
Strengths	– Maximum resolution – Unique diphenyl chemistry	– Designed for mAbs – Unique diphenyl chemistry	– Smaller globular proteins – Higher throughput update to ZORBAX 300SB methods	– Robust workhorse – Unique diphenyl chemistry	– Recommended for MS – excellent peak shape with both FA & TFA – PEEK-lined SS hardware option

Hydrophobic interaction chromatography

– **AdvanceBio HIC columns** are a native-mode alternative to reversed-phase separations, based on a unique 450 Å pore, totally porous ZORBAX particle. Intact, natively folded proteins are eluted from least to most hydrophobic.

Hydrophilic interaction chromatography

– **ZORBAX RRHD 300 Å HILIC columns** are made from fully porous 1.8 µm particles with complementary selectivity to reversed phase, making them particularly useful for hydrophilic samples such as glycopeptides and glycoproteins.

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Reversed-Phase Column Selection

Application	Agilent Columns	Notes	
Monoclonal antibodies and mAb fragments	AdvanceBio RP-mAb	Based on Poroshell technology featuring superficially porous particles that reduce diffusion distances and allow higher flow rates and steeper gradients to be used thus reducing run times—even on 600 bar systems. 450 Å pore size provides full access to the bonded phase by large molecules ensuring the best possible chromatography. Robust bonded phases designed for monoclonal antibody separations provide a range of selectivities that allow resolution to be optimized.	AB
	C4 SB-C8 Diphenyl		
	PLRP-S 1000 Å	Macroporous polymeric PLRP-S delivers excellent mAb separations, especially with formic acid mobile phases for MS detection.	
Intact proteins, monoclonal antibodies, mAb fragments and polypeptides	ZORBAX 300 Å, 1.8 µm	Optimized packing processes achieve stability up to 1200 bar for use with the 1290 Infinity II LC. RRHD 1.8 µm columns are available in 50 and 100 mm lengths for fast or high resolution separations of the most complex samples. StableBond C18 is ideal for complex protein and protein digest separations.	AB
	RRHD 300SB-C18 RRHD 300SB-C8 RRHD 300SB-C3 RRHD 300-Diphenyl		
	ZORBAX 300 Å, 3.5, and 5 µm	Ideal for use with HPLC systems. StableBond C3 and CN are useful for larger, more hydrophobic compounds.	
	300SB-C18 300SB-C8 300SB-C3 300SB-CN		
	PLRP-S	Polymeric PLRP-S offers alternate selectivity to silica-based columns, along with excellent peak shape in formic acid mobile phases.	
	100 Å 300 Å 1000 Å 4000 Å		
	Poroshell 300	5 µm Poroshell particles with 300 Å pores enable rapid HPLC separations of intact proteins.	AB
	300SB-C18 300SB-C8 300SB-C3 300Extend-C18		
Oligonucleotide analysis	PLRP-S 100 Å, 300 Å, 1000 Å, 4000 Å PL-SAX 1000 Å, 4000 Å	Multiple pore size options depending on oligonucleotide length. High temperature stability. Scalable from analytical to preparative separations. For more information on PL-SAX, please see **page 84**.	
	AdvanceBio Oligonucleotide	Ideal for high-resolution analytical and prep scale separations. 2.7 µm and 4 µm superficially porous particles for high resolution analysis and purification of oligonucleotides and their impurities	AB

AB Part of the AdvanceBio family

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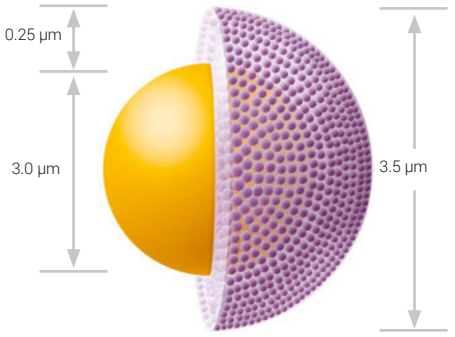
AdvanceBio RP-mAb



- **Improved accuracy:** superficially porous particles (3.5 µm) with wide pores (450 Å) increase mAb resolution while maintaining compatibility with all LC instruments.
- **Speed:** shorter analysis times compared to columns packed with fully porous particles.
- **Flexible method development:** range of chemistries—SB-C8, C4, and diphenyl.
- **Lower costs:** robust Poroshell packed bed and 2 µm inlet frit extend column lifetime by helping prevent inlet blockage.

Reversed-phase columns developed for the unique challenges of monoclonal antibody characterization

Analysis of intact and reduced monoclonal antibodies are critical measurements for characterizing therapeutic proteins and understanding their efficacy and stability. Poor chromatographic separations can result in rework and even compromise the accuracy of the characterization.

Long analysis times negatively impact the throughput of a laboratory and lead to delays in making decisions based on the results of characterization. To eliminate these problems, Agilent has developed a new reversed-phase column to optimize the performance of intact and reduced mAb analysis. The AdvanceBio RP-mAb column is based on Poroshell technology with unique engineering for pore size and bonded phases.

Robust particles designed specifically for fast and high resolution mAb separations

Large biomolecules, such as monoclonal antibodies, are typically separated slowly to reduce the potential peak broadening of these slow diffusing analytes. However, the Poroshell technology used in AdvanceBio RP-mAb columns features superficially porous particles made with a thin layer of porous silica, 0.25 µm thick, on a 3.0 µm solid silica core. This morphology reduces the diffusion distance, allowing higher flow rates and steeper gradients to be used—even on 600 bar systems. The wide 450 Å diameter of the pores in the thin layer provides full access to the bonded phase by the large monoclonal antibody molecules ensuring the best possible chromatography. A choice of robust bonded phases designed for monoclonal antibody separations, C4, SB-C8, and a unique diphenyl, provide a range of selectivities that allow resolution to be optimized.

AdvanceBio RP-mAb columns deliver higher resolution and shorter run times to provide fast, accurate, and reproducible results when analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications.

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Column Specifications

Bonded Phase	Pore Size	Temperature Limits*	pH Range*	Endcapped
AdvanceBio RP-mAb C4	450 Å	90 °C	1.0–8.0	Yes
AdvanceBio RP-mAb SB-C8	450 Å	90 °C	1.0–8.0	No
AdvanceBio RP-mAb Diphenyl	450 Å	90 °C	1.0–8.0	Yes

Specifications represent typical values only.

* Columns are designed for optimal use at low pH. At pH 6–8, highest column stability for all silica-based columns is obtained by operating at temperatures <40 °C and using low buffer concentrations in the range of 0.01–0.02 m.

Tips and tools

For more information on the characterization of monoclonal antibody primary structure, see: Reversed-Phase LC Primary Structure Characterization Workflow (publication **5991-6321EN**).

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High speed, high resolution separation of Trastuzumab Variant IgG1

Column: **AdvanceBio RP-mAb C4**
795775-904
2.1 x 100 mm, 3.5 µm

Mobile phase: A: 0.1% TFA in water:IPA (98.2)
B: IPA:ACN:mobile phase A (70:20:10)

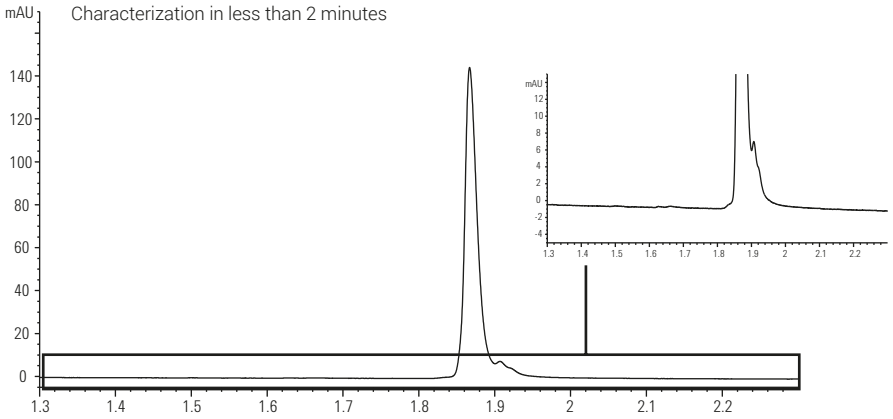
Flow rate: 1.0 mL/min

Gradient: 10–58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B

Temperature: 80 °C

Detector: UV, 254 nm

Sample: 5 µL injection of humanized recombinant Trastuzumab Variant IgG1 intact from Creative Biolabs (1 mg/mL)



AdvanceBio RP-mAb C4 provides a sharp peak and resolves fine detail in less than 2 minutes.

Selective diphenyl phase

Column: **AdvanceBio RP-mAb Diphenyl**
795775-944
2.1 x 100 mm, 3.5 µm

Mobile phase: A: 0.1% TFA in water: IPA (98:2)
B: IPA:ACN:mobile phase A (70:20:10)

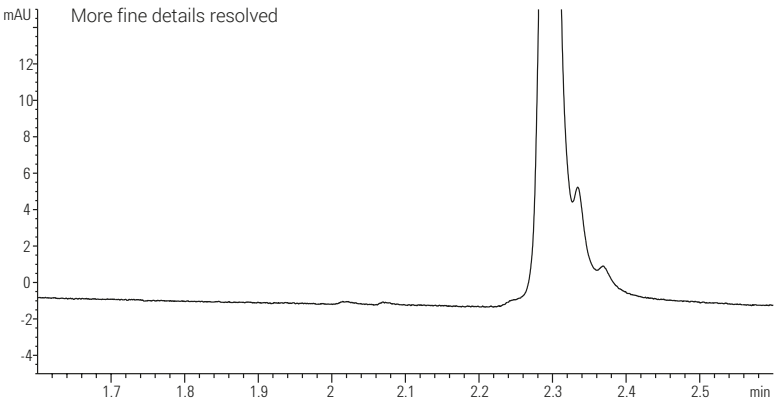
Flow rate: 1.0 mL/min

Gradient: 10–58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B

Temperature: 80 °C

Detector: UV, 254 nm

Sample: 5 µL injection of humanized recombinant Trastuzumab Variant IgG1 intact from Creative Biolabs (1 mg/mL)



The unique selectivity of AdvanceBio RP-mAb Diphenyl resolves even more fine detail.

Separation of intact humanized recombinant Trastuzumab IgG1

Column: **AdvanceBio RP-mAb C4**
795775-904
2.1 x 100 mm, 3.5 µm

Mobile phase: A: 0.1% TFA in water:IPA (98:2)
B: IPA:ACN:mobile phase A (70:20:10)

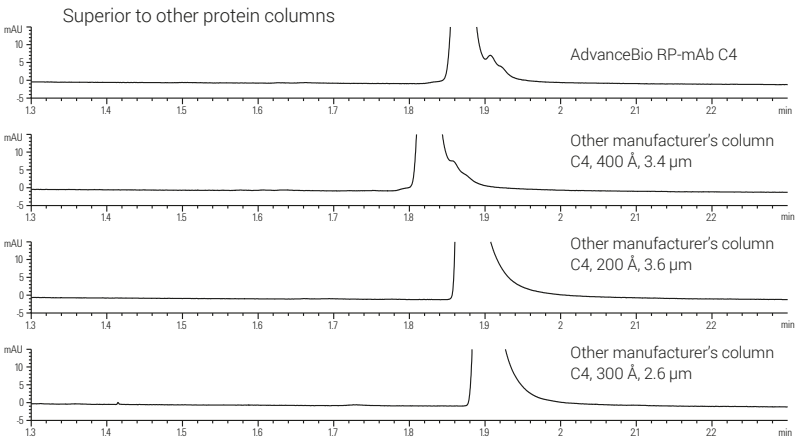
Flow rate: 1.0 mL/min

Gradient: 10–58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B

Temperature: 80 °C

Detector: UV, 254 nm

Sample: 5 µL injection of humanized recombinant Trastuzumab Variant IgG1 intact from Creative Biolabs (1 mg/mL)



Specifically designed for mAb separations, AdvanceBio RP-mAb provides superior peak shape and resolution when compared to other columns used for intact protein separations.

The Poroshell advantage

Column: **AdvanceBio RP-mAb SB-C8**
785775-906
2.1 x 100 mm, 3.5 µm

Mobile phase: A: 0.1% TFA in water
B: n-Propanol:ACN:mobile phase A (80:10:10)

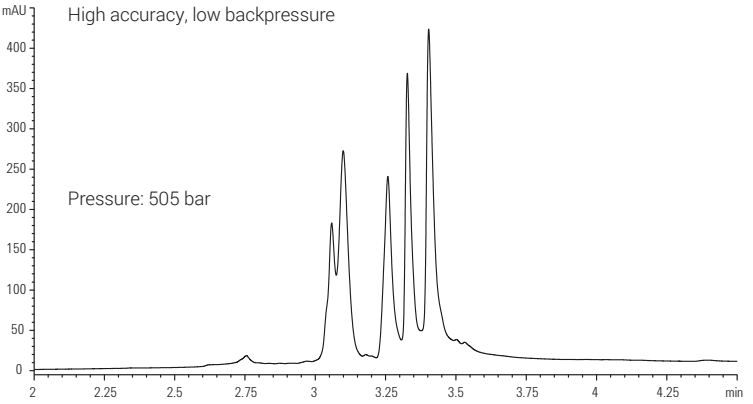
Flow rate: 0.8 mL/min

Gradient: 5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B

Temperature: 60 °C

Detector: UV, 220 nm

Sample: 1 µL injection of Fc/Fab, papain-digested humanized recombinant Trastuzumab Variant IgG1 from Creative Biolabs (2 mg/mL)



The wide-pore Poroshell technology of the AdvanceBio RP-mAb column delivers high efficiency, a short analysis time, and low pressure, at temperatures below 80 °C—the typical temperature of many reversed-phase methods.

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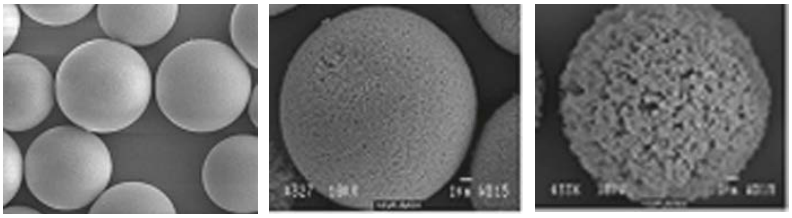
PLRP-S

- Contain durable and resilient polymer particles that deliver reproducible results over longer lifetimes
- Thermally and chemically stable
- Comply with USP L21 designation
- Pore sizes (100 Å to 4000 Å) for separations of small molecules to large complexes and polynucleotides

The PLRP-S family of columns consists of a range of pore sizes and particle sizes, all with identical chemistry and fundamental adsorptive characteristics. The particles are inherently hydrophobic. Therefore, no bonded phase or alkyl ligand is required for reversed-phase separations. This gives a highly reproducible material that is free from silanols and heavy metal ions. Columns within the extensive product range are suitable for micro separations, including bottom-up and top-down proteomics, analytical separations, and preparative purifications. In addition, process columns can be packed with bulk media.

For intact proteins, pore sizes larger than 300 Å are typical, with 1000 Å being a recommended starting option for PLRP-S. The smaller 100 Å pore size is commonly used for peptides or reversed phase separations of non-biological small molecules. While all pore sizes can be useful for oligonucleotide separations. Please see **162** for more information on PLRP-S for oligonucleotide separations.



A B C

Scanning electron micrographs (SEM) of PLRP-S 10 µm particles.

The difference in pore size is clearly seen.
A is the small pore 100 Å
B the larger pore 300 Å
C the gigaporous 4000 Å

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Column Specifications

pH range	1-14
Buffer content	Unlimited
Organic modifier	1-100%
Temperature limits	200 °C
Maximum pressure	3 µm: 275 bar/4000 psi
	5 µm, 8 µm, and 10 µm: 207 bar/3000 psi
	10–15 µm, 15–20 µm, and 30 µm: 103 bar/1500 psi

PLRP-S Applications

Pore size	Application
100 Å	Small molecules, synthesis and peptides
300 Å	Recombinant peptides/proteins
1000 Å	Large proteins and monoclonal antibodies
4000 Å	Large oligonucleotides or high speed separations

Exploiting chemical stability—TFA concentration

Column: **PLRP-S 100 Å**
PL1512-5500
4.6 x 250 mm, 5 µm

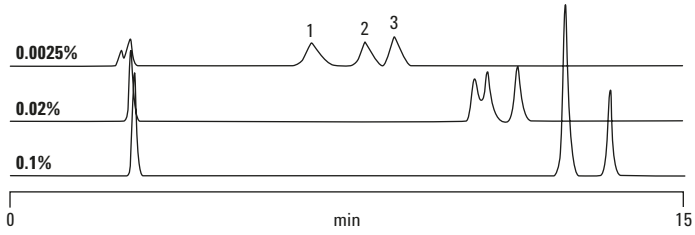
Mobile phase: A: TFA (various %) in water
B: TFA (various %) in ACN

Flow rate: 1.0 mL/min

Gradient: Linear 12–40% B in 15 min

Detector: ELS (neb=75 °C, evap=85 °C, gas=1.0 SLM)

1. Angiotensin III
2. Angiotensin II
3. Angiotensin I



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Exploiting chemical stability—NH_4OH concentration

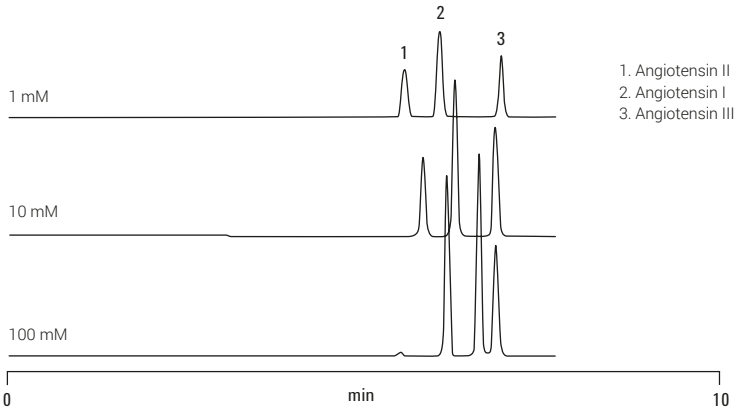
Column: **PLRP-S 100 Å**
PL1512-5500
4.6 x 250 mm, 5 µm

Mobile phase: A: NH_4OH (various mM) in water
B: NH_4OH (various mM) in ACN

Flow rate: 1.0 mL/min

Gradient: Linear 10–100% B in 15 min

Detector: ELS (neb=80 °C, evap=85 °C, gas=1.0 SLM)



Alberta Peptide Institute test mix

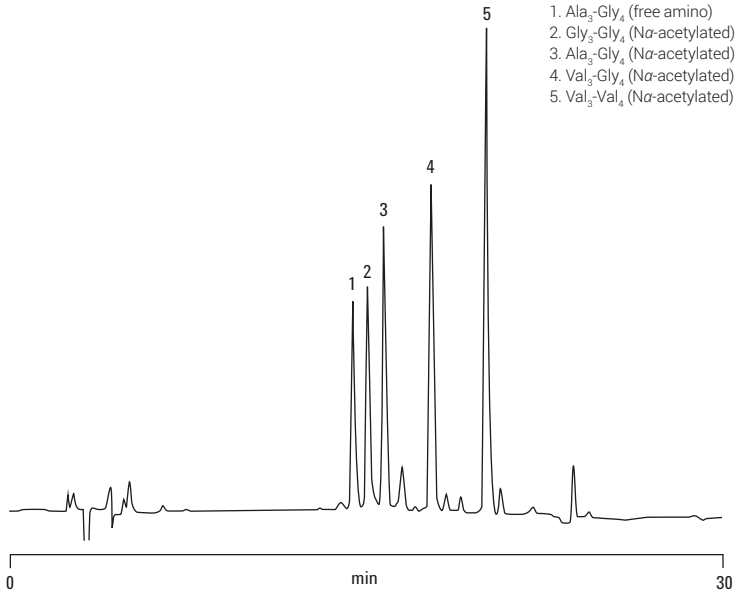
Column: **PLRP-S 100 Å**
PL1512-5500
4.6 x 250 mm, 5 µm

Mobile phase: A: 0.1% TFA in 99% water:1% ACN
B: 0.1% TFA in 70% water:30% ACN

Flow rate: 1 µL/min

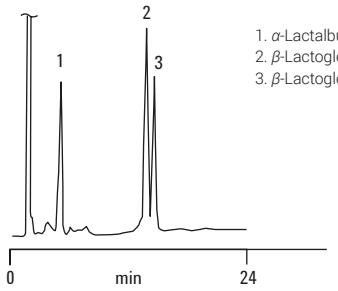
Gradient: 0–100% B in 30 min

Detector: UV, 220 nm



Whey proteins in dairy samples—milk

Column:	**PLRP-S 300 Å** **PL1512-3801** **4.6 x 150 mm, 8 µm**
Mobile phase:	A: 0.1% TFA in 99% water: 1% ACN B: 0.1% TFA in 1% water: 99% ACN
Flow rate:	1.0 mL/min
Injection volume:	10 µL
Gradient:	36–48% B, 0–24 min, 48–100% B, 24–30 min 100% B, 30–35 min, 100-36% B, 35–40 min
Detector:	UV, 220 nm

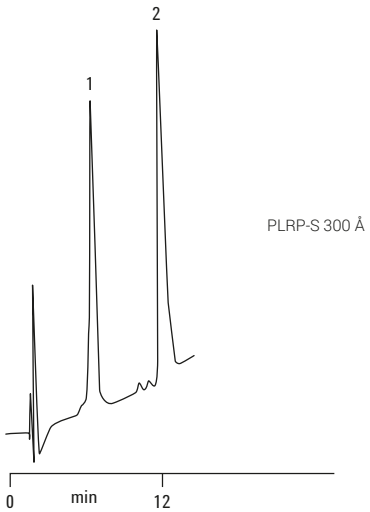


1. α-Lactalbumin
2. β-Lactoglobulin (B chain)
3. β-Lactoglobulin (A chain)

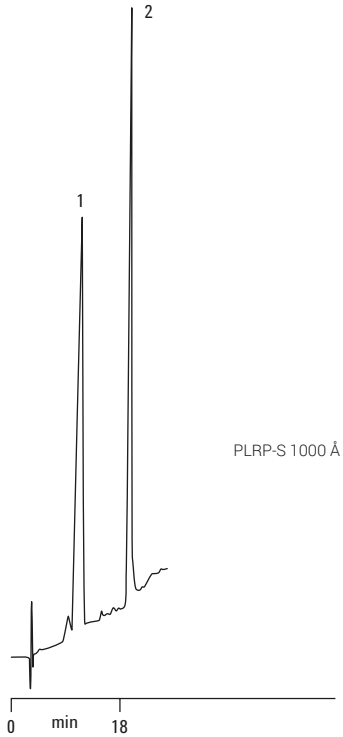
Large fibrous proteins

Column:	**PLRP-S 300 Å** **PL1512-3801** **4.6 x 150 mm, 8 µm**
Mobile phase:	A: 0.25% TFA in water B: 0.25% TFA in 5% water:95% ACN
Flow rate:	1.0 mL/min
Injection volume:	10 µL
Gradient:	20–60% B in 15 min
Detector:	UV, 220 nm

1. Collagen (120,000 mol wt)
2. Fibrinogen (340,000 mol wt)



PLRP-S 300 Å



PLRP-S 1000 Å

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AdvanceBio Desalting-RP

AdvanceBio Desalting-RP, p/n PL1612-1102 and cartridge holder, p/n 820999-901

Reversed-phase desalting for online removal of salt ions before MS detection

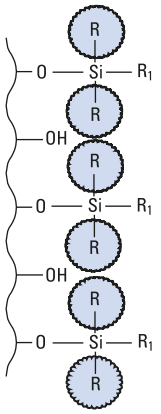
Affinity, ion exchange, and size exclusion chromatography are commonly used techniques in the analysis of proteins such as monoclonal antibodies. However, these techniques require aqueous mobile phases that contain nonvolatile salts. These nonvolatile salts present a problem when using MS detection as they cause signal suppression and can contaminate the MS detector through salt deposition leading to increased maintenance and instrument downtime. AdvanceBio Desalting-RP cartridges solve this problem, enabling fast and efficient online removal of salt ions before MS detection. These cartridge-style columns can be used on their own on any LC system to desalt collected fractions. Or on 1290 Infinity II 2D-LC system as the second dimension to desalt after the first dimension separation.

Tips and tools

For examples of the effective use of the AdvanceBio Desalting-RP cartridges in 2D-LC/MS, see application note **5991-7066EN**.

ZORBAX 300 Å StableBond

Agilent ZORBAX 300 Å StableBond columns are an ideal choice for the reproducible separations of proteins and peptides for two key reasons. First, wide-pore, 300 Å columns are necessary for an efficient separation of proteins and peptides, or other large molecules, to allow these analytes to completely access the bonded phase. Second, 300StableBond columns are unmatched in their durability at low pH, such as with TFA-containing mobile phases typically used for protein and peptide separations. For LC/MS separations at low pH, 300StableBond columns can also be used with formic acid and acetic acid mobile phase modifiers. These columns are available in five different bonded phases (C18, C8, C3, CN, and diphenyl (DP)) for selectivity and recovery optimization of proteins and polypeptides. To further increase sample recovery and improve efficiency for difficult proteins, 300StableBond columns can be used up to 80 °C. StableBond 300SB-C18 and 300SB-C8 columns are an ideal choice for complex protein and protein digest separations. These columns are also available in capillary (0.3 and 0.5 mm id) and nano (0.075 and 0.10 mm id) dimensions for reversed-phase LC/MS separations of protein digests. Capillary and nano columns can be used for either 1D or 2D proteomics separations.



Sterically Protected 300StableBond bonded phase

Column Specifications

Bonded Phase	Pore Size	Temp Limits*	pH Range*	Endcapped
ZORBAX RRHD 300SB-C18	300 Å	90 °C	1.0–8.0	No
ZORBAX RRHD 300SB-C8	300 Å	80 °C	1.0–8.0	No
ZORBAX RRHD 300SB-C3	300 Å	80 °C	1.0–8.0	No
ZORBAX RRHD 300-Diphenyl	300 Å	80 °C	1.0–8.0	Yes
ZORBAX 300SB-C18	300 Å	80 °C	1.0–8.0	No
ZORBAX 300SB-C8	300 Å	80 °C	1.0–8.0	No
ZORBAX 300SB-C3	300 Å	80 °C	1.0–8.0	No
ZORBAX 300SB-CN	300 Å	80 °C	1.0–8.0	No

Specifications represent typical values only.

* 300StableBond columns are designed for optimal use at low pH. At pH 6–8, the highest column stability for all silica-based columns is obtained by operating at temperatures <40 °C and using low buffer concentrations in the range of 0.01–0.02 M. At mid or high pH, 300Extend-C18 is recommended.

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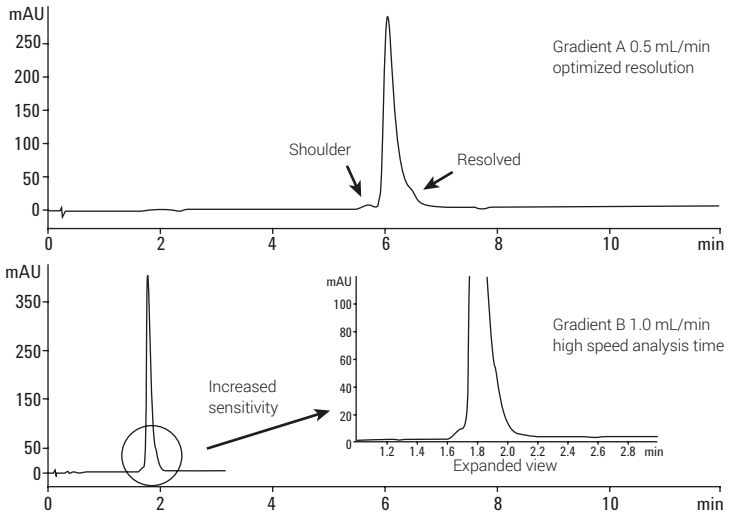
Higher resolution of intact monoclonal antibody

Column:	**ZORBAX RRHD 300SB-C8 857750-906 2.1 x 50 mm, 1.8 µm**
Mobile phase:	A: H_2O:IPA (98:2) + 0.1% TFA (v/v) B: IPA:ACN:H_2O (70:20:10) + 0.1% TFA (v/v)
Flow rate:	Between 0.5 mL/min and 1.0 mL/min
Gradient:	Multisegmented and linear elution
Temperature:	80 °C
Detector:	1290 Infinity LC with autosampler, binary pump and thermostatted column compartment, and diode array detector (DAD)
Sample:	UV, 225 nm



Tips and tools

For more information on the characterization of monoclonal antibody primary structure, see: Better Characterization of Biomolecules using Agilent AdvanceBio Reversed-Phase Columns (publication number **5991-2032EN**)

Reduced and alkylated mAb—separation of light chain and heavy chain variants

Column: **ZORBAX RRHD 300SB-C8**
866750-906
2.1 x 150 mm, 1.8 µm

Mobile phase: A: H_2O + 0.1% TFA (v/v)
B: n-propanol:ACN:H2O (80:10:10) + 0.1% TFA (v/v)

Flow rate: 0.5 mL/min

Injection: 3 µL (from 2.5 mg/mL sample)

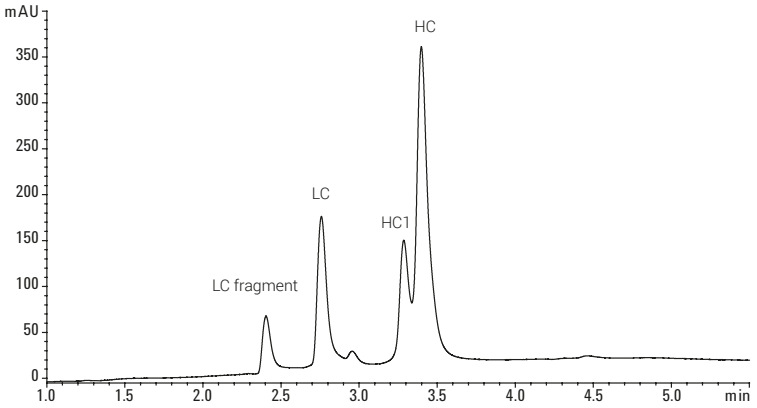
Gradient:

Time (min)	%Solvent B
0	20
3	35
4	40
5	40
5.1	90
5.5	90
6	25

Temperature: 75 °C

Detector: UV, 225 nm

Instrument: 1290 Infinity LC with autosampler, binary pump, thermostatted column compartment, and diode array detector (DAD)



For consecutive chromatographic runs, a 2 minute postrun was added to re-equilibrate the column.

Tips and tools

Typical mobile phases for protein and peptide separations combine a very low pH with TFA (or other acids) to solubilize proteins. StableBond columns have extremely long lifetimes under these conditions. They are available in 300 Å pore size for proteins up to 100–500 kDa.

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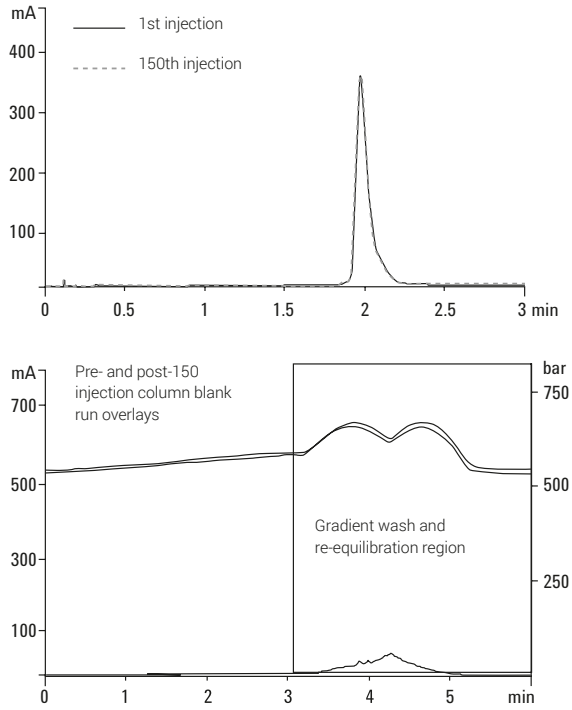
Improved reproducibility of monoclonal antibodies

Column:	**ZORBAX RRHD 300SB-C8 857750-906 2.1 x 50 mm, 1.8 µm**
Mobile phase:	A: H_2O:IPA (98:2) + 0.1% TFA (v/v) B: IPA:ACN:H_2O (70:20:10) + 0.1% TFA
Flow rate:	1.0 mL/min
Temperature:	80 °C
Detector:	1290 Infinity LC with diode array detector at 225 nm
Sample:	mAb

Gradient

Time, min	%B
0.00	25
3.00	35
4.00	90
5.00	25

Excellent column reproducibility and protein recovery using ZORBAX 300SB-C8.



Unique selectivity choices for mAb characterization

Columns:

ZORBAX RRHD 300SB-C18
858750-902
2.1 x 100 mm, 1.8 µm

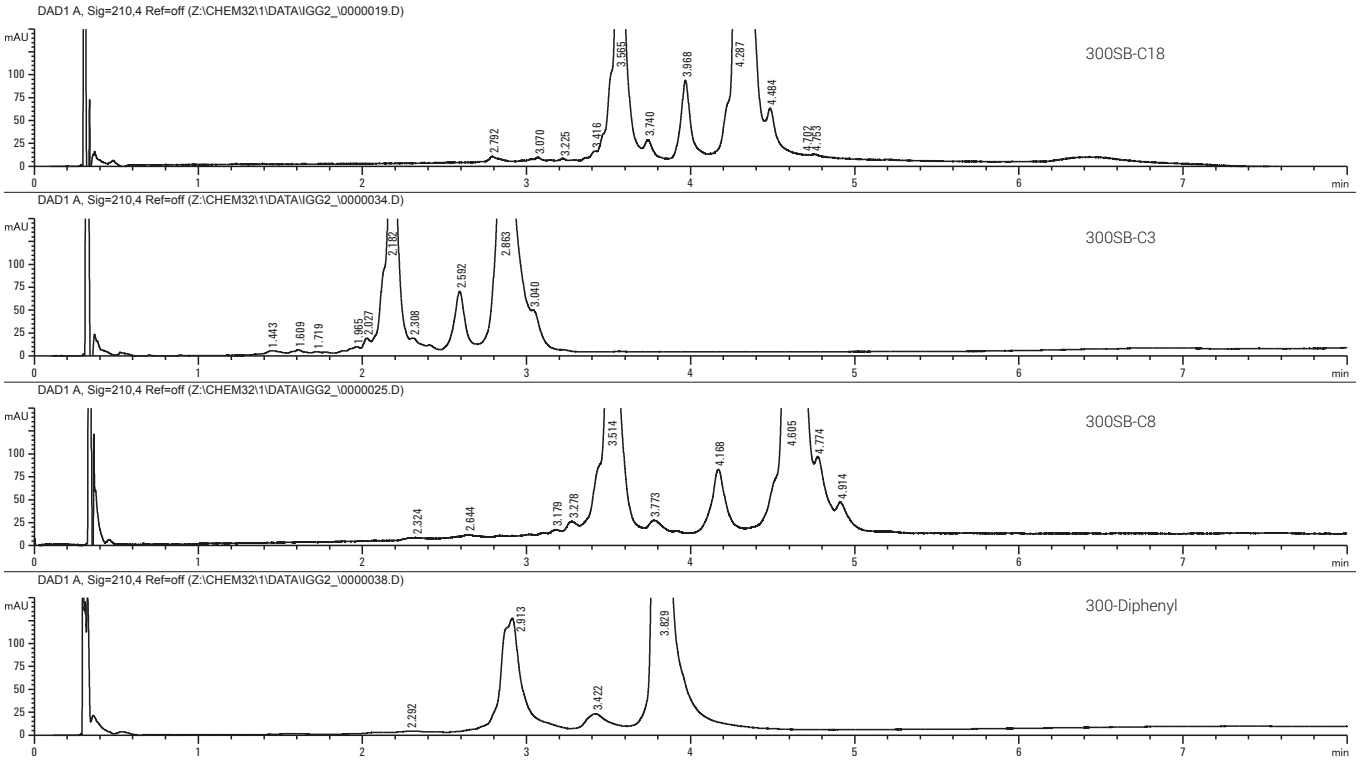
ZORBAX RRHD 300SB-C3
858750-909
2.1 x 100 mm, 1.8 µm

ZORBAX RRHD 300SB-C8
858750-906
2.1 x 100 mm, 1.8 µm

ZORBAX RRHD 300-Diphenyl
858750-944
2.1 x 100 mm, 1.8 µm

Mobile phase:	A: H_2O (0.1% TFA) (v/v) B: 80% nPA:10% ACN:10% H_2O (0.08% TFA) (v/v)
Injection volume:	3 µL (from 2.5 mg/mL sample)
Flow rate:	1.0 mL/min (3.5 µm*), 1.0 mL/min (1.8 µm)
Gradient:	25-35% B, 90% wash
Temperature:	80 °C
Detector:	UV, 215 nm

*Broad peaks at lower flow rates



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Peptides/proteins: effect of elevated temperature

Column: **ZORBAX 300SB-C3**
883995-909
4.6 x 150 mm, 5 µm

Mobile phase: A: 5:95 ACN:water with 0.10% TFA (v/v%)
B: 95:5 ACN:water with 0.085% TFA (v/v%)

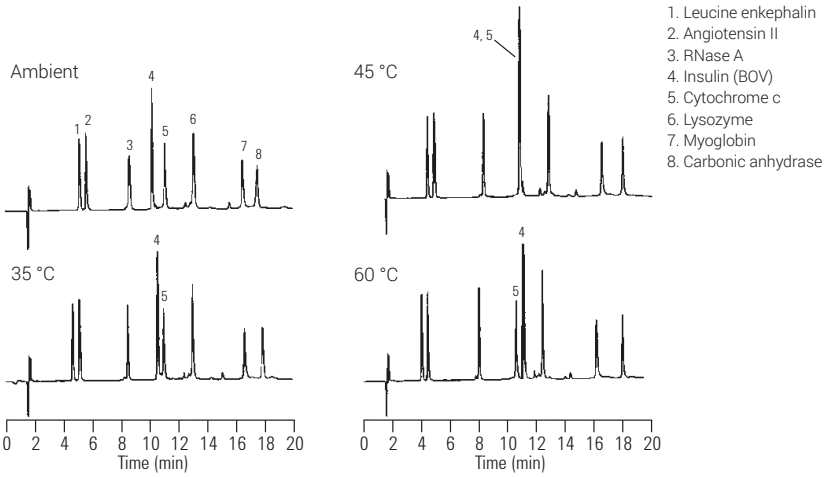
Flow rate: 1.0 mL/min

Gradient: 15–53% in 20 min, posttime 12 min

Temperature: Ambient–60 °C

Detector: UV, 215 nm

Sample: Polypeptides



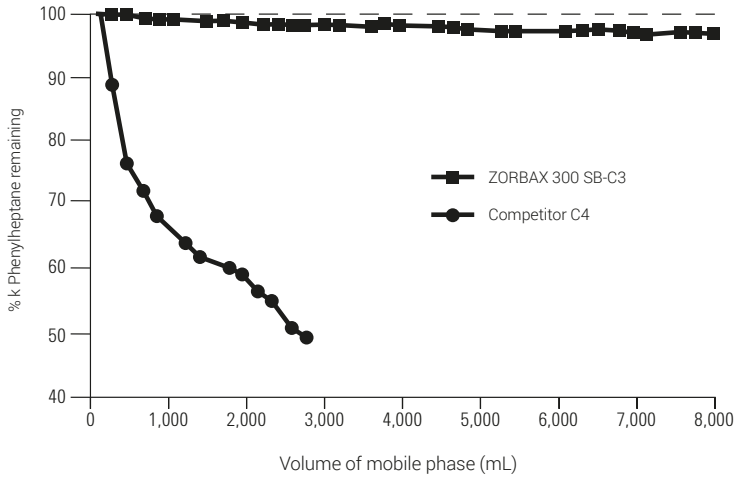
Short-chain ZORBAX 300SB-C3 is stable at low pH, high temperature

Column: **ZORBAX 300SB-C3**
883995-909
4.6 x 150 mm, 5 µm

Mobile phase: Gradient 0-100% B in 80 min
A: 0.5% TFA in water
B: 0.5% TFA in acetonitrile
Isocratic retention test conditions:
1-phenylheptane 50% A, 50% B

Flow rate: 1.0 mL/min

Temperature: 60 °C



Four different 300SB bonded phases optimize separation of large polypeptides

Column A: ZORBAX 300SB-C18
883995-902
4.6 x 150 mm, 5 µm

Column B: ZORBAX 300SB-C8
883995-906
4.6 x 150 mm, 5 µm

Column C: ZORBAX 300SB-C3
858750-909
4.6 x 150 mm, 5 µm

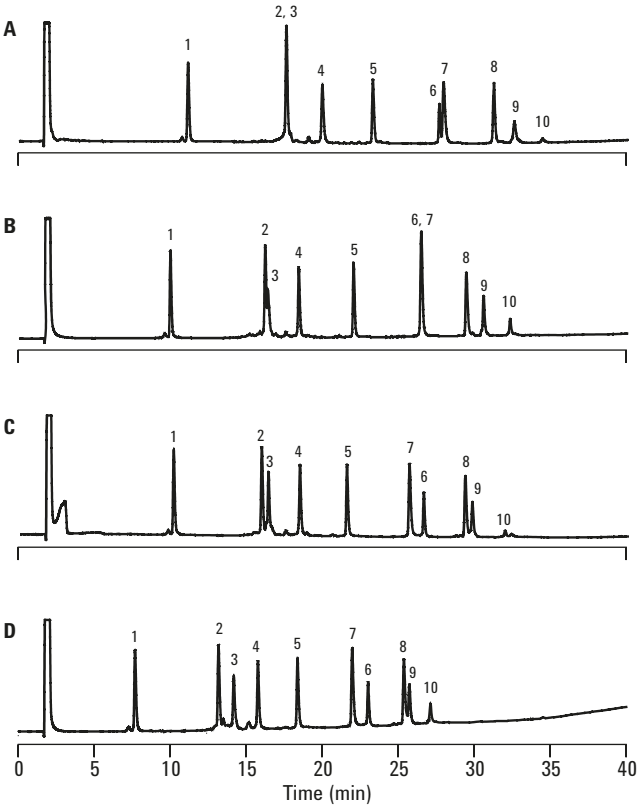
Column D: ZORBAX 300SB-CN
858750-905
4.6 x 150 mm, 5 µm

Mobile phase: Linear gradient, 25–70% B in 40 min
A: 0.1% TFA in water
B: 0.09% TFA in 80% acetonitrile:20% water

Flow rate: 1.0 mL/min

Temperature: 60 °C

Sample: 3 µg each protein



1. RNase
2. Insulin
3. Cytochrome c
4. Lysozyme
5. Parvalbumin
6. CDR
7. Myoglobin
8. Carbonic anhydrase
9. S-100β
10. S-100α

The 300SB-C18, C8, C3, and CN bonded phases all provide a different separation of this group of polypeptides. This adds an important parameter for quickly optimizing protein separations. The 300SB-CN column offers unique selectivity for more hydrophilic polypeptides.

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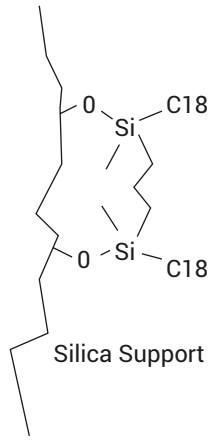
ZORBAX 300 Å Extend-C18

- Rugged, high and low pH separations of polypeptides and peptides from pH 2-11.5
- Different selectivity possible at high and low pH
- High efficiency and good recovery of hydrophobic peptides at high pH
- Ideal for LC/MS with ammonium-hydroxide-modified mobile phase

ZORBAX 300 Å Extend-C18 is a wide-pore HPLC column for high-efficiency separations of peptides from pH 2-11.5. The unique, bidentate bonded phase provides excellent lifetime and reproducibility at high and low pH. At high pH, retention and selectivity of peptides and polypeptides can change dramatically as a result of changes in charge on molecules. Excellent recoveries of hydrophobic polypeptides have been achieved at room temperature and high pH. LC/MS sensitivity of peptides and polypeptides can also be improved at high pH using a simple ammonium-hydroxide-containing mobile phase.



Novel bidentate C18-C18 bonding for Extend-C18 bonded phase

UHPLC Column Specifications

Bonded Phase	Pore Size	Temp Limits*	pH Range*	Endcapped
ZORBAX 300 Å Extend-C18	300 Å	60 °C	2.0-11.5	Double

Specifications represent typical values only

*Temperature limits are 60 °C up to pH 8, 40 °C from pH 8-11.5.

Tips and tools

Selecting the right column is only part of the total solution. Don't forget key supplies such as our wide range of LC lamps. Visit: **www.agilent.com/chem/lamps** for more.

Long life at high pH

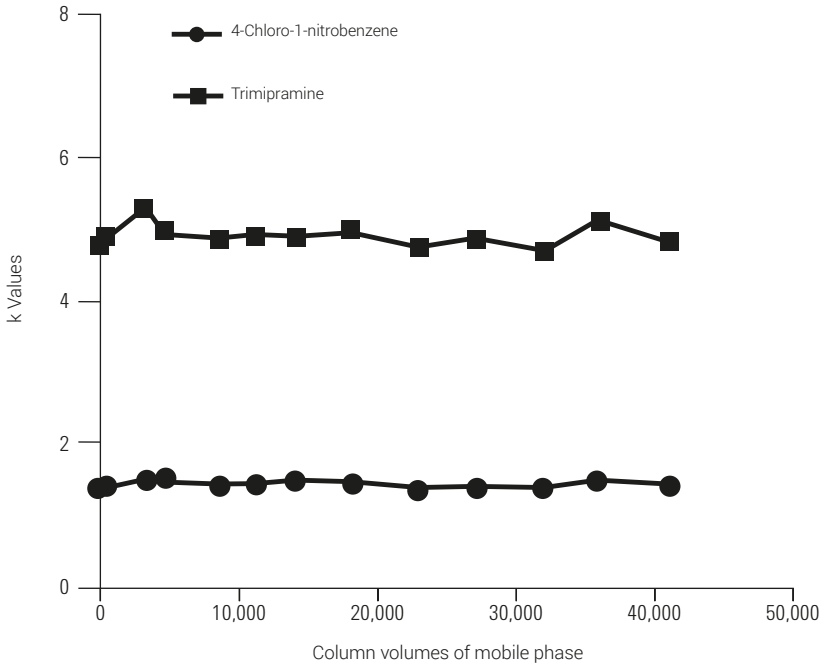
Column: **ZORBAX Extend-C18**
773450-902
4.6 x 150 mm, 5 µm

Mobile phase: 20% 20 mM NH_4OH, pH 10.5
80% methanol

Flow rate: 1.5 mL/min

Temperature: Aging 24 °C
Tests 40 °C

Each 10,000 column volume is approximately one working month.



Use ZORBAX Extend-C18 for alternate selectivity at high pH

Column: **ZORBAX Extend-C18**
773700-902
2.1 x 150 mm, 5 µm

Mobile phase: A: 0.1% TFA in water
B: 0.085% TFA in 80% ACN

A: 20 mM NH_4OH in water
B: 20 mM NH_4OH in 80% ACN

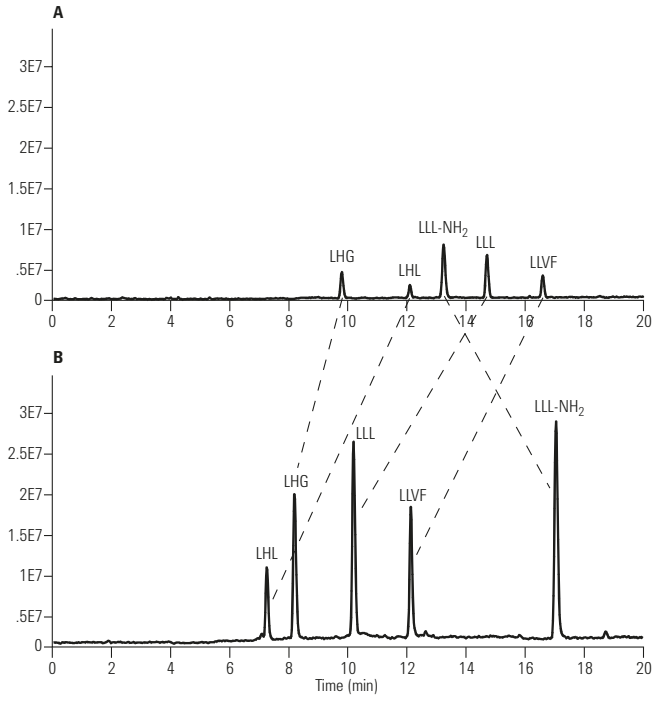
Flow rate: 0.25 mL/min

Gradient: 5-60% B in 20 min

Temperature: 25 °C

MS conditions: Pos. ion ESI; Vf 70 V, Vcap 4.5 kV,
N_2, 35 psi, 12 L/min, 300 °C
4 µL (50 ng each peptide)

The Extend column can be used for high pH separations of peptides. At high and low pH, very different selectivity can occur. Just by changing pH, a complementary method can be developed and it is possible to determine if all peaks are resolved. The Extend column can be used at high and low pH, and so the complementary separation can be investigated with one column. Better MS sensitivity for this sample is also achieved at high pH.



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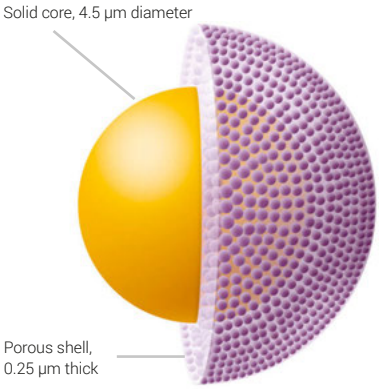
Poroshell 300 AB

- High speed separations of biomolecules with superficially porous particles
- 300 Å pores provide high efficiency and recovery with proteins (up to 1,000 kDa)
- Achieve long lifetime at low pH with Poroshell 300SB and at high pH with 300Extend-C18
- Optimize recovery and selectivity with four different bonded phases—300SB-C18, 300SB-C8, 300SB-C3, and 300Extend-C18



Poroshell 300 columns are ideal for fast separations of proteins and peptides because the 5 µm diameter superficially porous particle allows for fast flow rates to be used while maintaining sharp, efficient peaks. Poroshell columns with StableBond bonded phases provide excellent stability and selectivity choices with TFA and formic acid mobile phases. The Poroshell 300Extend-C18 column can be used from pH 2-11 for unique separations. These columns can also be used for analytical protein separations and LC/MS separations.

Peptides and proteins are typically separated slowly to reduce the potential peak broadening of these slow diffusing analytes. However, Poroshell columns use a superficially porous particle made with a thin layer of porous silica, 0.25 µm thick, on a solid core of silica. This reduces the diffusion distance for proteins, making rapid HPLC separations of peptides and proteins up to 500-1,000 kDa possible with 400/600 bar HPLC systems, including the 1260 Infinity II bio-inert LC.

HPLC Column Specifications

Bonded Phase	Pore Size	Temp Limits*	pH Range*	Endcapped
Poroshell 300SB-C18, C8, C3	300 Å	90 °C	1.0–8.0	No
Poroshell 300Extend-C18	300 Å	40 °C above pH 8 60 °C below pH 8	2.0–11.0	Yes

Specifications represent typical values only

* 300StableBond columns are designed for optimal use at low pH. At pH 6–8, the highest column stability for all silica-based columns is obtained by operating at temperatures < 40 °C and using low buffer concentrations in the range of 0.01–0.02 M. At mid or high pH, 300Extend-C18 is recommended.

Poroshell 300 columns

AB Part of the AdvanceBio family

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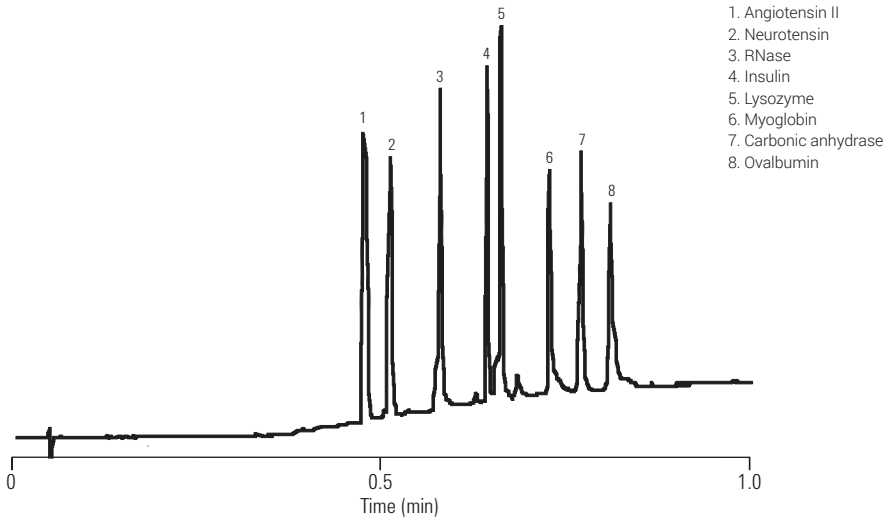
Poroshell 300 columns separate proteins and peptides in seconds

Column:	**Poroshell 300SB-C18** **660750-902** **2.1 x 75 mm, 5 µm**
Mobile phase:	A: 0.1% TFA in H_2O B: 0.07% TFA in ACN
Flow rate:	0.3 mL/min
Gradient:	5–100% B in 1.0 min
Temperature:	70 °C, 260 bar
Detector:	UV, 215 nm
Sample:	Proteins and peptides



This separation of eight polypeptides and proteins is completed in less than 60 seconds. Each peak is sharp and efficient.

Tips and tools

Further information can be found in:

Characterization of Glycosylation in the Fc Region of Therapeutic Recombinant Monoclonal Antibody (publication **5991-2323EN**)

Using the High-pH Stability of ZORBAX Poroshell 300Extend-C18 to Increase Signal-to-Noise in LC/MS (publication **5989-0683EN**)

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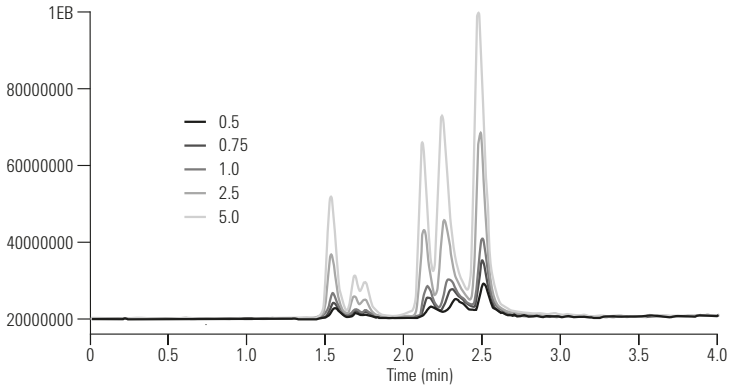
MicroBore Poroshell 300 columns provide maximum sensitivity for LC/MS

Column:	**Poroshell 300SB-C18 661750-902 1.0 x 75 mm, 5 µm**	
Mobile phase:	A: Water + 0.1% formic acid B: ACN + 0.1% formic acid	
Flow rate:	600 µL/min	
Gradient:	20–100% B in 5.5 min	
Temperature:	80 °C	
MS conditions:	LC/MS:	Pos. ion ESI; Vcap 6,000 V
	Drying gas flow:	12 L/min
	Drying gas temperature:	350 °C
	Nebulizer:	45 psi
	Fragmentor voltage:	140 V
	Scan:	600-2,500
	Stepsize:	0.15 amu
	Peak width:	0.06 min
Sample:	1 µL	



With narrow bore diameters of 2.1 mm, 1.0 mm, and 0.5 mm, Poroshell columns make an ideal LC/MS partner. When the sample is very limited, the 1.0 mm or 0.5 mm id Poroshell columns are an excellent choice for high sensitivity LC/MS analyses. Sensitive MS molecular weight determinations are possible with as little as 0.5 to 5 pmole of protein on Poroshell columns. The columns have also been used for rapid MS identification of intact proteins, even in the presence of stabilizers and tissue culture media.

Tips and tools

Agilent offers an extensive selection of vials and sample containment solutions including polypropylene and deactivated and siliconized glass. To see the full range, see publication **5994-4803EN**.

www.agilent.com/chem/vials-productivity

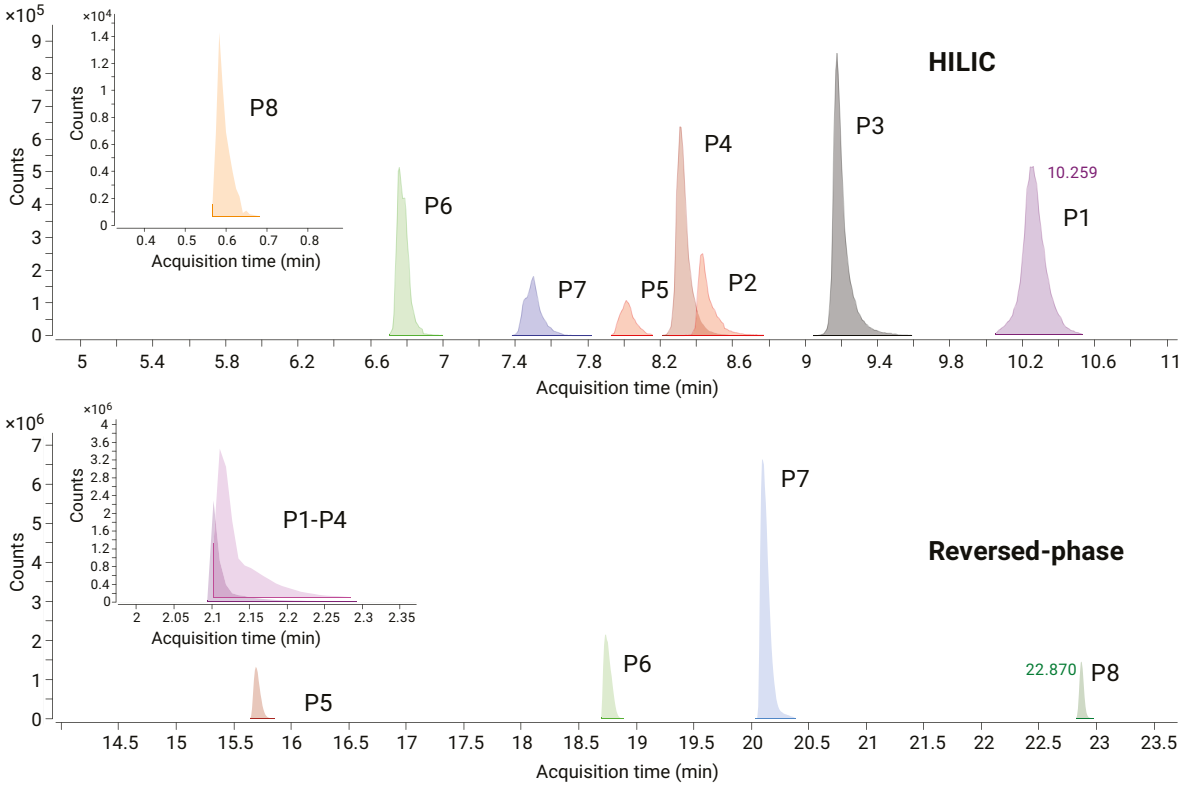
ZORBAX 300 Å HILIC

Agilent ZORBAX 300 Å HILIC columns are based upon the same totally porous, 1.8 µm particle as the reversed phase ZORBAX RRHD columns, with excellent stability at low pH and at high pressure for UHPLC separations. HILIC selectivity is highly complementary to reversed phase separations, with the most hydrophobic analytes eluting first rather than last. HILIC is also able to retain analytes that are often not retained by reversed phase, such as very hydrophilic peptides or proteins, such as those that are glycosylated. The 300 Å pore makes these HILIC columns suitable for intact proteins, while still being applicable to smaller peptides.

Column Specifications

Bonded Phase	Particle Size	Pore Size	pH Range	Pressure Maximum
HILIC	1.8 µm	300 Å	1-8	1200 bar

Alternate selectivity for peptide and protein separations



Comparing eight peptides from both columns for their retention times and resolutions

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HILIC conditions

Columns: **Agilent ZORBAX Rapid Resolution High Definition 300-HILIC, 2.1 x 100 mm, 1.8 µm (p/n 858750-901)**

Eluent: A: 95% ACN + 5% water;
B: 50 mM ammonium formate, pH 4.0

Flow rate: 0.4 mL/min

Gradient:

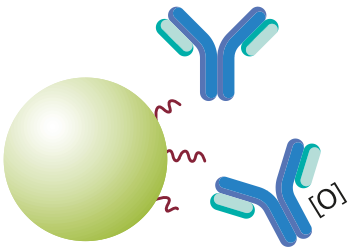
Time (min)	% B
0	0
15	100
15.1	0
20	0

Temperature: 55 °C

Peptide	Sequence	Hydrophobicity	RP retention time (min)	HILIC retention time (min)
P1	APPR	1.83	2.103	10.259
P2	GKLK	3.84	2.118	8.437
P3	ALGAQK	4.57	2.119	9.181
P4	AVSGLR	9.15	2.13	8.316
P5	YLLEAK	19.64	15.698	8.014
P6	VYSNFLRGK	23.14	18.742	6.583
P7	SLTTLLR	24.79	20.109	7.492
P8	VNFYAWKR	27.64	22.87	0.587

Peptides common to both columns.

Intact Analysis Using Hydrophobic Interaction Chromatography



AdvanceBio HIC

AdvanceBio HIC columns deliver high resolution, robust, and reproducible separations of native proteins at the intact level.

Built using the capabilities of the ZORBAX fully porous particles and proprietary bonding technology, these columns provide new levels of hydrophobicity and versatile single chemistry to address particularly challenging molecules such as monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), and other recombinant proteins.

Together with the 1260 Infinity II bio-inert LC system, the AdvanceBio HIC provides uncompromised performance and data consistency during characterization and validation.

- Optimized selectivity: ideal for mAb oxidation and ADC DAR ratios.
- Single chemistry: reduces the need for multiple-column screening for different CQAs.
- Enhanced robustness: improved column lifetime for ultimate confidence in your data.
- Proven performance: every batch of media is tested with NIST mAb.
- High quality: each column is individually tested to ensure packing efficiency.
- Greater productivity: shorter columns reduce analysis time while maintaining separation performance.

Column Specifications

Pore Size	Particle Size	Temperature Limit	pH Range	Pressure Limit	Flow Rate*
450 Å	3.5 µm	60 °C (at pH 7)	2.0–8.0 (at 35 °C)	400 bar (typical operating pressure <200 bar)	0.5–1.0 mL/min (4.6 mm id)

*In some cases, lowering the flow rate to 0.3 mL/min and extending gradient time may further improve resolution.

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Column: **AdvanceBio HIC 4.6 x 100 mm, 3.5 µm**

Eluent A: 2 M Ammonium Sulfate, 50 mM Sodium Phosphate, pH 7.0

Eluent B: 50 mM Sodium Phosphate, pH 7.0

Gradient:

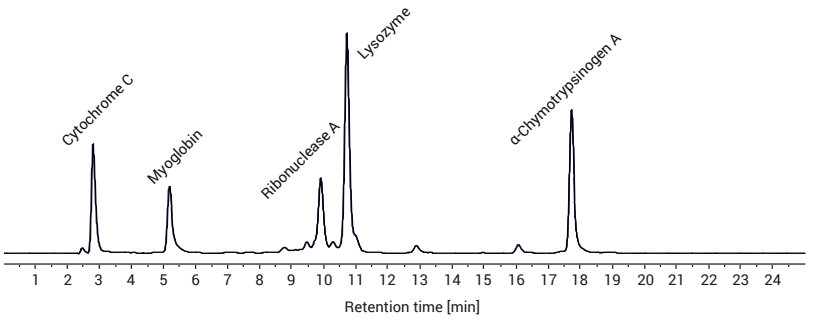
Time (min)	%A	%B
0	100	0
20	0	100
25	0	100
30	100	0
40	100	0

Flow rate: 0.5 mL/min

Temperature: 30 °C

Injection volume: 5 µL

Detection: UV, 220 nm



Column: **AdvanceBio HIC 4.6 x 100 mm, 3.5 µm**

Eluent A: 2 M Ammonium Sulfate, 50 mM Sodium Phosphate, pH 7.0

Eluent B: 50 mM Sodium Phosphate, pH 7.0

Gradient:

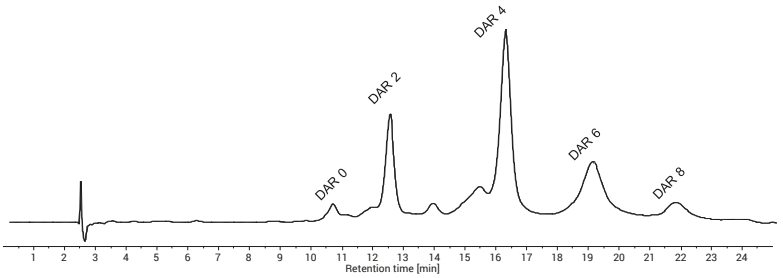
Time (min)	%A	%B	%C
0	50	45	5
20	0	75	25
25	0	75	25
30	50	45	5
40	50	45	5

Flow rate: 0.5 mL/min

Temperature: 30 °C

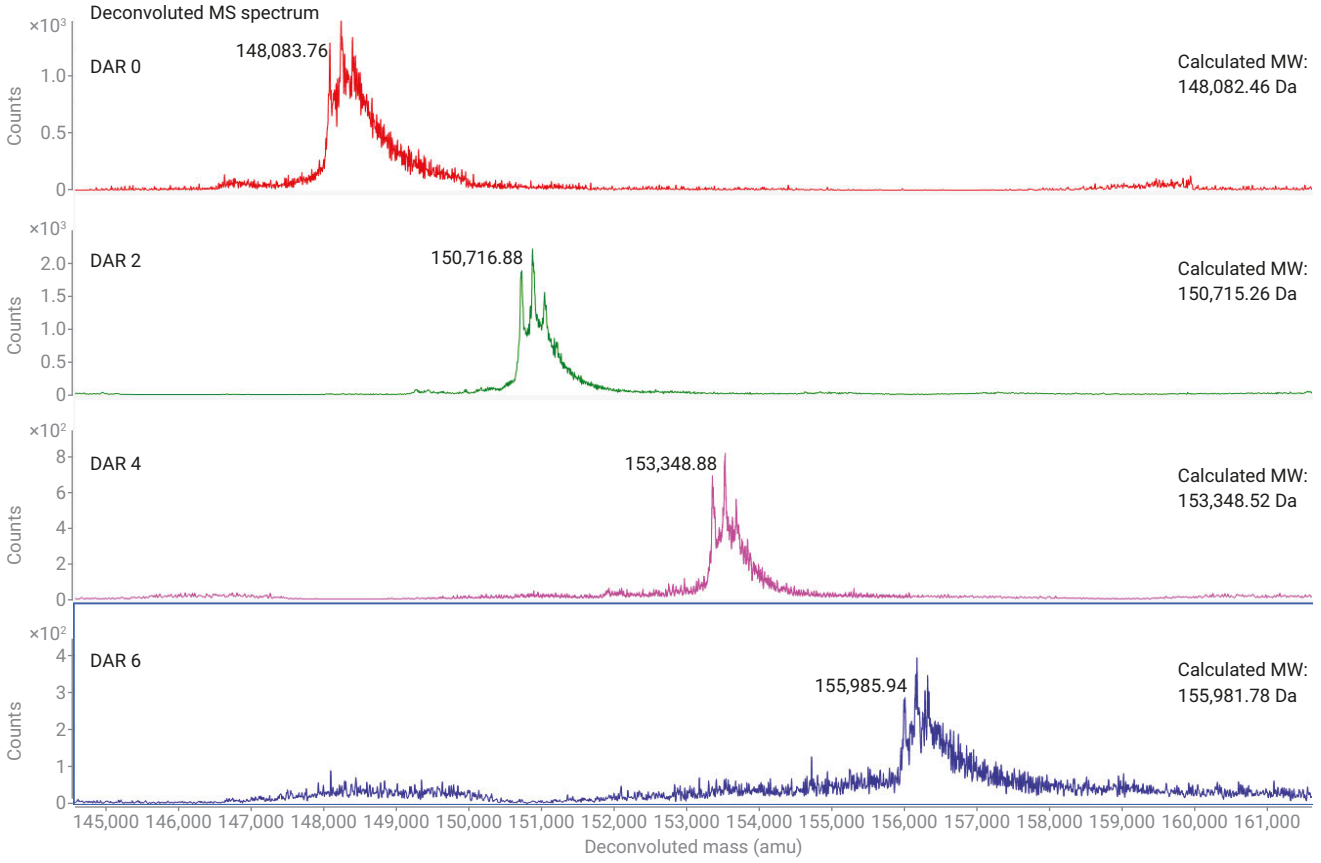
Injection volume: 5 µL

Detection: UV, 220 nm



AdvanceBio HIC DAR value is accurately confirmed by MS analysis

LC-native MS methods also enable determination of cysteine linked ADC DAR. Agilent has developed a 2D-LC/MS method for the characterization of intact cysteine linked DARs under native LC/MS conditions. The workflow uses the Agilent AdvanceBio HIC column in the first dimension, the Agilent AdvanceBio SEC column in the second dimension as the desalting column, and highly sensitive MS method to accurately determine intact mass for all ADCs with various DARS.



The deconvoluted MS spectrum of native SEC LC/MS analysis of various brentuximab vedotin DARs (DAR 0 to DAR 6).

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Product ordering information

AdvanceBio RP-mAb Columns

Dimensions (mm)	Particle Size (µm)	AdvanceBio RP-mAb C4 USP L26	AdvanceBio RP-mAb SB-C8 USP L7	AdvanceBio RP-mAb Diphenyl USP L11
4.6 x 150	3.5	793975-904	783975-906	793975-944
4.6 x 100	3.5	795975-904	785975-906	795975-944
4.6 x 50	3.5	799975-904	789975-906	799975-944
2.1 x 150	3.5	793775-904	783775-906	793775-944
2.1 x 100	3.5	795775-904	785775-906	795775-944
2.1 x 75	3.5	797775-904	787775-906	797775-944
2.1 x 50	3.5	799775-904	789775-906	799775-944

PLRP-S HPLC Columns

Dimensions (mm)	Particle Size (µm)	PLRP-S 100 Å USP L21	PLRP-S 300 Å USP L21	PLRP-S 1000 Å USP L21	PLRP-S 4000 Å USP L21
4.6 x 250	8	PL1512-5800	PL1512-5801	PL1512-5802	
4.6 x 150	8	PL1512-3800	PL1512-3801	PL1512-3802	PL1512-3803
4.6 x 50	8		PL1512-1801	PL1512-1802	PL1512-1803
4.6 x 250	5	PL1512-5500	PL1512-5501		
4.6 x 150	5	PL1111-3500	PL1512-3501		
4.6 x 50	5	PL1512-1500	PL1512-1501	PL1512-1502	PL1512-1503
4.6 x 150	3	PL1512-3300	PL1512-3301		
4.6 x 50	3	PL1512-1300	PL1512-1301		
2.1 x 250	8		PL1912-5801		
2.1 x 150	8		PL1912-3801	PL1912-3802	PL1912-3803
2.1 x 50	8		PL1912-1801	PL1912-1802	PL1912-1803
2.1 x 250	5	PL1912-5500	PL1912-5501		
2.1 x 150	5	PL1912-3500	PL1912-3501		
2.1 x 50	5	PL1912-1500	PL1912-1501	PL1912-1502	PL1912-1503
2.1 x 150	3	PL1912-3300	PL1912-3301		

(Continued)

Tips and tools

Visit our online store to directly purchase our columns and consumables: **www.agilent.com/chem/store**

PLRP-S HPLC Columns

Dimensions (mm)	Particle Size (µm)	PLRP-S 100 Å USP L21	PLRP-S 300 Å USP L21	PLRP-S 1000 Å USP L21	PLRP-S 4000 Å USP L21
2.1 x 50	3	PL1912-1300	PL1912-1301		
1.0 x 50	8			PL1312-1802	PL1312-1803
1.0 x 50	5	PL1312-1500	PL1312-1501	PL1312-1502	PL1312-1503
1.0 x 10	5			PL1C12-2502	
1.0 x 150	3	PL1312-3300			
1.0 x 50	3	PL1312-1300	PL1312-1301		
PLRP-S guard cartridges for 3.0 x 5.0 mm, 2/pk		PL1612-1801	PL1612-1801	PL1612-1801	PL1612-1801
Guard cartridge holder for 3.0 x 5.0 mm cartridges		PL1310-0016	PL1310-0016	PL1310-0016	PL1310-0016

ZORBAX 300 Å StableBond Columns

Description	Dimensions (mm)	Particle Size (µm)	300SB-C18 USP L1	300SB-C8 USP L7	300SB-CN L10	300SB-C3 USP L56	300-Diphenyl USP L11
4.6 mm i.d. Analytical Columns & Guards							
Analytical	4.6 x 250	5	880995-902	880995-906	880995-905	880995-909	
Analytical	4.6 x 150	5	883995-902	883995-906	883995-905	883995-909	
Analytical	4.6 x 50	5	860950-902	860950-906	860950-905	860950-909	
Guard Cartridge, 4/pk*	4.6 x 12.5	5	820950-921	820950-918	820950-923	820950-924	
Rapid Resolution	4.6 x 150	3.5	863973-902	863973-906	863973-905	863973-909	
Rapid Resolution	4.6 x 100	3.5	861973-902	861973-906			
Rapid Resolution	4.6 x 50	3.5	865973-902	865973-906	865973-905	865973-909	
3.0 mm i.d. Analytical Columns & Guards							
Solvent Saver Plus	3.0 x 150	3.5	863974-302	863974-306		863974-309	
Solvent Saver Plus	3.0 x 100	3.5		861973-306			
2.1 mm i.d. Analytical Columns & Guards							
Narrow Bore	2.1 x 250	5	881750-902				
Narrow Bore	2.1 x 150	5	883750-902	883750-906	883750-905	883750-909	
Recommended Guard cartridge, 4/pk*	2.1 x 12.5	5	821125-918	821125-918	821125-924	821125-924	

*Requires guard hardware kit part number 820999-901

(Continued)

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ZORBAX 300 Å StableBond Columns

Description	Dimensions (mm)	Particle Size (µm)	300SB-C18 USP L1	300SB-C8 USP L7	300SB-CN L10	300SB-C3 USP L56	300-Diphenyl USP L11
Narrow Bore RR	2.1 x 150	3.5		863750-906			
Narrow Bore RR	2.1 x 100	3.5	861775-902	861775-906			
Narrow Bore RR	2.1 x 50	3.5	865750-902	865750-906			
Narrow Bore RRHD	2.1 x 150	1.8	863750-902	866750-906		863750-914	863750-944
Narrow Bore RRHD	2.1 x 100	1.8	858750-902	858750-906		858750-909	858750-944
Narrow Bore RRHD	2.1 x 50	1.8	857750-902	857750-906		857750-909	857750-944
1.0 mm i.d. Analytical Columns & Guards							
MicroBore	1.0 x 250	5	861630-902				
MicroBore RR	1.0 x 150	3.5	863630-902	863630-906			
MicroBore RR	1.0 x 50	3.5	865630-902	865630-906			
MicroBore RRHD	1.0 x 50	1.8					965600-944
Semipreparative Column & Guard							
Semipreparative	9.4 x 250	5	880995-202	880995-206	880995-205	880995-209	
Recommended Guard cartridge, 2/pk*	9.4 x 15	7	820675-124	820675-124	820675-124	820675-124	
PrepHT Cartridge Columns (require end fittings kit 820400-901) & Guard							
PrepHT cartridge	21.2 x 250	7	897250-102	897250-106	897250-105	897250-109	
PrepHT cartridge	21.2 x 150	7	897150-102	897150-106		897150-109	
PrepHT cartridge	21.2 x 150	5	895150-902	895150-906		895150-909	
PrepHT cartridge	21.2 x 100	5	895100-902	895100-906		895100-909	
PrepHT cartridge	21.2 x 50	5	895050-902	895050-906		895050-909	
PrepHT guard, 2/pk*	17.0 x 7.5	5	820212-921	820212-918		820212-924	
Capillary glass-lined columns							
Capillary	0.5 x 150	5	5064-8264				
Capillary	0.5 x 35	5	5064-8294				
Capillary RR	0.5 x 150	3.5	5064-8268				

*Requires guard hardware kit part number 820999-901

(Continued)

Tips and tools

To get the most out of your Agilent instruments, visit: **www.agilent.com/chem/biolc-columns-user-guides**

ZORBAX 300 Å StableBond Columns

Description	Dimensions (mm)	Particle Size (µm)	300SB-C18 USP L1	300SB-C8 USP L7	300SB-CN L10	300SB-C3 USP L56	300-Diphenyl USP L11
Capillary RR	0.5 x 35	3.5	5065-4459				
Capillary	0.3 x 150	5	5064-8263				
Capillary	0.3 x 35	5	5064-8295				
Trap/guard, 5/pk**	0.3 x 5	5	5065-9913	5065-9914			
Capillary RR	0.3 x 150	3.5	5064-8267	5065-4460			
Capillary RR	0.3 x 100	3.5	5064-8259	5065-4461			
Capillary RR	0.3 x 50	3.5	5064-8300	5065-4463			
Nano columns (PEEK fused silica)							
Nano RR	0.1 x 150	3.5	5065-9910				
Nano RR	0.075 x 150	3.5	5065-9911				
Nano RR	0.075 x 50	3.5	5065-9924	5065-9923			

*Requires guard hardware kit part number 820999-901
**Requires guard hardware kit part number 5065-9915

ZORBAX 300 Å Extend-C18 Columns

Description	Dimensions (mm)	Particle Size (µm)	Part No.
Analytical	4.6 x 250	5	770995-902
Analytical	4.6 x 150	5	773995-902
Rapid Resolution	4.6 x 150	3.5	763973-902
Rapid Resolution	4.6 x 100	3.5	761973-902
Rapid Resolution	4.6 x 50	3.5	765973-902
Narrow Bore RR	2.1 x 150	3.5	763750-902
Narrow Bore RR	2.1 x 100	3.5	761775-902
Narrow Bore RR	2.1 x 50	3.5	765750-902
Guard cartridge, 4/pk	4.6 x 12.5	5	820950-932
Guard cartridge, 4/pk	2.1 x 12.5	5	821125-932
Guard hardware kit			820999-901

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Poroshell 300 Columns

Description	Dimensions (mm)	Partical Size (µm)	Poroshell 300SB-C18	Poroshell 300SB-C8	Poroshell 300SB-C3	Poroshell 300Extend-C18
Narrow Bore	2.1 x 75	5	660750-902	660750-906	660750-909	670750-902
MicroBore	1.0 x 75	5	661750-902	661750-906	661750-909	671750-902
Capillary	0.5 x 75	5		5065-4468		
Guard cartridge, 4/pk*	2.1 x 12.5	5	821075-920	821075-918	821075-924	

*Requires guard hardware kit part number 820999-901

ZORBAX 300 Å HILIC Columns

Description	Part No.
ZORBAX 300 Å HILIC, 2.1 x 100 mm, 300 Å, 1.8 µm	858750-901
ZORBAX 300 Å HILIC, 2.1 x 50 mm, 300 Å, 1.8 µm	857750-901

Agilent AdvanceBio HIC Columns

Description	Part No.
AdvanceBio HIC, 4.6 x 100 mm, 450 Å, 3.5 µm	685975-908
AdvanceBio HIC, 4.6 x 100 mm, 3.5 µm, method validation kit	685975-908K
AdvanceBio HIC, 4.6 x 30 mm, 450 Å, 3.5 µm	681975-908
AdvanceBio HIC, 4.6 x 30 mm, 3.5 µm, method validation kit	681975-908K

AdvanceBio Desalting-RP Cartridges

Description	Part No.
AdvanceBio Desalting-RP, 2.1 x 12.5 mm, 2/pk	PL1612-1102
Cartridge holder	820999-901

Tips and tools

Guard columns and filters help protect your column and instruments from particulates that can cause blockages, which increase system pressure and negatively impact performance—interrupting your daily workflow. Agilent's new Fast guards for UHPLC and Bio LC columns help protect the column, leading to longer column lifetimes, minimizing interruptions in your workflow.

For examples of Fast guards, click **here** and **here**.

Peptide Mapping and Analysis

Accurately determine amino acid sequence, post-translational modifications,and synthesis impurities

For full characterization of a protein, such as a monoclonal antibody, it is necessary to look at the primary amino acid sequence and any post-translational modifications (PTMs) to the sequence that may have occurred during the purification or formulation steps of manufacture. Reversed-phase separations are typically the technique of choice, both for its ability to resolve single amino acid modifications within a peptide based on hydrophobicity and its compatibility with MS detection for mass confirmation.

Agilent has multiple column options for peptide analysis to meet each user's separation needs, whether that be a global peptide mapping analysis, a detailed look at deamidation PTMs, UV or MS detection, or high or low pH mobile phase conditions.

Reversed-phase column selection

Application	Agilent Columns	Notes
Peptides in protein digest	AdvanceBio Peptide Mapping	An ideal 120 Å pore size for identifying a wide molecular weight range of peptides. Tested with a challenging peptide mix to ensure performance. The unique Agilent Poroshell technology enables shorter run times and better resolution of the full peptide sequence.
Synthetic peptide impurity analysis Host cell protein analysis	AdvanceBio Peptide Plus	Charged surface C18 chemistry with alternate selectivity to AdvanceBio Peptide Mapping. This column excels at separation of deamidated peptide variants as well as under formic acid mobile phase and high mass load conditions.
Synthetic peptide purification or impurity analysis	PLRP-S 100 Å, 300 Å	Scalable from analytical to preparative separations. Superior pH stability enables extreme pH use, including for sanitization.

Tips and tools

For HILIC separations of peptides, consider ZORBAX RRHD 300Å HILIC. Please see **page 59** for more information.

For more information about PLRP-S please see the PLRP-S section in the Intact Protein Analysis section on **page 55**.

For part number and ordering information please see the tables on **page 71**.

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AdvanceBio Peptide Mapping

- **Greater analytical confidence:** each batch of AdvanceBio Peptide Mapping media is tested with a rigorous peptide mix to ensure suitability and reproducibility, and to enable the identification of key peptides in complex peptide maps.
- **Save time:** two to three times faster than fully porous HPLC columns.
- **Every instrument works harder:** 4.6, 3.0, and 2.1 mm id columns are stable to 600 bar, enabling you to get the most from your UHPLC instruments. They can also deliver excellent performance for your legacy 400 bar instruments, too.
- **Increased flexibility:** achieve increased MS sensitivity with formic acid mobile phases on any HPLC.

These advanced biocolumns feature a 120 Å pore size with superficially porous 2.7 µm particles. They are specially tested with a challenging peptides mix to ensure reliable peptide mapping performance. In addition, AdvanceBio Peptide Mapping columns deliver exceptional resolution and speed for UHPLC, and excellent results for conventional HPLC.

Column Specifications

Bonded Phase	Pore Size	Temp Limits	pH Range	Endcapped
EC-C18	120 Å	60 °C	2.0–8.0	Double

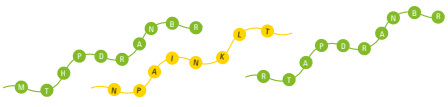
Specifications represent typical values only

Tips and tools

Do you want to know how scientists are using the AdvanceBio Peptide Mapping column? See:

Amano, M. *et al.* Detection of Histidine Oxidation in a Monoclonal Immunoglobulin gamma (IgG) 1 Antibody. *Analytical Chemistry*, 2014, 86 (15): 7536 -7543

Leah G. Luna and Katherine Coady, Identification of *X. laevis* Vitellogenin Peptide Biomarkers for Quantification by Liquid Chromatography Tandem Mass Spectrometry. J. *Anal Bioanal Tech*, 2014, 5:3



High resolution peptide map of erythropoietin digest

Column: **AdvanceBio Peptide Mapping 651750-902 2.1 x 250 mm, 2.7 µm**

Mobile phase: A: H_2O + 0.1% formic acid (v/v)
B: Acetonitrile + 0.1% formic acid (v/v)

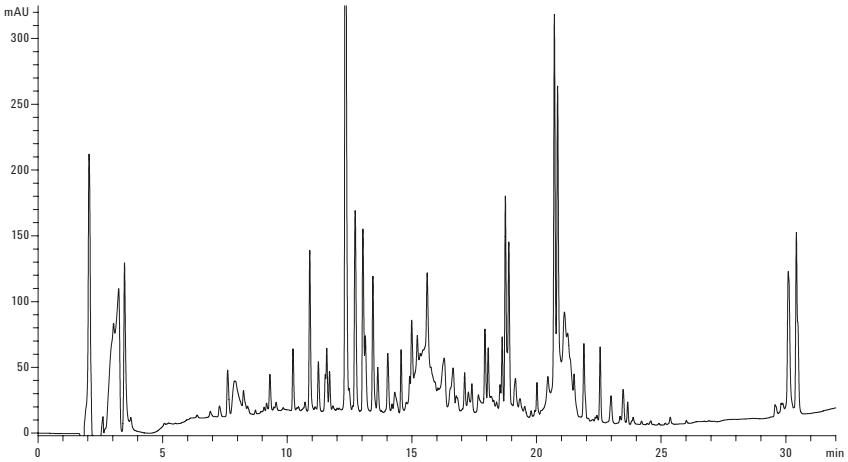
Flow rate: 0.4 mL/min

Gradient:

Time (min)	% B
0	3
28	45
33	60
34	95

Temperature: 55 °C

Sample: 5 µL (2 µg/µL)



Fast and efficient peptide mapping of IgG

Column: **AdvanceBio Peptide Mapping 655750-902 2.1 x 100 mm, 2.7 µm**

AdvanceBio Peptide Mapping 653750-902 2.1 x 150 mm, 2.7 µm

Mobile phase: A: H_2O + 0.1% FA (v/v)
B: 90% ACN + 0.1% FA (v/v)

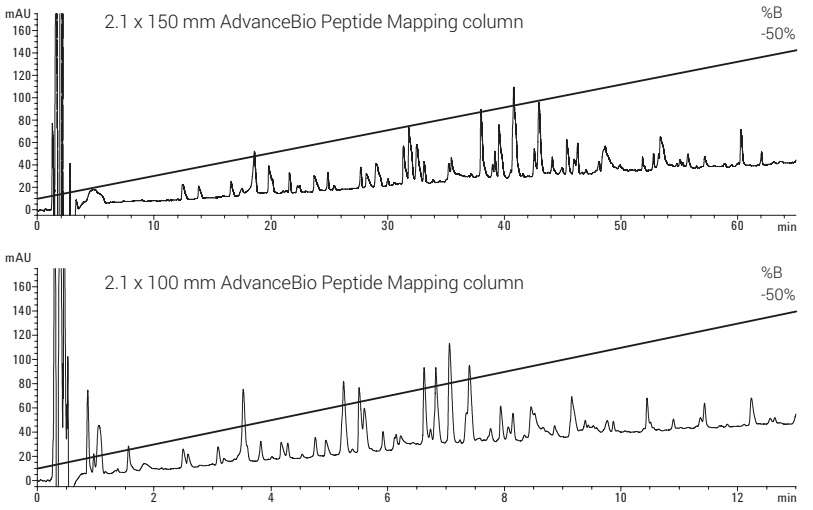
Flow rate: Various

Injection volume: 15 µL

Temperature: 40 °C

Detector: UV, 215/220 nm

Sample: 1290 Infinity LC and 6530 accurate mass quadrupole time-of-flight LC/MS



AdvanceBio Peptide Mapping column optimization for achieving a faster peptide mapping analysis. Gradient 10–40% B, DAD: 215 nm, 40 °C. Top panel, 75 min separation on a 2.1 x 150 mm column generated 59 peptide peaks (flow rate 0.2 mL/min, 211 bar). Bottom panel, optimized 14 min separation on a 2.1 x 100 mm column generated 57 peptide peaks (flow rate 0.6 mL/min, 433 bar).

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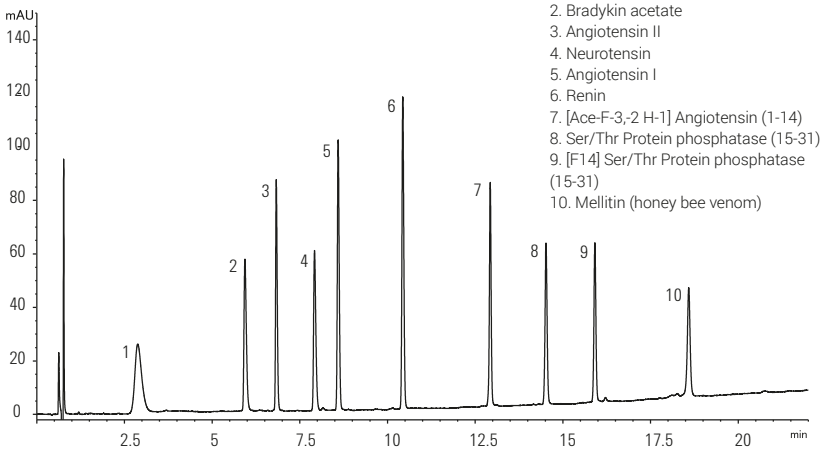
Quality assurance testing with Agilent peptide mix

Column:	**AdvanceBio Peptide Mapping 653750-902 2.1 x 150 mm, 2.7 µm**
Flow rate:	3 µL
Gradient:	A, H_2O (0.1% TFA), B, ACN (0.1% TFA), 0–25 min, 15–65% B; 25–26 min, 65–95% B
Temperature:	55 °C
Detector:	220 nm
Sample:	Peptide Mapping Standards mix (0.5-1.0 µg/µL per peptide) p/n 5190-0583



Test mix used for every batch of AdvanceBio Peptide Mapping media. The mixture contains 10 hydrophilic, hydrophobic, and basic peptides, ranging in molecular weight from 757 to 2845 Da. Every column is also tested with a small-molecule probe to ensure efficiency.

Real stories from the lab

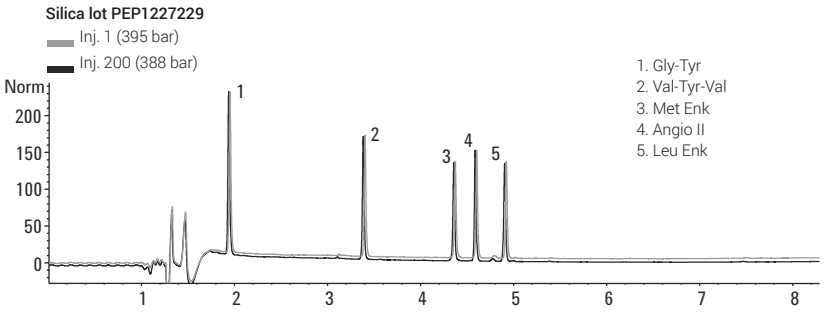
Bio bright spot

Learn how this lab manager was able to dramatically reduce downtime and improve user confidence.

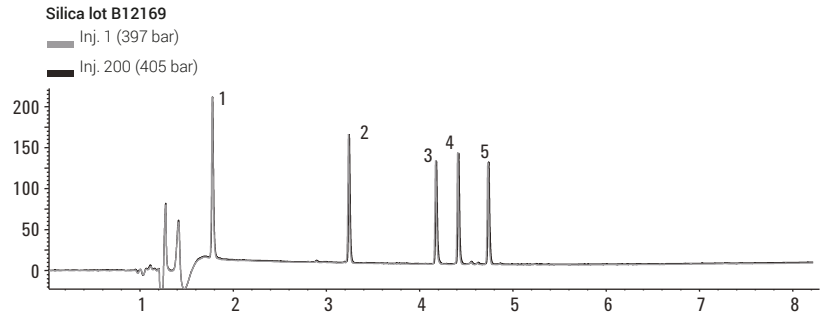
www.agilent.com/chem/story25

Lot-to-lot reproducibility after 200 injections

Column:	**AdvanceBio Peptide Mapping 651750-902 2.1 x 250 mm, 2.7 µm**
Flow rate:	0.5 mL/min
Injection:	1 µL
Gradient:	A, H_2O (0.1% TFA), B, ACN (0.08% TFA), 0–8 min, 10–60% B; 8.1–9 min, hold 95% B
Temperature:	55 °C
Detector:	220 nm
Sample:	Sigma HPLC peptide standards



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.39	4.36	4.59	4.90
200	3.52	4.48	4.70	5.02
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.020	0.021	0.020	0.022
200	0.020	0.021	0.019	0.021



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.36	4.29	4.52	4.85
200	3.24	4.18	4.41	4.74
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.019	0.020	0.019	0.020
200	0.019	0.020	0.019	0.020

Superior reproducibility, lot-to-lot and run-to-run. A 2.1 x 250 mm AdvanceBio Peptide Mapping column was used for maximum resolution.

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Agilent peptide quality control standard

Use the Agilent 10-peptide quality control standard, the same standard Agilent uses to QC its columns, to evaluate your column performance over its lifetime. It can be used for HPLC or LC/MS. Approximately 20 injections per vial.

AdvanceBio Peptide Plus

AdvanceBio Peptide Plus columns are reversed-phase, superficially porous particle HPLC columns optimized for the separation of target peptides, impurities, and post translational modifications. These columns feature a hybrid endcapped C18 stationary phase on a 100 Å pore size, 2.7 µm particle modified to have a charged surface. A charged, **surface C18 column dramatically improves the resolution of deamidated peptides from their unmodified variants** enhances selectivity for deamidated variants of peptides versus their unmodified forms when compared to a traditional C18 column.

Deamidation involves asparagine gets converted to aspartic acid or isoaspartic acid. The deamidated peptide often have mass shift of 1 Dalton so LC/MS technique is often used to detect such small change in mass. The deamidated peptides sometimes coelute with their unmodified forms since conversion of asparagine to the corresponding carboxylic acids does not result in a large change in hydrophobicity at low pH. This can impact the quantitation of deamidation, and in some cases, may even prevent detection of the deamidated variant. Keep in mind that Mass spec itself cannot resolve deamidated peptides so separation is crucial.

The most important aspect for achieving a reliable, well-resolved peptide mapping separation is the selection of a suitable column. For peptide separations, the preferred column pore sizes range from 100 Å to 120 Å, optimum phase selection is typically C18. superficially porous columns have become increasingly popular for biological separations – especially among the biopharmaceutical industry – because they address the limitations of protein and peptide mass diffusion. These columns offer a shorter diffusion path allowing the separations of larger molecules at high linear velocities without the system backpressure increases associated with the smaller particles. Reversed-phase separations of peptides are commonly carried out at low pH (pH<3) and elevated temperatures (>40 °C).

PTMs or impurities
Resolution of deamidated variants – Positively charged surface C18 enhances selectivity of deamidated variants

Data accuracy
Peak shape and MS sensitivity – Charged surface enables good peak shape with formic acid

Excel in higher mass load
Identify low abundant peptides – Charged surface C18 phase more tolerant of high mass loads

Data quality
Vigorous QC process to meet the needs of the most discerning scientists

Enhanced selectivity for deamidated variants

Parameter	Agilent 1290 Infinity II LC
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, (p/n 695775-949) Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, (p/n 653750-902)
Column Temperature	60 °C
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	0.4 mL/min
Gradient	Time (min) % B 0 3 2 3 40 40 50.5 100 53 3
Post Time	7 minutes

Parameter	Agilent 6546 Q-TOF
Source	Agilent Jet Stream
Gas Temperature	323 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	m/z 300 to 1,700
MS Scan Rate (spectra/s)	5
Acquisition Mode	Positive mode, extended dynamic range (2 GHz) Centroid data format

Peptide	Sequence (Nondeamidated Form)	m/z of $[M+2H]^{2+}$
A	NQVSLTCLVK	581.8103
B	FNWYVDGVEVHNAK	839.4047
C	VVSVLTVLHQDWLNGK	904.5071
D	NTAYLQMNSLR	655.8300
E	GLEWVGYIDPSNGETTYNQK	1136.0323

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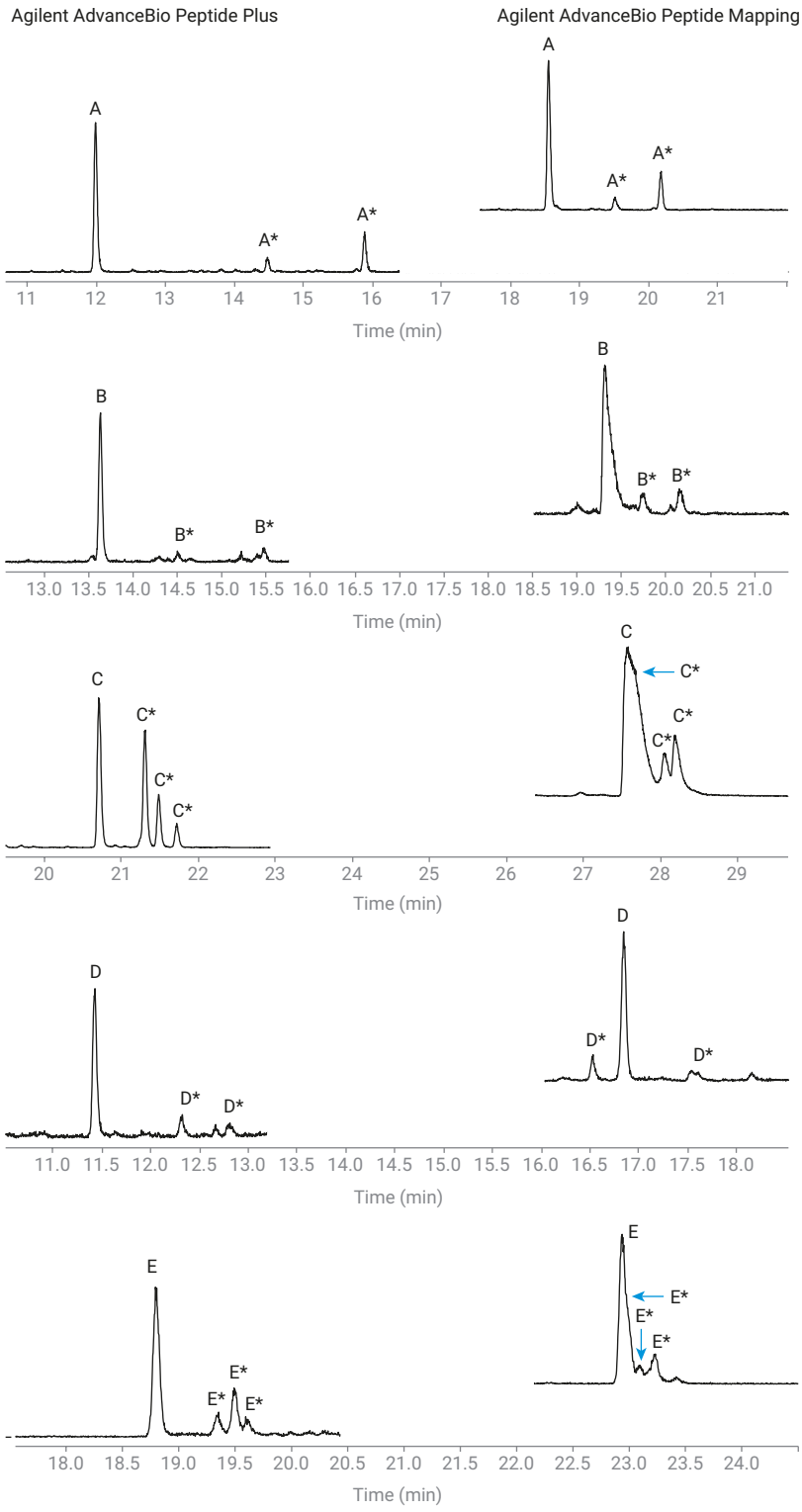

Separation of peptides and their deamidated variants (indicated by *) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.

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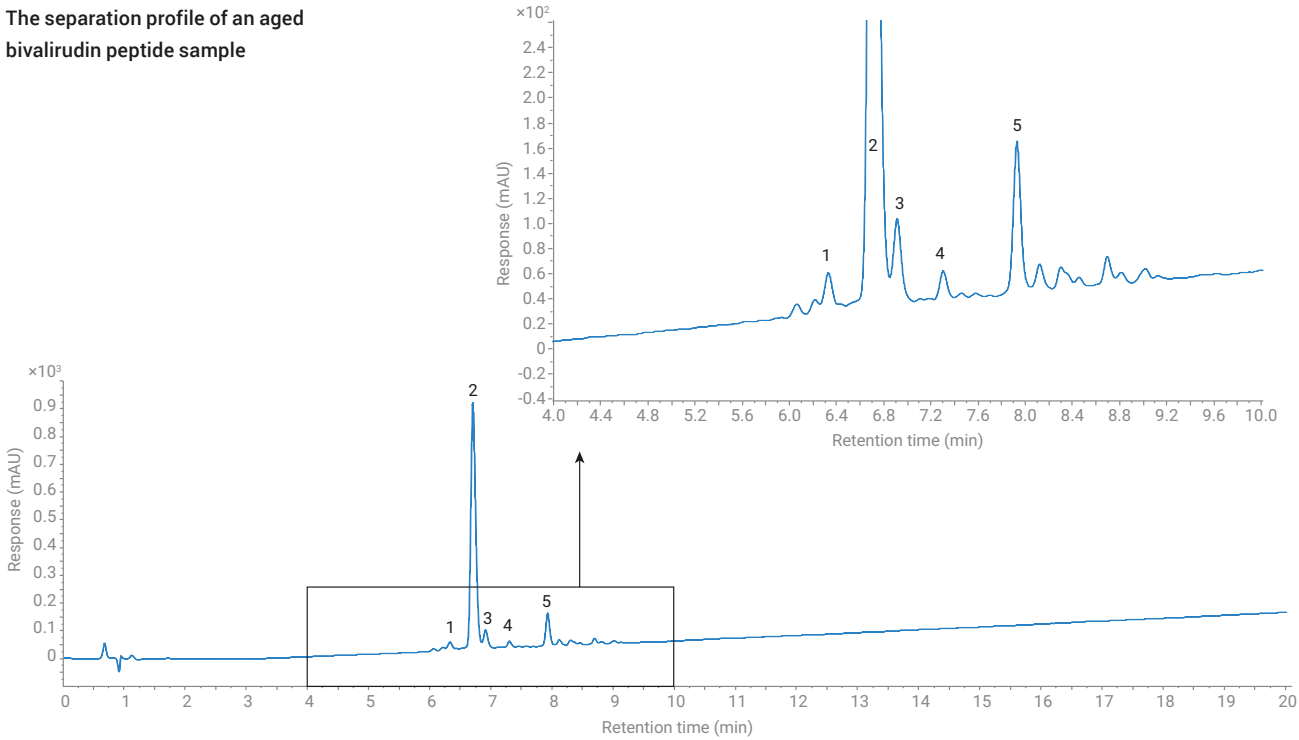
HPLC Conditions

Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm (p/n 695775-949)
Mobile Phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Gradient	0 min: 17% B 2 min: 17% B 22 min: 37% B 24 min: 95% B 26 min: 95% B 26.1 min: 17% B
Post Time	5 min
Flow Rate	0.4 mL/min
Column Temperature	60 °C
Injection Volume	5 µL (UV); 1 µL (MS)

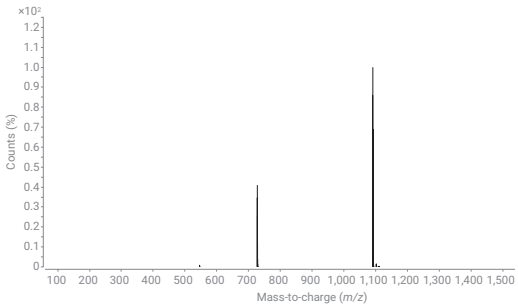
Parameter	Agilent 6545XT AdvanceBio LC/Q-TOF
Source	Dual Agilent Jet Stream
Gas Temperature	350 °C
Drying Gas Flow	10 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	m/z 100 to 1,700 (MS); m/z 50 to 1,700 (MS/MS)
MS Scan Rate	8 spectra/s
MS/MS Scan Rate	3 spectra/s
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Collision Energy	3.6 × (m/z)/100 – 4.8

The separation profile of an aged bivalirudin peptide sample

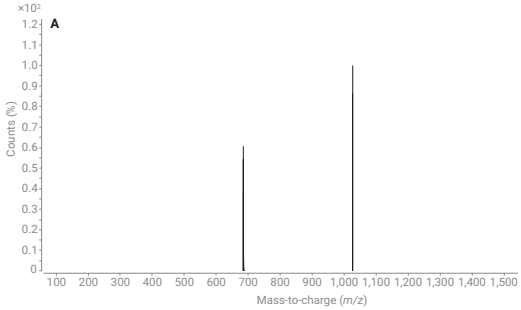


Peak 1 lost glutamic acid at position 17 or 18

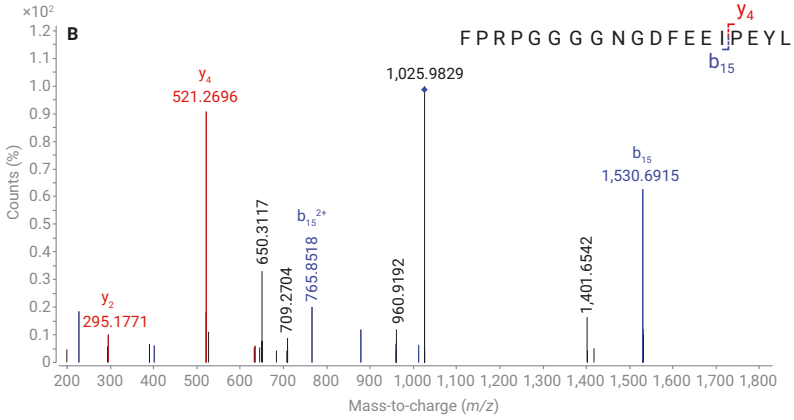
MS spectrum of main product (peak 2), FPRPGGGGNGDFEEIPEEYL



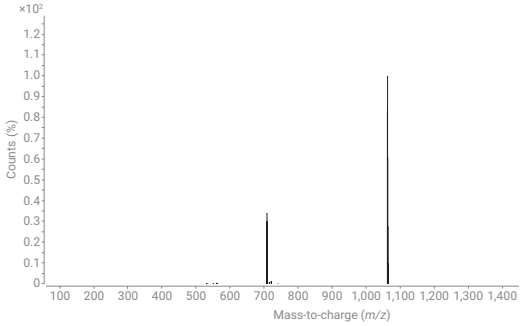
MS spectrum of impurity (peak 1)



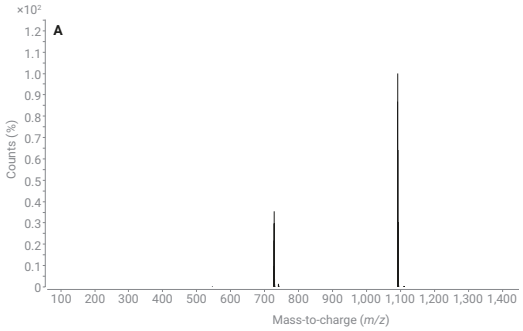
MS/MS spectrum of impurity (peak 1), revealing the location of missing glutamic acid residue



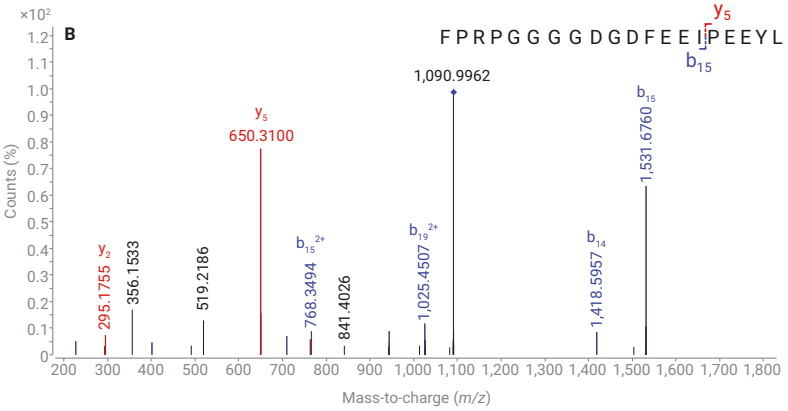
MS spectrum of main product (peak 2), FPRPGGGGNGDFEEIPEEYL



MS spectrum of impurity (peak 5)



MS/MS spectrum of impurity (peak 1), revealing the location ofAsn deamidation



Peak	Mass (Da)	Peak ID	Target Mass (Da)	Mass error (ppm)
1	2,049.95	Deletion of Glu	2,049.94	1.71
2	2,178.99	Product	2,178.99	1.65
5	2,179.97	Deamidation	2,179.97	2.02

Peak 5 indicates deamidation – Asn at position 9 has been converted to Asp

Tips and tools

Peptide mapping is a powerful technique and the most widely used identity test for proteins, particularly those produced by recombinant means. There are several considerations to be made in addition to column selection for reproducible and accurate peptide maps, including protein digestion, sample preparation, method optimization, and so on.

For fundamental techniques used in peptide mapping procedures and considerations when optimizing your peptide mapping separations, see: Keys for Enabling Optimum Peptide Characterizations: A Peptide Mapping "How to" Guide (publication **5991-2348EN**).

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Product ordering information

AdvanceBio Peptide Mapping

Description	Part No.
4.6 x 150 mm, 2.7 µm	653950-902
3.0 x 150 mm, 2.7 µm	653950-302
2.1 x 250 mm, 2.7 µm	651750-902
2.1 x 150 mm, 2.7 µm	653750-902
2.1 x 100 mm, 2.7 µm	655750-902
1 x 150 mm, 2.7 µm	863600-911
0.075 x 150 mm, 2.7 µm	5065-9925
4.6 x 5 mm, Fast guard*	850750-911
3.0 x 5 mm, Fast guard*	853750-911
2.1 x 5 mm, Fast guard*	851725-911
0.3 x 50 mm, Fast guard*	5065-9946

*Fast guards extend column lifetime without slowing down the separation or affecting resolution.

Agilent Peptide Quality Control Standard

Description	Part No.
Peptide quality control standard, 71 µg in 2 mL vial	5190-0583

AdvanceBio Peptide Plus

Description	Part No.
AdvanceBio Peptide Plus 2.1 x 150 mm, 2.7 µm	695775-949
AdvanceBio Peptide Plus 2.1 x 250 mm, 2.7 µm	693775-949
AdvanceBio Peptide Plus 2.1 x 50 mm, 2.7 µm	699775-949
AdvanceBio Peptide Plus 3.0 x 150 mm, 2.7 µm	693975-349
AdvanceBio Peptide Plus 4.6 x 150 mm, 2.7 µm	693975-949
AdvanceBio Peptide Plus, 2.1 x 150 mm ,2.7 µm, method validation kit	695775-949K
UHPLC Guard, AdvanceBio Peptide Plus, 2.1 mm, 2.7 µm, 3 pack	821725-954
UHPLC Guard, AdvanceBio Peptide Plus, 3.0 mm, 2.7 µm, 3 pack	823750-952
UHPLC Guard, AdvanceBio Peptide Plus, 4.6 mm, 2.7 µm, 3 pack	820750-940

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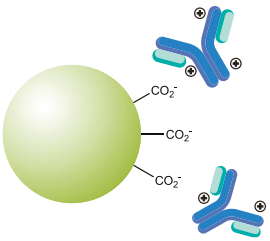
Charge Variant Analysis



Purify proteins and other charged molecules

Ion-exchange chromatography (IEX) is a highly sensitive technique that allows you to separate ions and polar molecules based on their charge. Like SEC, IEX can be used to separate proteins in their native state.

Applying IEX to charge variant analysis

During production and purification, antibodies can exhibit changes in charge heterogeneity as a result of amino acid substitutions, glycosylation, phosphorylation, and other post-translational or chemical modifications.

In protein analysis, charge variations at a given pH indicate a change in the primary molecular structure—resulting in additional forms of the protein in question. These are called isoforms (or charge variants).

Because these changes can impact stability and activity—or cause immunologically adverse reactions—the analysis of charge variants is critical to biopharmaceuticals.

As a leading supplier to the biopharmaceutical industry, Agilent understands that quality and consistency are critical to providing safe, highly efficacious therapeutics. Agilent ion-exchange BioHPLC columns offer the speed, resolution, and reproducibility you need to quickly and cost-effectively get life-changing products into the hands of those who need them.

The pages that follow describe the Agilent family of weak and strong ion-exchangers—both anionic and cationic.

- Nonporous Bio IEX columns are designed for high resolution, high efficiency, and high recovery separations.
- Bio MAb columns are optimized for separating charge isoforms of monoclonal antibodies.
- Porous IEX columns (PL-SAX and PL-SCX) are chemically stable, and are available in two pore sizes, allowing you to separate peptides, oligonucleotides, and very large proteins.
- Bio-Monolith IEX columns are uniquely suited for separating antibodies, viruses, and DNA.
- Buffer Advisor software is an ideal solution for automated protein separation by ionic strength gradients.

Tips and tools

For more information about the Agilent Buffer Advisor software, see publication **5991-1408EN**.

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Ion Exchange Column Selection

Application	Agilent Columns	Notes
Monoclonal antibodies	Bio MAb	Thorough characterization of monoclonal antibodies includes the identification and monitoring of acidic and basic isoforms. Bio MAb HPLC columns feature a unique resin specifically designed for high resolution charge-based separations of monoclonal antibodies.
Peptides and proteins	Bio IEX	Bio IEX columns are packed with polymeric, nonporous, ion-exchange particles. The Bio IEX columns are designed for high resolution, high recovery, and highly efficient separations.
Proteins, peptides, and deprotected synthetic oligonucleotides	PL-SAX • 1000 Å • 4000 Å	The strong anion-exchange functionality, covalently linked to a fully porous chemically stable polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. The 5 µm media deliver separations at high resolution while the 30 µm media are used for medium pressure liquid chromatography
Globular proteins and peptides	PL-SAX 1000 Å	
Very large biomolecules/high speed	PL-SAX 4000 Å	
Small peptides to large proteins and very large biomolecules	PL-SCX 1000 Å	PL-SCX is a macroporous PS/DVB matrix with a very hydrophilic coating and strong cation-exchange functionality. This process is controlled to provide the optimum density of strong cation-exchange moieties for the analysis, separation, and purification of a wide range of biomolecules. The 5 µm media deliver separations at higher resolution while the 30 µm media are used for medium pressure liquid chromatography.
Antibodies (IgG, IgM), plasmid DNA, viruses, phages, and other macro biomolecules	Bio-Monolith • Bio-Monolith QA • Bio-Monolith DEAE • Bio-Monolith SO_3	Strong cation-exchange, strong and weak anion-exchange phases. Bio-Monolith HPLC columns are compatible with InfinityLab LC series.

Bio MAb HPLC columns

- A packing support composed of a rigid, spherical, highly cross-linked polystyrene divinylbenzene (PS/DVB) nonporous bead
- Particles grafted with a hydrophilic polymeric layer, virtually eliminating nonspecific binding of antibody proteins
- A different process is used to layer the weak cation-exchange phase to the particle giving it a higher density than the Bio WCX column particles
- Specifically designed for the separation of charge isoforms of monoclonal antibodies

Thorough characterization of monoclonal antibodies includes the identification and monitoring of acidic and basic isoforms. Bio MAb HPLC columns feature a unique resin specifically designed for high resolution, charge-based separations of monoclonal antibodies. These columns are compatible with aqueous solution buffers, acetonitrile/acetone/methanol, and water mixtures. Commonly used buffers are phosphate, tris, MES, and acetate.

Bio MAb columns are available in 1.7, 3, 5, and 10 µm sizes, providing higher resolution with smaller particles.

Column Specifications

Bonded Phase	Inner Diameter	Particle Size	pH Stability	Operating Temperature Limit	Flow Rate
Weak cation-exchange (carboxylate)	2.1 and 4.6 mm	1.7, 3, 5, and 10 µm	2–12	80 °C	0.1–1.0 mL/min
	10 mm	5 µm	2–12	80 °C	2.3–4.7 mL/min
	21.2 mm	5 µm	2–12	80 °C	10.6–21.2 mL/min

Tips and tools

Are you looking to increase your throughput for charge variant analysis of monoclonal antibodies? If so, see: Reducing Cycle Time for Charge Variant Analysis of Monoclonal Antibodies (publication **5991-4722EN**).

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Consistent ion-exchange mAb separation

Column: **Bio MAb, PEEK**
5190-2407
4.6 x 250 mm, 5 µm

Mobile phase: A: Sodium phosphate 10 mM, pH 5.5
B: A + sodium chloride 0.5 M

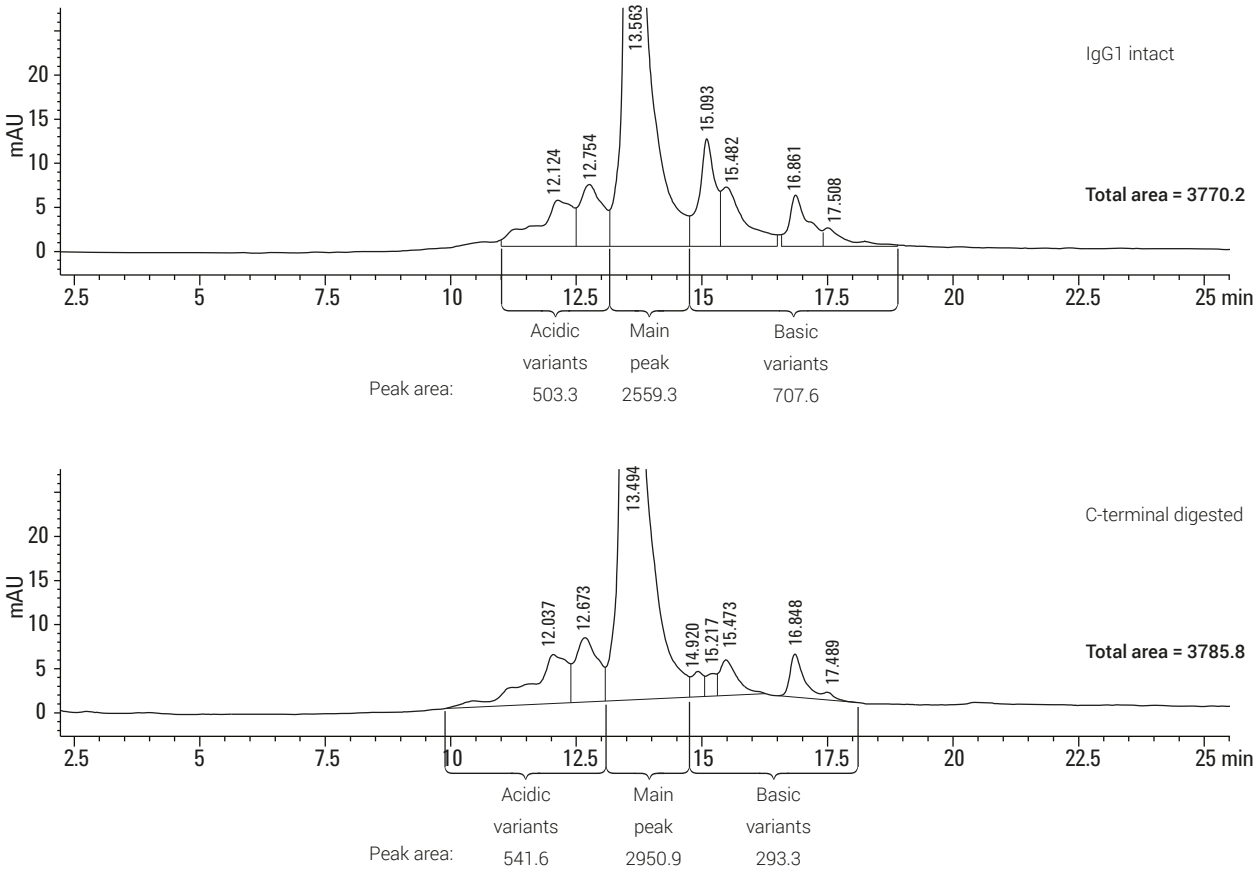
Flow rate: 0.85 mL/min

Gradient: 10 to 35% B from 0 to 25 min
(unless otherwise stated)

Detector: UV, 225 nm

Sample: 5 µg of 1 mg/mL of intact or C-terminal digested IgG1

Instrument: 1260 Infinity bio-inert quaternary LC or
1100 Series LC



Calculation of C-terminal digested IgG1 using a Bio MAb 5 µm column on the 1260 Infinity bio-inert quaternary LC.

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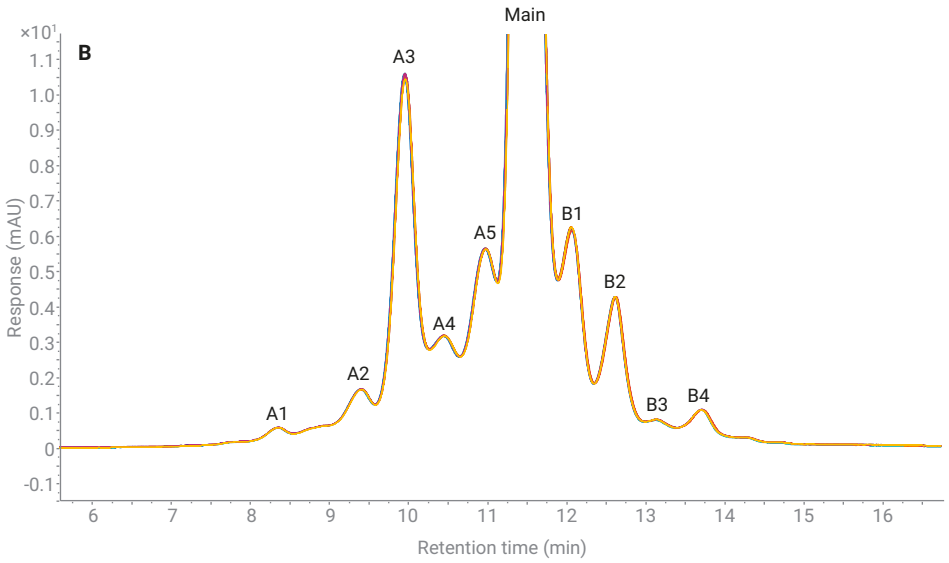
Reproducibility for trastuzumab charge variant separation



Precision in RSD	RT (%)	Area (%)
A1	0.033	1.793
A2	0.016	0.701
A3	0.026	0.403
A4	0.023	0.813
A5	0.032	0.327
Main	0.033	0.313
B1	0.038	0.329
B2	0.048	0.254
B3	0.046	3.549
B4	0.051	0.812

Reproducibility studies with seven subsequent runs for charge variant separation of trastuzumab with 0.66% B/min (3.3 mM/min) gradient slope.

WCX separation of Fab and Fc fragments of Trastuzumab

Column: **Bio MAb, PEEK**
5190-2411
2.1 x 250 mm, 5 µm

Mobile phase: A: 20 mM MES, pH 5.6
B: 20 mM MES, pH 5.6 + 300 mM NaCl

Flow rate: 170 µL/min

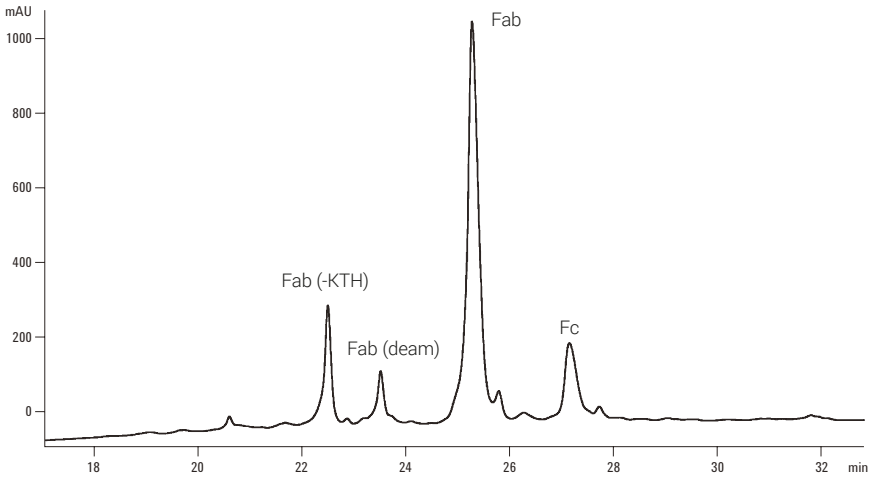
Injection volume: 16 µL

Gradient:

Time (min)	% B
0	0
39.5	80
40	100
50	100
50.5	2
60	2

Temperature: 30 °C

Instrument: 1100 series



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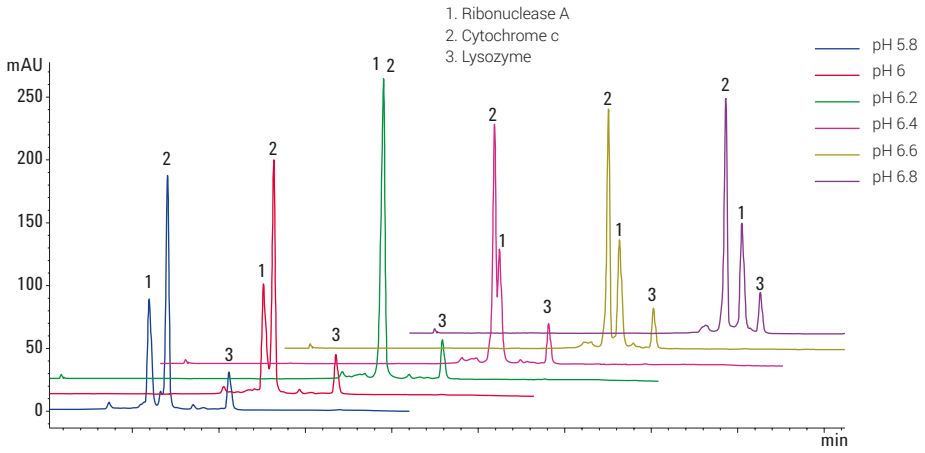
Method development using Buffer Advisor software—determination of optimum pH

Column: **Bio MAb, PEEK**
5190-2407
4.6 x 250 mm, 5 µm

Instrument:	1260 Infinity Bio-inert Quaternary LC
Buffer:	A: H_2O B: NaCl 3 M C: MES (2-N-morpholino) ethanesulfonic acid monohydrate) 60 mM D: MES-Na (2-(N-morpholino) ethanesulfonic acid sodium salt) 35 mM
Sample:	Mix of three proteins, dissolved in PBS (phosphate buffered saline), pH 7.4 Ribonuclease A: 13,700 Da, pI 9.6 Cytochrome c: 12,384 Da, pI 10-10.5 Lysozyme: 14,307 Da, pI 11.35
Flow rate:	1 mL/min
Gradient:	0 min–20 mM NaCl 5 min–20 mM NaCl 30 min–500 mM NaCl 35 min–1,000 mM NaCl 36 min–20 mM NaCl
Injection volume:	10 µL
Thermostat:	4 °C
Temperature TCC:	25 °C
DAD:	280 nm/4 nm Reference: OFF
Peak width:	>0.05 min (1.0 s respose time)(5 Hz)

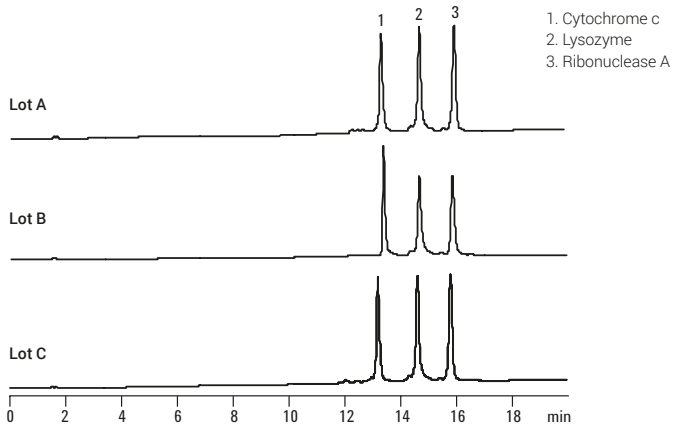


pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients

Virtually eliminate retention time variations

Column:	**Bio MAb, stainless steel** **5190-2413** **4.6 x 250 mm, 10 µm**
Mobile phase:	A: 10 mM Sodium phosphate, pH 6.0 B: A + 1.0 M Sodium chloride
Flow rate:	1.0 mL/min
Gradient:	0–100% B in 42 min
Temperature:	25 °C
Detector:	UV, 214 nm

The combination of well-controlled resin production, column surface chemistry, and column packing virtually eliminates retention time variations from column-to-column and lot-to-lot.



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Bio IEX HPLC columns

- Highly cross-linked and rigid nonporous poly(styrene divinylbenzene) (PS/DVB) particles are grafted with a hydrophilic polymeric layer, eliminating nonspecific binding
- Uniform, densely packed ion-exchange functional groups are chemically bonded to the hydrophilic layer (multiple ion-exchange groups per anchor) to increase column capacity
- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Multiple ion-exchange groups are captured on one anchor to increase capacity

Bio IEX HPLC columns are packed with polymeric, nonporous, ion-exchange particles and are designed for high resolution, high recovery, and highly efficient separations of peptides, oligonucleotides, and proteins.

The Bio IEX family includes strong cation-exchange (SCX), weak cation-exchange (WCX), strong anion-exchange (SAX), and weak anion-exchange (WAX) phases. All phases are available in 1.7, 3, 5, and 10 µm nonporous particles.

Column Specifications

Bonded Phase	Inner Diameter	Particle Size	pH Stability	Operating Temperature Limit	Flow Rate
SCX (strong cation-exchange)–SO_3H	2.1 and 4.6 mm	1.7, 3, 5, and 10 µm	2–12	80 °C	0.1–1.0 mL/min
WCX (weak cation-exchange)–COOH	10 mm	5 µm	2–12	80 °C	2.3–4.7 mL/min
SAX (strong anion-exchange)–$N(CH_3)_3$	21.2 mm	5 µm	2–12	80 °C	10.6–21.2 mL/min
WAX (weak cation-exchange)–$N(C_2H_5)_2$					

Tips and tools

For further information on optimizing your charged variant analysis, refer to the Agilent Biocolumns Application Compendium – Charge Variant Analysis, including page 5 of the compendium featuring a “how-to” guide (publication **5994-0034EN**).

Shortened and simplified charge variant workflow

Column:
Bio WCX, stainless steel
5190-2443
4.6 x 50 mm, 3 µm

Bio SCX, stainless steel
5190-2423
4.6 x 50 mm, 3 µm

Mobile phase:
A: Water
B: Sodium chloride 1.5 M
C: Monosodium phosphate 40 mM
D: Disodium phosphate 40 mM
By combining predetermined proportions of C and D as determined by the Buffer Advisor software, buffer solutions at the desired pH range and strength were created.

Flow rate: 1.0 mL/min

Injection volume: 10 µL

Gradient: Conditions for chromatograms shown:
pH 5.0 to 7.0, 10 to 25 mM buffer strength
sodium chloride (NaCl) 0 to 500 mM, 0 to 15 min
sodium chloride (NaCl) 500 mM, 15 to 20 min
DOE experiments
pH 5.0 to 7.0
0 to 200 mM, 0 to 250 mM, and 0 to 300 mM NaCl

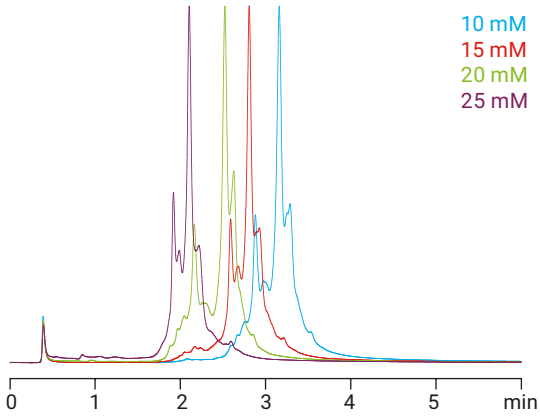
Temperature: Ambient

Detector: UV, 220 nm

Sample: IgG monoclonal antibody

Sample conc: 2 mg/mL (in sodium phosphate buffer 20 mM, pH 6.0)

Instrument: 1260 Infinity bio-inert quaternary LC



Optimizing buffer strength at pH 6.5 from the screening chromatograms of a monoclonal IgG separation

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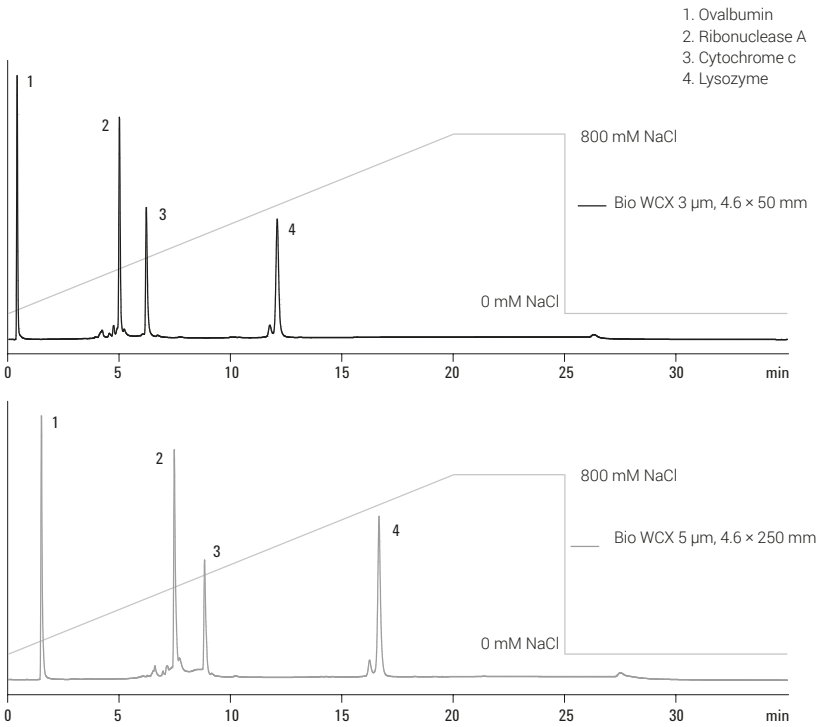
Achieve faster analysis time with smaller particles and shorter column lengths—speed up your separation by 30%

Column:	**Bio WCX, stainless steel** **5190-2445** **4.6 x 250 mm, 5 µm** **Bio WCX, stainless steel** **5190-2443** **4.6 x 50 mm, 3 µm**
Mobile phase:	A: Sodium phosphate 20 mM, pH 6.5 B: A + sodium chloride 1.6 M
Flow rate:	1.0 mL/min
Injection volume:	10 µL
Gradient:	0 to 50% B
Temperature:	Ambient
Detector:	UV, 220 nm
Sample conc:	0.5 mg/mL
Instrument:	1260 Infinity bio-inert quaternary LC



Protein separation on a Bio WCX 4.6 x 50 mm, 3 µm column versus a Bio WCX 4.6 x 250 mm, 5 µm column (flow rate 1.0 mL/min). Faster analysis times were achieved through smaller particle size and shorter column length—samples eluted from the longer column in 17 min, and in just 12 min from the shorter column.

Tips and tools

For further information see:

Optimizing protein separations with Agilent weak cation-exchange columns (publication **5990-9628EN**)

Faster separations using Agilent weak cation-exchange columns (publication **5990-9931EN**)

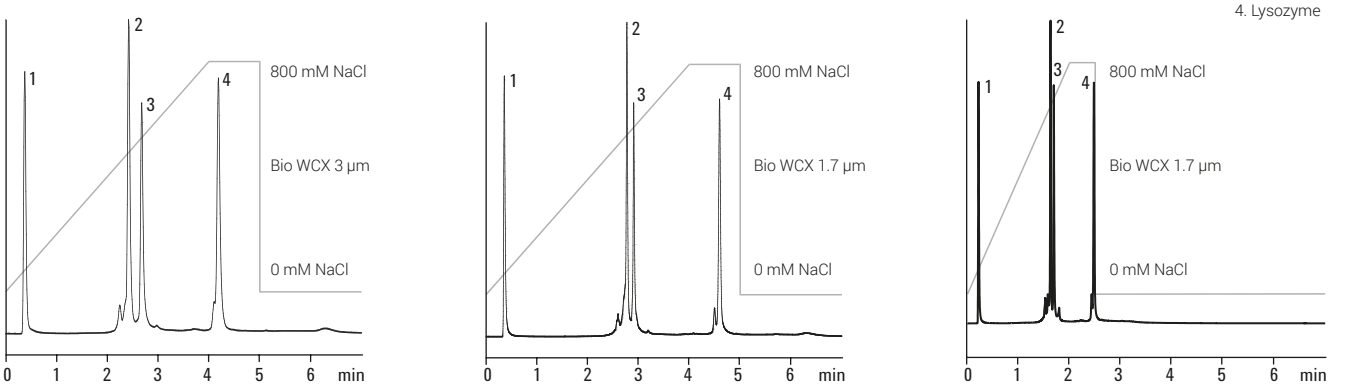
pH Gradient elution for improved separation of monoclonal antibody variants (publication **5990-9629EN**)

Optimizing protein separations with cation-exchange chromatography using Agilent Buffer Advisor (publication **5991-0565EN**)

Smaller particle sizes provide increased resolution

Column:	**Bio WCX, stainless steel** **5190-2443** **4.6 x 50 mm, 3 µm**
	Bio WCX, stainless steel **5190-2441** **4.6 x 50 mm, 1.7 µm**
Mobile phase:	A: Sodium phosphate 20 mM, pH 6.5 B: A + sodium chloride 1.6 M
Injection volume:	10 µL
Gradient:	0 to 50% B
Temperature:	Ambient
Detector:	UV, 220 nm
Sample conc:	0.5 mg/mL
Instrument:	1260 Infinity bio-inert quaternary LC

1. Ovabumin
2. Ribonuclease A
3. Cytochrome c
4. Lysozyme



Left and middle: Protein separation on a Bio WCX 3 µm column versus a Bio WCX 1.7 µm column (flow rate 1.0 mL/min).
Right: By increasing the flow rate to 1.7 mL/min, the separation time was reduced to less than 3 minutes. (A Bio WCX column was used.)
Reduce analysis time—without sacrificing peak shape and resolution—by increasing flow rate.

Tips and tools

Use Agilent solvent filters to remove particles from self-prepared buffers and mobile phases.

Visit: **www.agilent.com/chem/solvent-filters-degassers**

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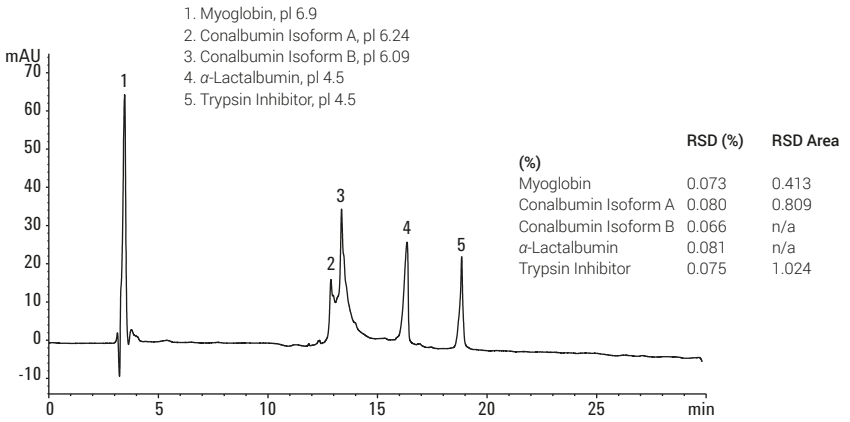
Analysis of proteins by anion-exchange columns using the Agilent 1260 Infinity bio-inert quaternary LC system

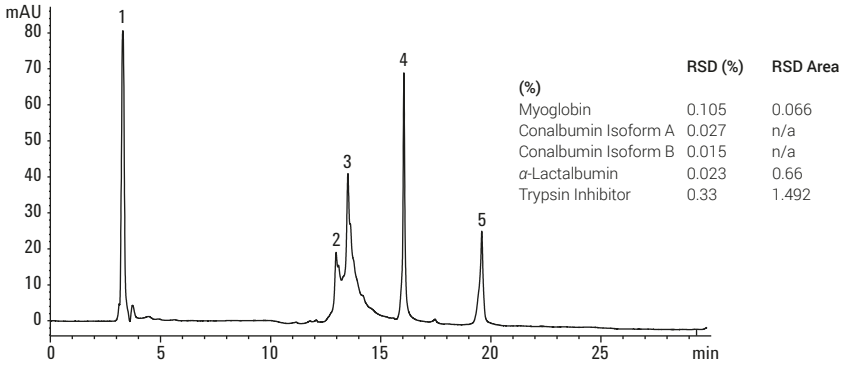
Column:	**Bio WAX, PEEK** **5190-2487** **4.6 x 250 mm, 5 µm**
Buffer:	A: 20 mM tris, pH 7.6 B: 20 mM tris, pH 7.6 + 2 M NaCl, 1 M KCl, 1 M CH_3COONa, 1 M $[(CH_3)_4N]Cl$
Gradient 1 M:	5 min–100% A 20 min–70% B 25 min–100% B
Gradient 2 M:	5 min–100% A 20 min–35% B 25 min–50% B 25.01 min–100% B
Stop time:	30 min
Posttime:	20 min
Temperature:	25 °C
Flow rate:	0.5 mL/min
Injection volume:	5 µL
DAD:	280 nm
Peak width:	0.025 min (0.5 s response time) (10 Hz)

For further information see application note
5990-9614EN



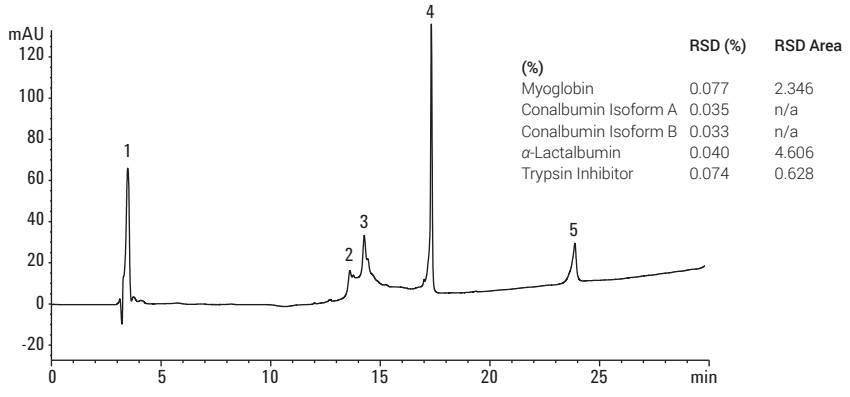
(%)	RSD (%)	RSD Area
Myoglobin	0.073	0.413
Conalbumin Isoform A	0.080	0.809
Conalbumin Isoform B	0.066	n/a
α-Lactalbumin	0.081	n/a
Trypsin Inhibitor	0.075	1.024

Figure 1. Protein separation by AEX by a linear gradient using 2 M NaCl as eluting salt.



(%)	RSD (%)	RSD Area
Myoglobin	0.105	0.066
Conalbumin Isoform A	0.027	n/a
Conalbumin Isoform B	0.015	n/a
α-Lactalbumin	0.023	0.66
Trypsin Inhibitor	0.33	1.492

Figure 2. Protein separation by AEX by a linear gradient using 1 M KCl as eluting salt.



(%)	RSD (%)	RSD Area
Myoglobin	0.077	2.346
Conalbumin Isoform A	0.035	n/a
Conalbumin Isoform B	0.033	n/a
α-Lactalbumin	0.040	4.606
Trypsin Inhibitor	0.074	0.628

Figure 3. Protein separation by AEX by a linear gradient using 1 M $[(CH_3)_4N]Cl$) as eluting salt.

PL-SAX Strong Anion-Exchange columns

- Small particles deliver excellent chromatographic performance
- Wide range of particle sizes and two pore sizes for flexible analysis to scale up purification
- Exceptional stability for long column lifetime

PL-SAX -$N(CH_3)_3^+$ is ideal for the anion-exchange HPLC separations of proteins, peptides, and deprotected synthetic oligonucleotides under denaturing conditions. The strong anion-exchange functionality, covalently linked to a chemically stable fully porous polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. PL-SAX delivers improved chromatography for self-complementary or G-rich sequences that may associate to form aggregates or hairpin structures. The 5 µm material provides high-efficiency separations of n and n-1 sequences. A wide range of particle sizes and column geometries permits analysis scale-up to purification. The strong anion-exchange functionality provides a material with exceptional chemical and thermal stability, even with sodium hydroxide eluents, leading to long column lifetime.

Column Specifications

Bonded Phase	Inner Diameter (mm)	Particle Size (µm)	Pore Size (Å)	pH Stability	Operating Temperature Limit
Strong anion-exchange	2.1, 4.6, 7.5, 25, 50, and 100	5, 8, 10, and 30	1000 Å and 4000 Å	1–14	80 °C

Tips and tools

For more information on oligonucleotide separations, see Chapter 12 of this catalog.
For more details on PL-SAX at the preparative level, see **5994-4723EN**.

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Standard ion-exchange protein separation

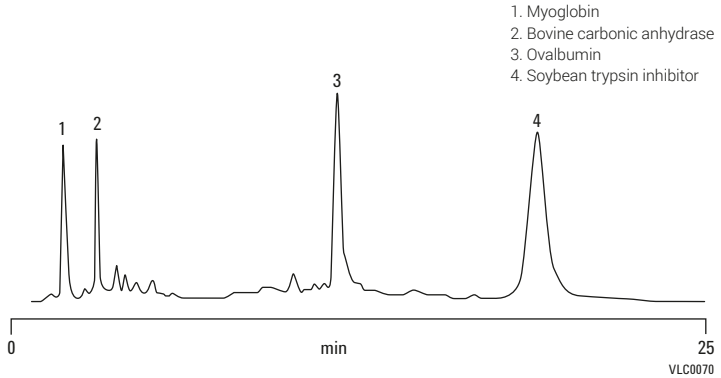
Column: **PL-SAX 1000 Å**
PL1551-1502
4.6 x 50 mm, 5 µm

Mobile phase: A: 10 mM tris HCl, pH 8
B: A + 350 mM sodium chloride, pH 8

Gradient: 0–100% B in 20 min

Flow rate: 1.0 mL/min

Detector: UV, 220 nm



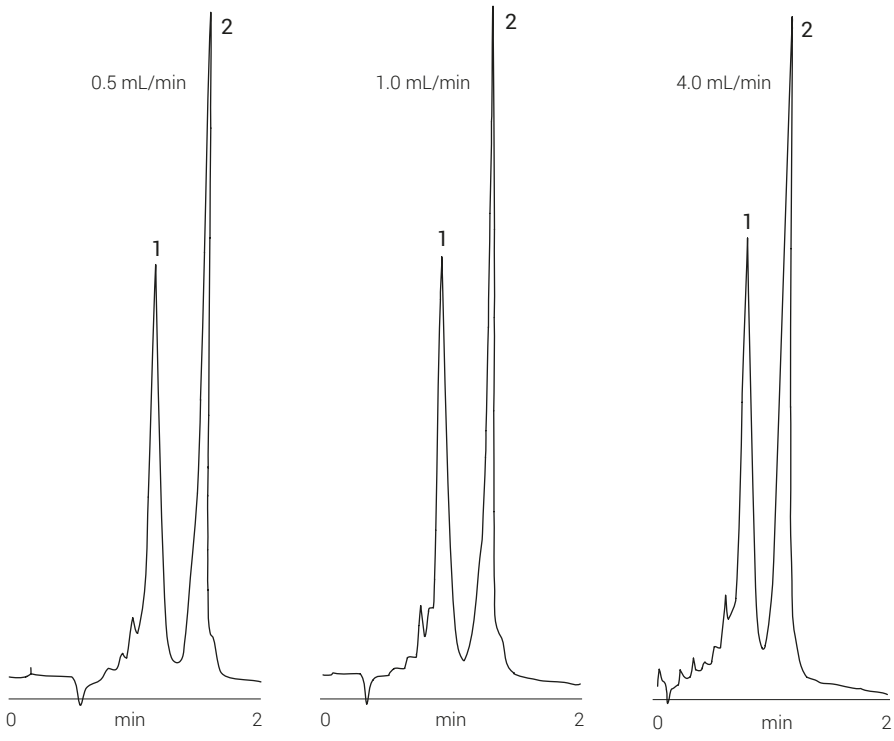
Separation of ovalbumin and soyabean trypsin inhibitor

Column: **PL-SAX 1000Å 8 µm**

Buffer: 0.01 M Tris, pH 8, eluting salt NaCl

Flow rate: 1.0 mL/min

Flow Rate (mL/min)	R_s
0.5	3.79
1.0	4.27
1.5	4.46
2.0	3.68
3.0	3.37
4.0	3.09



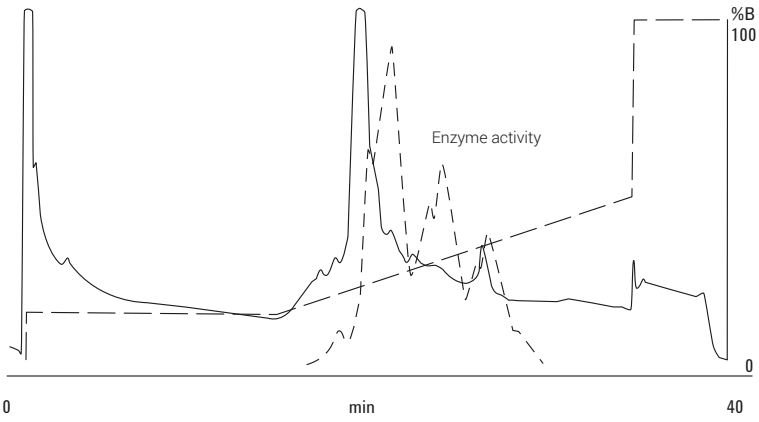
Analysis of choline kinase

Column: **PL-SAX 4000 Å**
PL1551-1803
4.6 x 50 mm, 8 µm

Mobile phase: A: 20 mM tris 5% ethylene glycol, pH 7.5
(The following are required to retain enzyme activity)
1.0 mM ethylene glycol tetraacetic acid
2.0 mM ß-mercaptoethanol
0.2 mM phenylmethylsulfonyl fluoride
B: A + 1 M potassium chloride

Flow rate: 3.0 mL/min

Detector: UV, 280 nm



Separation courtesy of T Porter, Purdue University, USA

Analysis of representative whey proteins

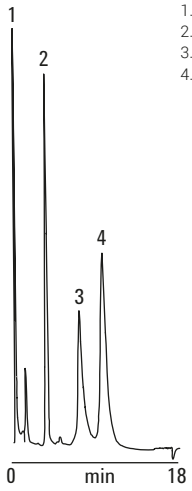
Column: **PL-SAX 1000 Å**
PL1551-1802
4.6 x 50 mm, 8 µm

Mobile phase: A: 20 mM tris HCl, pH 7
B: A + 500 mM sodium acetate, pH 7

Flow rate: 1.0 mL/min

Gradient: Linear 0-50% B in 10 min

Detector: UV, 280 nm



1. Carbonic anhydrase
2. α-Lactalbumin
3. β-Lactoglobulin B
4. β-Lactoglobulin A

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PL-SCX Strong Cation-Exchange columns

- Optimal design for effective separation of biomolecules
- Pore sizes allow use of a range of solute sizes
- Exceptional stability for long column lifetime

PL-SCX -SO_3 is a macroporous PS/DVB matrix with a very hydrophilic coating and strong cation-exchange functionality. This process is controlled to provide the optimum density of strong cation-exchange moieties for the analysis, separation, and purification of a wide range of biomolecules, from small peptides to large proteins. The 5 µm media deliver separations at higher resolution with the 30 µm media used for medium pressure liquid chromatography.

Column Specifications

Bonded Phase	Inner Diameter (mm)	Particle Size (µm)	Pore Size	pH Stability	Operating Temperature Limit
Strong cation-exchange	2.1, 4.6, 7.5, 25, 50, and 100	5, 8, 10, and 30	1000 Å	1–14	80 °C

Standard protein separation

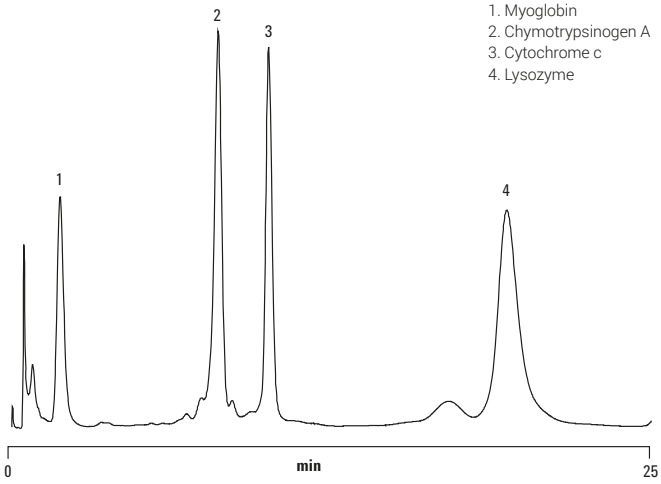
Column: **PL-SCX 1000 Å**
PL1545-1502
4.6 x 50 mm, 5 µm

Mobile phase: A: 20 mM potassium dihydrogen phosphate, pH 6.0
B: A + 1 M sodium chloride

Gradient: 0–100% B in 20 min

Flow rate: 1.0 mL/min

Detector: UV, 280 nm



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Bio-Monolith Ion-Exchange HPLC columns

- Polymer-based, monolith HPLC columns designed for macro biomolecule separations
- Flow-rate independent separations; no diffusion, no pores, and no void volume make transport between mobile and stationary phase very rapid
- Monolith disk is 5.2 x 4.95 mm (100 µL column volume) with continuous channels, eliminating diffusion mass transfer
- Extremely fast separations speed up method development time and decrease costs; locking in method parameters takes significantly less time and buffer

Bio-Monolith Ion-Exchange HPLC columns provide high resolution and rapid separations of antibodies (IgG, IgM), plasmid DNA, viruses, phages, and other macro biomolecules. The product family offers strong cation-exchange, strong and weak anion-exchange. Bio-Monolith HPLC columns are compatible with InfinityLab LC series.

Bio-Monolith Ion-Exchange HPLC column

Bio-Monolith HPLC Column Selection Guide

Column	Description	Key Applications
Bio-Monolith QA	The quaternary amine bonded phase (strong anion-exchange) is fully charged over a working pH range of 2–13, binding negatively charged biomolecules.	– Adenovirus process monitoring and quality control – IgM purification monitoring and quality control – Monitoring DNA impurity removal – Monitoring endotoxin removal – HSA purity
Bio-Monolith DEAE	The diethylaminoethyl bonded phase (weak anion-exchange) offers increased selectivity of biomolecules with negative charge over a working pH range of 3–9.	– Process monitoring and quality control of bacteriophage manufacturing and purification – Process monitoring and quality control of plasmid DNA purification
Bio-Monolith SO_3	The sulfonyl bonded phase (strong cation-exchange) is fully charged over a working pH range of 2–13, binding positively charged biomolecules.	– Fast and high resolution analytical separations of large molecules such as proteins and antibodies – Hemoglobin A1c fast analytics

Column Specifications

Dimensions	5.2 mm x 4.95 mm
Column volume	100 µL
Maximum pressure	150 bar (15 MPa, 2,200 psi)
Temperature min/max	Operating: 2–40 °C
	Storage: 2–8 °C
Recommended pH	Operating range: 2–13
	Cleaning-in-place: 1–14
Materials of construction	Hardware: stainless steel
	Packing: poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous monolith
Color ring identifier	Bio-Monolith QA: blue
	Bio-Monolith DEAE: green
	Bio-Monolith SO_3: red
Shelf life/expiration date	SO_3, QA, DEAE: 24–36 months

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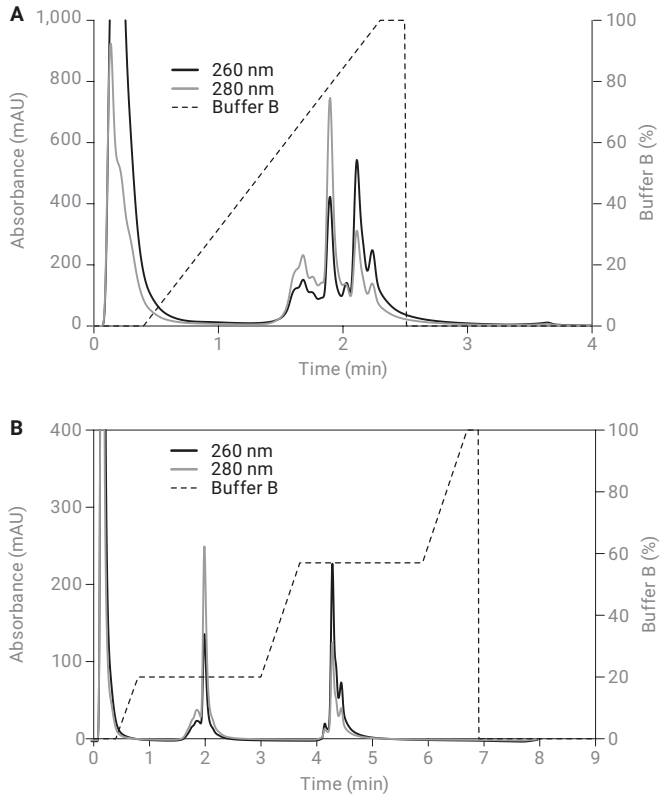
Analysis of Adenovirus Type 5 particles

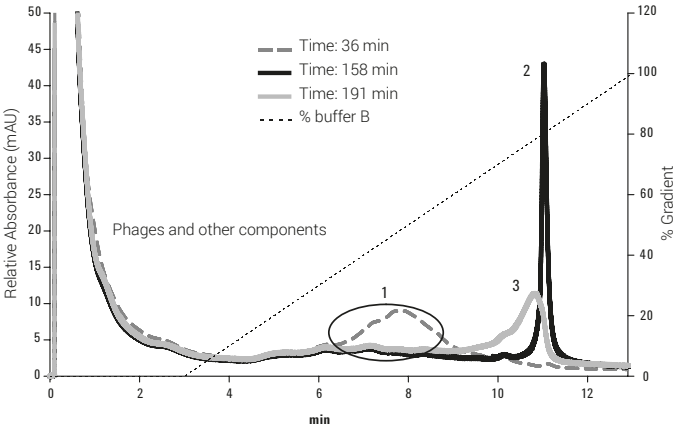
Column:	**Agilent Bio-Monolith Column QA, 5.2 mm id × 4.95 mm (p/n 5069-3635)**
Mobile phase:	A / B: See figure legends
Flow rate:	1 mL/min
Gradient:	See figure legends
Detector:	UV at 260 and 280 nm
Instrument:	Agilent 1100 series HPLC system fitted with a diode array detector (DAD)



Analysis of Ad5 cellular lysate on an Agilent Bio-Monolith QA column. The solid blank line is absorbance at 260 nm and the grey line is absorbance at 280 nm. Conditions: (A) Buffer A: 20 mM Tris, pH 7.5; Buffer B: 20 mM Tris, 1.5 m NaCl, pH 7.5. Linear gradient of 0 to 100% Buffer B over 19 column volumes (CV), followed by a 2 CV hold at 100% B. (B) Buffer A: 20 mM Tris-HCl buffer + 0.1 M NaCl, pH 7.5; Buffer B: 20 mM Tris-HCl buffer + 2 M NaCl, pH 7.5. Step gradient: a 22 CV hold at 20% buffer B and a 22 CV hold at 57% Buffer B. Flow rate: 1 mL/min. Sample: Ad5 cellular lysate. Injection volume: 25 µL.

Monitor phage production during fermentation

Column:	**Bio-Monolith DEAE 5069-3636 5.2 x 4.95 mm**
Mobile phase:	A: 125 mM sodium phosphate buffer, pH 7.0 B: 125 mM sodium phosphate buffer + 1 M sodium chloride, pH 7.0
Flow rate:	1 mL/min
Gradient:	100% buffer A (2.5 min) 0–100% buffer B (10 min) 100% buffer A (2 min)
Detector:	UV, 280 nm
Instrument:	High pressure gradient HPLC system, Agilent 1200 Infinity series



As phage proliferation progresses, the genomic DNA (gDNA) concentration increases as the host cells are lysed. In the late stages of fermentation, gDNA begins to degrade into fragments. These gDNA fragments cannot be easily removed by purification media, and so it is critical to stop the fermentation cycle before the degradation of the gDNA. The chromatogram above represents three samples taken from the bioreactor at 36, 158, and 191 minutes. Peak 1 represents phage, media, and host cells, peak 2 the intact gDNA, and peak 3 the fragmented gDNA.

Product ordering information

Bio MAb HPLC Columns

Dimensions (mm)	Particle Size (µm)	Bio MAb PEEK	Pressure Limit	Bio MAb Stainless Steel	Pressure Limit
21.2 x 250	5			5190-6885	275 bar, 4000 psi
10 x 250	5			5190-6884	275 bar, 4000 psi
4.6 x 250	10	5190-2415	275 bar, 4000 psi	5190-2413	275 bar, 4000 psi
4.6 x 50	10	5190-2416	275 bar, 4000 psi		
4.6 x 250	5	5190-2407	400 bar, 5800 psi	5190-2405	400 bar, 5800 psi
4.6 x 50	5	5190-2408	400 bar, 5800 psi		
4.6 x 50	3			5190-2403	551 bar, 8000 psi
4.6 x 50	1.7			5190-2401	600 bar, 8700 psi
4.0 x 10, guard	10			5190-2414	275 bar, 4000 psi
4.0 x 10, guard	5			5190-2406	413 bar, 6000 psi
4.0 x 10, guard	3			5190-2404	551 bar, 8000 psi
4.0 x 10, guard	1.7			5190-2402	600 bar, 8700 psi
2.1 x 250	10	5190-2419	275 bar, 4000 psi		
2.1 x 50	10	5190-2420	275 bar, 4000 psi		
2.1 x 250	5	5190-2411	400 bar, 5800 psi		
2.1 x 50	5	5190-2412	400 bar, 5800 psi		

Bio IEX HPLC Columns, PEEK

Dimensions (mm)	Particle Size (µm)	Pressure Limit	Bio SCX Part No.	Bio WCX Part No.	Bio SAX Part No.	Bio WAX Part No.
4.6 x 250	10	275 bar, 4000 psi	5190-2435	5190-2455	5190-2475	5190-2495
4.6 x 50	10	275 bar, 4000 psi	5190-2436	5190-2456	5190-2476	5190-2496
4.6 x 250	5	400 bar, 5800 psi	5190-2427	5190-2447	5190-2467	5190-2487
4.6 x 50	5	400 bar, 5800 psi	5190-2428	5190-2448	5190-2468	5190-2488
2.1 x 250	10	275 bar, 4000 psi	5190-2439	5190-2459	5190-2479	5190-2499
2.1 x 50	10	275 bar, 4000 psi	5190-2440	5190-2460	5190-2480	5190-2500
2.1 x 250	5	400 bar, 5800 psi	5190-2431	5190-2451	5190-2471	5190-2491
2.1 x 50	5	400 bar, 5800 psi	5190-2432	5190-2442	5190-2462	5190-2492

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Bio IEX HPLC Columns, Stainless Steel

Dimensions (mm)	Particle Size (µm)	Pressure Limit	Bio SCX Part No.	Bio WCX Part No.	Bio SAX Part No.	Bio WAX Part No.
21.2 x 250	5	413 bar, 6000 psi	5190-6879	5190-6881	5190-6883	5190-6877
10 x 250	5	413 bar, 6000 psi	5190-6878	5190-6880	5190-6882	5190-6876
4.6 x 250	10	275 bar, 4000 psi	5190-2433	5190-2453	5190-2473	5190-2493
4.6 x 250	5	413 bar, 6000 psi	5190-2425	5190-2445	5190-2465	5190-2485
4.6 x 150	3	551 bar, 8000 psi				5190-6875
4.6 x 50	3	551 bar, 8000 psi	5190-2423	5190-2443	5190-2463	5190-2483
4.6 x 50	1.7	600 bar, 8700 psi	5190-2421	5190-2441	5190-2461	5190-2481
4.0 x 10, guard	10	275 bar, 4000 psi	5190-2434	5190-2454	5190-2474	5190-2494
4.0 x 10, guard	5	413 bar, 6000 psi	5190-2426	5190-2446	5190-2466	5190-2486
4.0 x 10, guard	3	551 bar, 8000 psi	5190-2424	5190-2444	5190-2464	5190-2484
4.0 x 10, guard	1.7	600 bar, 8700 psi	5190-2422	5190-2442	5190-2462	5190-2482

Bio-Monolith HPLC Column Selection Guide

Column	Part No.
Bio-Monolith QA	5069-3635
Bio-Monolith DEAE	5069-3636
Bio-Monolith SO_3	5069-3637

PL-SAX Strong Anion-Exchange Columns

Dimensions (mm)	Particle Size (µm)	Pressure Limit	PL-SAX 1000 Å	PL-SAX 4000 Å
100 x 300	30	207 bar, 3000 psi	PL1851-3102	PL1851-3103
100 x 300	10	207 bar, 3000 psi	PL1851-2102	PL1851-2103
50 x 150	30	207 bar, 3000 psi	PL1751-3702	PL1751-3703
50 x 150	10	207 bar, 3000 psi	PL1751-3102	PL1751-3103
25 x 150	30	207 bar, 3000 psi	PL1251-3702	PL1251-3703
25 x 150	10	275 bar, 4000 psi	PL1251-3102	PL1251-3103
25 x 50	10	207 bar, 3000 psi	PL1251-1102	PL1251-1103
7.5 x 150	8	207 bar, 3000psi	PL1151-3802	PL1151-3803
7.5 x 150	8	207 bar, 3000psi	PL1151-1802	PL1151-1803
4.6 x 250	30	207 bar, 3000 psi	PL1551-5702	PL1551-5703
4.6 x 150	30	207 bar, 3000 psi	PL1551-3702	PL1551-3703
4.6 x 250	10	207 bar, 3000 psi	PL1551-5102	PL1551-5103
4.6 x 150	10	207 bar, 3000 psi	PL1551-3102	PL1551-3103
4.6 x 150	8	207 bar, 3000 psi	PL1551-3802	PL1551-3803
4.6 x 50	8	207 bar, 3000 psi	PL1551-1802	PL1551-1803
4.6 x 50	5	207 bar, 3000 psi	PL1551-1502	PL1551-1503
2.1 x 150	8	207 bar, 3000 psi	PL1951-3802	PL1951-3803
2.1 x 50	8	207 bar, 3000 psi	PL1951-1802	PL1951-1803
2.1 x 50	5	207 bar, 3000 psi	PL1951-1502	PL1951-1503

Tips and tools

Visit our online store to directly purchase our columns and consumables: **www.agilent.com/chem/store**

PL-SAX Strong Anion-Exchange Bulk Media

Amount	Particle Size (µm)	Pressure Limit	PL-SAX 1000 Å	PL-SAX 4000 Å
10 g	30	207 bar, 3000 psi	PL1451-2702	PL1451-2703
10 g	10	207 bar, 3000 psi	PL1451-2102	PL1451-2103
100 g	30	207 bar, 3000 psi	PL1451-4702	PL1451-4703
100 g	10	207 bar, 3000 psi	PL1451-4102	PL1451-4103
1 kg	30	207 bar, 3000 psi	PL1451-6702	PL1451-6703
1 kg	10	207 bar, 3000 psi	PL1451-6102	PL1451-6103

PL-SCX Strong Cation-Exchange Columns

Dimensions (mm)	Particle Size (µm)	Pressure Limit	PL-SCX 1000 Å
100 x 300	30	207 bar, 3000 psi	PL1845-3102
100 x 300	10	207 bar, 3000 psi	PL1845-2102
50 x 150	30	207 bar, 3000 psi	PL1745-3703
50 x 150	10	207 bar, 3000 psi	PL1745-3102
25 x 150	30	207 bar, 3000 psi	PL1245-3702
25 x 150	10	207 bar, 3000 psi	PL1245-3102
25 x 50	10	207 bar, 3000 psi	PL1245-1102
7.5 x 50	8	207 bar, 3000psi	PL1145-1802
4.6 x 250	30	207 bar, 3000 psi	PL1545-5703
4.6 x 150	30	207 bar, 3000 psi	PL1545-3702
4.6 x 250	10	207 bar, 3000 psi	PL1545-5102
4.6 x 150	10	207 bar, 3000 psi	PL1545-3102
4.6 x 150	8	207 bar, 3000 psi	PL1545-3802
4.6 x 50	8	207 bar, 3000 psi	PL1545-1802
4.6 x 50	5	207 bar, 3000 psi	PL1545-1502
2.1 x 150	8	207 bar, 3000 psi	PL1945-3802
2.1 x 50	8	207 bar, 3000 psi	PL1945-1802
2.1 x 50	5	207 bar, 3000 psi	PL1945-1502

PL-SCX Strong Cation-Exchange Bulk Media

Amount	Particle Size (µm)	Pressure Limit	PL-SCX 1000 Å
100 g	30	207 bar, 3000 psi	PL1445-4702
100 g	10	207 bar, 3000 psi	PL1445-4102
1 kg	30	207 bar, 3000 psi	PL1445-6702
1 kg	10	207 bar, 3000 psi	PL1445-6102

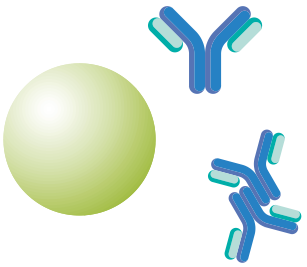
Tips and tools

Agilent workflow ordering guides provide you with tips and tricks, starting conditions, and part numbers for columns and accessories for specific applications.

Current ion exchange workflow ordering guides include publication **5994-4636EN** and publication **5994-6053EN** for oligo purification with PL-SAX columns and charge variant of mAbs with the Bio MAb column.

Aggregation and Fragment Analysis

Accurately determine biomolecule aggregation and fragmentation



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

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

Which SEC column is right for your application?

As a leading manufacturer of SEC columns and instruments for over 30 years, Agilent is continually developing new SEC products that will provide even higher resolution and quicker separations. This section highlights Agilent's broad family of SEC columns for protein biopolymer analysis:

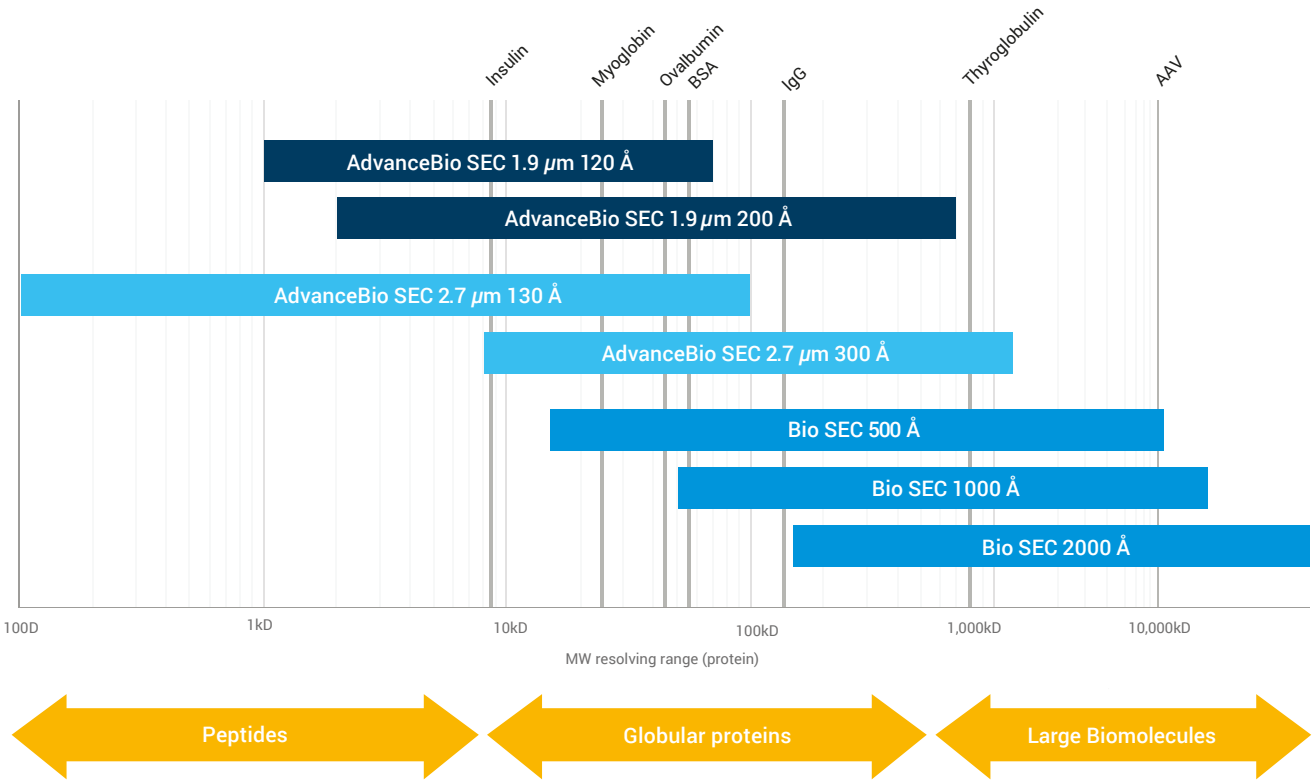
- AdvanceBio SEC is available in two particle sizes for aggregate and fragment analysis of proteins and peptides. The 2.7 µm particle columns are recommended for routine SEC-UV or SEC-LS measurements. The 1.9 µm particles are stable up to 600 bar, ensuring they are robust for high resolution and high throughput SEC-UV or SEC-MS analysis. Both particles have a hydrophilic polymer coating, resulting in minimal secondary interactions.
- Bio SEC-3 and Bio SEC-5 columns are available in a variety of pore sizes, diameters up to 21.2 mm, and are well suited for protein analysis with UV or MS detection. Note that 3 µm Bio SEC-3 columns provide higher resolution than the 5 µm Bio SEC-5 columns.
- Pore sizes from 500 Å to 2000 Å are available in the Bio SEC-5 columns for SEC analysis of macromolecular structures such as AAVs, virus-like particles, and large oligonucleotides.
- PROTEEMA and MAB columns are diol SEC columns recommended where L20 USP designation is required. Pore sizes range from 100 to 1000 Å and bio-inert column hardware is available.

Size Exclusion Chromatography (SEC)

Application	Agilent Columns	Notes	USP Designation
Peptides, proteins, aggregate analysis	AdvanceBio SEC 2.7 µm	Robust hydrophilic coating yielding minimal secondary interactions. Available in two pore sizes, 130 Å and 300 Å.	L59
	AdvanceBio SEC 1.9 µm	The same robust hydrophilic coating, but in a smaller particle format for higher resolution and throughput. 1.9 µm particles are robust up to 620 bar, and available in two pore sizes, 120 Å and 200 Å.	L59
	Bio SEC-3	Higher resolution and faster separation from 3 µm particles, with 100 Å, 150 Å, or 300 Å pore sizes. Scalable to 21.2 mm i.d.	L59
	PROTEEMA	Diol SEC phase with 3 µm particles and 100 Å, 300 Å, or 1000 Å pore sizes.	L20
	MAB	Mixed bed diol SEC phase with 3 µm particles	L20
Large biomolecules such as AAVs and VLPs, or samples with multiple molecular weight components	Bio SEC-5	More pore size options (100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, and 2000 Å) to cover a wide range of analytes. Scalable to 21.2 mm i.d.	L59

Pore size selection

Agilent has a wide selection of SEC columns that give you the choices you need to perfect separations based on your analytes and method parameters. This chart offers 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 AdvanceBio SEC columns.



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AdvanceBio SEC

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

AdvanceBio SEC 2.7 µm columns are robust columns for routine SEC needs, with a unique hydrophilic coating to minimize secondary interactions between the sample and stationary phase without adding extra salt or organic solvent to the mobile phase. Even for sticky samples such as ADCs, this column has minimized secondary interactions for accurate answers. Available in two pore sizes, 300 Å and 130 Å, they are ideal for sensitive, highly reproducible aggregate analysis of proteins and peptides.

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

For SEC-MS and your most challenging SEC applications, AdvanceBio SEC 1.9 µm columns are also available in PEEK-lined stainless steel hardware in a 2.1 mm i.d. This bio-inert, metal-free flow path is ideal for metal-sensitive or native-mode SEC-MS analyses.

- Increased analytical speed to help you meet vital deadlines
- Increased resolution for more accurate quantitation
- Increased sensitivity for quantitate aggregates, even at low levels
- Increased reproducibility to eliminate rework
- Uniquely designed standards for AdvanceBio SEC columns, providing optimal calibration and performance verification
- 2.7 and 1.9 µm particle sizes, each with two pore size options
- PEEK-lined stainless steel hardware available for a bio-inert flow path

Column Specifications

Particle Size	Pore Size	Molecular Weight Range	pH Range	Max Pressure	Flow Rate
2.7 µm	130 Å	100–120,000 Da	2–8.5	400 bar (typical operating pressure < 200 bar)	0.1–2.0 mL/min (7.8 mm id) 0.1–0.7 mL/min (4.6 mm id)
2.7 µm	300 Å	5,000–1,250,000 Da	2–8.5	400 bar (typical operating pressure < 200 bar)	
1.9 µm	120 Å	1,000–80,000 Da	2–8.5	620 bar	0.1– 0.5 mL/min (4.6 x 300 mm) 0.1– 0.7 mL/min (4.6 x 150 mm) 0.05-0.1 mL/min (2.1 mm id)
1.9 µm	200 Å	2,000–700,000 Da	2–8.5	620 bar	

Real stories from the lab

Reducing complexity drives better lab economics

Learn how the CrossLab team helped a large pharma lab manage different types of instruments, from different manufacturers.

www.agilent.com/chem/story92

Tips and tools

Use Agilent solvent filters to remove particles from self-prepared buffers and mobile phases.

Visit: **www.agilent.com/chem/solvent-filters-degassers**

For recommended instrument configuration as well as initial mobile phase conditions with accompanying chromatograms, see the quick start guides for the AdvanceBio SEC 2.7 µm and 1.9 µm columns (publications **5994-4620EN** and **5994-4621EN**, respectively).

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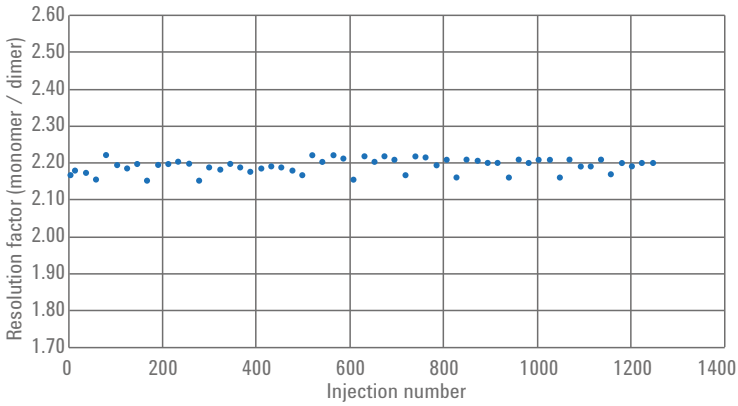
Long column lifetime with robust AdvanceBio SEC columns

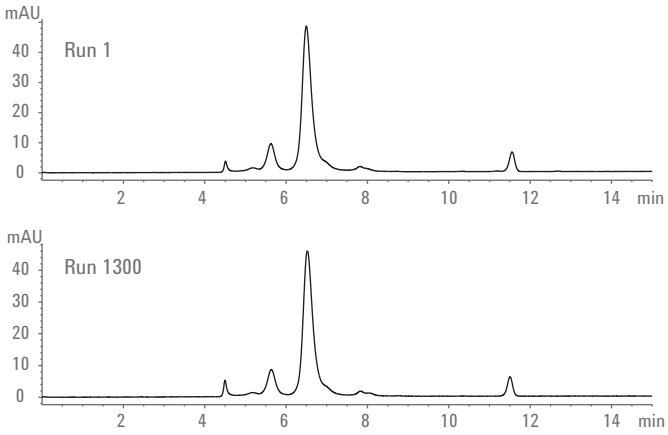
Column: **AdvanceBio SEC 2.7 µm 300 Å, 7.8 x 300 mm**

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.





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

Hydrophilic SEC media ideal for antibody: drug conjugate (ADC) analysis

Column: **AdvanceBio SEC 2.7 µm 300 Å, 7.8 x 300 mm**

Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC System

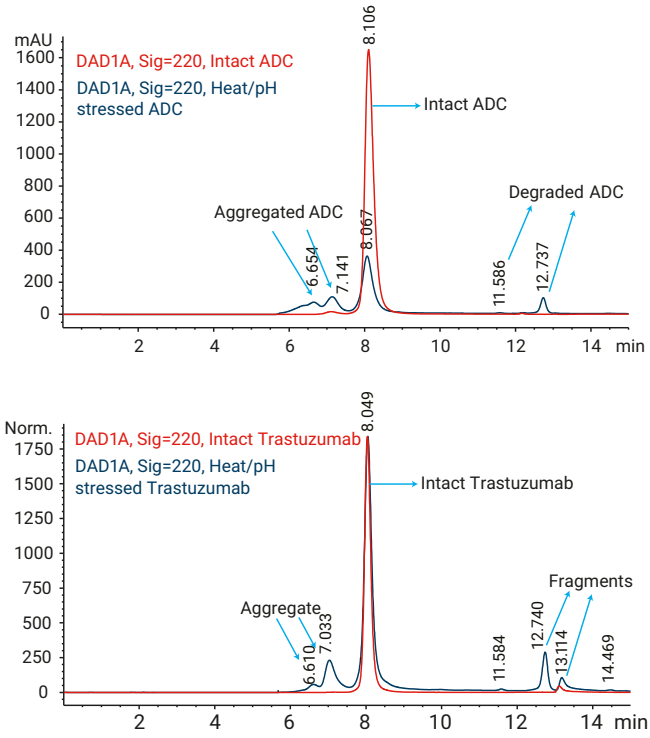
Mobile phase: PBS, 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4

TCC Temp.: Ambient

Injection: 10 µL

Flow rate:: 0.8 mL/min

Detector: UV, 220 nm



AdvanceBio SEC 2.7 µm Operating Parameters

Mobile Phase Compatibility	150 mM phosphate buffer, pH 7.0 (recommended starting conditions) Other aqueous buffers with high and low salt can be used. Mixtures of water and acetonitrile can be used. (Check solubility of buffer components and system pressure.)
pH Stability	2 to 8.5
Injection Volume	1–5 µL (recommended) Maximum 1% column volume
Operating Temperature	20–30 °C (recommended) 80 °C (maximum)
Typical Operating Pressure	< 200 bar (2,900 psi) (single column)
Maximum Pressure	400 bar (5,800 psi)
Working Flow Rate	0.1 to 2.0 mL/min for 7.8 mm i.d. columns (recommended) 0.1 to 0.7 mL/min for 4.6 mm i.d. columns (recommended) For two columns in series, lower flow rates may be necessary to ensure maximum pressure does not exceed 400 bar (5,800 psi).

Note: Working at extremes of the operating parameters is likely to reduce column lifetime.

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AdvanceBio SEC 1.9 µm Operating Parameters

Parameter	Value
Shipping Solvent/Long-Term Storage Solution	pH 6.7 100 mM sodium phosphate buffer with 0.02% NaN_3
Working Flow Rate	for 4.6 × 150 mm 0.1 to 0.7 mL/min for 4.6 × 300 mm 0.1 to 0.5 mL/min for 2.1 mm id columns 0.05 to 0.10 mL/min
Injection Volume	1–5 µL (recommended) Maximum 1% column volume
Maximum Pressure	620 bar (9,000 psi)
pH Stability	2 to 8.5
Salt Concentration	≤0.5 M
Mobile Phase Compatibility	Compatible with all the SEC mobile phases for UV. Phosphate buffer, pH 7.0 with different salt concentrations and denaturing and native mode SEC-MS mobile phases.
Operating Temperature	20 to 40 °C (recommended), 80 °C (maximum)

Note: Working at extremes of the operating parameters may reduce column lifetime.

High resolution, high throughput mAb separations

Column: **AdvanceBio SEC 1.9 µm 200 Å, 4.6 x 150 mm**

Instrument: Agilent 1260 Infinity II LC

Mobile phase: 50 mM sodium phosphate, 200 mM NaCl, pH 7.0

Column Temp.: 25 °C

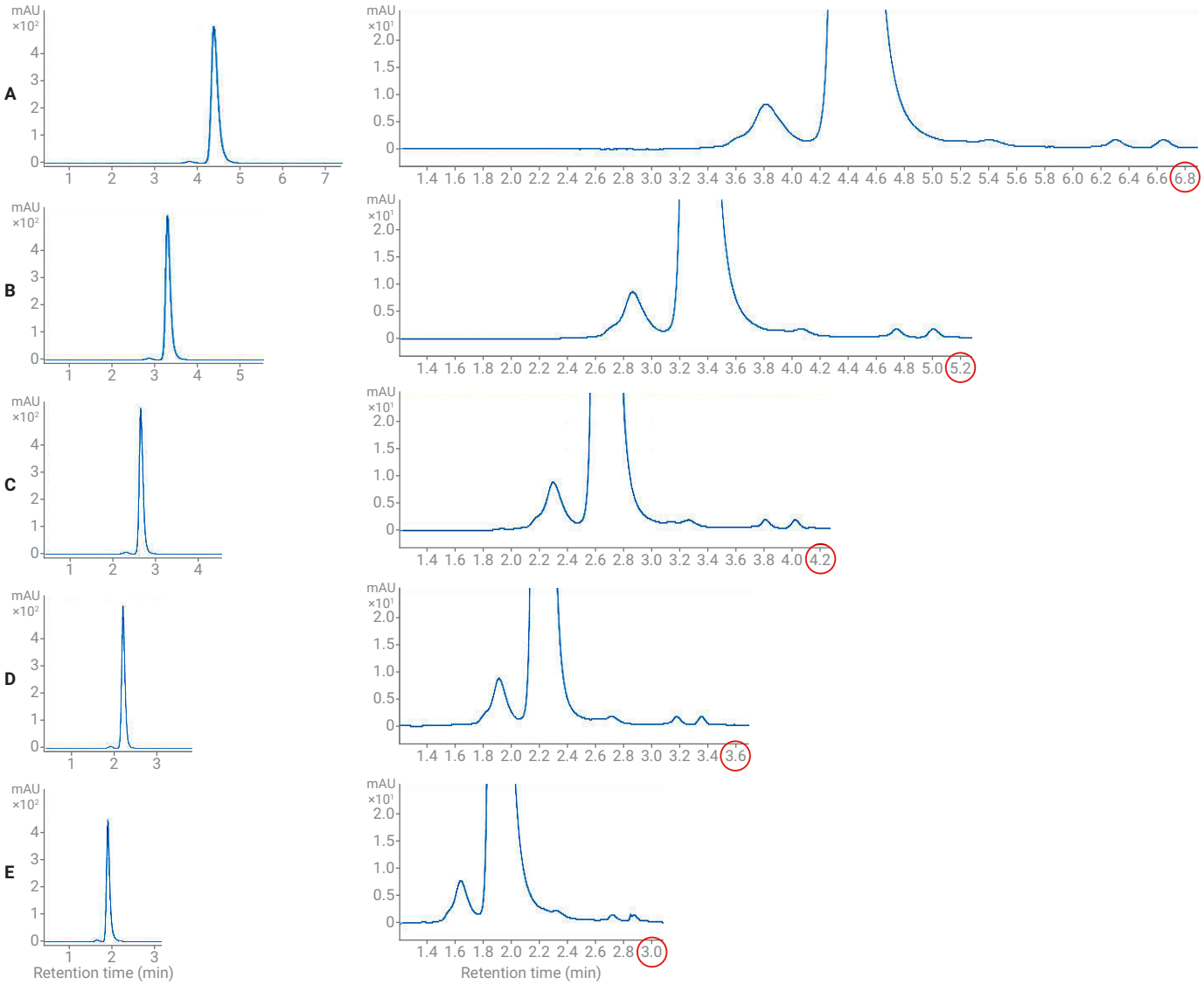
Injection: 1 µL

Flow rate: 0.3 to 0.7 mL/min

Detector: UV, 220 nm

Effect of flow rate on resolution, monomer area percentage, and sample throughput

Flow Rate (mL/min)	Run time (min)	Backpressure (bar)	Resolution (Dimer/Monomer)	Dimer Area %	Samples Per Hour	Samples Per Day (24 hours)
0.3	6.8	164	1.81	2.33	8-9	211
0.4	5.2	218	1.79	2.35	11-12	276
0.5	4.2	272	1.78	2.35	14	342
0.6	3.6	324	1.77	2.39	16-17	400
0.7	3.0	380	1.58	2.30	20	480



Size exclusion chromatograms of SigmaMAb using 4.6 × 150 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.3 mL/min; B) 0.4 mL/min; C) 0.5 mL/min; D) 0.6 mL/min; E) 0.7 mL/min.

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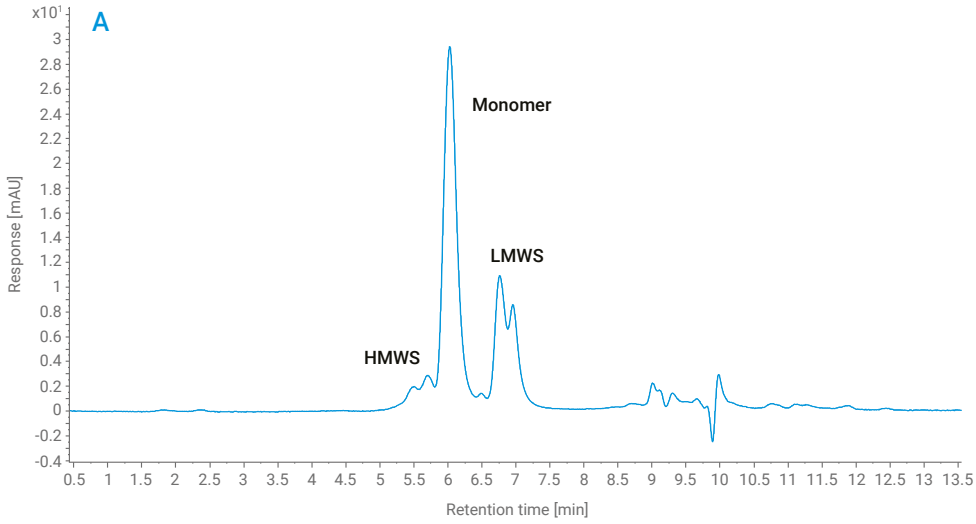
Size exclusion separations of human growth hormone

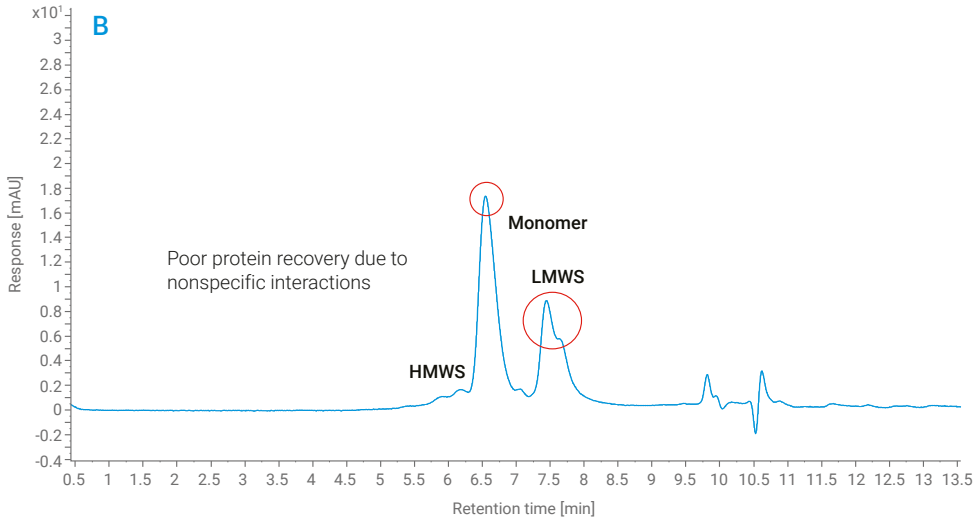
Instrument:	Agilent 1260 Infinity II bio-inert LC system
Software:	Agilent OpenLab CDS
Flow rate:	0.35 mL/min
Eluent:	150 mM phosphate, pH 7
Sample:	Concentration 1 mg/mL
Temperature:	25 °C
Injection volume:	2 µL
Detection:	UV, 220 nm

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

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



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



Bio SEC

A versatile portfolio providing 100 Å to 2000 Å pores, and dimensions up to 21.2 mm id, Agilent Bio SEC supports a range of protein sizes and injection volume requirements.

Agilent Bio SEC columns offer exceptional resolution for larger biomolecules with improved peak capacity and resolution due to specially designed packing that increases pore volume.

Bio SEC features

- High resolution: sharper peaks and better protein recovery
- Exceptional loading capacity and recovery due to proprietary hydrophilic layer
- 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: 100 Å up to 2000 Å pore size for small proteins, vaccines, and high molecular weight biomolecules
- Robust particles compatible with multi-detectors including light scattering
- MS-compatible

Bio SEC-3 columns are 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.

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

Column Specifications

Pore Size	Particle Size	Mol Wt Range	pH Range	Max Pressure
100 Å	3 µm	100–100,000	2–8.5	137 bar, 2000 psi
150 Å	3 µm	500–150,000	2–8.5	137 bar, 2000 psi
300 Å	3 µm	5,000–1,250,000	2–8.5	137 bar, 2000 psi
100 Å	5 µm	100–100,000	2–8.5	137 bar, 2000 psi
150 Å	5 µm	500–150,000	2–8.5	137 bar, 2000 psi
300 Å	5 µm	5,000–1,250,000	2–8.5	137 bar, 2000 psi
500 Å	5 µm	15,000–5,000,000	2–8.5	137 bar, 2000 psi
1000 Å	5 µm	50,000–7,500,000	2–8.5	137 bar, 2000 psi
2000 Å	5 µm	>10,000,000	2–8.5	137 bar, 2000 psi

Flow Rate

Column ID	Recommended Flow Rate
4.6 mm	0.1-0.4 mL/min
7.8 mm	0.2-1.2 mL/min
21.2 mm	1.0-10.0 mL/min

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Pore size choice

The choice of media pore size will influence the resolution in SEC. As the separation is based on differences in molecular size in solution, the sample must be able to permeate the porous structure of the particles. If the pore size is too small, the samples will be excluded from the pores and elute in the void volume of the column, and if too large, then all will be able to fully permeate the particles and so there will be very little separation.

Column A: **Bio SEC-3, 100 Å**
5190-2503
4.6 x 300 mm, 3 µm

Column B: **Bio SEC-3, 150 Å**
5190-2508
4.6 x 300 mm, 3 µm

Column C: **Bio SEC-3, 300 Å**
5190-2513
4.6 x 300 mm, 3 µm

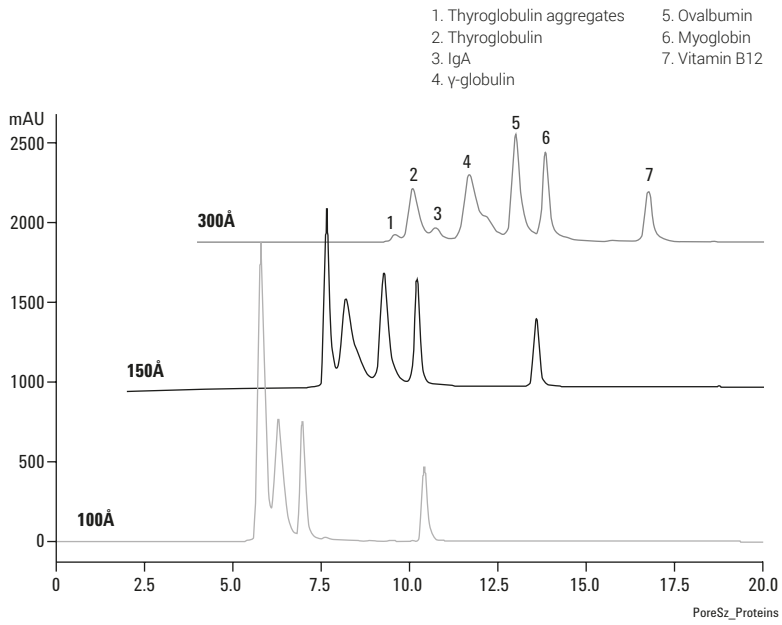
Mobile phase: Sodium phosphate 100 mH, sodium chloride 150 mM, pH 6.8

Flow rate: 0.35 mL/min

Gradient: 10–58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B

Detector: UV, 220 nm

Sample: Bio-Rad gel filtration standards mix



Tips and tools

Deactivated/silanized vials have inert surfaces that will not interact with metals, biologicals or proteins, and will not cause pH shifts. Avoid standard polypropylene vials for biological or light-sensitive compounds.

Visit our **vials and closures** shop to boost your lab productivity.

Comparing Bio SEC-3 and Bio SEC-5

Analysis of monoclonal antibody

Column: **Bio SEC-3, 300 Å**
5190-2511
7.8 x 300 mm, 3 µm

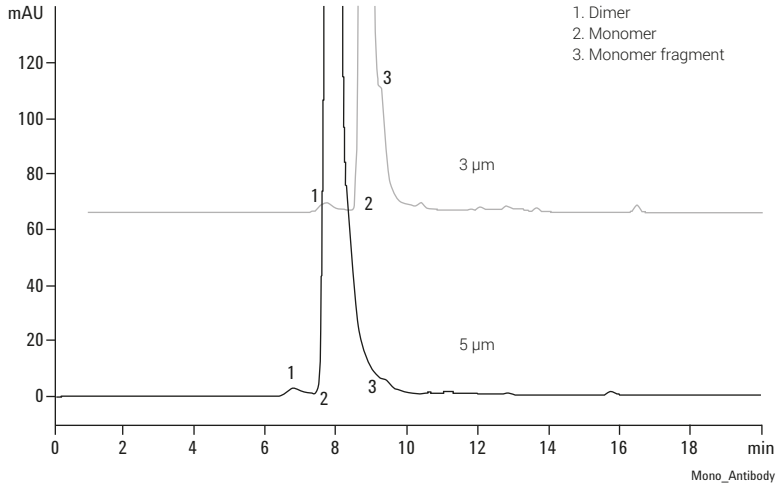
Column: **Bio SEC-5, 300 Å**
5190-2526
7.8 x 300 mm, 5 µm

Mobile phase: Sodium phosphate 150 mM, pH 7.0

Flow rate: 1 mL/min

Detector: UV, 220 nm

Sample: Humanized monoclonal antibody



The 3 µm column gives higher definition of the fragmentation plan.

Tips and tools

There are many things to consider when developing aggregation analysis for your proteins; the effect of solute size and molecular weight, column selection choices, important mobile phase considerations, and more. For a guide on all of the above, see:

Size exclusion chromatography for biomolecule analysis: A "How to" guide (publication **5991-3651EN**)

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Calibration curves—Bio SEC-5

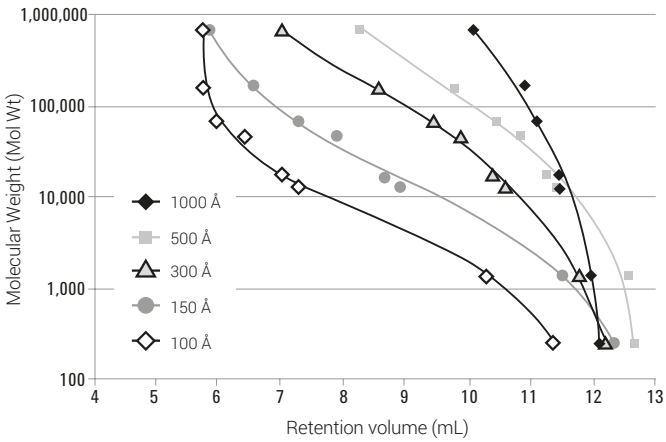
Column: **Bio SEC-5**
7.8 x 300 mm, 5 µm

Mobile phase: Sodium phosphate 150 mM, pH 7.0

Flow rate: 1.0 mL/min

Detector: UV, 214 nm

		Retention Volume				
Proteins	**Mol Wt**	**1000 Å**	**500 Å**	**300 Å**	**150 Å**	**100 Å**
Thyroglobulin	670,000	10.07	8.23	7.03	5.82	5.77
γ-Globulin	150,000	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	17,00 0	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 B12	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



Virus-like particle aggregate analysis

Columns: **Agilent Bio SEC-5, 7.8 × 300 mm, 5 µm, 2000 Å (p/n 5190-2541)**
Agilent Bio SEC-5, 7.8 × 300 mm, 5 µm, 1000 Å (p/n 5190-2536)

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

Flow rate: 0.6 mL/min

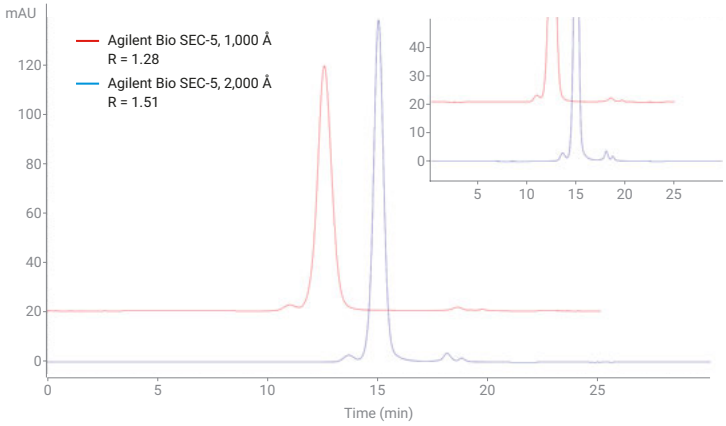
Column Temperature: Room temperature

Sample Volume: 5 µL

Detection Wavelength: 220 nm

Run Time: 30 min

HPLC System: Agilent 1260 Infinity II LC system with quaternary pump



The same virus-like particle was analyzed using Agilent Bio SEC-5, 2,000 Å and Bio SEC-5, 1,000 Å columns.

PROTEEMA columns

PROTEEMA columns are well suited for aqueous GPC/SEC analysis of natural and synthetic proteins, peptides, enzymes, and gelatins/collagens with molecular weights ranging from 100 to 7,500,000 Da. Multiple column configurations are available to match your specific application, including two particle sizes, 3 and 5 µm, three porosities, 100, 300, and 1,000 Å, and both stainless steel and bio-inert hardware options. This diol SEC phase is suitable for separations requiring USP designation L20.

Column Specifications

Particle Size	Pore Size	Molecular Weight Range*	pH Range	Max Pressure	Max Temperature	Max Flow Rate
3 or 5 µm	100 Å 300 Å 1000 Å	100-150,000 Da 1000-1,200,000 Da 1000-7,500,000 Da	2-8	200 bar (3 µm) 150 bar (5 µm)	70 °C	3 mL/min

*Based on proteins

Calibration curves–pullulan (buffer)

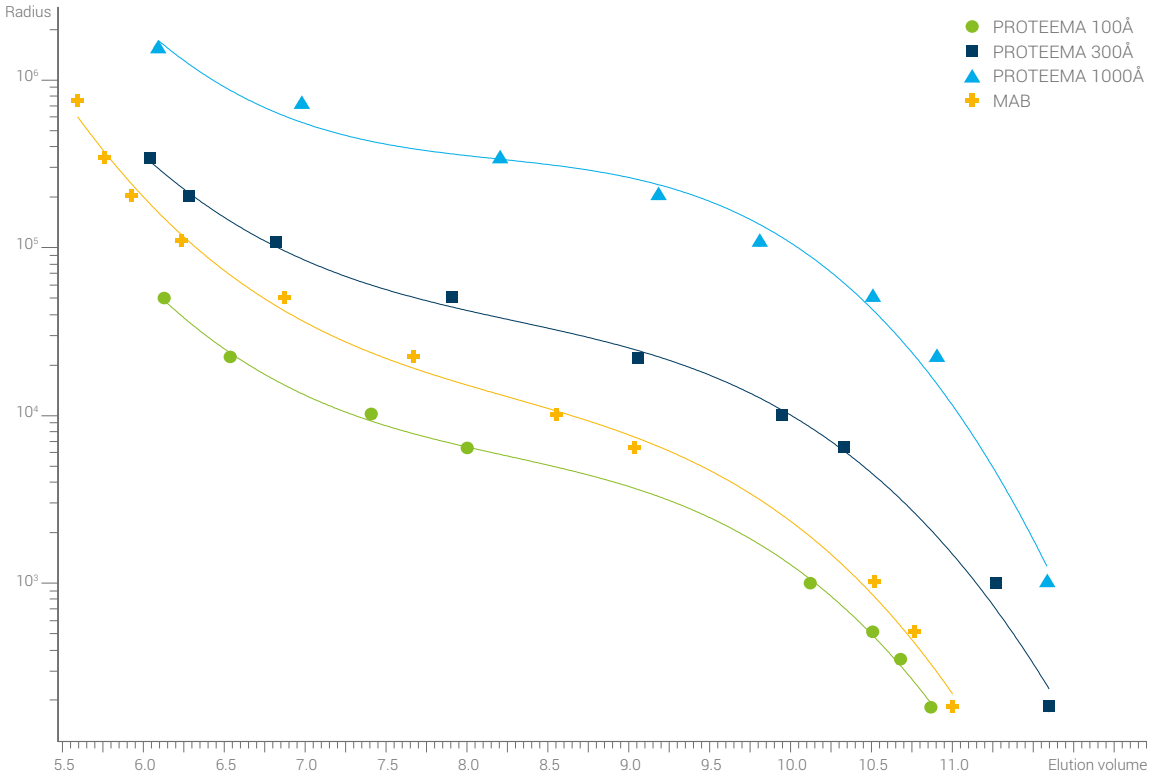
Column: **PROTEEMA 100 Å, 300 Å, 1000 A**
MAB, each 8 x 300 mm, 3 µm

Mobile phase: Phosphate buffer pH 6.6 (34 mmol) + 0.5M NaCl

Flow rate: 1 mL/min

Temperature: 23 °C

Detector: RI



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MAB

MAB columns are useful for the aqueous GPC/SEC analysis of monoclonal antibodies, IgG, and proteins with molecular weights ranging from 100 to 1,000,000 Da. The mixed bed was designed for separation of monoclonal antibody aggregates and fragments and optimized and pre-equilibrated for quick use with light scattering detection. These silica-based, diol SEC columns are categorized as USP designation L20. MAB columns are available in 3 µm particle size to best fit your protein analysis.

Column Specifications

Particle Size	Pore Size	Molecular Weight Range	pH Range	Max Pressure	Max Temperature	Max Flow Rate
3 µm	Mixed	100-1,000,000 Da	2-8	150 bar	70 °C	3 mL/min

Aggregate analysis of immunoglobulin G

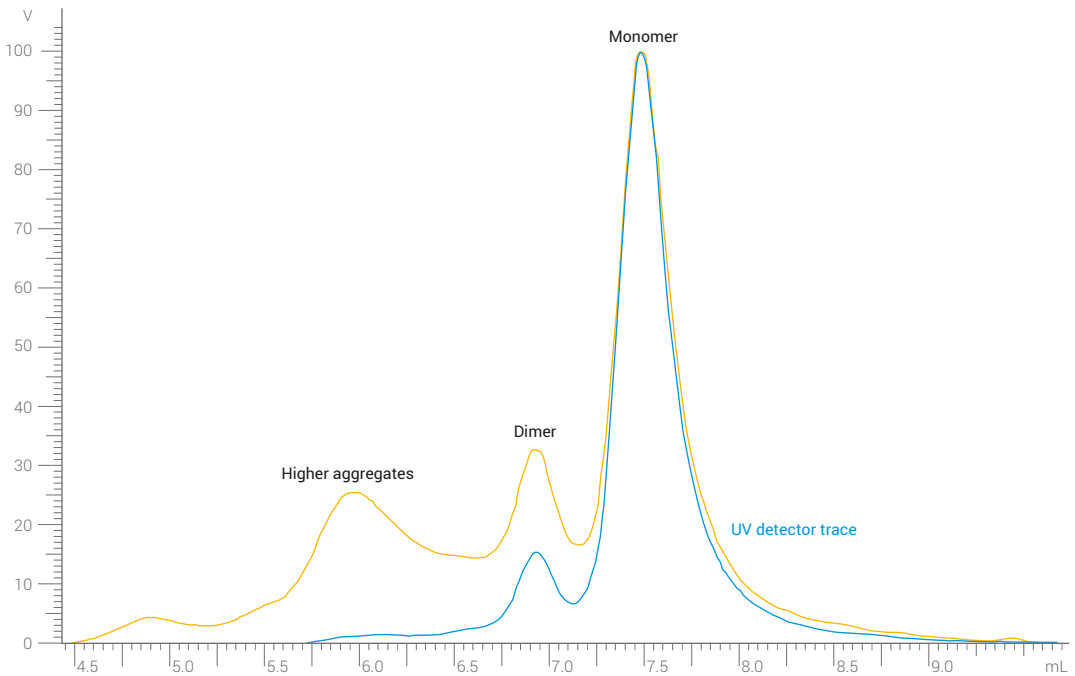
Column: **MAB, 8 x 300 mm, 3 µm**

Flow rate: 1 mL/min

Sample concentration: 2.5 g/L

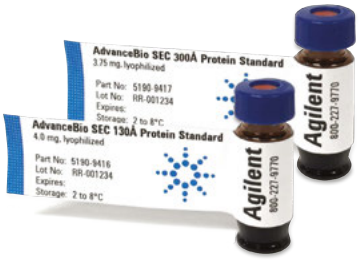
Injection volume: 20 µL

Detection: UV, 280 nm
MALLS (90°)



Low concentration of higher aggregates, detected using MALLs (yellow signal)

Protein Standards for SEC



AdvanceBio SEC protein standards, p/n 5190-9416 and p/n 5190-9417

130 Å AdvanceBio SEC protein standards

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

300 Å AdvanceBio SEC protein standards

A protein mix consisting of five carefully selected proteins (thyroglobulin, g-globulin, ovalbumin, myoglobin, angiotensin II) designed to calibrate Agilent's 300 Å or 200 Å AdvanceBio size exclusion columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

Tips and tools

Regularly checking your HPLC system with a well characterized performance standard increases confidence in results and helps identify problems quickly. While many scientists use a mixture of proteins like the AdvanceBio SEC standards, some people also use a monoclonal antibody standard. **Agilent-NISTmAb** is available in small aliquots for convenience.

See the aggregation analysis chapter (publication **5994-2069EN**) of the Agilent NISTmAb application compendium (publication **5994-1501EN**) for sample methods and chromatograms.

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SEC molecular weight standard separations

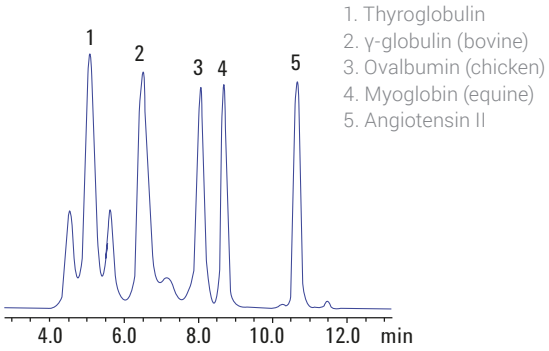
Column: **AdvanceBio SEC 2.7 µm 7.8 x 300 mm**

Sample: AdvanceBio SEC Protein Standards

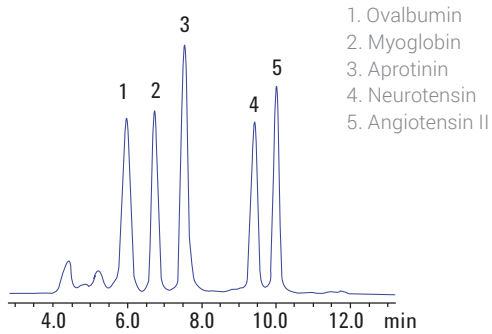
Mobile phase: 150 mM sodium phosphate, pH 7.0

Flow rate: 1.0 mL/min

Agilent AdvanceBio SEC Protein Standards



AdvanceBio SEC 300Å Protein Standard separation on AdvanceBio SEC 300Å column



AdvanceBio SEC 130Å Protein Standard separation on AdvanceBio SEC 130Å column

Tips and tools

Column user guides are excellent resources, with instructions for use and column care, as well as suggested starting methods:

www.agilent.com/chem/biolc-columns-user-guides

Protein standards kits

Kitted protein standards enable users to choose the best protein standards for a given analysis or troubleshooting circumstance. PSS-PROKIT contains 10 individually bottled, lyophilized standards ranging in molecular weight from 243 Da to 670 kDa. PSS-PROKITR1 contains five each of three different vials, containing 3-4 lyophilized standards already mixed. These kits give users the flexibility to design their own mixture, troubleshoot with individual proteins, or use ready-mixed combinations of fully resolved standards.

PSS-PROKIT components

Component Name	Molecular Weight	Hydrodynamic Radius (nm)	Quantity
Cytidine	243 Da		1 x 0.1 g
Vitamin B12	1.4 kDa		1 x 0.1 g
Aprotinin	6.5 kDa		1 x 0.1 g
Cytochrome C	12 kDa	1.5	1 x 0.1 g
Myoglobin	17.5 kDa	1.8	1 x 0.1 g
B-lactoglobulin	35 kDa	2.8	1 x 0.1 g
Albumin (chicken)	44 kDa	3.5	1 x 0.1 g
Albumin (bovine)	67 kDa	4.4	1 x 0.1 g
Gamma-globulins	158 kDa	5.8	1 x 0.1 g
Thyroblobulin	670 kDa	9.1	1 x 0.1 g

Tips and tools

Our **application method ordering guides** make it easy to find the exact consumables you need for your analysis without spending time digging through multiple catalogs.

Check out ordering guides for the analysis of **mAb dimers and fragments**, or for **SEC-MS**, to find all the tools you need, plus useful resources such as selection criteria, application notes, and best practice tips.

ReadyCal-Kit components

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

Overlay of ReadyCal Kit Protein Standards

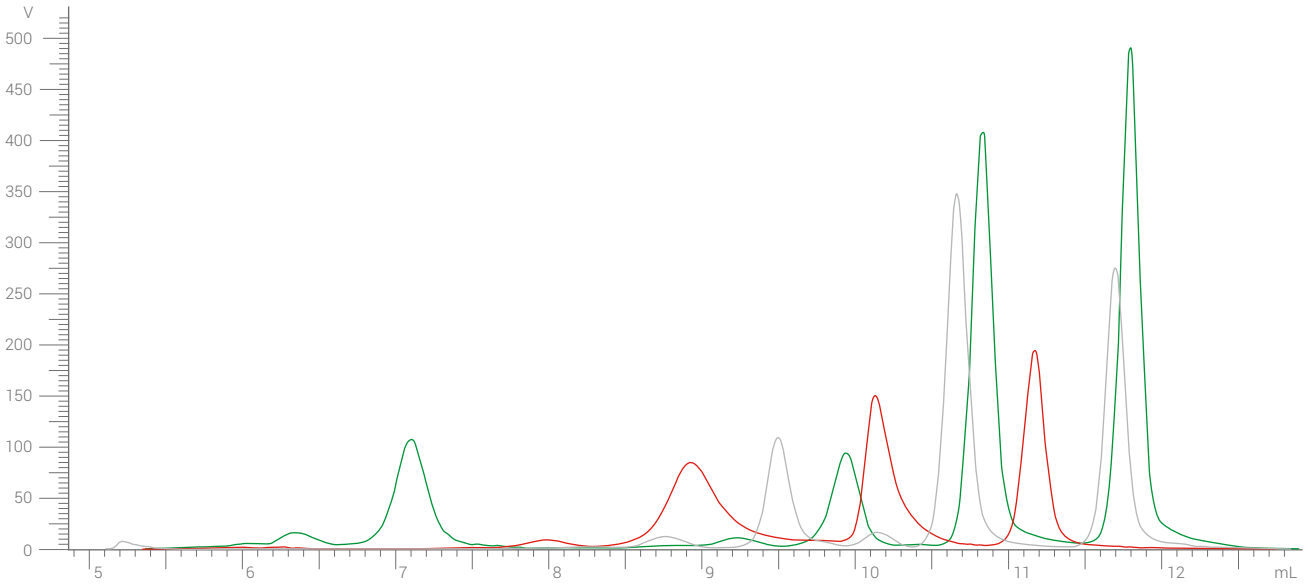
Column: **PROTEEMA 300 A, 8 x 300 mm, 3 µm**

Mobile Phase: Phosphate buffer pH 6.6 (34 mmol), 0.5M NaCl

Flow rate: 1 mL/min

Temperature: 23 °C

Detection: UV, 280nm



Product ordering information

AdvanceBio SEC Columns, 2.7 µm

Dimension (mm)	Particle Size (µm)	130 Å	300 Å
4.6 x 300	2.7	PL1580-5350	PL1580-5301
4.6 x 150	2.7	PL1580-3350	PL1580-3301
4.6 x 50, guard	2.7	PL1580-1350	PL1580-1301
7.8 x 300	2.7	PL1180-5350	PL1180-5301
7.8 x 150	2.7	PL1180-3350	PL1180-3350
7.8 x 50, guard	2.7	PL1180-1350	PL1180-1301

AdvanceBio SEC Columns, 1.9 µm

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

Bio SEC-3 Columns

Dimension (mm)	Particle Size (µm)	Bio SEC-3 100 Å	Bio SEC-3 150 Å	Bio SEC-3 300 Å
21.2 x 300	3	5190-6850	5190-6851	5190-6852
21.2 x 50, guard	3	5190-6854	5190-6855	5190-6856
7.8 x 300	3	5190-2501	5190-2506	5190-2511
7.8 x 150	3	5190-2502	5190-2507	5190-2512
7.8 x 50, guard	3	5190-2505	5190-2510	5190-2515
4.6 x 300	3	5190-2503	5190-2508	5190-2513
4.6 x 150	3	5190-2504	5190-2509	5190-2514
4.6 x 50, guard	3	5190-6846	5190-6847	5190-6848

Bio SEC-5 Columns

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

PROTEEMA columns

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

MAB columns

Dimensions (mm)	Particle Size (µm)	Stainless Steel	Bio-Inert
4.6 x 30, guard	3	MAM050303	MAM050303BI
4.6 x 250	3	MAM052503MC	MAM052503MCBI
8 x 50, guard	3	MAA080503	MAA080503BI
8 x 300	3	MAA083003MC	MAA083003MCBI

AdvanceBio SEC Standards

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

PSS Protein Standards

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

ZORBAX GF-250 and GF-450 gel filtration columns

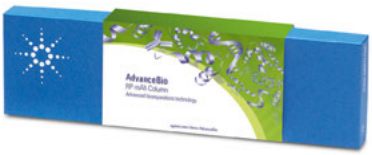
Description	Dimensions (mm)	Particle Size (µm)	Part No.
GF-250, 150 Å	9.4 x 250	4	884973-901
GF-250, 150 Å	4.6 x 250	4	884973-701
GF-450, 300 Å	9.4 x 250	6	884973-902
Guard Columns (hardware required)			
GF-450 Diol, guard cartridge, 2/pk	9.4 x 15	6	820675-111
GF-250 Diol, guard cartridge, 4/pk	4.6 x 12.5	6	820950-911
GF-450 Diol, guard cartridge, 2/pk	9.4 x 15	6	820675-111
Prep guard hardware kit			840140-901
Guard hardware kit			820999-901
PrepHT Columns			
PrepHT GF-250, 150 Å	21.2 x 250	6	877974-901
PrepHT GF-450, 300 Å	21.2 x 250	6	877974-910
PrepHT endfittings, 2/pk			820400-901
PrepHT guard cartridge, 2/pk	17.0 x 7.5	5	820212-911
Guard cartridge hardware			820444-901

Tips and tools

Update your SEC technology to AdvanceBio SEC 300 Å for higher resolution and fewer secondary interactions.

See **page 97** for more information.

Glycosylation Characterization



The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA).

N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD), and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation.

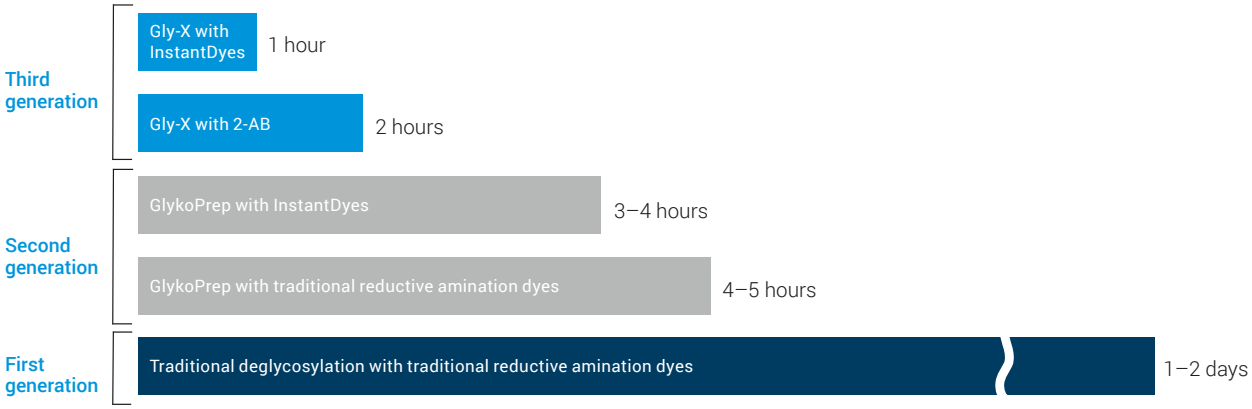
There are a number of analytical methods that are used to obtain information about the structure and form of protein glycosylation.

Methods for characterization of glycosylation

- For structural identification, including identification of glycosylation sites, mass spectrometry detection is used with reversed-phase and hydrophilic interaction chromatography (HILIC).
- For released N-glycan analysis, glycans are derivatized with a fluorescent label to allow fluorescence detection and separated by HILIC.
- The sialic acid containing glycans will also impart more charge to the protein and can be characterized by ion-exchange chromatography. Agilent also offers solutions enabling preparation of fluorescently-labeled released sialic acids for LC/FLD or colorimetric analysis.

Having characterized the glycoprotein and glycopeptide fragments to obtain information about the number and position of the glycosylation sites, it is then necessary to identify and quantify the individual glycans. To do this, the glycans must be cleaved from the protein and analyzed using HILIC columns. As glycans have no chromophore, derivatization with a fluorophore is carried out to enable FLD detection to characterize and quantify the glycans.

In addition to columns, software, and instrumentation, Agilent also offers a full line of N-glycan and sialic acid sample preparation kits as well as tools to aid in N-glycan identification such as enzymes, standards, and libraries.



The evolution of N-glycan sample preparation (times are shown on the right).

Hydrophilic Interaction Column Selection

Application	Agilent Columns	Notes	
Glycans cleaved from a glycoprotein including monoclonal antibodies	AdvanceBio Glycan Mapping	Amide bonded phase for rapid equilibration and enhanced selectivity for glycans.	AB
	1.8 µm	Based on a fully porous particle for high-speed separations and high throughput applications. Stability to 1200 bar for use with the 1290 Infinity II LC.	
	2.7 µm	Based on Poroshell technology to give a superficially porous particle with reduced diffusion distances. This gives high-resolution separations at lower pressures, and enables the use of longer column lengths for increased separation efficiency.	
Hydrophilic peptides and glycopeptides	ZORBAX RRHD 300 Å, 1.8 µm	A 300 Å silica particle to provide an orthogonal separation to the ZORBAX RRHD 300 Å, 1.8 µm reversed-phase columns.	AB
	AdvanceBio Glycan Mapping	The amide bonded phase provides an alternative HILIC functionality for small hydrophilic peptides and glycopeptides.	

AB Part of the AdvanceBio family

N-glycan sample preparation

AdvanceBio Gly-X technology

Agilent AdvanceBio Gly-X (formerly ProZyme) is a next generation N-glycan preparation platform that provides a simplified in-solution workflow. Combined with InstantPC dye along with an efficient vacuum plate cleanup step to remove excess label and denaturant, samples are ready for UHPLC or LC/MS in 60 minutes or less.

Tips and tools

Learn more about N-glycan sample preparation

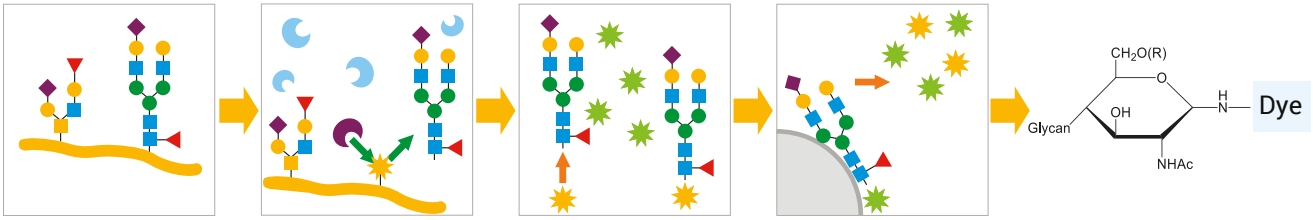
Workflow ordering guide – N-Glycan Analysis of Biotherapeutic Glycoproteins (publication **5994-3926**)

Workflow ordering guide – N-Glycan Analysis of Biotherapeutic Glycoproteins Using AdvanceBio Gly-X 2-AB (publication **5994-4158**)

Application note – Gly-X InstantPC vs Waters RapiFluor-MS comparison (publication **5994-5653**)

Application note – Streamlined Workflows for N-Glycan Analysis of Biotherapeutics with LC/FLD/MS (publication **5994-1348**)

Gly-X N-glycan sample preparation workflow



Denature
3 min at 90 °C

Deglycosylate
PNGase F,
5 min at 50 °C

Label
InstantDye, 1 min
5 min at 50 °C
InstantPC
Reductive amination
dye, 60 min (no
dry down) 2-AB

Clean up
15-20 min at RT

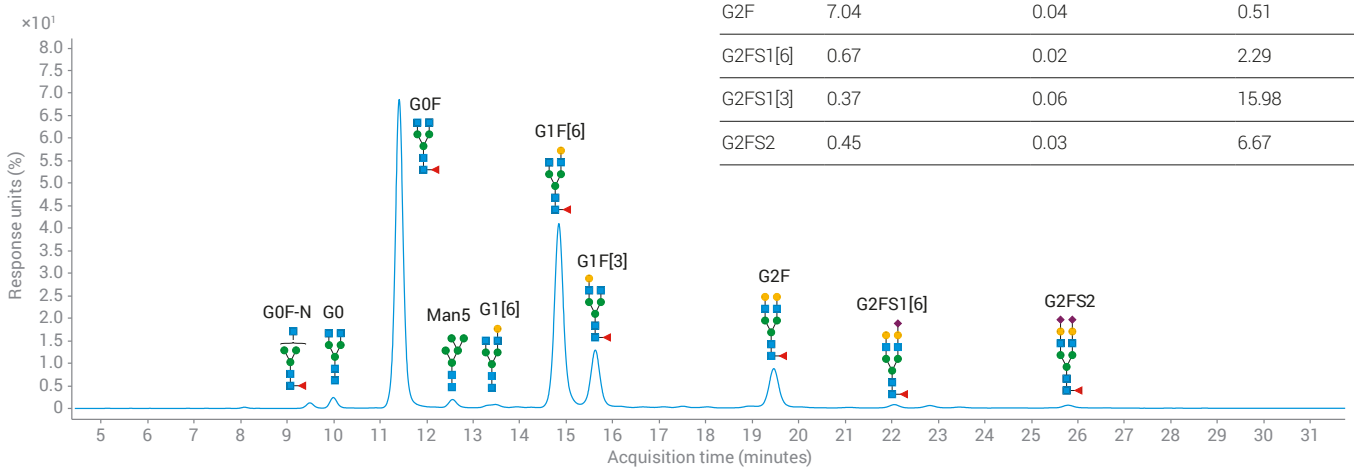
Labeled N-glycans
LC-FLR, LC-MS, CE

Gly-X N-glycan sample preparation workflow. The recommended starting sample amount is 1 to 40 µg, which is a higher maximum sample amount than similar workflows. You may also be able to use more protein, depending on the molecule. For further sample considerations, please see the individual Gly-X product manuals.

Rituximab N-glycans, Gly-X InstantPC

Relative %area, SD, and %CV values for Rituxan N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67



GlykoPrep and GlykoPrep-plus N-glycan sample preparation

Released in 2012, ProZyme GlykoPrep solid phase cartridges were the first platform to use “instant” glycan labeling. The cartridges streamlined and standardized N-glycan sample preparation in both spin and automation (AssayMAP Bravo) formats. Reproducibility was demonstrated in two interlaboratory studies using LC and CE.

GlykoPrep-plus kits enable automation with the Agilent AssayMAP Bravo to provide out-of-the-box automateion for increase precision and walk-away time. Although GlykoPrep has been superseded by Gly-X, we continue to support existing GlykoPrep customers.

Traditional Methods for N-glycan sample preparation

Older N-glycan sample preparation workflows include native or denaturant-driven digestion with PNGase F, purification of released glycans, labeling with a fluorophore, and purification of labeled glycans. These workflows take 1 to 2 days, and are not suitable for high-throughput applications or automation.

With the introduction of Gly-X, we can help you transition from a 2-AB workflow to faster sample preparation techniques with higher throughput. However, we will continue to support traditional workflows with a range of glycan labeling and cleanup tools.

AdvanceBio sialic acid profiling and quantitation

Sialic acid analysis simplified – with options for profiling and quantitation of sialic acids, Agilent has your sialic acid analysis needs covered.

Tips and tools

Learn more about the Agilent AdvanceBio sialic acid profiling and quantitation

Sialic Acid Analysis of Biotherapeutic Glycoproteins Using AdvanceBio Sialic Acid Profiling and Quantitation Kit and LC/FLD/MS (publication **5994-4201EN**)

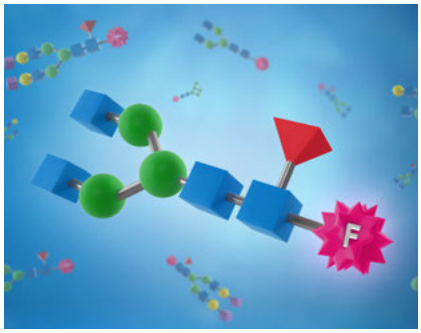
Total Sialic Acid Quantitation of Biotherapeutic Glycoproteins (publication **5994-4383EN**)

Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit – Rapid quantitation of NANA and NGNA species (publication **5994-2788EN**)

Agilent AdvanceBio Sialic Acid Quantitation Kit – Streamlined sample processing (publication **5994-2789EN**)

An Improved Workflow for Profiling and Quantitation of Sialic Acids in Biotherapeutics (publication **5994-2352EN**)

Glycobiology standards and libraries



Glycan standards to help you achieve Trusted Answers - N-glycan standards and libraries available unlabeled or pre-labeled with InstantPC, 2-AB, InstantAB, APTS, and 2-AA.

Many common glycan types seen in biotherapeutics are covered, including complex biantennary neutral and sialylated, high mannose, and alpha gal. You can also choose from several glycan libraries for glycoproteins such as:

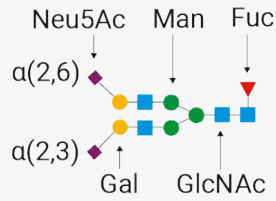
- Human IgG
- Bovine RNase B
- Bovine fetuin
- Human α1-acid glycoprotein (AGP)
- Recombinant monoclonal IgG (mAb) made in Chinese hamster ovary (CHO) cells
- Triantennary and tetraantennary sialylated N-glycan libraries

In addition, we offer standards containing either α(2,3)- or α(2,6)-linked sialic acids:

- The α(2,3) sialic acid linkage is found on glycoproteins produced in CHO cells. Glycans with α(2,3) sialylation have shorter HILIC retention times than isomeric N-glycans with α(2,6) sialic acid linkages.
- The α(2,6) sialic acid linkage is found on glycoproteins such as human intravenous immunoglobulin (IVIG)

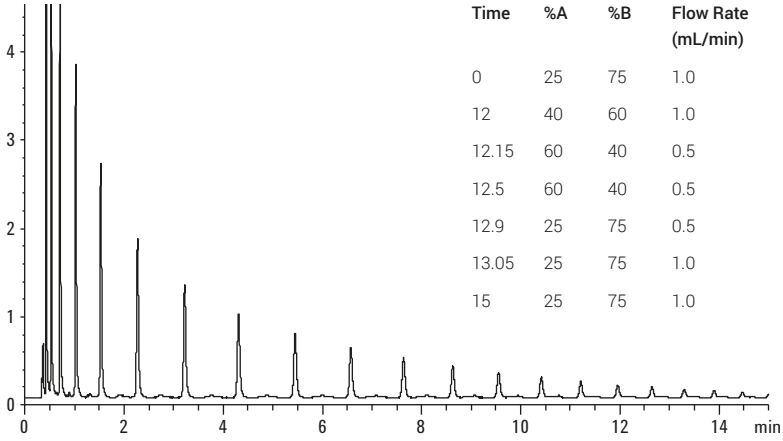
See glycan standards selection guide **5994-2202EN**

Glycan structure key



Separation of a 2-AB labeled dextran ladder

Column:	**AdvanceBio Glycan Mapping 859700-913 2.1 x 150 mm, 1.8 µm**
Mobile phase:	A: 100 mM NH4Fc, pH 4.5 B: ACN
FLD:	Excitation = 260 nm Emission = 430 nm
Injection volume:	2 µL (10 pmol total glycan/1 µL 75:25 ACN:water)
Sample:	2-AB (p/n GKSB-503) labeled dextran ladder



Time	%A	%B	Flow Rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

This analysis uses the Agilent dextran ladder standard, together with an AdvanceBio Glycan Mapping column to correlate retention times of unknown glycans.

Glycobiology enzymes

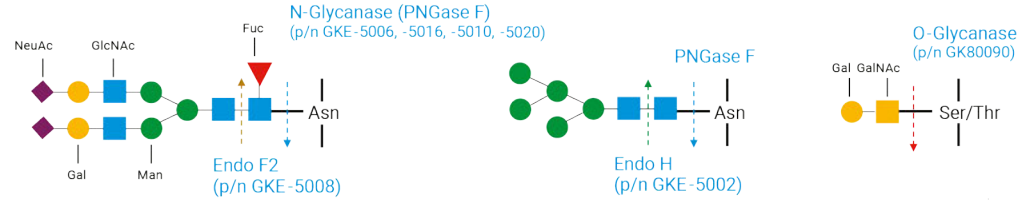
Agilent now offers a variety of glycoenzymes to support your released glycan and other analytical workflows. A selection of our enzymes is shown in Figure 8. Please visit the Agilent website for our full range.

- Endoglycosidases cleave within a glycan structure. N-glycanase (PNGase F, technically an asparagine amidase) is widely used to study released glycans and to generate de-N-glycosylated protein, because it releases most intact N-glycans.
- Exoglycosidases cleave exposed or "terminal" monosaccharide residue from glycans. Commonly used exoglycosidases include galactosidase for degalactosylation and sialidase (neuraminidase) for desialylation of released glycans, glycoproteins, or cells.

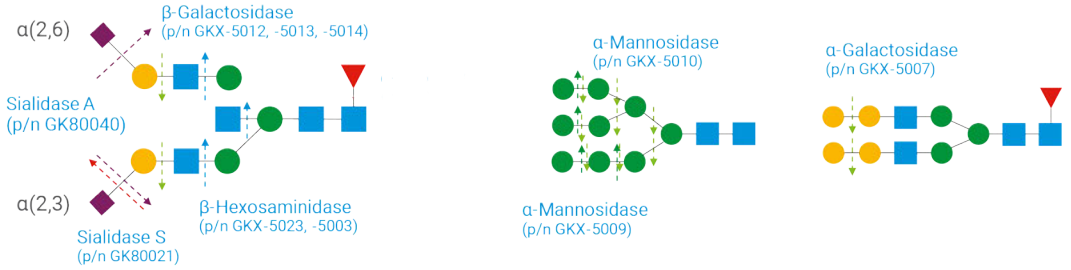
See the enzyme selection guide, publication number **5994-2202EN**

Specificities for selected endoglycosidases (A) and exoglycosidases (B)

A. Endoglycosidases



B. Exoglycosidases



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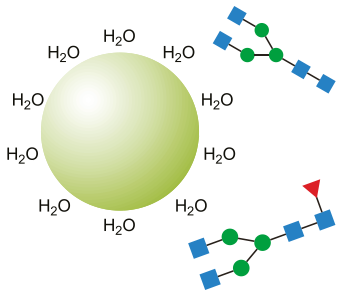
AdvanceBio Glycan Mapping columns



AdvanceBio Glycan Mapping columns, standards, and sample preparation products for the selective removal of the N-glycans from a glycoprotein, including monoclonal antibodies.

Speed of analysis—1.8 µm columns provide high throughput N-glycan analysis where speed is the primary concern either due to the number of samples or to the immediate requirement for data.

Resolution—high resolution separations are achieved using the 2.7 µm particles packed in the 250 mm column. This increased resolution enables accurate quantitation of target glycans and changes to the protein glycosylation profile, which may have occurred during expression.

Comprehensive methods—for sample preparation, chromatographic analysis, and data interpretations to ensure reproducibility, and accuracy of identification and quantitation

Simplicity of ordering—a single part number to order the full sample preparation workflow for protein solubilization to purification of 2-AB labeled glycans, plus kits for each part of the sample preparation workflow for versatility.

Column Specifications

Bonded Phase	Inner Diameter (mm)	Particle	Endcapped	pH Stability	Operating Temperature	Pressure Limit
Amide HILIC	2.1 and 4.6	1.8 µm, fully porous	No	2–7	40 °C	1200 bar
Amide HILIC	2.1 and 4.6	2.7 µm, superficially porous	No	2–7	40 °C	600 bar

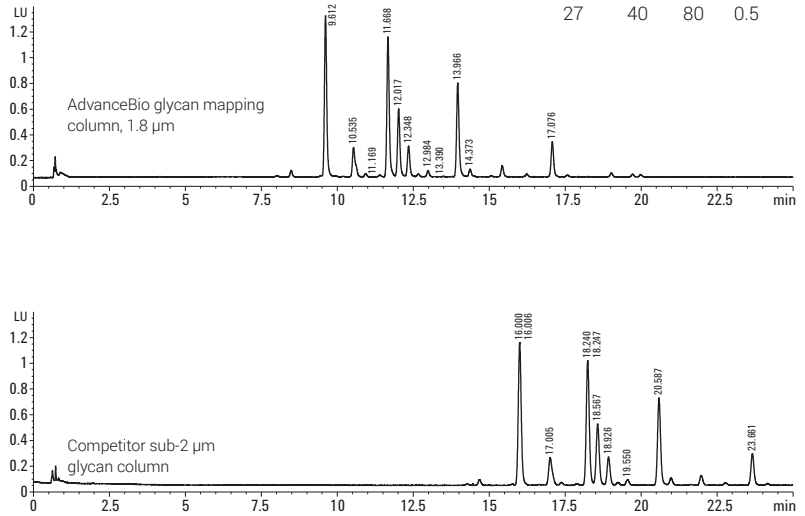
Speed of analysis

The AdvanceBio Glycan Mapping 1.8 µm columns are recommended for high throughput analysis where short run times are required.

Superior results—in 40% less time than the competition

Column:	**AdvanceBio Glycan Mapping 859700-913 2.1 x 150 mm, 1.8 µm**
Column B:	Competitor sub-2 µm glycan column
Mobile phase:	100 mM NH_4Formate, pH 4.5 B: ACN
Injection volume:	2 µl in 70:30 ACN: 100 mM NH_4Formate
Sample thermostat:	10 °C
FLD:	Excitation = 260 Emission = 430
Flow rate:	0.35 mL/min
Instrument:	1290 Infinity LC with 1260 Infinity fluorescence detector (FLD)
Sample:	2-AB labeled N-linked Human IgG glycan library (p/n GKSB-005)

Time	%A	%B	Flow Rate mL/min
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	40	80	0.5



The AdvanceBio Glycan Mapping column delivers better resolution, narrower bands, and higher peak capacity than the non-Agilent sub-2 µm column in a 2.1 x 150 mm configuration.

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Resolution

The AdvanceBio Glycan Mapping 2.7 µm media, in longer column lengths, are recommended for high resolution separations.

Superior results—in 40% less time than the competition

Column: **AdvanceBio Glycan Mapping 859700-913 2.1 x 150 mm, 1.8 µm**

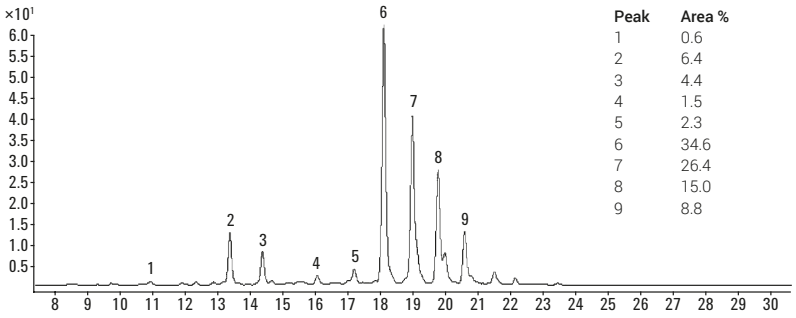
Instrument: 1290 Infinity binary LC

Buffer: A: 100 mM ammonium formate in water, pH 4.5

B: Acetonitrile

MS conditions:

Gas temperature:	250 °C
Sheath gas temperature:	250 °C
Gas flow:	8 L/min
Sheath gas flow:	8 L/min
Nebulizer:	25 psi
Vcap:	3,500 V
Nozzle:	1,000 V
Fragmentor:	200 V
Skimmer:	45 V
Oct 1 RF Vpp:	550
Collision energies:	15 and 30 V
Mode:	MS and targeted MS/MS



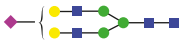
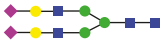
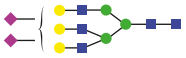
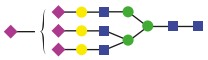
Peak	Area %
1	0.6
2	6.4
3	4.4
4	1.5
5	2.3
6	34.6
7	26.4
8	15.0
9	8.8

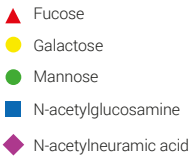
The N-glycans cleaved from fetuin using PNGase F were analyzed after 2-AB derivatization using UHPLC —FLD. The peak assignment by MS shows that the N-glycans cleaved from fetuin are complex biantennary and triantennary glycans containing N-acetylneuramic acid (NeuAc) but no fucose. Fetuin 2-AB N-glycans analyzed using HILIC-UHPLC with peak assignments determined by MS.

Instrument conditions

	Fetuin Gradient
Starting flow rate	0.5 mL/min
Gradient	0 min 75% B
	45 min 50% B
	47 min 40% B, flow 0.5 mL/min
	47.01 min, flow 0.25 mL/min
	49 min 0% B
	51 min 0% B
	51.01 min 75% B, flow 0.25 mL/min
	52.00 min, flow 0.5 mL/min flow
Stop time	52 min
Posttime	20 min
Injection volume	1 µL
Thermostat autosampler	5 °C
FLD	Excitation = 260 nm Emission = 430 nm
Peak width	>0.013 min (0.25 s resp. time) (37.04 Hz)

Detailed information of Fetuin N-glycan structures

Peak	Oxford	Structure
1	A2G2S1	
2, 3	A2G2S2	
4	A3GGS2	
5	A3G3S3, A3G3S2 (trace)	
6	A3G3S3, A3G3S2 (trace)	
7	A3G3S3, A3G3S4 (trace)	
8	A3G3S4, A3G3S3	
9	A3G3S4	



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Hydrophilic and glycopeptide analysis

Peptide analysis demands high selectivity and run-to-run reproducibility as provided by reversed-phase chromatography. However, reversed-phase columns have limited retention and selectivity for hydrophilic peptides, including glycopeptides. The ZORBAX RRHD 300-HILIC, 1.8 µm columns provide increased retention of hydrophilic and glycopeptides compared to reversed-phase columns so that valuable information is not lost when doing peptide mapping experiments.

The two techniques are orthogonal and provide complementary information for protein primary structure analysis.

- A ZORBAX 300 Å particle for analysis across the range of peptide sizes
- The 1.8 µm particle delivers UHPLC performance with 1200 bar stability
- Provides UHPLC orthogonality when used with the ZORBAX RRHD 300 Å reversed-phase columns

Column Specifications

Bonded Phase	Inner Diameter (mm)	Particle Size	Endcapped	pH Stability	Operating Temperature	Pressure Limit
Silica	2.1	1.8, fully porous	No	1-8	40 °C	1200 bar

Peptide mapping is used for characterization and impurity profiling of protein biotherapeutics. Reversed-phase UHPLC/HPLC is routinely used but when the digest contains hydrophilic peptides, such as glycopeptides, valuable information may be missed. The ZORBAX RRHD 300-HILIC column retains the hydrophilic glycopeptides and, when coupled with mass spectrometry, provides identification of this important group of protein fragments.

Glycopeptide identification in a protein tryptic digest

Column: **ZORBAX RRHD 300-HILIC**
858750-901
2.1 x 100 mm, 1.8 µm

Mobile phase: A: 100% ACN
B: 50 mM ammonium formate, pH 4.5

Flow rate: 0.4 mL/min

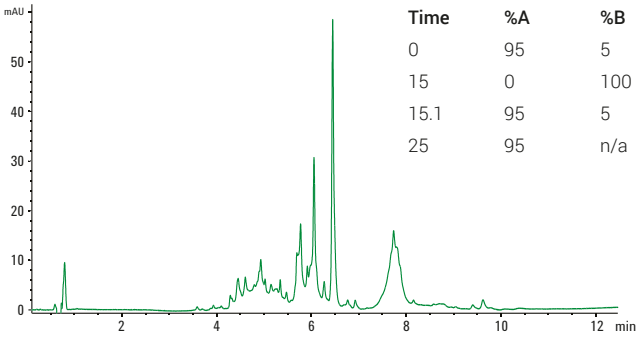
Injection: 5 µg

Detector: UV, 280 nm

Instrument: 1290 Infinity LC, 6224 accurate-mass time-of-flight, dual ESI source in positive ion mode

Sample: Glycopeptide from digested EPO protein (1 mg/mL)

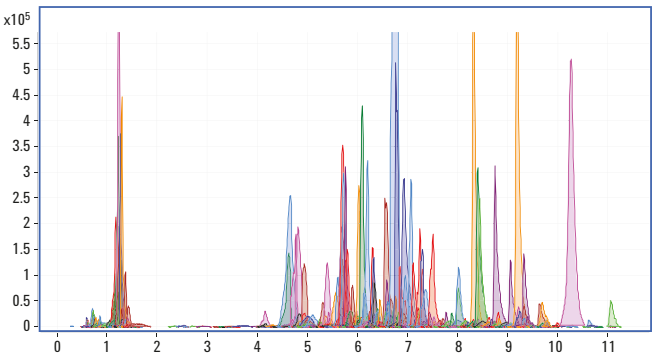
UV



Gradient

Time	%A	%B
0	95	5
15	0	100
15.1	95	5
25	95	n/a

MS



UV shows the separation of an erythropoietin (EPO) peptide map using the ZORBAX RRHD 300-HILIC 2.1 x 100 mm column and **MS** shows the extracted compound chromatograms of matched EPO. Seven peptides were identified from the HILIC-MS data that were not identified by RP-MS. HILIC is orthogonal to RP and provides additional resolution of the hydrophilic glycopeptides, in a protein enzyme digest.

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Product ordering information

AdvanceBio Glycan Mapping, 1.8 µm, Stable to 1200 Bar

Size (mm)	Part No.
2.1 x 150	859700-913
2.1 x 100	858700-913
2.1, 1.8 µm, Fast Guard	821725-905

AdvanceBio Glycan Mapping, 2.7 µm, Superficially Porous, Stable to 1200 Bar

Size (mm)	Part No.
4.6 x 250	680975-913
4.6 x 150	683975-913
4.6 x 100	685975-913
2.1 x 250	651750-913
2.1 x 150	683775-913
2.1 x 100	685775-913
2.1, 2.7 µm, Fast Guard	821725-906

GlykoPrep

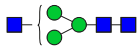
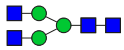
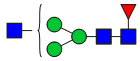
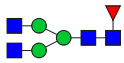
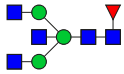
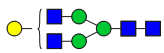
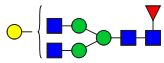
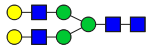
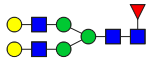
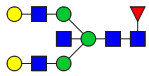
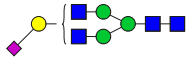
Description	Part No.
GlykoPrep Rapid N-Glycan Prep with InstantPC, 24-ct	GP24NG-PC
GlykoPrep Rapid N-Glycan Prep with 2-AB, 24-ct	GP24NG-AB
GlykoPrep Rapid N-Glycan Prep with InstantPC, 96-ct	GP96NG-PC
GlykoPrep Rapid N-Glycan Prep with 2-AB, 96-ct	GP96NG-AB
GlykoPrep Rapid N-Glycan Prep with InstantPC, 96-ct, AssayMAP Bravo Automated	GPPNG-PC
GlykoPrep Rapid N-Glycan Prep with 2-AB, 96-ct, AssayMAP Bravo Automated	GPPNG-AB
GlykoPrep Starter Labware Set	AM200
GlykoPrep Microtube Adapter Set	AM400

Gly-X

AdvanceBio Gly-X N-Glycan Prep with InstantPC, 24-ct	GX24-IPC
AdvanceBio Gly-X N-Glycan Prep with 2-AB Express, 24-ct	GX24-2AB
AdvanceBio Gly-X N-Glycan Prep with InstantPC, 96-ct	GX96-IPC
AdvanceBio Gly-X N-Glycan Prep with 2-AB Express, 96-ct	GX96-2AB
AdvanceBio Gly-X Manifold Spacer	GX100

Sialic Acid Analysis

AdvanceBio Sialic Acid Profiling and Quantitation Kit, 24-ct	GS24-SAP
AdvanceBio Total Sialic Acid Quantitation Kit, 48-ct	GS48-SAQ
AdvanceBio Total Sialic Acid Quantitation Kit, 96-ct	GS96-SAQ

Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
Complex-type Native N-Glycans									
G0-N	NGA2-N	A1			GKPC-401		GKSB-401		GKSP-401
G0	NGA2	A2		GKC-004300	GKPC-301	GKIB-301	GKSB-301	GKSA-301	GKSP-301
G0F-N	NGA2F-N	F(6)Al			GKPC-402		GKSB-402		GKSP-402
G0F	NGA2F	F(6)A2		GKC-004301	GKPC-302	GKIB-302	GKSB-302	GKSA-302	GKSP-302
G0FB	NGA2FB	F(6)A2B		GKC-004311			GKSB-303		
G1	NA2G1	A2G1		GKC-014300	GKPC-317	GKIB-317	GKSB-317		GKSP-317
G1F	NA2G1F	F(6)A2G1		GKC-014301	GKPC-316	GKIB-316	GKSB-316	GKSA-316	GKSP-316
G2	NA2	A2G(4)2		GKC-024300	GKPC-304	GKIB-304	GKSB-304	GKSA–304	GKSP-304
G2F	NA2F	F(6)A2G(4)2		GKC-024301	GKPC-305	GKIB-305	GKSB-305	GKSA-305	GKSP-305
G2FB	NA2FB	F(6)A2BG(4)2		GKC-024311			GKSB-306		
G1S1 α(2,3)		A2G(4)1S(3)1			GKPC-329				

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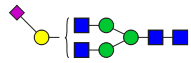
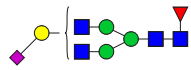
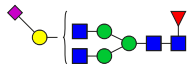
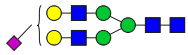
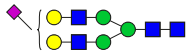
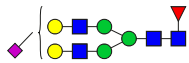
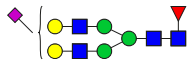
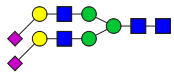
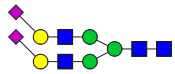
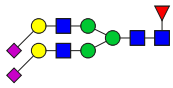
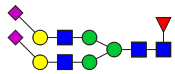
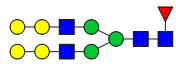
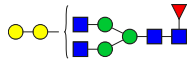
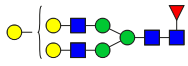
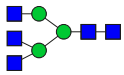
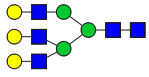
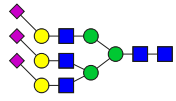
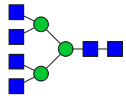
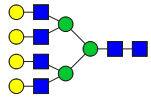
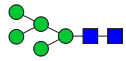
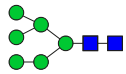
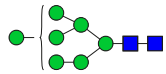
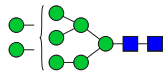
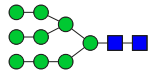
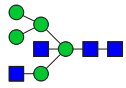
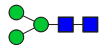
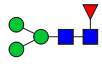
Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
G1S1 α(2,6)		A2G(4)1S(6)1			GKPC-319				
G1FS1 α(2,3)		FA2G(4)1S(3)1			GKPC-330				
G1FS1 α(2,6)		FA2G(4)1S(6)1			GKPC-320				
G2S1 α(2,3)	A1(α2,3)	A2G(4)2S(3)1			GKPC-321				
G2S1 α(2,6)	A1(α2,6)	A2G(4)2S(6)1		GKC-124300	GKPC-311	GKIB-311	GKSB-311	GKSA-311	GKSP-311
G2FS1 α(2,3)	A1F(α2,3)	F(6)A2G(4)2S(3)1			GKPC-325				
G2FS1 α(2,6)	A1F(α2,6)	F(6)A2G(4)2S(6)1		GKC-124301	GKPC-315	GKIB-315	GKSB-315	GKSA-315	GKSP-315
G2S2 α(2,3)	A2(α2,3)	A2G(4)2S(3)2			GKPC-322				
G2S2 α(2,6)	A2(α2,6)	A2G(4)2S(6)2		GKC-224300	GKPC-312	GKIB-312	GKSB-312	GKSA-312	GKSP-312
G2FS2 α(2,3)	A2F(α2,3)	F(6)A2G(4)2S(3)2			GKPC-323				
G2FS2 α(2,6)	A2F(α2,6)	F(6)A2G(4)2S(6)2		GKC-224301	GKPC-313	GKIB-313	GKSB-313	GKSA-313	GKSP-313
G2F w/2 α-gal	NA2Ga2F	F(6)A2G(4)2Ga(3)2			GKPC-318		GKSB-318		GKSP-318
G1F w/1 α-gal	NA2G 1FGa1	F(6) A2G(4)1Ga(3)1			GKPC-403				
G2F w/1 α-gal	NA2FGa1	F(6)A2G(4)2Ga(3)1			GKPC-404				
A3	NGA3	A3		GKC-005300		GKIB-307	GKSB-307	GKSA-307	
G3	NA3	A3G(4)3		GKC-035300			GKSB-308	GKSA-308	
G3S3 α(2,6)	A3(a2,6)	A3G(4)3S(6)3		GKC-335300			GKSB-314		

Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
A4	NGA4	A4		GKC-006300			GKSB-309	GKSA-309	
G4	NA4	A4G(4)4		GKC-046300			GKSB-310		
High Mannose-type Native N-Glycans									
Man5	MAN-5	M5		GKM-002500	GKPC-103	GKIB-103	GKSB-103	GKSA-103	GKSP-103
Man6	MAN-6	M6		GKM-002600	GKPC-104	GKIB-104	GKSB-104	GKSA-104	GKSP-104
Man7	MAN-7	M7		GKM-002700	GKPC-105	GKIB-105	GKSB-105	GKSA-105	GKSP-105
Man8	MAN-8	M8		GKM-002800	GKPC-106	GKIB-106	GKSB-106	GKSA-106	GKSP-106
Man9	MAN-9	M9		GKM-002900	GKPC-107	GKIB-107	GKSB-107	GKSA-107	GKSP-107
Hybrid-type Native N-Glycan									
Hybrid	HYBR	M5A1B					GKSB-111		
Native N-Glycan Cores									
NF	NF			GKR-001001					
NN	NN			GKR-002000			GKSB-100		
NNF	NNF			GKR-002001					
Man1	MNN	M1		GKR-002100					
Man1F	MNNF	F(6)M1		GKR-002101					
Man3				GKR-002300			GKSB-101		
Man3F				GKR-002301			GKSB-102		

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Glycans	Unlabeled	InstantPC	InstantAB	2-AB	2-AA	APTS
N-Glycan Libraries						
Human IgG N-Glycan Library	GKLB-005	GKPC-005	GKIB-005	GKSB-005	GKSA-005	GKSP-005
CHO mAb N-Glycan Library		GKPC-020				
CHO mAb N-Glycan Library plus CHO mAb Glycoprotein		GKPC-020-P				
Human α1-acid glycop rotein N-Glycan Library	GKLB-001		GKIB-001	GKSB-001	GKSA-001	
Bovine Fetuin N-Glycan Library	GKLB-002		GKIB-002	GKSB-002	GKSA-002	
RNase B N-Glycan Library (High Mannose)			GKIB-009			
Biantennary and High Mannose Partitioned Library			GKIB-520	GKSB-520		GKSP-520
Sialylated Biantennary N-Glycan Library			GKIB-232	GKSB-232		GKSP-232
α(2,6) Sialylated Biantennary N-Glycan Library				GKSB-262		GKSP-262
α(2,3) Sialylated Triantennary N-Glycan Library		GKPC-233	GKIB-233	GKSB-233		GKSP-233
α(2,6) Sialylated Triantennary N-Glycan Library		GKPC-263		GKSB-263		GKSP-263
α(2,3) Sialylated Tetraantennary N-Glycan Library		GKPC-234	GKIB-234	GKSB-234		GKSP-234
α(2,6) Sialylated Tetraantennary N-Glycan Library		GKPC-264		GKSB-264		GKSP-264
Alignment Standards						
Glucose Unit (GU) Ladder		GKPC-503	GKIB-503	GKSB-503	GKSA-503	GKSP-503
Internal Migration Standards for Capillary Electrophoresis (CE)						GKSP-500

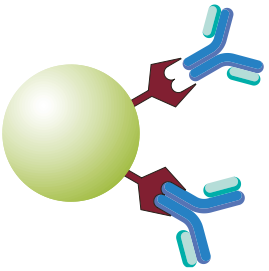
ZORBAX RRHD 300-HILIC 1.8 µm Columns

Size (mm)	Inner Diameter (mm)	Particle Size (µm)	Part No.
ZORBAX RRHD 300-HILIC	2.1 x 100	1.8	858750-901
ZORBAX RRHD 300-HILIC	2.1 x 50	1.8	857750-901

Tips and tools

Visit our online store to directly purchase our columns and consumables: **www.agilent.com/chem/store**

Titer Determination



Affinity chromatography is a powerful technique that takes advantage of highly specific molecular interactions, frequently between specific proteins (for example, antigen/antibody). Agilent offers several specialty affinity products, monolithic Protein A and monolithic Protein G columns for the isolation and quantitation of IgG and a series of multiple affinity removal systems for the elimination of high abundance proteins in biological samples.

Bio-Monolith HPLC columns

- Designed for the analytical separation of all IgG (human and mouse)
- Flow rate independent separations; no diffusion, no pores, and no void volume make transport between mobile and stationary phase very rapid
- Extremely fast separations speed up method development time and decrease costs
- Locking in method parameters takes significantly less time and buffer

Bio-Monolith Protein A and Protein G HPLC columns are part of the Bio-Monolith column family. Protein A and Protein G Bio-Monolith columns are compatible with HPLC and preparative LC systems, including 1100, 1200, and 1260 bio-inert quaternary LC.

Bio-Monolith Protein A column, 5069-3639

Tips and tools

For more information on salt tolerance for mAb binding and acidic buffers compatibility for mAb elution on Bio-Monolith Protein A columns, see publication **5991-2990EN**.

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Column Specifications	
Dimensions	5.2 mm x 4.95 mm
Column volume	100 µL
Maximum pressure	150 bar (15 MPa, 2,200 psi)
Temperature min/max	Operating: 2-40 °C
	Storage: 2-8 °C
Recommended pH	Operating range: 2-13
	Cleaning-in-place: 1-14
Materials of construction	Hardware: stainless steel
	Packing: poly (glycidyl methacrylate-co-ethylene dimethacrylate) highly porous monolith
Color ring identifier	Bio-Monolith Recombinant Protein A and Native Protein A: white Bio-Monolith Protein G: orange
Shelf life/expiration date	12 months

Tips and tools

Further information can be found in:

Robust, Reliable Recombinant Protein A Monolith Column for Antibody Titer Determination (publication **5994-3088EN**)
mAb Titer Determination in 60 Seconds Using the Agilent Bio-Monolith rProtein A Column (publication **5994-3969EN**)
mAb Titer Analysis with the Agilent Bio-Monolith Protein A Column (publication **5991-5135EN**)
Agilent Bio-Monolith Protein A Monitors Monoclonal Antibody Titer from Cell Cultures (publication **5991-2990EN**)
Cell Clone Selection Using the Agilent Bio-Monoltih Protein A Column and LC/MS (publication **5991-5124EN**)
Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS (publication **5991-5125EN**)

Binding Affinity of Bio-Monolith Protein A and G for Different IgG Subclasses

Antibody	Protein A	Protein G
Human		
Human IgG1	++++	++++
Human IgG2	++++	++++
Human IgG3	–	++++
Human IgG4	++++	++++
Human IgA	++	–
Human IgD	++	–
Human IgE	++	–
Human IgM	++	–
Mouse		
Mouse IgG1	+	++
Mouse IgG2a	++++	++++
Mouse IgG2b	++++	+++
Mouse IgG3	+	+++
Mouse IgM	+/–	–
Antibody fragments		
Human Fab	+	+
Human F(ab')2	+	+
Human scFv	+	+
Human Fc	+	+
Human κ	+	+
Human λ	+	+

++++ = Strong affinity
+++ = Moderate affinity
++ = Weak affinity
+ = Slight affinity
– = No affinity

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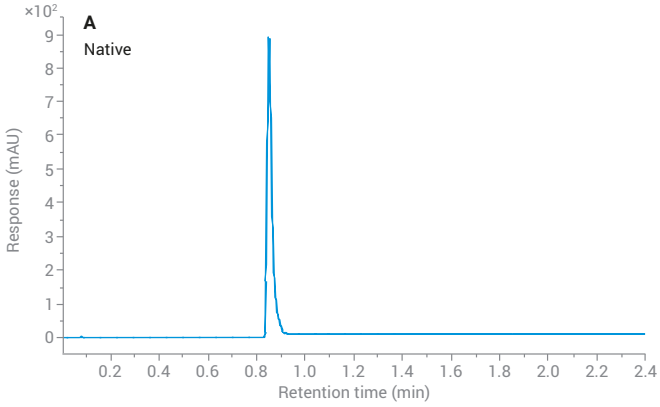
Bridging study between the recombinant Protein A and native Protein A column

Column:	**Bio-Monolith Recombinant Protein A 5190-6903 4.95 × 5.2 mm** **Bio-Monolith Protein A 5069-3639 4.95 x 5.2 mm**
Mobile phase:	A: Sodium phosphate buffer, 50 mM, pH 7.4 B: Citric acid, 0.1 M, pH 2.6
Flow rate:	1.5 mL/min
Injection volume:	5-50 µL (25 µg loading)
Detector:	UV, 280 nm
Gradient:	0% B for 0.5 min, 100% B from 0.6-2.6 min, 0% B from 2.7-4 min
Temperature:	25 °C
Sample:	Recombinant IgG in Chinese Hamster Ovary (CHO) cell culture supernatant
Instrument:	1290 Infinity II Bio LC system

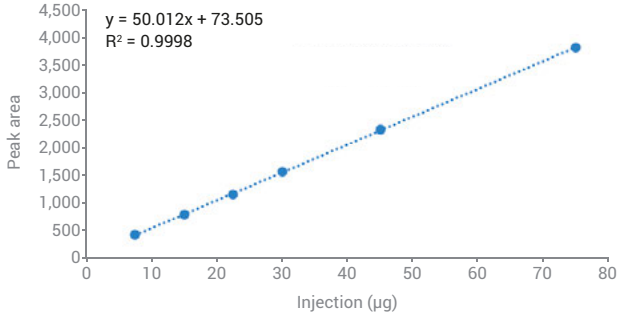
Retention time



	Native	rProtein A
Retention Time (Min)	0.850 ± 0.001	0.850 ± 0.001
Peak Height	880.9 ± 8.1	881.8 ± 5.3

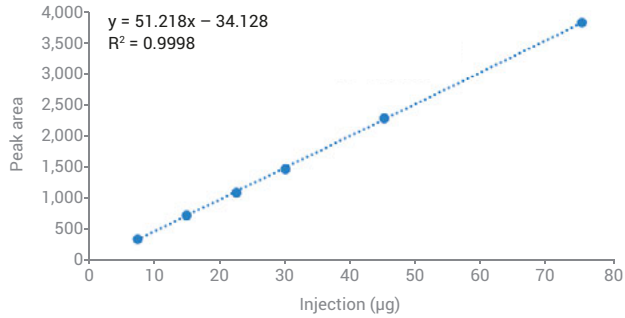
Chromatogram and mAb peak result comparison between Native and rProtein A columns.

Linearity Response



Spiked sample	Measured	% Deviation
25 µg (pure)	25.25 µg	0.99%
25 µg (in sup.)	26.09 µg	4.35%

rProtein A column: Linearity response



Spiked sample	Measured	% Deviation
25 µg (pure)	24.94 µg	-0.23%
25 µg (in sup.)	25.78 µg	3.12%

Native Protein A column: Linearity response

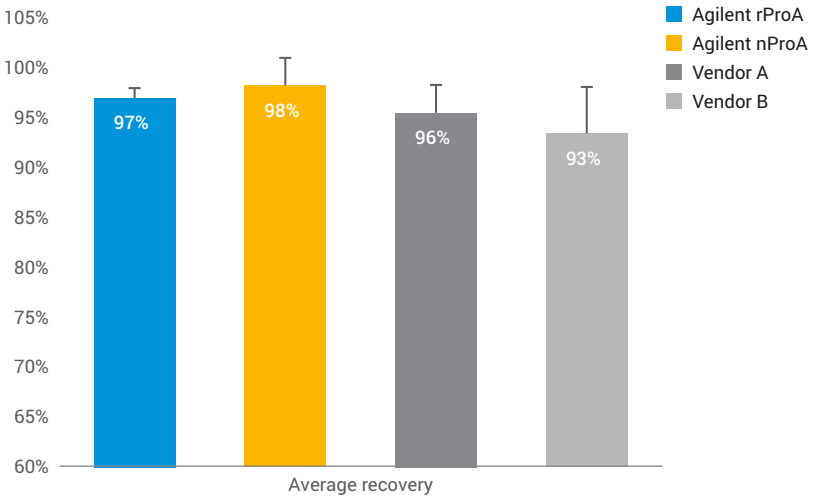
Recovery analysis

In addition to comparing the recovery between native and the rProtein A columns, two non-Agilent rProtein A columns were included in this study. Flow rate was adjusted to 2 mL/min to accommodate a non-Aglient column's operating flow rate. Addition mAb samples were included:

- Agilent-NISTmAb (P/N: 5191-5744)
- Sigma SiLu mAb from Sigma-Aldrich (SiLu Lite, P/N: MSQC4)

Baseline area under the curve (AUC) of mAb peak was obtained by injecting purified mAb sample, which was diluted with mobile phase B, without column (with a union). Column was applied and AUC of eluted mAb was obtained. Same amount of mAb sample as baseline AUC was used.

Recovery % = (AUC of eluted mAb / Baseline AUC) X 100



mAb recovery results comparison

The average recovery of the rProtein A column was 1% lower than the native protein A column but still showed better recovery than the two non-Agilent columns. While the nProtein A column took the slight lead in recovery, it was the rProtein A column that demonstrated the most robust recovery across the three mAb samples.

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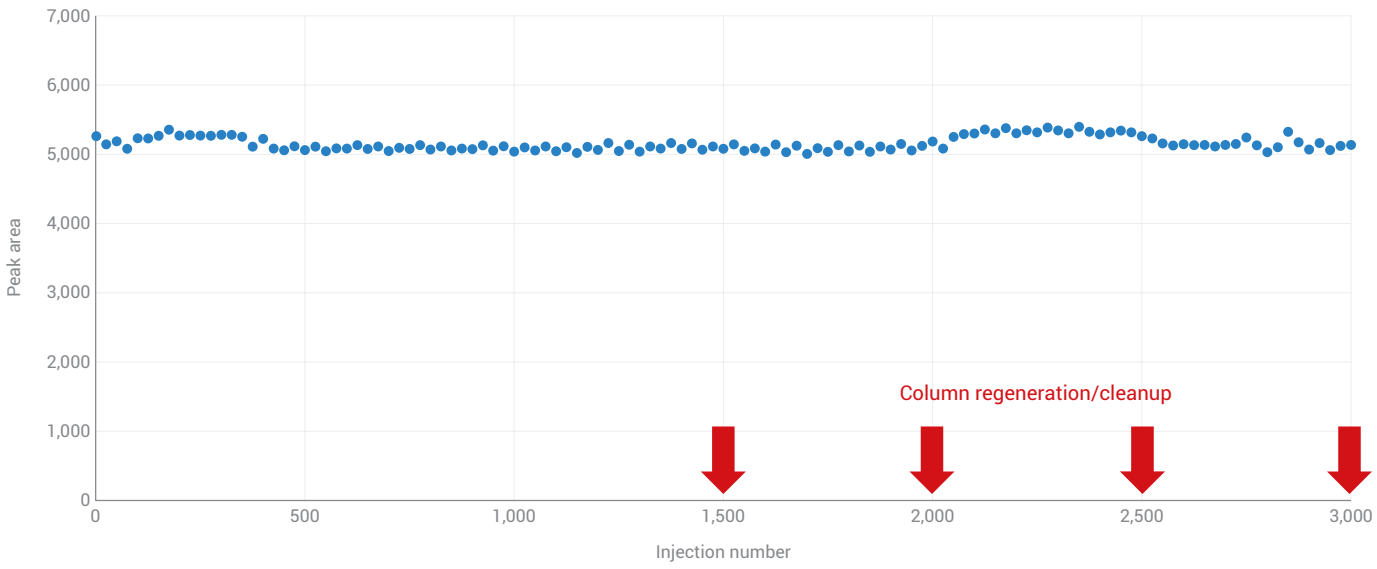
Robust, reliable antibody titer determination

Method conditions

HPLC Conditions	
Column	Bio-Monolith Protein A, 4.95 × 5.2 mm (p/n 5190-6903)
Binding Buffer (Eluent A)	50 mM sodium phosphate, pH 7.4
Eluting Buffer (Eluent B)	100 mM citric acid, pH 2.6
Cleanup Buffer	1 M NaCl in 100 mM sodium phosphate, pH 7.4 20% isopropanol in 50 mM sodium phosphate, pH 7.4
Gradient Profile	Time %B 0.0 to 0.5 0 (binding) 0.6 to 1.8 100 (elution) 1.9 to 4.0 0 (reconditioning)
Flow Rate	1 mL/min
Column Temperature	24 °C
Detection	UV, 280 nm
Injection Volume	As required (1 to 20 µL)



Rapid humanized monoclonal antibody quantitation

Column: **Bio-Monolith Protein A**
5069-3639
5.2 x 4.95 mm

Mobile phase: A: 50 mM phosphate, pH 7.4
B: 100 mM citric acid, pH 2.8

Flow rate: 1 mL/min

Injection volume: Variable (50 µL, optimized for CHO cell culture supernatant contains IgG1)

Gradient:

Time (min)	%A	%B	
0 to 0.5	100	0	Binding
0.6 to 1.7	0	100	Eluting
1.8 to 3.5	100	0	Re-equilibrating

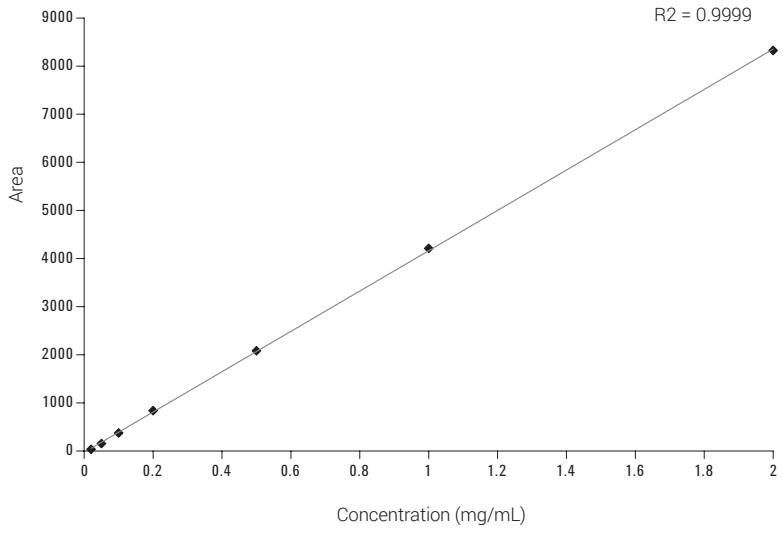
Temperature: Ambient

Detector: UV, 280 nm

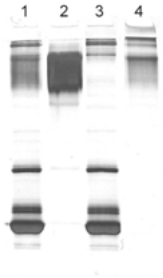
Fraction collection: Time-based

Sample: IgG1 (1-20 mg/mL) and CHO cell supernatant contains IgG1 (up to 20 mg/mL total protein)

	RT (min)	Peak Area
1	383	1.666
2	372	1.666
3	365	1.665
4	389	1.667
5	383	1.666
6	378	1.666
7	379	1.668
8	377	1.666
9	376	1.667
10	377	1.667
Mean	378	1.667
S	6.52	0.001
%RSD	1.73	0.060

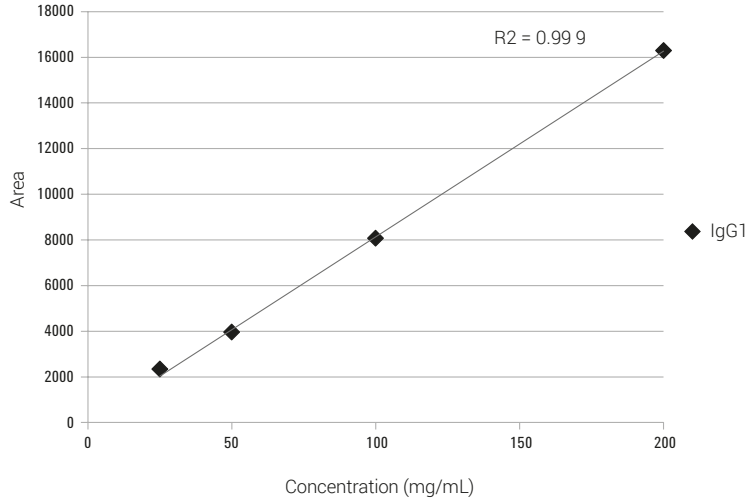


Calibration curves of modified humanized Trastuzumab (panel A: 0-2 mg/mL, and B: 25-200 mg/mL)



Key:
Lane 1: Whole serum before separation
Lane 2: IgG standard
Lane 3: Peak 1 (flow-through fraction)
Lane 4: Peak 2 (Protein A-bound fraction; for example, IgG1)

SDS PAGE analysis of fractions from the separation



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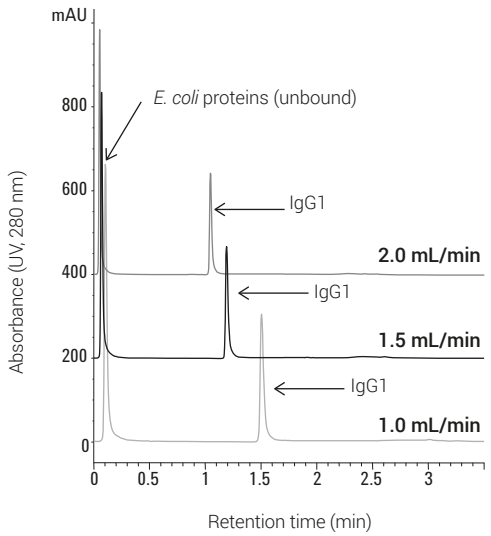
No impact on binding efficiency with high flow rate

Column:	**Bio-Monolith Protein A** **5069-3639** **5.2 x 4.95 mm**
Mobile phase:	A: Sodium phosphate buffer, 20 mM, pH 7.4 B: Citric acid, 0.1 M, pH 2.8
Flow rate:	1.0, 1.5, and 2.0 mL/min
Injection:	4 µL (from 2.5 mg/mL IgG1 spiked with 20 mg/mL of E.coli supernatant) detector: UV, 280 nm
Gradient:	0% B for 0.5 min, 100% B from 0.6-1.7 min, 0% B from 1.8-3 min
Temperature:	25 °C
Sample:	Humanized IgG1 and *E.coli* lysate
Instrument:	1260 Infinity bio-inert LC



Binding of IgG1 with the Bio-Monolith Protein A column evaluated at several flow rates. More sample was loaded for this study to easily observe changes in chromatogram and signal integration.

Flow Rate Versus Peak Relative Area on Unbound Proteins and IgG1

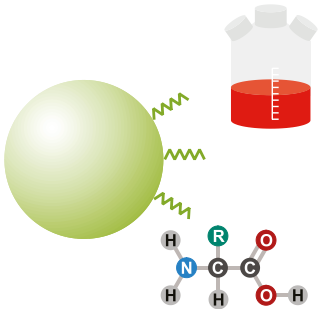
Flow Rate (mL/min)	Unbound Area (mAu/S)	IgG1 Area (mAu/S)	Unbound Relative area (%)	IgG1 Relative area (%)	Pressure (bar)
1.0	1230	738	63	37	32
1.5	840	492	63	37	47
2.0	636	363	64	36	68

Product ordering information

Bio-Monolith Protein A and Protein G

Description	Part No.
Bio-Monolith Recombinant Protein A, 4.95 x 5.2 mm	5190-6903
Bio-Monolith Protein G, 4.95 x 5.2 mm	5190-6900
Bio-Monolith Protein A, 4.95 x 5.2 mm	5069-3639

Cell Culture and Amino Acid Analysis



AdvanceBio columns from Agilent make it easier for your biotech lab to analyze amino acids and other small metabolites in spent cell culture media—with or without sample derivatization. Columns for both solutions are tested with amino acids to ensure quality and performance. Simply choose the workflow that suits your needs.

Choose the Agilent AdvanceBio Amino Acid Analysis kit for industry-standard LC/UV analysis

- Get automated online derivatization of amino acids with reverse-phase LC separation and UV detection.
- Use any Agilent LC system.
- Minimize your investment in instrumentation and expertise.

Choose Agilent AdvanceBio MS Spent Media columns for fast, underivatized LC/MS analysis

- Analyze amino acids and other cell culture metabolites with a single method: HILIC LC separation with MS detection.
- Eliminate the need to derivatize your sample.
- Use any LC/MS system.
- No need for baseline chromatographic resolution with MS detection.

Tips and tools

Agilent InfinityLab well plates and sealing mats are the ideal sample containers for your high-throughput LC/MS applications.

Visit: **www.agilent.com/chem/well-plates**

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Agilent solutions for spent media analysis

Derivatization?

Yes

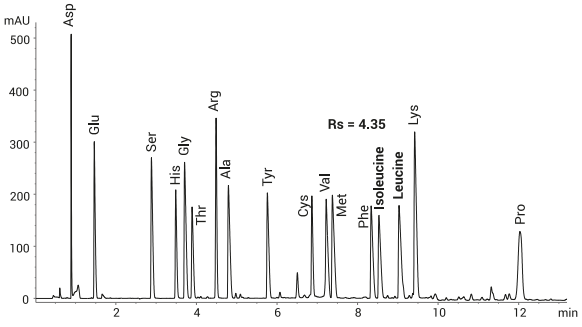
No

AdvanceBio
Amino Acid Analysis
Ready to use reagents

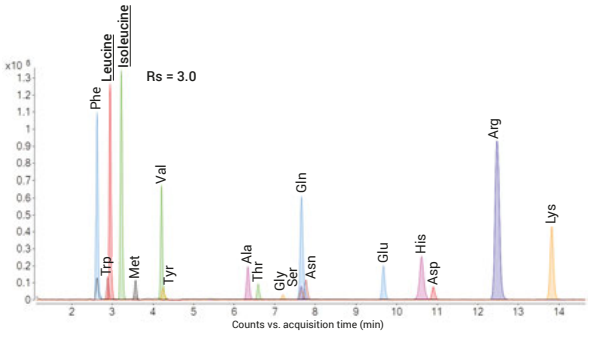
AdvanceBio Amino Acid Analysis
Reversed Phase LC/UV

Fast, reliable results

AdvanceBio MS Spent Media
HILIC LC/MS



Amino acids



Amino acids and cell culture metabolites

AdvanceBio Amino Acid Analysis (AAA)

Agilent AdvanceBio Amino Acid Analysis (AAA) columns deliver fast, sensitive, and reproducible separations of amino acids in protein hydrolysates and cell culture media.

AdvanceBio AAA includes proven reagents for amino acid derivatization, a ready-to-use amino acid standards kit, columns based on Agilent's innovative Poroshell technology, and expert support from Agilent. Alongside Agilent InfinityLab LC Series instruments, AdvanceBio AAA provides a complete solution for amino acid analysis.

These columns are part of the Agilent AdvanceBio family—designed as innovative solutions for biomolecule characterization.

- **Reliable results:** high resolution separations delivered by efficient Poroshell particle morphology.
- **Reduced costs:** long column lifetimes from robust, high-pH resistant, chemically modified silica.
- **Increased flexibility:** compatibility with both HPLC and UHPLC systems via 2.7 µm diameter particles.
- **Quality control:** AdvanceBio AAA columns are batch tested with amino acid standards to ensure quality.
- **Easy ordering:** standards and reagents available as kits.
- **Automated online derivatization:** with Agilent analytical injection systems.

Column Specifications

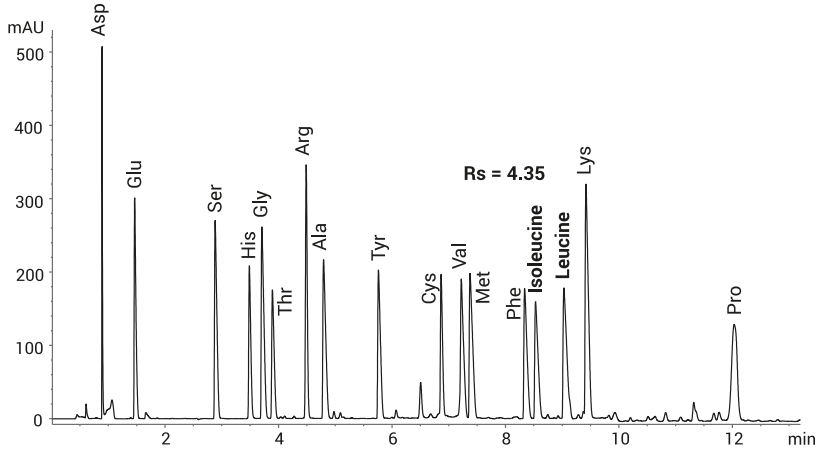
Bonded Phase	Particle Size	Pore Size	Temp Limit	pH Range	Endcapped	Pressure Limit
C18	2.7 µm	100 Å	60 °C	3.0–11.0	Double	600 bar

Tips and tools

For step-by-step instructions and to learn about Agilent's end-to-end solution, visit:
www.agilent.com/chem/aaa-how-to-guide

LC/UV

Column:	**Agilent AdvanceBio Amino Acid Analysis 4.6 x 100 mm p/n 655950-802**
Column temperature:	30 °C
Mobile phase:	Low pH, positive ion mode MS detection: A = 10 mM Na_2HPO_4, 10 mM Na2B4O7, pH 8.2 B = acetonitrile:methanol:water, 45:45:10 (v:v:v)
Flow rate:	1.5 mL/min
Gradient:	Time (min) %B 0 2 0.35 2 13.4 57 13.5 100 15.7 100 15.7 2 18 end
Sample:	Protein hydrolysate
Detection:	Agilent 1260 Infinity II DAD WR



Ultraviolet chromatogram of amino acids from protein hydrolysate. The resolution between leucine and isoleucine is 4.35, which easily meets the European Pharamacopoeia requirement that resolution be greater than 1.5. [European Pharmacopoeia 9.0 (2.2.56) Amino Acid Analysis.]

AdvanceBio Amino Acid Analysis standards and kit

All necessary derivatization reagents and amino acid standards for quantitation are included in a single part number. Individual components may be reordered as needed.

Each amino acid standard contains the following amino acids:

- Glycine
- L-cysteine
- L-histidine
- L-tyrosine
- L-leucine
- L-methionine
- L-serine
- L-alanine
- L-phenylalanine
- L-glutamic acid
- L-proline
- L-isoleucine
- L-arginine
- L-threonine
- L-valine
- L-lysine
- L-aspartic acid

AdvanceBio MS Spent Media

Agilent AdvanceBio MS Spent Media columns are HILIC columns that deliver fast, sensitive, and reproducible separations of underivatized amino acids and other polar metabolites found in bioprocessor cell culture media for mass spectrometric detection.

Alongside Agilent InfinityLab LC series instruments and Agilent MS instruments, AdvanceBio MS Spent Media provides a complete solution for spent media analysis.

AdvanceBio MS Spent Media analysis is a new addition to the Agilent AdvanceBio family, designed as innovative solutions for biomolecule production and characterization.

- Fast, MS-based workflow
- No sample derivatization needed, saving time and resources
- PEEK-lined stainless steel column hardware for an inert flow path to achieve excellent peak shape and recovery of challenging ionic metabolites
- Baseline chromatographic resolution of leucine and isoleucine isomers
- Columns are tested with amino acids to ensure quality and performance
- Excellent analytical sensitivity with MS-friendly mobile phases
- Compatibility with both HPLC and UHPLC systems via 2.7 µm Poroshell particle

Column Specifications

Bonded Phase	Pore Size	Particle Size	Temperature Limit	pH Range	Pressure Limit
HILIC-Z	100 Å	2.7 µm	80 °C (at pH 7)	3.0–11.0 (at 35 °C)	600 bar

Tips and tools

For HILIC best practices please see Hydrophilic Interaction Chromatography Method Development and Troubleshooting (publication **5991-9271EN**).

LC/MS

Column: **Agilent AdvanceBio MS Spent Media 2.1 x 100 mm p/n 675775-901**

Column temperature: 30 °C

Mobile phase: Low pH, positive ion mode MS detection:
A = 10% 200 mM ammonium formate in water pH 3, 90% water
B = 10% 200 mM ammonium formate in water pH 3, 90% acetonitrile
Final salt concentration is 20 mM.
We recommend preparing mobile phases from a concentrated buffer stock to ensure robust and consistent mobile phases.

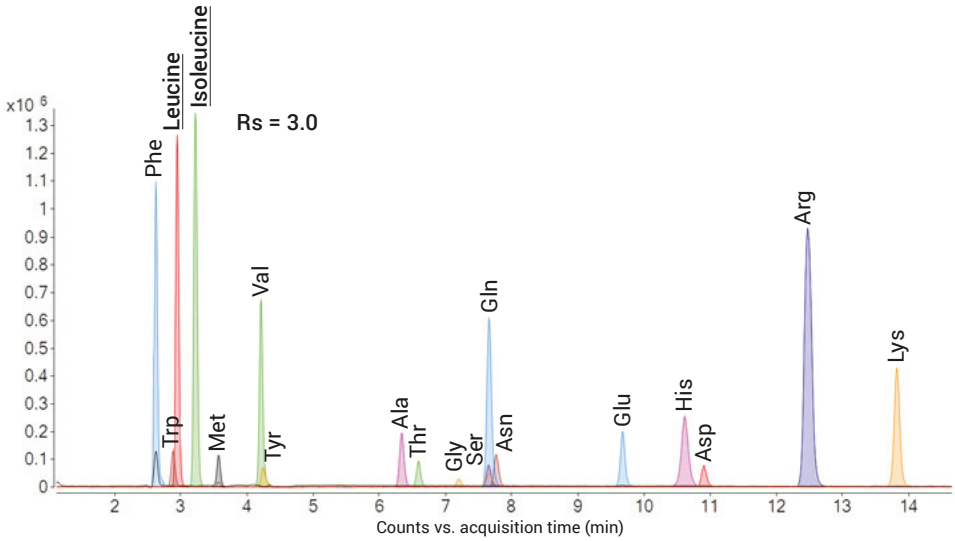
Flow rate: 0.5 mL/min

Gradient:

Time (min)	%B (Low pH, positive ion mode))	% B (High pH, negative ion mode)
0	1.00	100
15	80	80
15.5	100	100
20	100	100

Sample: Cell culture media, diluted five-fold with mobile phase B

Detection: Agilent 6230 time-of-flight LC/MS



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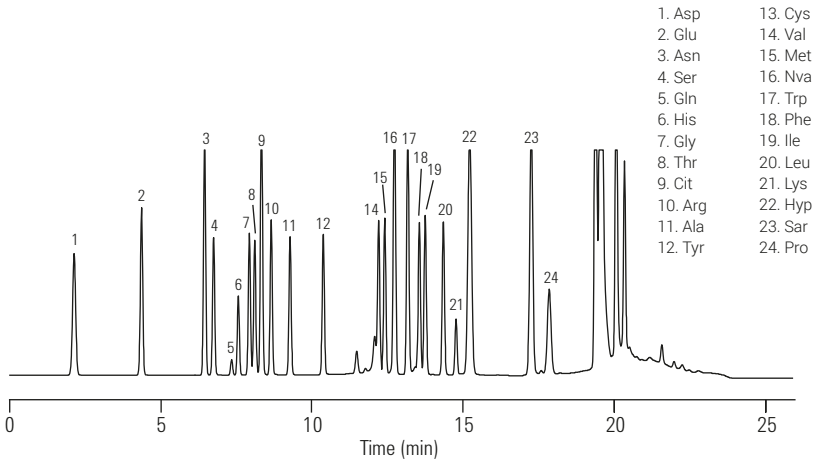
High resolution of 24 amino acids

Column:	**ZORBAX Eclipse AAA 963400-902 4.6 x 150 mm, 3.5 µm**
Mobile phase:	A: 40 mM Na_2HPO_4, pH 7.8
	B: ACN:MeOH:water, 45:45:10 v/v
Flow rate:	2 mL/min
Temperature:	40 °C
Detector:	Fluorescence
Sample:	24 amino acids



This high resolution separation of 24 amino acids is achieved in 18 minutes. If the Rapid Resolution 4.6 x 75 mm Eclipse AAA column is selected, these amino acids are resolved in 9 minutes.

Tips and tools

Quick reference guides list the common supplies you should have on hand to keep your Agilent InfinityLab LC series operating at peak efficiency. Download your free copy at **www.agilent.com/chem/getguides**

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Product ordering information

AdvanceBio Amino Acid Analysis (AAA) Columns

Description	Part No.
AdvanceBio Amino Acid Analysis 100 Å, 3.0 x 100 mm, 2.7 µm	695975-322
AdvanceBio Amino Acid Analysis 100 Å, 4.6 x 100 mm, 2.7 µm	655950-802
AdvanceBio Amino Acid Analysis 100 Å, 3.0 x 5 mm, 2.7 µm (3-pack guards)	823750-946
AdvanceBio Amino Acid Analysis 100 Å, 4.6 x 5 mm, 2.7 µm (3-pack guards)	820750-931

AdvanceBio Amino Acid Analysis (AAA) Standards and Reagents

Description	Part No.
Standards and reagents kit	5190-9426
Kit contents (can be ordered separately)	
Buffer, borate, 100 mL	5061-3339
FMOC reagent, 10 ampoules, 1 mL each, for AAA	5061-3337
OPA reagent, 10 mg/mL, 6 ampoules, 1 mL each	5061-3335
Dithiodiproprionic acid (DTDPA), 5g	5062-2479
AA standard, 1 nmol, 10/pk	5061-3330
AA standard, 250 pmol, 10/pk	5061-3331
AA standard, 100 pmol, 10/pk	5061-3332
AA standard, 25 pmol, 10/pk	5061-3333
AA standard, 10 pmol, 10/pk	5061-3334
AA supplement, 1 g each	5062-2478

AdvanceBio MS Spent Media

Description	Part No.
AdvanceBio MS Spent Media 100 Å, 2.1 x 50 mm, 2.7 µm	679775-901
AdvanceBio MS Spent Media 100 Å, 2.1 x 100 mm, 2.7 µm	675775-901
AdvanceBio MS Spent Media 100 Å, 2.1 x 150 mm, 2.7 µm	673775-901

ZORBAX Columns

Description	Part No.
ZORBAX Eclipse Amino Acid Analysis, 80 Å, 4.6 x 150 mm, 5 µm	993400-902
ZORBAX Eclipse Amino Acid Analysis, 80 Å, 4.6 x 150 mm, 3.5 µm	963400-902
ZORBAX Eclipse Amino Acid Analysis, 80 Å, 4.6 x 75 mm, 3.5 µm	966400-902
ZORBAX Eclipse Amino Acid Analysis, 80 Å, 3.0 x 150 mm, 5 µm	961400-302
ZORBAX Eclipse Amino Acid Analysis 80 Å, 4.6 x 12.5 mm, 5 µm (4-pack guards)	820950-931
Guard hardware kit	820999-901
ZORBAX Eclipse Plus RRHD, 95 Å, 2.1 x 50, 1.8 µm, 1200 bar	959757-902
ZORBAX Eclipse Plus RRHT, 95 Å, 2.1 x 50, 1.8 µm, 600 bar	959741-902

Buffer, borate, 100 mL,
5061-3339

AA supplement, 1 g each,
5062-2478

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Protein Depletion

To more easily isolate and identify proteins in biological samples, such as serum, plasma, and cerebrospinal fluid (CSF), the multiple affinity removal system is designed to chromatographically eliminate interfering high-abundance proteins from biological samples. Removal of these abundant proteins improves the subsequent LC/MS and electrophoretic analysis of the sample by effectively expanding the dynamic range.

Agilent protein fractionation system and proteomics reagents

- LC/MS analysis of biological samples
- Preparation for electrophoretic analysis
- Sample preparation for biomarker discovery
- Instrument and workflow validation
- Cost-effective immunodepletion
- Sample desalting, concentration, and fractionation

For sample fractionation and desalting, the Agilent mRP-C18 high-recovery protein column is designed to simultaneously desalt, concentrate, and fractionate in one easy step with extremely high recovery of samples as compared to conventional RP-HPLC columns that are fully compatible with LC/MS analysis.

In addition, validated reagents for sample preparation in biomarker discovery and other proteomics applications are also available, including a complex standard, and proteomics grade trypsin. For your convenience, these reagents are fully compatible with Agilent LC/MS methods and require no additional sample pretreatments.

Large volume requirements and custom column dimensions can also be fulfilled with our custom configurations.

Tips and tools

Learn more about the complete Agilent services portfolio at **www.agilent.com/chem/services**

Multiple affinity removal system

The multiple affinity removal system enables the identification and characterization of high-value, low abundant proteins and biomarkers found in serum, plasma, and other biological fluids.

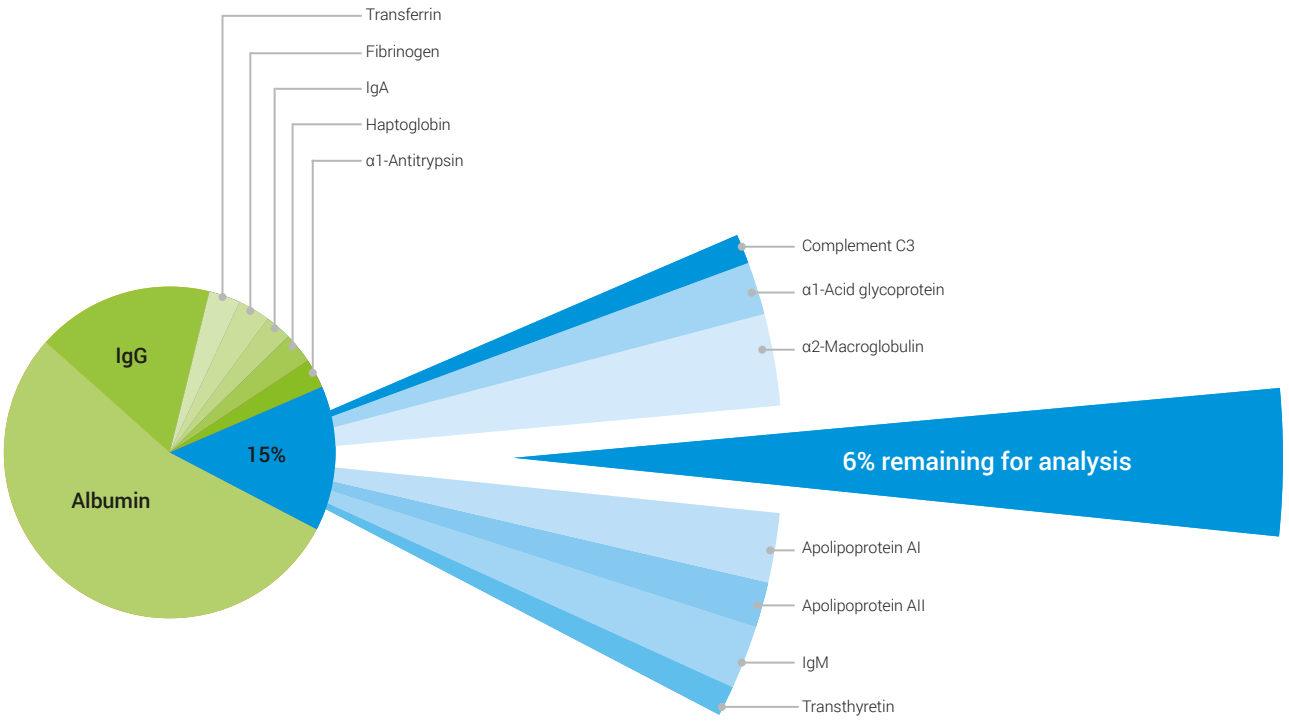
The multiple affinity removal system reproducibly and specifically removes up to fourteen high-abundant proteins found in human biological fluids and three high-abundant proteins found in mouse biological fluids.

The multiple affinity removal system is available in a variety of LC column dimensions and in spin cartridge format. When combined with Agilent optimized buffers, convenient spin filters, and concentrators, the multiple affinity removal system creates an automated, integrated depletion solution compatible with most LC instruments (columns), and bench-top centrifuges (spin cartridges).

Samples depleted using the multiple affinity removal system are ready for downstream analyses such as 2D gel electrophoresis, LC/MS, and other analytical techniques.

Multiple affinity removal system

High abundance proteins removed by Agilent multiple affinity removal columns and spin cartridges



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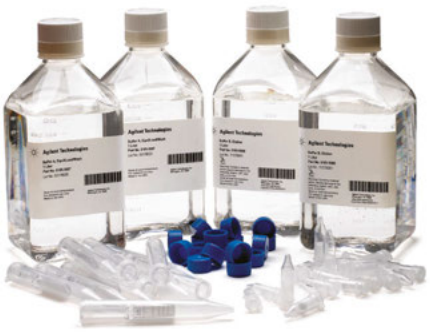
Multiple affinity removal system starter kits

The LC Column and Spin Cartridge reagent Starter Kits include all the required supplies to use with the multiple affinity removal system. These buffers provide optimal conditions for column longevity and sample reproducibility.

- The kits provide enough Buffer A and Buffer B for approximately 200 sample depletions using the 4.6 x 50 mm LC columns, approximately 100 sample depletions using the 4.6 x 100 mm LC columns and 200 spin cartridge uses.
- Buffer A, the loading buffer, minimizes protein-protein interactions, allowing low-abundant proteins often bound to high-abundant proteins to pass through the column, while the targeted high-abundant proteins bind to their associated antibodies.
- Buffer B, the elution buffer, then disrupts the antibody-protein interaction eluting the high-abundant proteins off the column.



LC column reagent starter kit, 5185-5986

Tips and tools

For more information on how to reduce your cycle time for affinity chromatography, see:
Reducing Cycle Time for Affinity Removal of High-Abundant Proteins in Human Plasma. Alternating Column Regeneration Using an Agilent 1200 Infinity Series Quick-Change Bio-inert 2-position/10-port Valve and an Agilent 1290 Infinity Flexible Cube (publication **5991-4721EN**).

Product ordering information

Multiple Affinity Removal System Selection Guide

Product	Proteins Removed	Total Protein Removed	Dimensions	Load Capacity	Part No.
MARS Human-14	Albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein. All, complement C3, transthyretin	94%	Spin cartridge	8–10 µL	5188-6560
			4.6 x 50 mm	20 µL	5188-6557
			4.6 x 100 mm	40 µL	5188-6558
			10.0 x 100 mm	250 µL	5188-6559
MARS Human-7	Albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin, fibrinogen	88–92%	Spin cartridge	12–14 µL	5188-6408
			4.6 x 50 mm	30–35 µL	5188-6409
			4.6 x 100 mm	60–70 µL	5188-6410
			10.0 x 100 mm	250–300 µL	5188-6411
MARS Human-6	Albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin	85–90%	Spin cartridge	7–10 µL	5188-5230
			4.6 x 50 mm	15–20 µL	5185-5984
			4.6 x 100 mm	30–40 µL	5185-5985
MARS Human-High Capacity	Albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin	85–90%	Spin cartridge	14–16 µL	5188-5341
			4.6 x 50 mm	30–40 µL	5188-5332
			4.6 x 100 mm	60–80 µL	5188-5333
			10.0 x 100 mm	up to 340 µL	5188-5336
MARS Human-2	Albumin, IgG	69%	Spin cartridge	50 µL	5188-8825
			4.6 x 50 mm	100 µL	5188-8826
MARS Human-1	Albumin	50–55%	Spin cartridge	65 µL	5188-5334
			4.6 x 50 mm	130 µL	5188-6562
MARS Mouse-3	Albumin, IgG, transferrin	80%	Spin cartridge	25–30 µL	5190-2534
			4.6 x 50 mm	37–50 µL	5188-5217
			4.6 x 100 mm	75–100 µL	5188-5218

Tips and tools

Visit our online store to directly purchase our columns and consumables: **www.agilent.com/chem/store**

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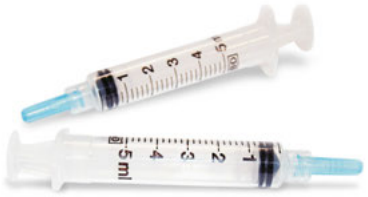
Multiple Affinity Removal System Starter Kits

Description	Part No.
High concentration sample dilution buffer, 50 mL	5188-8283
LC column reagent starter kit includes:	5185-5986
Buffer A, for loading, washing, and equilibrating, 1 L	5185-5987
Buffer B, for eluting, 1 L	5185-5988
0.22 µm cellulose acetate, 25/pk, 1 L	5185-5990
Spin concentrators, 5K MWCO, 4 mL, 25/pk	5185-5991
Multiple affinity removal spin cartridge reagent kit includes:	5188-5254
Buffer A, for loading, washing, and equilibrating, 1 L	5185-5987
Buffer B, for eluting, 1 L	5185-5988
2 x spin filters, 0.22 µm cellulose acetate, 25/pk	5185-5990
Spin concentrators, 5K MWCO, 4 mL, 25/pk	5185-5991
Luer-Lok adapters, 2/pk	5188-5249
Plastic syringe, 5 mL, Luer-Lok, 2/pk	5188-5332
6 x microtube, 1.5 mL, screw top, 100/pk	5188-5251
Caps and plugs, 6/pk	5188-5252
PTFE needles, Luer-Lok, 10/pk	5188-5253



Luer-Lok syringe, 5188-5250

Luer-Lok adapters, 5188-5249

Luer-Lok needles, 5188-5253

Oligonucleotides

Introduction

The interest in oligonucleotides has widened considerably in recent years and includes both DNA and RNA in a wide range of sizes, from 10 – 20 nucleotides in length to many hundreds or thousands of nucleotides. The smaller molecules may be prepared synthetically and can contain a variety of impurities, including closely related sequences with omissions or insertions, to sequences containing chemical modifications, either intentionally or inadvertently due to side reactions. Larger sequences such as mRNA are produced enzymatically and can contain different impurities as a result.

Consequently, analysis and purification of oligonucleotides by liquid chromatography presents a unique set of challenges. The phosphate backbone means the molecules are very hydrophilic which means reversed-phase chromatography is not as straightforward as might be expected. Also, the ability to form strong hydrogen bonds with complementary sequences – essential for creating the classic double helix structure that is so familiar – means it may be necessary to carry out chromatography at elevated temperatures to dissociate these secondary structures.

Purification considerations

Oligonucleotides are used for a variety of different purposes and the level of purity needed may determine the approach needed for purification. In some case, as PCR (polymerase chain reaction) primers for example, the size of the oligonucleotide is relatively small (around 20 – 30 nucleotides) and purification can be accomplished using a solid phase extraction approach known as “trityl on” purification. For other applications, or for larger oligonucleotides where impurities are closely related to the product, HPLC approaches are preferred.

For HILIC column options, we suggest the AdvanceBio Glycan Mapping column, an amide-based HILIC stationary phase on **page 123**, or the AdvanceBio MS Spent Media columns, zwitterionic HILIC phase on **page 146**.

The most popular chromatographic techniques for oligonucleotide analysis and purification are:

- Ion-pair reversed-phase – ionically pairing a cationic base such as triethylamine acetate (TEAA) with the negatively charged phosphate backbone results in a complex that is sufficiently hydrophobic to be retained on a traditional reversed-phase column. The resultant complex is then easily retained on traditional reversed-phase columns. A very shallow gradient of increasing organic modifier will cause the oligonucleotides to elute with very good resolution.
- Ion exchange – a strong anion exchanger will interact with the negatively charged phosphate backbone of oligonucleotides. A gradient of increasing salt concentration will ensure the oligonucleotide species elute sequentially and well separated.

DNA and RNA oligonucleotide column selection

DNA and RNA Oligonucleotide Column Selection

Technique	Agilent Columns	Notes
Ion-pair reversed-phase	AdvanceBio Oligonucleotide	High efficiency 2.7 µm and 4 µm superficially porous particles for high resolution analysis of oligonucleotides and their impurities.
	PLRP-S	Macroporous polymeric particles in a wide range of particle sizes and pore sizes, offering the greatest pH and temperature stability.
Anion exchange	Agilent Bio IEX	Non-porous polymeric particles with hydrophilic coating eliminating mass transfer issues, providing exceptional performance. Available in a range of particle sizes for analytical and lab scale preparative capabilities.
	PL-SAX	Fully porous polymeric particles with strong anion exchange functionality, available from analytical to prep scale, including bulk media.

When choosing the most appropriate technique to use, several things should be considered. Deciding if the technique will be needed for preparative scale purification means selecting the column that will provide the highest capacity and still be able to deliver the necessary purity, but also requiring a column or stationary phase that can be scaled up to the necessary size. If it is intended to use MS detection then it will be necessary to use an approach where the mobile phase is compatible.

Since oligonucleotides come in a wide range of different sizes, choosing a column stationary phase that has pores large enough to allow all molecules to diffuse is essential. If the pore size is too large, some of the separation capability will be lost due to lower surface area. If the pore size is too small, larger molecules will not be able to permeate into the particles. An exception to this is when non-porous particles are available for analytical purposes.

Tips and tools

To learn more about Agilent's portfolio of LC columns for oligonucleotide separations, including workflow ordering guides and training materials, see **explore.agilent.com/oligonucleotide-chromatography-solutions**.

Ion-pair reversed-phase separation

AdvanceBio Oligonucleotide

To successfully separate deprotected synthetic oligonucleotides, you need columns that have high resolving power and are robust enough to withstand challenging conditions.

Agilent AdvanceBio Oligonucleotide columns feature high efficiency, 2.7 µm and 4 µm superficially porous Poroshell particles available in 2.1 mm ID analytical dimensions through 21.2 mm ID preparative columns. Using technology exclusive to Agilent, we chemically modify the particles to make them exceedingly resistant to high pH mobile phases. We also bond them with an end-capped C18 phase that delivers excellent selectivity for oligonucleotides. What's more, we test every batch of AdvanceBio oligonucleotide media with a resolution standard to ensure consistent performance.

The family of AdvanceBio products are designed to deliver consistent, exceptional performance for the complete characterization of proteins, antibodies, conjugates, new biological entities and biopharmaceuticals.

The unique pore size distribution and shorter diffusion distance provided by these particles enable both LC-UV (LC-DAD) and LC-MS applications, or separations as the second dimension in a 2D-LC experiment, to be run on oligonucleotides up to around 100 – 150 nucleotides in length with high resolution.

Although the pores are not large enough to allow the analysis of very large oligonucleotides such as mRNA, which requires the use of wide pore PLRP-S materials, AdvanceBio Oligonucleotide columns can be used to analyze mRNA capping, a critical quality attribute in biotherapeutic mRNA-based vaccines.

Column Specifications

Bonded Phase	Particle Size	Pore Size	Temp Limit	pH Range	Endcapped	Pressure Limit
C18	2.7 µm	100 Å	65 °C	3.0–11.0	Double	600 bar
C18	4 µm	100 Å	65 °C	3.0–11.0	Double	600 bar

Tips and tools

To learn more about the impact of different ion pair reagents on oligonucleotide separations, see application note **5994-2957EN**.

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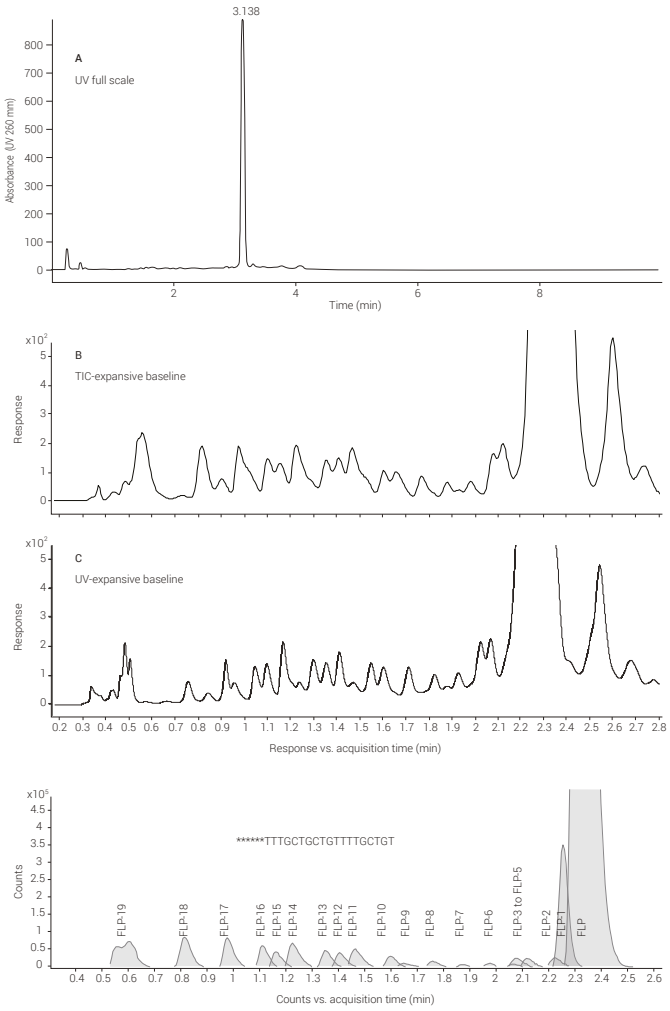
Column: **AdvanceBio Oligonucleotide 2.1 x 50 mm (p/n 659750-702)**

Mobile phase:	A: HFIP:TEA (400 mM:15mM) in water
	B: MeOH:mobile phase A (50:50)
Flow rate:	0.4 mL/min
Gradient:	30–40% B in 0.5 min; 40–70% B in 5 min
Sample:	25 mer DNA
Temperature:	65 °C
Detection:	UV at 260 nm
Detection:	MS
Min range:	400 m/z
Max range:	1,700 m/z
Scan rate:	3.00 spectra/s
Ion polarity:	-ve
VCap:	3,500
Nozzle voltage:	1,000 V
Fragmentor:	200



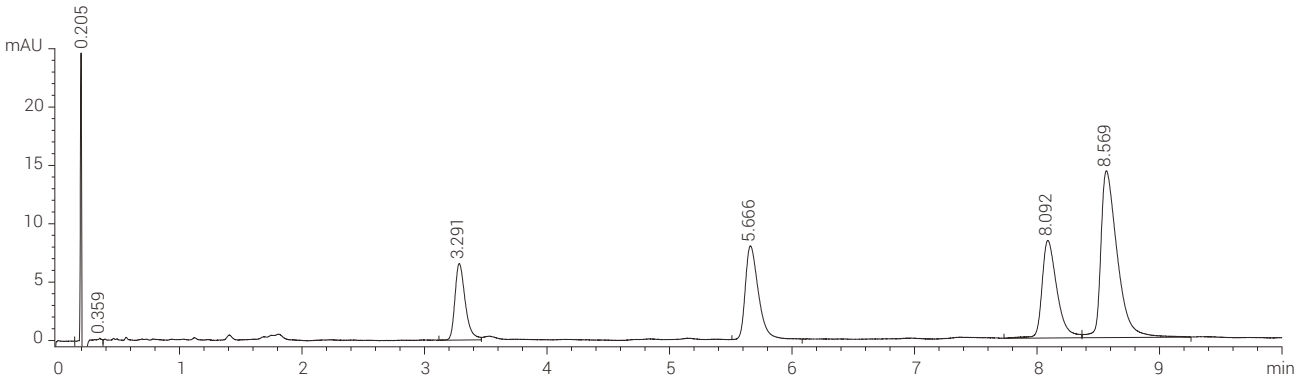
Deconvoluted data from TIC of a 25-mer DNA oligonucleotide separated by the AdvanceBio Oligonucleotide column

AdvanceBio Oligonucleotide standards

To ensure performance for your separations, every batch of AdvanceBio Oligonucleotide media is tested with the Agilent Oligonucleotide Resolution standard. The Oligonucleotide Resolution standard containing 14, 17, 20, and 21 mer synthetic oligonucleotides is designed to demonstrate N / N-1 resolution.

Column:	**AdvanceBio Oligonucleotide 2.1 x 50 mm (p/n 659750-702)**
Mobile phase:	A: 100 mM TEAA in water B: 100 mM TEAA in acetonitrile
Gradient:	6 to 8% B in 12 min
Stop time:	13 min
Post run:	5 min
Flow rate:	0.6 mL/min

Sample:	Agilent Oligonucleotide Resolution Standard (p/n 5190-9028)
Temperature:	65 °C
Injection:	0.5 µL
Detection:	UV at 260 nm



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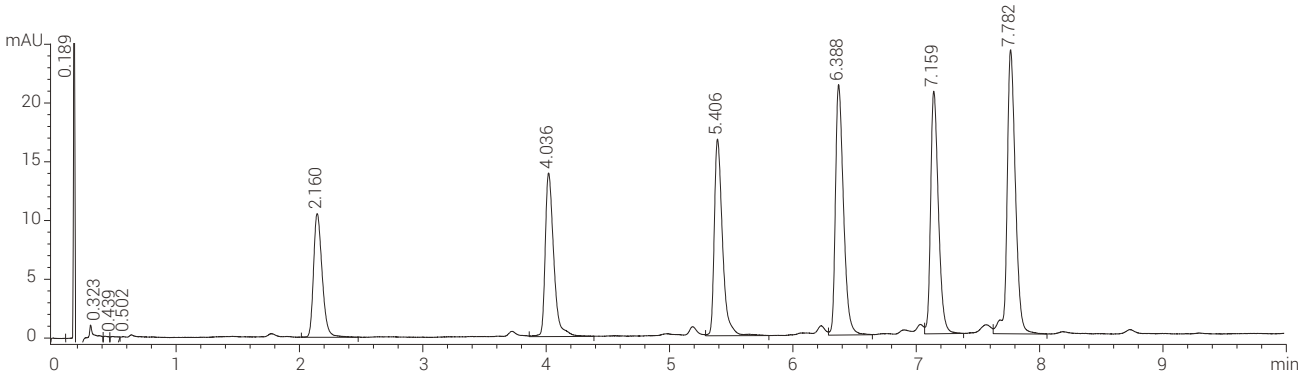
Agilent also offers an Oligonucleotide Ladder standard containing 15, 20, 25, 30, 35, and 40 mer synthetic oligodeoxythymidines, an excellent tool for demonstrating column selectivity and reproducibility.

Column: **AdvanceBio Oligonucleotide 2.1 x 50 mm (p/n 659750-702)**
Mobile phase: A: 100 mM TEAA in water
B: 100 mM TEAA in acetonitrile
Gradient: 10 to 14% B in 10 min
Stop time: 11 min
Post run: 5 min
Flow rate: 0.6 mL/min
Column temperature: 65 °C
Sample: Agilent Oligonucleotide Ladder standard (p/n 5190-9029)
Injection: 10 µL
Detection: UV at 260 nm



Tips and tools

Size exclusion chromatography – suitable for desalting, or for separating different classes of oligonucleotide, SEC is unable to resolve similar sized molecules. It is a non-interactive technique and is very straightforward to operate without a need for gradient elution. For SEC column options, we suggest the AdvanceBio SEC columns on **page 97**, or for larger pore sizes for longer oligonucleotides, the Agilent Bio SEC-5 columns on **page 104.**

HILIC (hydrophilic liquid interaction chromatography) – ideally suited for separating very hydrophilic molecules such as oligonucleotides. It is considered a "difficult" technique to master since the separation occurs in an aqueous layer absorbed on the surface of the stationary phase. Ensuring this absorbed layer is consistently formed and maintained is key, so extra attention to column conditioning is essential.

PLRP-S

- Contain durable and resilient polymeric particles that deliver reproducible results over longer lifetimes, even under harsh decontamination conditions for process scale separations
- Thermally and chemically stable
- Pore sizes (100 Å to 4000 Å) for separations of small oligonucleotides to large polynucleotides
- Fully scalable from analytical scale discovery stage to multi-kg CGMP production

PLRP-S columns and media are available in a range of pore sizes and particle sizes, all with identical chemistry and fundamental adsorptive characteristics. Inherently hydrophobic chemistry means there is no bonded phase, alkyl ligand required for reversed-phase separations.

This gives a highly reproducible material that is free from silanols and heavy metal ions. Within the extensive product range are polymeric reversed-phased columns suitable for analytical separations, and preparative purifications. In addition, process scale columns can be packed with bulk media.

PLRP-S polymeric chemistry features high pH and temperature compatibility, essential for your oligonucleotide purification and analysis.

Capacity and resolution are two key parameters for maximizing the throughput of a purification. With a wide choice of pore sizes and extended range of operating conditions, PLRP-S provides more options to achieve the optimum process. Particle sizes range from 3 µm to 50 µm for scale-up from the µg/mg discovery stage to multi-kg CGMP production. Excellent chemical stability, up to 1 M NaoH, permits decontamination and regeneration that increase column lifetime. PLRP-S media batch sizes of up to 600 L are available, providing single batch packing of multiple columns.

As part of our commitment to quality and continuity of supply, all manufacturing is carried out under a fully documented process, and facility audits are routinely conducted.

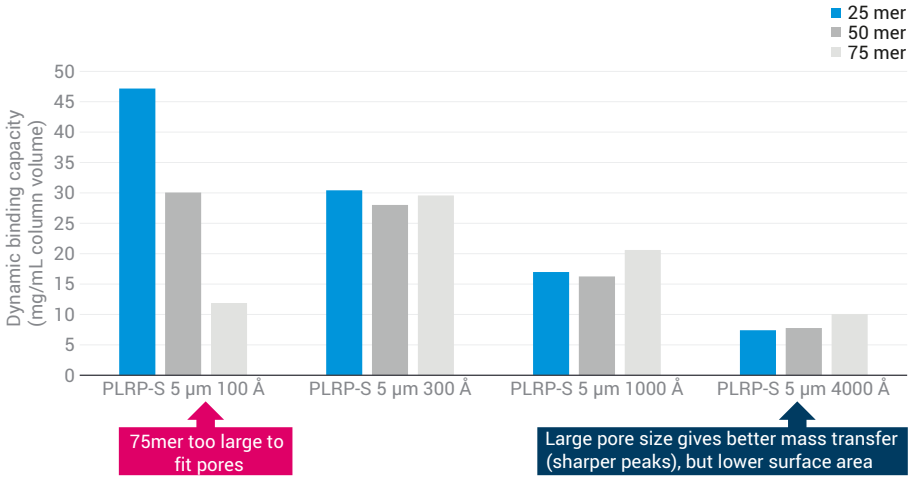
UHPLC Column Specifications

pH range	1–14
Buffer content	Unlimited
Organic modifier	1–100%
Temperature limits	200 °C
Maximum pressure	3 µm: 275 bar/4000 psi
	5 µm, 8 µm, and 10 µm: 207 bar/3000 psi
	10–15 µm, 15–20 µm, and 30 µm: 103 bar/1500 psi

PLRP-S Applications

Pore size	Application
100 Å, 300 Å	Small oligonucleotides up to CRISPR guide RNA
1000 Å	Large oligonucleotides
4000 Å	Very large oligonucleotides / mRNA / high speed

The correct choice of pore size should be made based on the size of molecule: larger molecules require larger pore sizes to enable accessibility and improved mass transfer. With fully porous particles such as PLRP-S, larger pore size means the internal surface area will be lower. The higher surface area of the lower pore sizes provides a potential for higher capacity, an important consideration for purification.



Comparison of binding capacity of different size oligonucleotides.

Tips and tools

For more information about Agilent oligonucleotide purification solutions, see brochure **5994-4286EN**.

Strong Anion Exchange

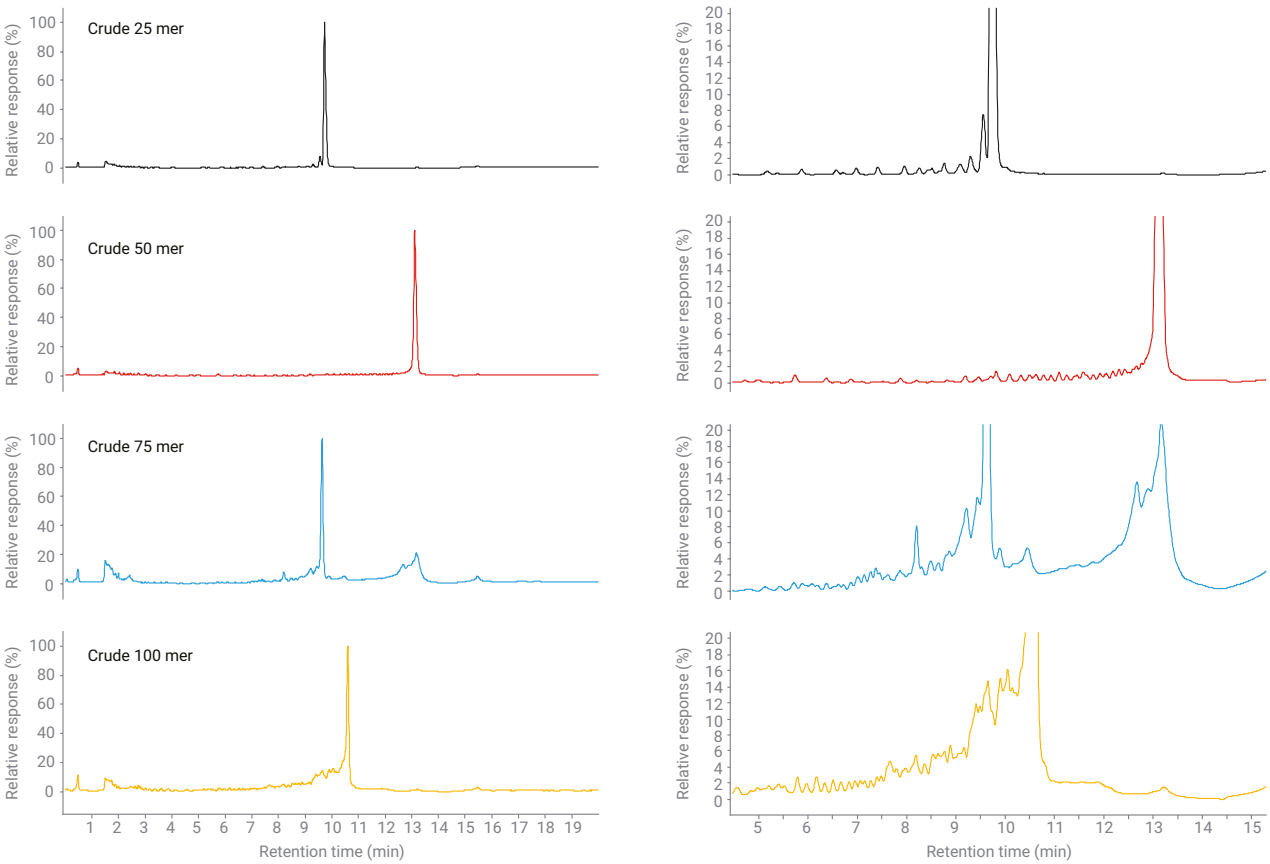
Agilent Bio-IEX

- Highly cross-linked and rigid nonporous poly(styrene divinylbenzene) (PS/DVB) particles are grafted with a hydrophilic polymeric layer, eliminating nonspecific binding
- Uniform, densely packed ion-exchange functional groups are chemically bonded to the hydrophilic layer (multiple ion-exchange groups per anchor) to increase column capacity
- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations

Bio IEX HPLC columns are packed with polymeric, nonporous, ion-exchange particles and are designed for high resolution, high recovery, and highly efficient separations of peptides, oligonucleotides, and proteins.

The Bio IEX family includes cation-exchange for protein analysis, however the negatively charged phosphate backbone of oligonucleotides means that strong anion-exchange (SAX) is the preferred choice. All phases are available in 1.7, 3, 5, and 10 µm nonporous particles.

The absence of a pore structure means accessibility and mass transfer issues are eliminated, improving the resolution for analytical separations; however, the overall capacity is greatly reduced.



Analytical separation of crude 25, 50, 75, and 100 mer using an Agilent Bio SAX 5 µm column (left) with zoomed regions (right).

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PL-SAX

- Small, fully porous particles deliver excellent chromatographic performance
- Wide range of particle sizes and two pore sizes for flexible analysis to scale up purification
- Exceptional stability for long column lifetime

PL-SAX is ideal for the anion-exchange HPLC separations of deprotected synthetic oligonucleotides under denaturing conditions, and other acidic peptides and proteins. The strong anion-exchange functionality, covalently linked to a chemically stable fully porous polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. PL-SAX delivers improved chromatography for self-complementary or G-rich sequences that may associate to form aggregates or hairpin structures. The 5 µm material provides high-efficiency separations of n and n-1 sequences. A wide range of particle sizes and column geometries permits analysis scale-up to purification.

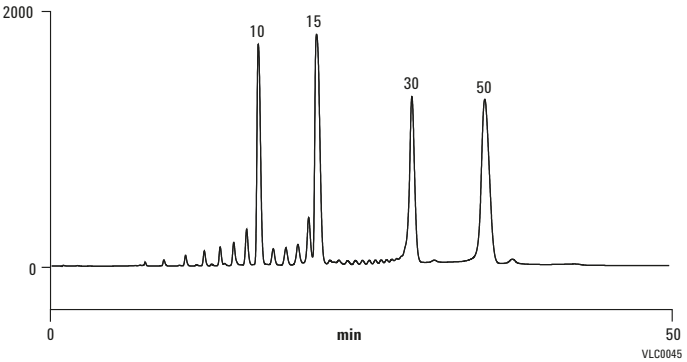
The strong anion-exchange functionality provides a material with exceptional chemical and thermal stability, even with sodium hydroxide eluents, leading to long column lifetime.

Column Specifications

Bonded Phase	Internal diameter (mm)	Particle Size (µm)	Pore Size (Å)	pH Stability	Operating Temperature Limit
Strong anion-exchange	2.1, 4.6, 7.5, 25, 50, and 100	5, 8, 10, and 30	1000 Å and 4000 Å	1–14	80 °C

Reliable separations of synthetic oligonucelotides—high resolution separation of a poly-t-oligonucleotide size standard spiked with 10-mer, 15-mer, 30-mer, and 50-mer (main peaks)

Column:	**PL-SAX 1000 Å** **PL1551-1802** **4.6 x 50 mm, 8 µm**
Mobile phase:	A: 7:93 v/v ACN: 100 mM TEAA, pH 8.5 B: 7:93 v/v ACN: 100 mM TEAA, 1 M ammonium chloride, pH 8.5
Gradient:	0–40% B in 10 min, followed by 40–70% B in 14 min and 70–100% B in 25 min
Flow rate:	1.0 mL/min
Temperature:	60 °C
Detector:	UV, 220 nm



High-resolution separation of poly-t-oligonucleotide. With the gradient used here, baseline separation of the n-1 from n was easily obtained up to the 15-mer.

Product ordering information

AdvanceBio Oligonucleotide Columns

Size (mm)	Particle Size (µm)	Part No.
2.1 x 150	2.7	653750-702
2.1 x 100	2.7	655750-702
2.1 x 50	2.7	659750-702
2.1 x 5, guard	2.7	821725-921
4.6 x 150	2.7	653950-702
4.6 x 100	2.7	655950-702
4.6 x 50	2.7	659950-702
4.6 x 5, guard	2.7	820750-921

AdvanceBio Oligonucleotide Columns

Size (mm)	Particle Size (µm)	Part No.
21.2 x 50	4	671050-702
21.2 x 150	4	671150-702
4.6 x 150	4	693971-702
4.6 x 100	4	695971-702
4.6 x 50	4	699971-702
4.6, guard	4	820750-941

AdvanceBio Oligonucleotide Standards

Description	Part No.
Oligonucleotide Resolution Standard	5190-9028
Oligonucleotide Ladder Standard	5190-9029

PLRP-S HPLC Columns

Size (mm)	Particle Size (µm)	PLRP-S 100 Å USP L21	PLRP-S 300 Å USP L21	PLRP-S 1000 Å USP L21	PLRP-S 4000 Å USP L21
4.6 x 250	8	PL1512-5800	PL1512-5801	PL1512-5802	
4.6 x 150	8	PL1512-3800	PL1512-3801	PL1512-3802	PL1512-3803
4.6 x 50	8		PL1512-1801	PL1512-1802	PL1512-1803
4.6 x 250	5	PL1512-5500	PL1512-5501		
4.6 x 150	5	PL1111-3500	PL1512-3501		
4.6 x 50	5	PL1512-1500	PL1512-1501	PL1512-1502	PL1512-1503
4.6 x 150	3	PL1512-3300	PL1512-3301		
4.6 x 50	3	PL1512-1300	PL1512-1301		
2.1 x 250	8		PL1912-5801		
2.1 x 150	8		PL1912-3801	PL1912-3802	PL1912-3803

(Continued)

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PLRP-S HPLC Columns

Size (mm)	Particle Size (µm)	PLRP-S 100 Å USP L21	PLRP-S 300 Å USP L21	PLRP-S 1000 Å USP L21	PLRP-S 4000 Å USP L21
2.1 x 50	8		PL1912-1801	PL1912-1802	PL1912-1803
2.1 x 250	5	PL1912-5500	PL1912-5501		
2.1 x 150	5	PL1912-3500	PL1912-3501		
2.1 x 50	5	PL1912-1500	PL1912-1501	PL1912-1502	PL1912-1503
2.1 x 150	3	PL1912-3300	PL1912-3301		
2.1 x 50	3	PL1912-1300	PL1912-1301		
1.0 x 50	8			PL1312-1802	PL1312-1803
1.0 x 50	5	PL1312-1500	PL1312-1501	PL1312-1502	PL1312-1503
1.0 x 150	3	PL1312-3300			
1.0 x 50	3	PL1312-1300	PL1312-1301		
PLRP-S guard cartridges for 3.0 x 5.0 mm, 2/pk		PL1612-1801	PL1612-1801	PL1612-1801	PL1612-1801
Guard cartridge holder for 3.0 x 5.0 mm cartridges		PL1310-0016	PL1310-0016	PL1310-0016	PL1310-0016

Prep to Process PLRP-S

Size (mm)	Particle Size (µm)	PLRP-S 100 Å	PLRP-S 300 Å	PLRP-S 1000 Å	PLRP-S 4000 Å
100 x 300	30			PL1812-3102	PL1812-3103
100 x 300	15-20	PL1812-6200	PL1812-6201		
100 x 300	10-15	PL1812-6400	PL1812-6401		
100 x 300	10	PL1812-6100	PL1812-6101		
100 x 300	8	PL1812-6800	PL1812-6801		
50 x 300	8	PL1712-6800	PL1712-6801		
50 x 150	30			PL1712-3702	PL1712-3703
50 x 150	15-20	PL1712-3200	PL1712-3201		
50 x 150	10-15	PL1712-3400	PL1712-3401		
50 x 150	10	PL1712-3100	PL1712-3101	PL1712-3102	PL1712-3103
50 x 150	8	PL1712-3800	PL1712-3801		
25 x 300	15-20	PL1212-6200	PL1212-6201		

(Continued)

Prep to Process PLRP-S

Size (mm)	Particle Size (µm)	PLRP-S 100 Å	PLRP-S 300 Å	PLRP-S 1000 Å	PLRP-S 4000 Å
25 x 300	10-15	PL1212-6400	PL1212-6401		
25 x 300	10	PL1212-6100	PL1212-6101		
25 x 300	8	PL1212-6800	PL1212-6801		
25 x 150	30			PL1212-3702	PL1212-3703
25 x 150	10	PL1212-3100	PL1212-3101	PL1712-3102	PL1712-3103
25 x 150	8	PL1212-3800	PL1212-3801		
25 x 50	10			PL1212-1102	PL1212-1103
PLRP-S Method Development Columns					
4.6 x 250	30			PL1512-5702	PL1512-5703
4.6 x 250	15-20	PL1512-5200	PL1512-5201		
4.6 x 250	10-15	PL1512-5400	PL1512-5401		
4.6 x 250	10	PL1512-5100	PL1512-5101	PL1512-5102	PL1512-5103
4.6 x 250	8	PL1512-5800	PL1512-5801		
4.6 x 150	30			PL1512-3702	PL1512-3703
4.6 x 150	15-20	PL1512-3200	PL1512-3201		
4.6 x 150	10-15		PL1512-3401		
4.6 x 150	10	PL1512-3100	PL1512-3101	PL1512-3102	PL1512-3103
4.6 x 150	8	PL1512-3800	PL1512-3801		

PLRP-S Bulk Media

Particle Size (µm)	Unit	PLRP-S 100 Å	PLRP-S 300 Å	PLRP-S 1000 Å	PLRP-S 4000 Å
50	1 kg	PL1412-6K00	PL1412-6K01	PL1412-6K02	
	100 g	PL1412-4K00	PL1412-4K01	PL1412-4K02	
30	100 g			PL1412-4702	PL1412-4703
15-20	1 kg	PL1412-6200	PL1412-6201		
	100 g	PL1412-4200	PL1412-4201		
10-15	1 kg	PL1412-6400	PL1412-6401		
	100 g	PL1412-4400	PL1412-4401		
10	1 kg	PL1412-6100	PL1412-6101		
	100 g	PL1412-4100	PL1412-4101	PL1412-4102	PL1412-4103
8	1 kg	PL1412-6800	PL1412-6801		

Custom column and bulk media ordering.

If you do not see the combination of pore size/particle size and column dimension or the bulk media quantity you require in these tables, contact your local sales office for assistance with our custom ordering process.

Bio SAX HPLC Columns, PEEK

Size (mm)	Particle Size (µm)	Pressure Limit	Part No.
4.6 x 250	10	275 bar, 4000 psi	5190-2475
4.6 x 50	10	275 bar, 4000 psi	5190-2476
4.6 x 250	5	400 bar, 5800 psi	5190-2467
4.6 x 50	5	400 bar, 5800 psi	5190-2468
2.1 x 250	10	275 bar, 4000 psi	5190-2479
2.1 x 50	10	275 bar, 4000 psi	5190-2480
2.1 x 250	5	400 bar, 5800 psi	5190-2471
2.1 x 50	5	400 bar, 5800 psi	5190-2462

Bio SAX HPLC Columns, Stainless Steel

Size (mm)	Particle Size (µm)	Pressure Limit	Part No.
21.2 x 250	5		5190-6883
10 x 250	5		5190-6882
4.6 x 250	10	275 bar, 4000 psi	5190-2473
4.6 x 250	5	413 bar, 6000 psi	5190-2465
4.6 x 150	3		
4.6 x 50	3	551 bar, 8000 psi	5190-2463
4.6 x 50	1.7	600 bar, 8700 psi	5190-2461
4.0 x 10, guard	10	275 bar, 4000 psi	5190-2474
4.0 x 10, guard	5	413 bar, 6000 psi	5190-2466
4.0 x 10, guard	3	551 bar, 8000 psi	5190-2464
4.0 x 10, guard	1.7	600 bar, 8700 psi	5190-2462

Tips and tools

Visit our online store to directly purchase our columns and consumables: **www.agilent.com/chem/store**

PL-SAX Strong Anion-Exchange Columns

Size (mm)	Particle Size (µm)	Pressure Limit	PL-SAX 1000 Å	PL-SAX 4000 Å
100 x 300	10	207 bar, 3000 psi	PL1851-2102	PL1851-2103
50 x 150	30	207 bar, 3000 psi	PL1751-3702	PL1751-3703
50 x 150	10	207 bar, 3000 psi	PL1751-3102	PL1751-3103
25 x 150	30	207 bar, 3000 psi	PL1251-3702	PL1251-3703
25 x 150	10	275 bar, 4000 psi	PL1251-3102	PL1251-3103
25 x 50	10	207 bar, 3000 psi	PL1251-1102	PL1251-1103
4.6 x 250	30	207 bar, 3000 psi	PL1551-5702	PL1551-5703
4.6 x 150	30	207 bar, 3000 psi	PL1551-3702	PL1551-3703
4.6 x 250	10	207 bar, 3000 psi	PL1551-5102	PL1551-5103
4.6 x 150	10	207 bar, 3000 psi	PL1551-3102	PL1551-3103
4.6 x 150	8	207 bar, 3000 psi	PL1551-3802	PL1551-3803
4.6 x 50	8	207 bar, 3000 psi	PL1551-1802	PL1551-1803
4.6 x 50	5	207 bar, 3000 psi	PL1551-1502	PL1551-1503
2.1 x 150	8	207 bar, 3000 psi	PL1951-3802	PL1951-3803
2.1 x 50	8	207 bar, 3000 psi	PL1951-1802	PL1951-1803
2.1 x 50	5	207 bar, 3000 psi	PL1951-1502	PL1951-1503

PL-SAX Strong Anion-Exchange Bulk Media

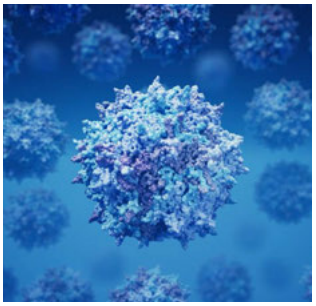
Size (mm)	Particle Size (µm)	Pressure Limit	PL-SAX 1000 Å	PL-SAX 4000 Å
100 g	30	207 bar, 3000 psi	PL1451-4702	PL1451-4703
100 g	10	207 bar, 3000 psi	PL1451-4102	PL1451-4103

Tips and tools

Column user guides are excellent resources, with instructions for use and column care, as well as suggested starting methods:

www.agilent.com/chem/biolc-columns-user-guides

Vector-Based Therapeutics Analysis



Characterization from intact vector down to single amino acid modification

Numerous biotherapeutic delivery platforms have emerged as exciting and promising new modalities in biopharmaceutical research. These range from protein-based capsids delivering oligonucleotides for cell and gene therapy, to protein-based virus-like particles for vaccines, and lipid-based nanoparticles delivering mRNA for vaccines.

Adeno-associated viruses (AAVs) are the most common type of viral vectors under investigation for cell and gene therapy, and have been successful in treating inherited retinal diseases and spinal muscular atrophy. As AAVs are explored for use as therapeutic delivery platforms, it is vital to ensure that all the critical quality attributes (CQAs) of the therapeutic product are monitored.

AAVs have both familiar and unique CQAs

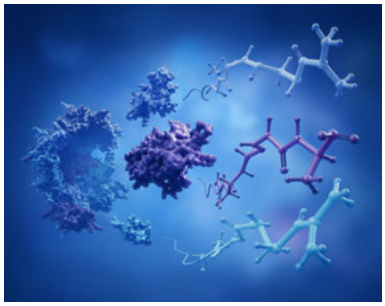
AAVs exist in several different forms called serotypes, each with similar higher order structure, but sufficient amino acid sequence differences to naturally target different types of tissue. This natural selectivity makes them useful vectors for the development of targeted biotherapeutics, but also make the implementation of platform analytical methods challenging.

Many AAV CQAs are familiar given their similarity to CQAs monitored for other single protein biotherapeutics, such as protein sequence confirmation via LC/MS or peptide mapping. While other CQAs, such as the proportion of capsids that are filled—and filled correctly—versus empty is unique. Some of these analyses are further complicated by the presence of the transgene encapsulated inside the AAV.

Tips and tools

To learn more about Agilent's portfolio of LC column for CQA analysis of AAVs, visit **explore.agilent.com/advancebio-aav**.

Capsid protein identity confirmation



AAV capsids comprise ~60 copies of three proteins, VP1, VP2, and VP3, in a stoichiometric ratio of approximately 1:1:10, respectively. Chromatographic separation of these three proteins is challenging because of high sequence homology, as all three are spliced from the same gene. SDS-PAGE gels with silver stain or antibody detection-based methods such as ELISA and immunoblotting have historically been used to assess the ratio of the three capsid proteins. However, these approaches are cumbersome and error-prone, and may require the generation of new antibodies specific to each type of AAV. Generating antibodies with the specificity required to distinguish can be difficult given the high degree of homology between AAV serotypes. Liquid chromatography mass spectrometry (LC/MS) overcomes these challenges with improved speed, specificity, and precision. Agilent provides workflow solutions for both intact protein and peptide mapping for identification and localization of post-translational modifications (PTMs).

Reversed-phase separations of AAVs

For intact protein separations of AAV capsids, ZORBAX RRHD wide pore columns are recommended. These 1.8 µm particle, UHPLC columns are available in 4 different chemistries. The sequence differences between AAV serotypes makes a single platform method challenging, but very similar methods can be used by taking advantage of these different phase chemistries. ZORBAX RRHD 300SB-C18 and 300-Diphenyl are currently recommended depending upon the serotype.

Peptide mapping is an essential method that determines protein sequences and identifies PTMs, with a higher level of granularity than intact mass measurements alone. This is often with MS detection during development and characterization stages, and with UV or MS detection for QC of released products.

AdvanceBio Peptide Mapping columns are recommended for sequence and PTM identification for AAV capsid proteins. The 2.7 µm superficially porous C18 stationary phase enable high resolution at modest back pressures, and strong overall performance for peptide maps that vary widely in terms of peptide size and hydrophobicity.

Tips and tools

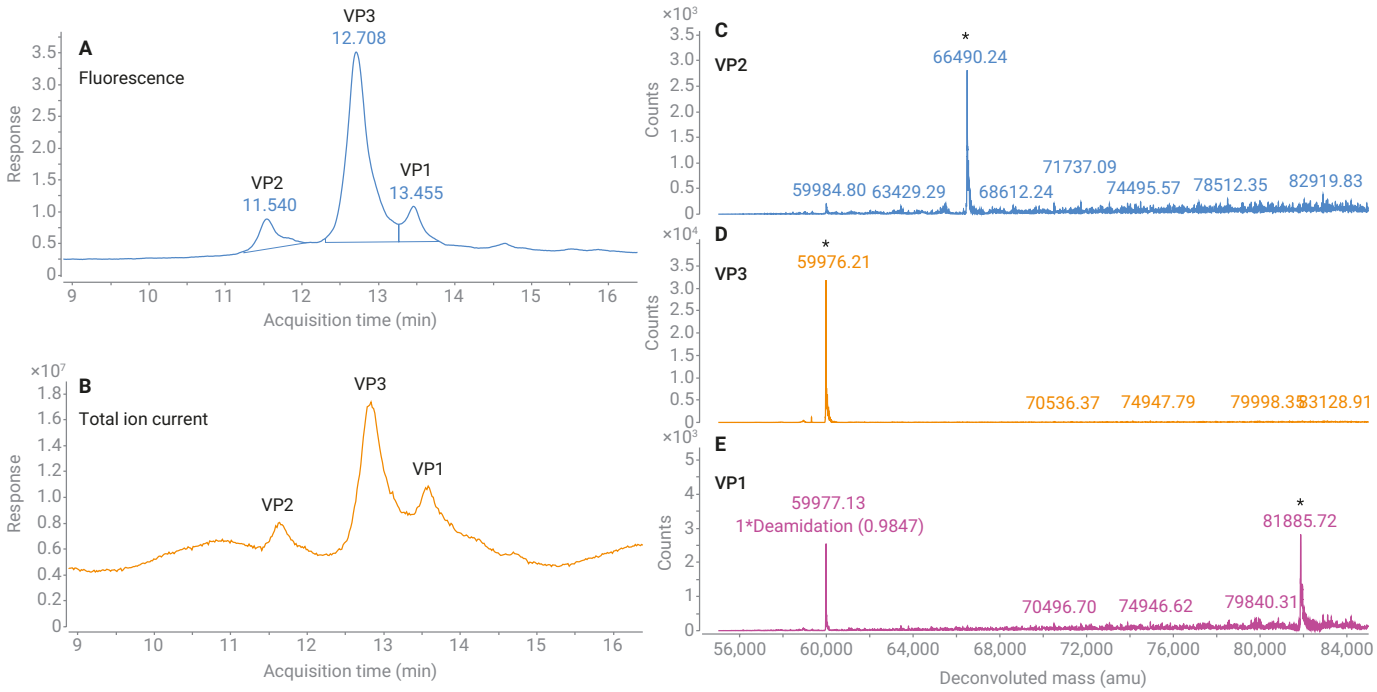
For further column selection and method detail and additional consumables recommendations, please see ordering guide **5994-4829EN**.

ZORBAX RRHD Wide Pore Column Specifications

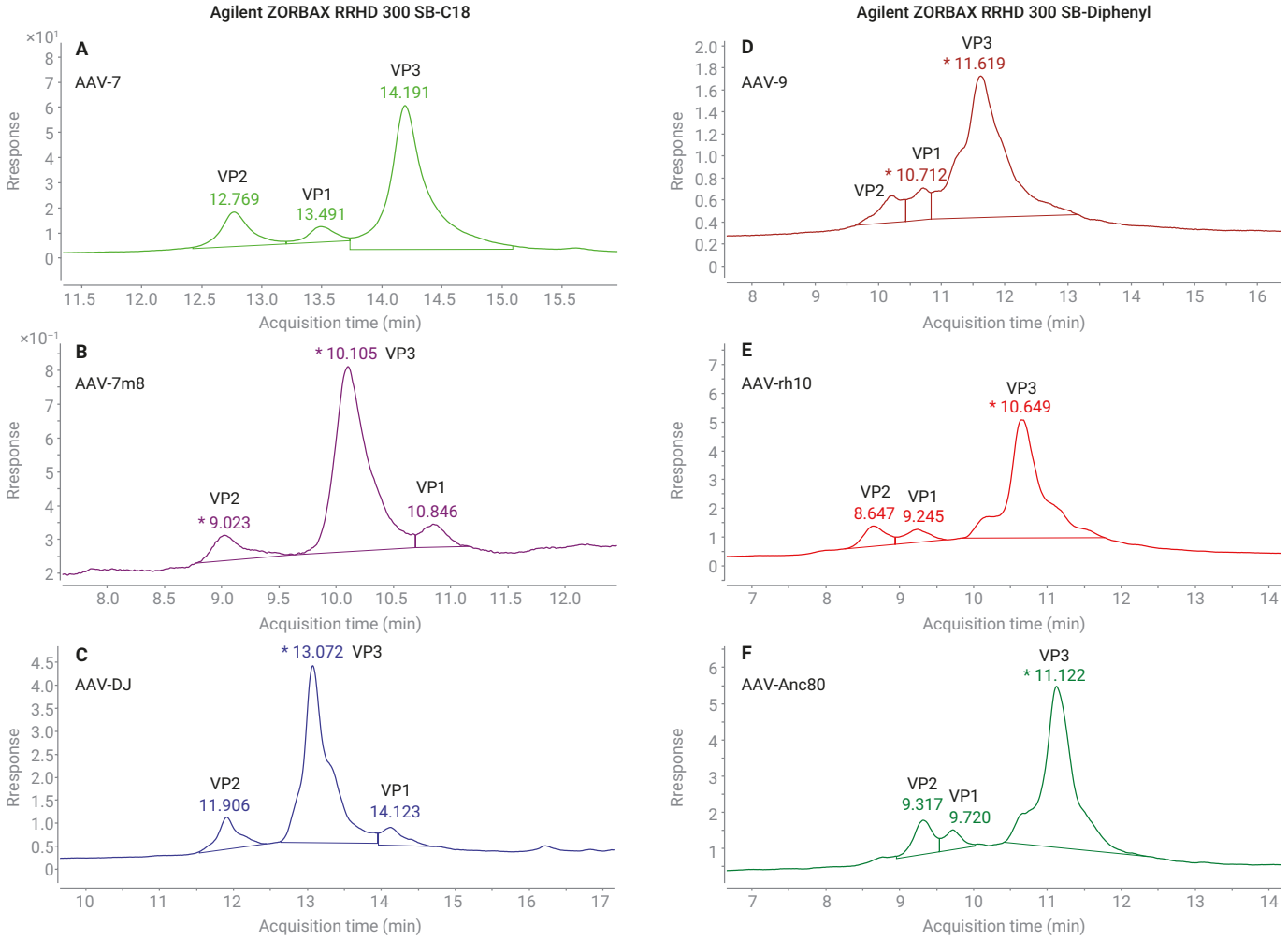
Bonded Phase	Particle Size	Pore Size	Temperature Limit	pH Range	Endcapped
300SB-C18	1.8 µm	300 Å	80 °C	1.0-8.0	No
300-Diphenyl	1.8 µm	300 Å	80 °C	1.0-8.0	Yes

AdvanceBio Peptide Mapping Column Specifications

Bonded Phase	Particle Size	Pore Size	Temperature Limit	pH Range	Endcapped
EC-C18	2.7 µm	120 Å	60 °C	2.0-8.0	Double



LC/MS of denatured AAV-2 capsid proteins using the optimized method on an Agilent ZORBAX RRHD 300Å SB-C18 column. (A) Fluorescence chromatogram depicting the three capsid proteins. (B) Total ion current. (C to E) Deconvoluted mass spectra of the three capsid proteins, with the relevant mass peaks marked by asterisks.



LC/MS of denatured AAV capsid proteins of six different serotypes on (A to C) Agilent ZORBAX RRHD 300 SB-C18, and (D to F) Agilent SB-Diphenyl columns.

Agilent 1290 Infinity II LC System

Column	Agilent ZORBAX RRHD 300 Å StableBond C18 2.1 x 100 mm, 1.8 µm p/n 858750-902	Agilent ZORBAX RRHD 300 Å Diphenyl 2.1 x 100 mm, 1.8 µm p/n 858750-944
Gradient	0 to 5 min, 28% B 23 min, 32.5% B 23.5 min, 80% B 26 min, 80% B	0 to 5 min, 33% B 21 min, 37% B 21.5 min, 80% B 23 min, 80% B
Solvent A	0.1% formic acid, 0.1% TFA in DI water	
Solvent B	90% isopropanol, 9.8% DI water, 0.1% formic acid, 0.1% TFA	
Column Temperature	80 °C	
Flow Rate	0.4 mL/min	
Sample Quantity	1.5 x 10^{11} viral genomes/injection	

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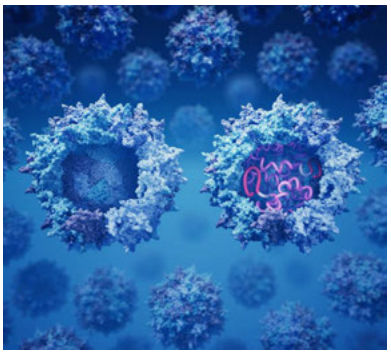
Empty/full analysis of adeno associated viruses



Payload analysis – both in terms of proportion of filled versus empty capsids, as well as payload identity – are critical quality attributes unique to capsid-based biotherapeutics such as AAVs. Differentiating filled versus empty capsids is challenging, and these measurements become even more challenging when considering the low concentrations typical of these samples. Electron microscopy and analytical ultracentrifugation are commonly used to assess the ratio of empty to full capsids, however these methods are slow, challenging and expensive driving users to continue the search for other approaches. Empty and full capsids are difficult to differentiate chromatographically, but the potential speed and relative ease of the techniques continue to make chromatography an appealing approach. Size exclusion with light scattering detection and anion exchange with fluorescence detection are two promising options. However with no universal solution, that is one that can be directly applied to all serotypes and payloads, no consensus has been reached on the ideal technique.

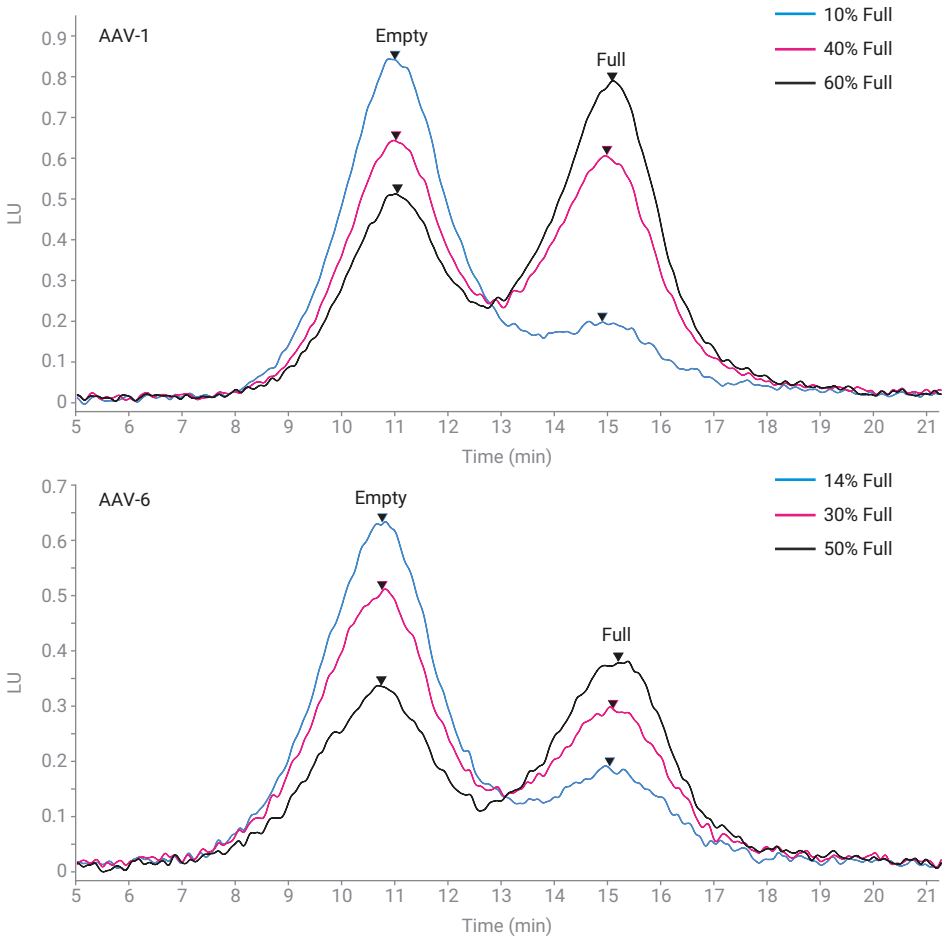
Anion exchange separations of AAVs

While anion exchange methods must be optimized for sufficiently different serotypes, they can show excellent linearity and reproducibility. Agilent Bio SAX columns have been demonstrated the necessary reproducible separation to be recommended for the quantitation of empty versus full AAV capsids.

Agilent Bio SAX Column Specifications

Bonded Phase	Particle Size	Temperature Limit	pH Range	Flow Rate
SAX (strong anion-exchange) – $N(CH_3)_3$	5 µm	80 °C	2.0–12.0	0.1 – 1.0 mL/min *0.1 mL/min recommended for best results for AAVs

Measuring mixtures of empty and full AAV capsids



Separation of AAV-1 and AAV-6 samples with different full/empty capsid ratios using the Agilent binary High-Speed Pump.

Agilent 1290 Infinity II Bio LC System

Column	Agilent Bio SAX, 2.1x50 mm, 5 µm PEEK hardware		
Solvent A	70 mM bis-tris propane, pH 9.0 2 mM $MgCl_2$		
Solvent B	70 mM bis-tris propane + 1M tetramethyl ammonium chloride, pH 9.0 2 mM $MgCl_2$		
Gradient	Time 0 min 25 min 25.1 min 28 min	%B 15% 27.5% 100% 100%	Flow Rate 0.1 mL/min 0.1 mL/min 0.3 mL/min 0.3 mL/min
Fluorescence Detection	λ_{ex} = 280 nm, λ_{em} = 340 nm		

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Aggregate analysis of vector-based therapeutics

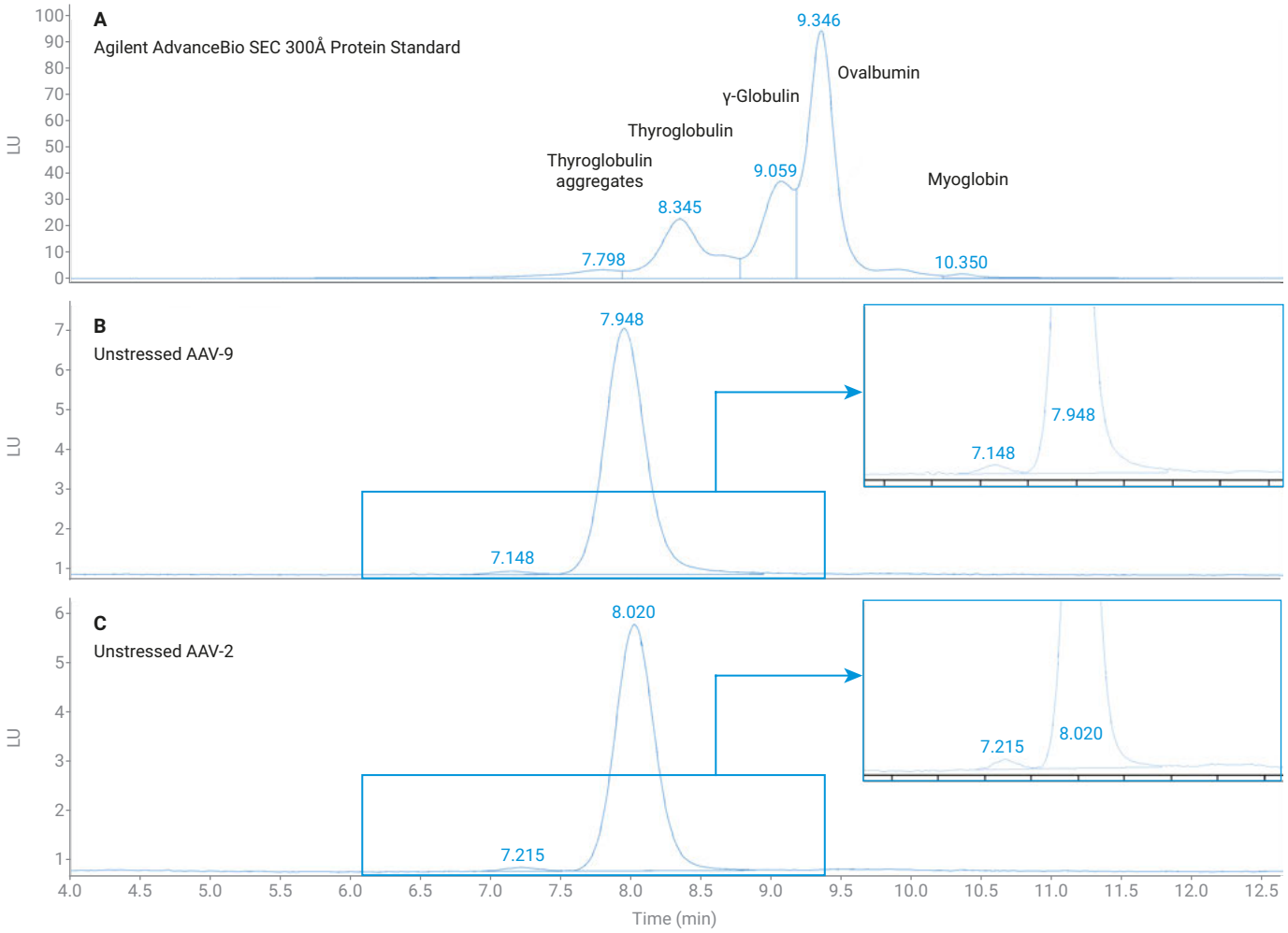
Aggregate analysis is a very commonly monitored CQA for AAVs just as it is for any other protein-based biotherapeutic. The key difference lies in the pore sizes necessary to accurately assess sample aggregation. AAV capsids are approximately 250 Å (25 nm) in diameter, making their aggregates too larger to be included in the pores of common size exclusion columns, which often have pore sizes of 300 Å or less. Conventional wisdom suggests that an SEC pore size should be at least three times larger than the molecule of interest, which would suggest a pore size ≥ 750 Å. While many users have adopted columns of pore size around 500 Å for AAVs, we have found improved monomer-dimer resolution using 1000 Å pores.

The Agilent line of Bio SEC-5 columns are available in pore sizes suitable for a wide range of macromolecular biotherapeutics, including 500, 1000, and even 2000 Å.

Agilent Bio SEC-5 Column Specifications

Pore Size	Particle Size	Mol Wt Range	pH Range	Max Pressure	Flow Rate
500 Å	5 µm	15,000–5,000,000	2–8.5	137 bar, 2000 psi	1.0–10.0 mL/min (21.2 mm id)
					0.2–1.2 mL/min (7.8 mm id)
					0.1–0.4 mL/min (4.6 mm id)
1000 Å	5 µm	50,000–7,500,000	2–8.5	137 bar, 2000 psi	1.0–10.0 mL/min (21.2 mm id)
					0.2–1.2 mL/min (7.8 mm id)
					0.1–0.4 mL/min (4.6 mm id)
2000 Å	5 µm	>10,000,000	2–8.5	137 bar, 2000 psi	1.0–10.0 mL/min (21.2 mm id)
					0.2–1.2 mL/min (7.8 mm id)
					0.1–0.4 mL/min (4.6 mm id)

Size exclusion chromatograms of AAV-9 and AAV-2



Fluorescence chromatograms of Agilent AdvanceBio SEC 300Å Protein Standard and unstressed AAV-9 and AAV-2. Note that the protein standard also contains angiotensin II, which is not shown because it contains no tryptophan amino acids and is therefore nonfluorescent under the chosen excitation and emission settings.

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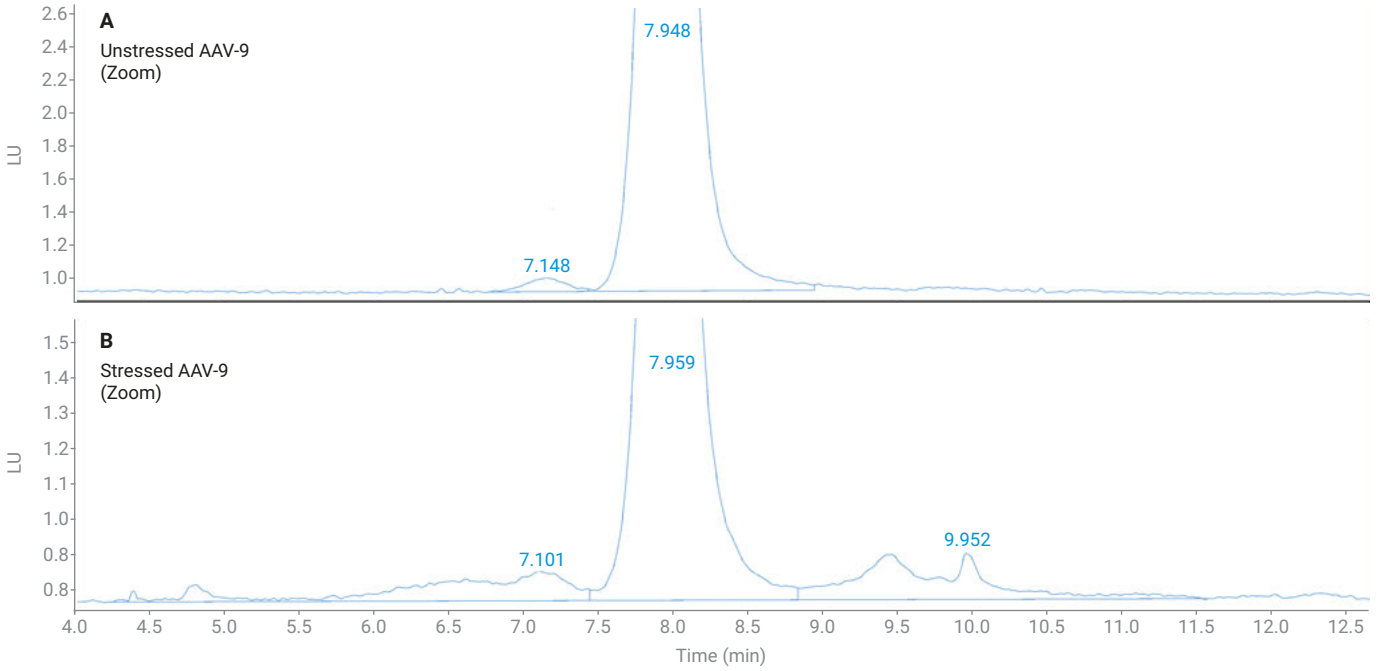
Aggregation of unstressed and stressed AAV-9



Fluorescence chromatograms of unstressed and stressed AAV-9.

Agilent 1290 Infinity II LC System

Column	Agilent Bio SEC-5 5 µm, 1000 Å, 4.6 x 300 mm p/n 5190-2538
Mobile Phase	50 mM phosphate buffer + 400 mM NaCl, pH 7.4
Column Temperature	80 °C
Flow Rate	0.4 mL/min
Detection	Fluorescence, λ_{ex} = 280 nm, λ_{em} = 340 nm
Injection Volume	20 µL

Product ordering information

ZORBAX RRHD Wide Pore Columns for Intact Protein Analysis

Description	Part No.
Agilent ZORBAX RRHD Diphenyl, 2.1 x 150 mm, 1.8 µm, 300 Å	863750-944
Agilent ZORBAX RRHD Diphenyl, 2.1 x 100 mm, 1.8 µm, 300 Å	858750-944
Agilent ZORBAX RRHD StableBond C18, 2.1 x 150 mm, 1.8 µm, 300 Å	863750-902
Agilent ZORBAX RRHD StableBond C18, 2.1 x 100 mm, 1.8 µm, 300 Å	858750-902

AdvanceBio Peptide Mapping Columns for Peptide Level Analysis

Description	Part No.
AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm	653750-902
AdvanceBio Peptide Mapping, 2.1 x 250 mm, 2.7 µm	651750-902
AdvanceBio Peptide Mapping guard column, 2.1 x 150 mm, 2.7 µm, 3/pk	851725-911

Bio SEC-5 Columns for Aggregate and Size Distribution Analysis

Size (mm)	Particle Size (µm)	Bio SEC-5 500 Å USP L59	Bio SEC-5 1000 Å USP L59	Bio SEC-5 2000 Å USP L59
21.2 x 300	5	5190-6866	5190-6867	5190-6868
21.2 x 50, guard	5	5190-6872	5190-6873	5190-6874
7.8 x 300	5	5190-2531	5190-2536	5190-2541
7.8 x 150	5	5190-2532	5190-2537	5190-2542
7.8 x 50, guard	5	5190-2535	5190-2540	5190-2545
4.6 x 300	5	5190-2533	5190-2538	5190-2543
4.6 x 150	5	5190-2534	5190-2539	5190-2544
4.6 x 50, guard	5	5190-6860	5190-6861	5190-6862

Method Development Guidelines

Primary structure analysis methods

This section on column selection strategy for primary structure analysis provides some critical details on method development for mAb, proteins, and peptides.

Peptides and polypeptides Mol Wt <10 kDa	Proteins Mol Wt >10 kDa	Reduced/fragmented Mol Wt <50 kDa	Intact Mol Wt >150 kDa
Initial bonded phase			
AdvanceBio Peptide Mapping Also consider: ZORBAX 300 Å StableBond C18	**ZORBAX 300 Å StableBond C8** Also consider: PLRP-S ZORBAX 300 Å StableBond C18 ZORBAX 300 Å StableBond C3 Poroshell 300 StableBond C18 Poroshell 300 StableBond C8 Poroshell 300 StableBond C3	**AdvanceBio RP-mAb StableBond C8** Also consider: PLRP-S AdvanceBio RP-mAb C4 AdvanceBio RP-mAb diphenyl ZORBAX 300 Å diphenyl ZORBAX 300 Å StableBond C8 ZORBAX 300 Å StableBond C3 ZORBAX 300 Å StableBond C18	**AdvanceBio RP-mAb C4** Also consider: PLRP-S AdvanceBio RP-mAb StableBond C8 AdvanceBio RP-mAb diphenyl Poroshell 300 StableBond C3 Poroshell 300 StableBond C8
Initial separation conditions			
Mobile phase: A: 95% H_2O:5% ACN with 0.1% TFA B: 5% H_2O:95% ACN with 0.085% TFA **Gradient:** 3 to 60% in 30 min **Temperature:** 40 °C	**Mobile phase:** A: 95% H_2O:5% ACN with 0.1% TFA B: 5% H_2O:95% ACN with 0.085% TFA **Gradient:** 5 to 70% in 20 min **Temperature:** 80 °C	**Mobile phase:** A: 100% H_2O with 0.1% TFA B: 10% H_2O:10% ACN:80% n-PA with 0.08% TFA **Gradient:** 5 to 40% in 10 min **Temperature:** 60 °C	**Mobile phase:** A: 98% H_2O:2% IPA with 0.1% TFA B: 10% H_2O:0% ACN:70% IPA with 0.08% TFA **Gradient:** 10 to 60% B in 5 min **Temperature:** 80 °C
2.1 mm id columns **Particle size** 2.7 µm SPP **Flow rate** 0.5 mL/min	**4.6 mm id columns** **Particle size** 2.7 µm SPP **Flow rate** 2.0 mL/min	**2.1 mm id columns** **Particle size** 1.8 µm TPP 3.5 µm TPP 5 µm TPP 5 µm SPP **Flow rate** 0.5 mL/min 0.2 mL/min 0.2 mL/min 1.0 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm TPP 5 µm TPP **Flow rate** 1.0 mL/min 1.0 mL/min	**2.1 mm id columns** **Particle size** 3.5 µm SPP 1.8 µm TPP 3.5 µm TPP 5 µm TPP **Flow rate** 0.8 mL/min 0.3 mL/min 0.2 mL/min 0.2 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm SPP 3.5 µm TPP 5 µm TPP **Flow rate** 4.0 mL/min 1.0 mL/min 1.0 mL/min	**2.1 mm id columns** **Particle size** 3.5 µm SPP 5 µm SPP **Flow rate** 1.0 mL/min	**4.6 mm id columns** **Particle size** 3.5 µm SPP **Flow rate** 5.0 mL/min

SPP = superficially porous particle, TPP = totally porous particle

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Start at low pH with simple aqueous/organic gradient

Typically, a water:acetonitrile with 0.1% trifluoroacetic acid (TFA) gradient is used to elute all components of interest. A typical high resolution gradient on a 300 Å pore size column requires 30 to 50 min. An AdvanceBio RP-mAb column requires a shorter analysis time and a higher flow rate, and still provides exceptional resolution. To improve resolution, increase the gradient time, decrease column length, or increase flow rate.
For LC/MS methods, TFA can reduce detector sensitivity and is often replaced with ammonium formate/formic acid.

Optimize sample solubility

For best peak shape and recovery at any pH, it is important to completely solubilize a sample. Highly acidic or neutral solvents can be used with AdvanceBio RP-mAb, ZORBAX 300 Å StableBond, Poroshell 300 StableBond, and AdvanceBio Peptide Mapping, while neutral solvents and dilute bases can be used with ZORBAX 300Extend-C18 and Poroshell 300Extend-C18.

Solvent choices to solubilize proteins and peptides

Water/phosphate buffer

Dilute acid (TFA, acetic acid or HCl)

Neutral pH, 6–8 M guanidine-HCl or isthiocyanate

Acetic acid 5%/6 M urea

Dilute acid + aqueous/organic solvents (ACE, MeOH, THF)

Dilute base (ammonium hydroxide)

DMSO or 0.1%–1% in DMSO

Formamide

Weakest

Strongest

Increase the temperature

Separations of proteins and peptides are influenced by temperature and higher column temperature can dramatically improve both resolution and recovery of proteins and hydrophobic and aggregating peptides.

AdvanceBio RP-mAb: Up to 90 °C
ZORBAX 300 StableBond, Poroshell 300 StableBond: Up to 80 °C
AdvanceBio Peptide Mapping: Up to 60 °C

Optimize mobile phase pH

Try mid and high pH if low pH does not work

If an optimized, low pH method does not provide an ideal separation, then mid or high pH mobile phase can be used. At high pH, selectivity is often very different because acidic amino acids become negatively charged and some basic amino acids may lose their charge. ZORBAX 300Extend-C18 is an excellent choice for mid to high pH separation.

Column:	**ZORBAX 300 Extend-C18 4.6 x 150 mm, 5 µm 773995-902**
Mobile phase:	A: 20 mM nH_4OH in H_2O B: 20 mM nH_4OH in 80% ACN
Gradient:	5–60% B in 30 min
Temperature:	25–30 °C (<60 °C)
Flow rate:	1 mL/min

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Reversed-phase LC/MS methods

LC/MS of proteins and peptides is used to provide information for protein characterization, to accurately identify post-translational modifications of proteins, and to determine the molecular weight of synthetic and natural peptides. LC/MS is also used to provide protein identification in 2D separations for proteomics applications. Therefore, LC/MS of proteins and peptides is a critical separation area, which requires some special column and mobile phase recommendations. Smaller column sizes are often used for LC/MS and TFA is generally not used in mobile phase because of reduced sensitivity in the MS with this mobile phase additive.

Analytical LC/MS applications

2.1 mm id columns provide good sensitivity when sample size is not limited.
With Poroshell columns, smaller 1 mm column ids may be used.

High sensitivity/proteomics applications

Capillary columns are used for high sensitivity protein and peptide applications. The 0.5 mm id columns are used for protein and protein digest separations, while the 0.3 mm id columns are most often used for protein digests. These can be analyzed at high pH with an ammonium hydroxide mobile phase. Nano columns (0.1 and 0.075 mm id) are often used in 2D LC/MS systems for proteomics and the initial choice is C18 bonded phase.

Charge variant analysis methods

This section on column selection strategy for charge variant analysis provides some critical details on method development for mAb, proteins, and peptides.

Bio MAb columns and monoclonal antibodies	Bio IEX columns, cation-exchange (SCX, WCX), monoclonal antibodies, proteins, peptides, and glycoproteins	Bio IEX columns, anion-exchange (SAX, WAX), oligonucleotides, and proteins

Recommended initial conditions

Mobile phase: A: 20 mM sodium phosphate, pH 5.5 B: Buffer A + 200 mM NaCl **Gradient:** 10–35% B in 50 min **Sample size:** 2 mg/mL, 5 µL injection **Temperature:** Ambient **Detection:** UV, 220 nm	**Mobile phase:** A: 20 mM sodium phosphate, pH 5.0 for WCX or pH 6.0 for SCX B: Buffer A + 500 mM NaCl **Gradient:** 1–100% B in 30 min for 50 mm columns, 60 min for 250 mm columns **Sample size:** 2 mg/mL, 5 µL injection **Temperature:** Ambient **Detection:** UV, 220/280 nm	**Mobile phase:** A: 20 mM Tris-HCl, pH 8.5 B: A + 500 mM NaCl **Gradient:** 1–100% B in 30 min for 50 mm columns, 60 min for 250 mm columns **Sample size:** 2 mg/mL, 5 µL injection **Temperature:** Ambient **Detection:** UV, 220/280 nm

Select flow rate based on column diameter and particle size

2.1 mm id columns

Particle size, µm	Flow rate, mL/min
5	0.1–0.5
1.7	0.1–0.8

4.6 mm id columns

Particle size, µm	Flow rate, mL/min
1.7	0.1–0.3
3	0.1–0.5
3.5	0.1–0.8
10	0.1–1.0

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Optimize conditions

Some separations may require a specific buffer, ionic strength, pH, and temperature.

Ionic strength

Certain ionic strength is required to sustain the function of columns. Usually, a minimal concentration of 10 to 20 mM salt is required. However, greater than 20 mM strength may prevent the adsorption of biomolecules onto the column. Commonly used salts are sodium and potassium chloride and acetate. For elution, a typical salt concentration is 400 to 500 mM.

Note: Never use water alone for washing columns as it causes a significant increase in backpressure.

Selection of buffers and pH

Buffers play a key role in the optimization of separations. Phosphate buffers are typically used for antibodies and many biomolecules. The following are also recommended: MES, tris, and ACES buffers. Use buffers of pH 5.0 to 6.5. pH can be adjusted usually by +/- 0.2 units. For some specific proteins, buffers with higher pH (>pH 6.5) may be needed. Phosphoric acid, acetic acid, HCl, and NaOH can be used to adjust pH.

pH gradients can also be used for elution.

Additives

Organic solvents
Acetonitrile, ethanol, methanol, and other similar solvents can be used up to 50%.

Detergents
Nonionic, anionic, and zwitterionic detergents can be used. Cationic detergents are not recommended.

Selection of buffers and pH

For anion-exchange, acetate and phosphate buffers of pH 8.0 to 9.0 are recommended. pH can be adjusted usually by +/- 0.2 units. For some specific proteins, buffers with higher or lower pH may be needed. Phosphoric acid, acetic acid, HCl, and NaOH can be used to adjust pH.

pH gradients can also be used for elution.

Additives

Organic solvents
Acetonitrile, ethanol, methanol, and other similar solvents can be used up to 50%.

Detergents
Nonionic, cationic, and zwitterionic detergents can be used. Anionic detergents are not recommended.

Temperature

Agilent Bio MAb and IEX columns are stable up to 80 °C. However, many proteins and biomolecules are heat labile. Be sure to establish the temperature stability of your sample before routinely using high temperature for separation.

Charge variant analysis methods with Agilent Buffer Advisor software

Agilent Buffer Advisor is a software tool that enables more reproducible and precise ion exchange methods. For example, the software can generate methods using quaternary mixing for a salt gradient with constant pH, or for a pH gradient with a salt gradient for cleanup. These methods can be directly imported into Agilent LC acquisition software.

Recommended initial conditions

Salt gradient (see application note: 5991-0656EN)

Columns:	Bio WCX, 4.6 x 250 mm, 10 µm Bio WCX, 4.6 x 250 mm, 5 µm
Mobile phase:	A: water B: 1.6 M NaCl C. 40.0 mM NaH_2PO_4 D. 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, 20 mM buffer solutions at the desired pH range are produced.
Gradient:	0 to 50% B, 0 to 20 min (constant pH, for example, pH 6.0) 50% B, 20 to 25 min 0% B, 25 to 35 min
Temperature:	Ambient
Injection volume:	10 µL
Detection:	UV, 220 nm
Instrument:	1260 Infinity bio-inert LC
Sample:	Ovalbumin, ribonuclease A, cytochrome c, lysozyme
Sample conc:	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)

pH gradient (see application note: 5990-9629EN)

Column:	Bio MAb, 4.6 x 250 mm, 5 µm
Mobile phase:	A: water B: 1.6 M naCl C. 40.0 mM NaH_2PO_4 D. 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, buffer solutions at the desired pH range are produced at the selected buffer strengths.
Gradient:	pH 6.0 to 8.0, 0 to 20 min 0 to 800 mM NaCl, 20 to 25 min 800 mM NaCl, 25 to 30 min
Temperature:	Ambient
Injection volume:	10 µL
Detection:	UV, 220 nm
Instrument:	1260 Infinity II bio-inert LC
Sample:	IgG monoclonal antibody
Sample conc:	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)

Note: Similarly, the above approaches can be applied for Agilent WAX and SCX columns with modifications.
To access the Critical Quality Attributes Application Compendium, visit: **www.agilent.com/chem/cqa-applications**

Select flow rate based on column diameter and particle size

2.1 mm id columns

Particle size, µm	Flow rate, mL/min
1.7	0.1–0.3
3	0.1–0.5
5	0.1–0.8
10	0.1–1.0

4.6 mm id columns

Particle size, µm	Flow rate, mL/min
1.7	0.1–0.3
3	0.1–0.5
5	0.1–0.8
10	0.1–1.0

Note: Always start with a low flow rate and default to the recommended operating limit of the column.

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Aggregation and fragment analysis methods

This section on column selection strategy for aggregation analysis provides some critical details on method development for mAb, proteins, and peptides.

Choose initial columns and conditions for size-based separation of biomolecules, aggregation analysis—peptides, polypeptides, and proteins

Peptides, polypeptides, proteins
Mol Wt >0.1–1,250 kda

Peptides, polypeptides, proteins
Mol Wt >0.1–10,000 kda

Select column based on molecular weight range and pore size

AdvanceBio SEC (2.7 µm)

Pore size	Mol Wt range, kDa
130 Å	0.1–120
300 Å	5–1,250

Bio SEC-3 (3 µm)

Pore size	Mol Wt range, kDa
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250

Bio SEC-5 (5 µm)

Particle size, µm	Flow rate, mL/min
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250
500 Å	15–5,000
1000 Å	50–7,500
2000 Å	>10,000

Recommended initial separation conditions

Columns: AdvanceBio SEC
Bio SEC (3 µm and 5 µm)

Mobile phase: Phosphate buffer 150 mM, pH 7.0*

Gradient: Isocratic in 15 to 60 min range

Temperature: Recommended 10 to 30 °C, maximum 80 °C

Flow rate: 0.1 to 0.4 mL/min for 4.6 mm id columns
0.1 to 1.25 mL/min for 7.8 mm id columns
1.0 to 10.0 mL/min for 21.2 mm id columns

Sample size: ≤5% of total column volume

*Other aqueous buffers with high and low salt can be used

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After the initial chromatogram, additional changes may be needed to improve the separation, maintain protein solubility, or to decrease sample interaction with the chromatographic media. The ionic strength of the mobile phase can be adjusted up or down to attain an optimized separation. pH can also be adjusted usually +/- 0.2 units. If further optimization is necessary, the upward or downward range should be expanded. A change of temperature or addition of an organic solvent can also be used.

For protocols requiring additional salt, these buffers are typical:

100 to 150 mM sodium chloride in 50 mM sodium phosphate, pH 7.0

100 to 150 mM sodium sulfate in 50 mM sodium phosphate, pH 7.0

50 to 100 mM urea in 50 mM sodium phosphate, pH 7.0

Other similar salts (for example, KCl) and guanidine hydrochloride can also be used.

pH range:

2.0 to 8.5

Potential organic solvent additions include:

5 to 10% ethanol (or other similar solvents) in 50 mM sodium phosphate, pH 7.0

5% DMSO in 50 mM sodium phosphate, pH 7.0

Particular care must be taken to avoid excessive pressure changes due to the high viscosity of some aqueous/organic solvent mixtures. Use reduced flow rate or increased temperature to help alleviate potential problems.

Temperature:

Typically, SEC separations are run at 20 to 30 °C. Separation of proteins and peptides may require higher temperatures to improve both resolution and recovery of proteins and hydrophobic peptides.

Maximum temperature of Bio SEC columns is 80 °C.

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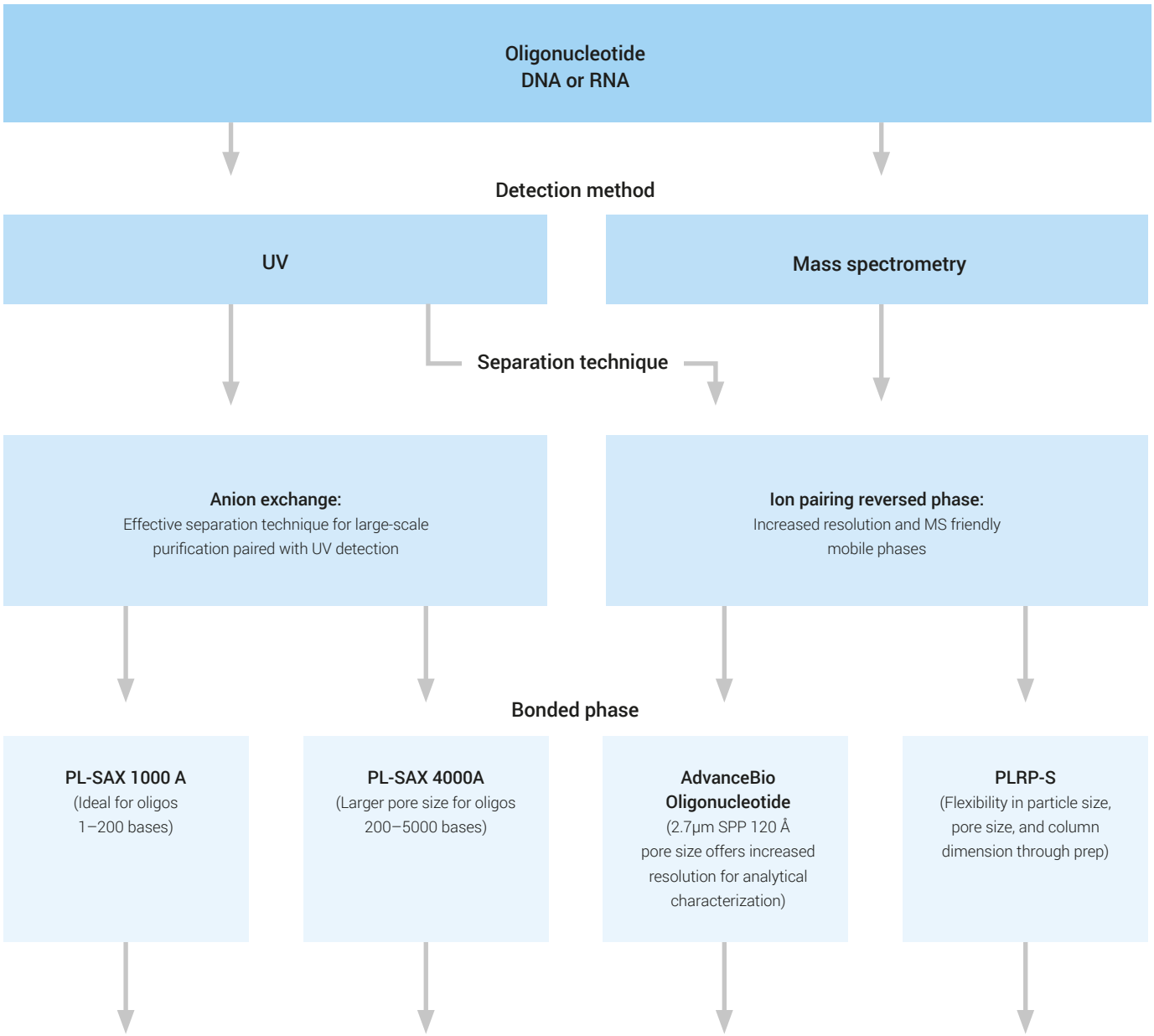
Oligonucleotide analysis



Initial separation conditions

Mobile Phase:
10mM Tris, pH 8.0 +10% ACN
Prepare Stock Solution of 1 M Tris, pH 8.0, adjusted with HCl

MP A
10 mL of stock buffer mixed with 100 mL of ACN
and 890 mL of water

MP B
10 mL of stock buffer mixed with 100 mL ACN,
2M NaCl, and water was added up to 1 L.

Gradient
20 to 40% B in 10 minutes

Column Temperature
30 °C

Column
2.7 µm, 50 x 2.1mm
(p/n 659750-702)

MP A
15 mM TEA and 400 mM
HFIP in Water

MP B
Methanol

Gradient
0 to 1 min, 10% B
1 to 10 min, 10 to 40% B
10 to 11 min, 40 to 95% B

Column Temperature
65 °C

Flow Rate
0.5 mL/min

Column
PLRP-S 5 µm, 50 x 2.1mm,
1000 Å

MP A
15 mM dibutylamine + 25 mM
HFIP in DI water

MP B
15 mM dibutylamine + 25 mM
HFIP in methanol

Gradient
0 to 1 min, 15% B
1.1 to 10.5 min, 45% B
10.6 to 11.5 min, 90% B

Column Temperature
80 °C

Flow Rate
0.4 mL/min

Method optimization variables

Variables that can be adjusted to optimize chromatography:

pH: Increasing pH with the addition of NaOH to pH 11 or 12 can help limit secondary interactions and sharpen peaks. Higher pH combined with increasing salt concentrations can help elute longer oligos.

Temperature: Increasing MP and column compartment temperature reduces MP viscosity, reduces secondary interactions, and thus can improve peak shape.

Organic MP modifiers: The addition of Acetonitrile mobile phase modifiers (10 to 15%) has been shown to improve chromatography.

*pH and temperature increases can cause unwanted sample degradation. Changes should be made step wise and monitored for undesired impurities.

Variables that can be adjusted to optimize chromatography:

Temperature: Increasing MP and column compartment temperature reduces MP viscosity, reduces secondary interactions, and thus can improve peak shape.

Ion-pairing reagent: IP-RP utilizes ion pairing reagents, generally amines with different hydrophobic alkyl chains. The amine interacts with the anionic oligos forming a pair that can then be retained through hydrophobic interactions with the RP stationary phase. The ion pair selected can be adjusted to improve separation and retention

Organic MP modifiers: Depending on the ion-pairing reagent chosen, changes in the organic concentration in the mobile phase can be used to optimize analyte retention (increase or decrease).

*pH and temperature increases can cause unwanted sample degradation. Changes should be made step wise and monitored for undesired impurities.

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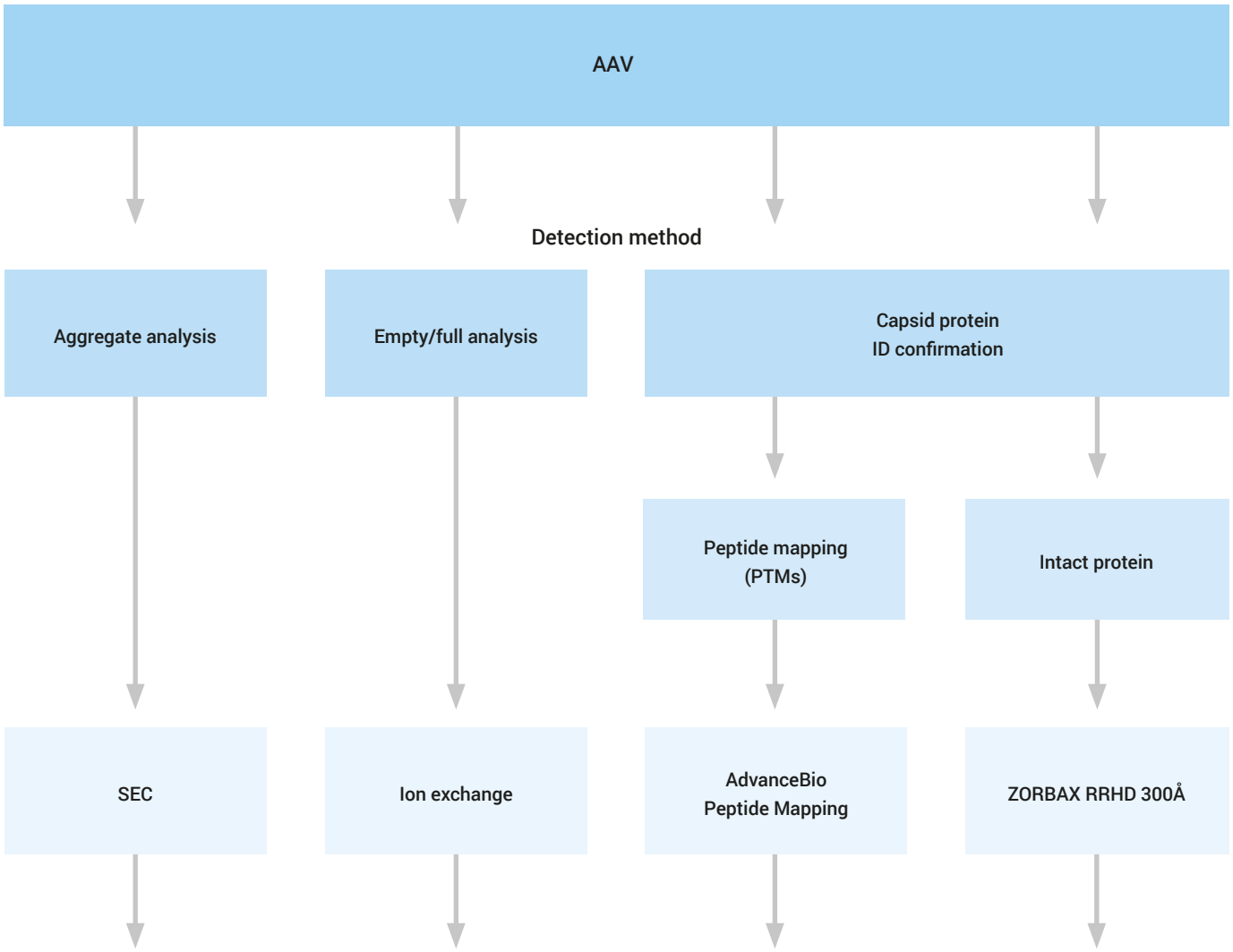
Adeno-associated virus (AAV) analysis



Initial Separation Conditions

Column
Agilent Bio SEC-5,
300 x 4.6 mm 5 µm, 1000 Å

Mobile Phase
50 mM phosphate buffer,
400 mM NaCl, pH 7.4

Column Temperature
80 °C

Flow Rate
0.4 mL/min

FLD
λ_{ex} = 280 nm, λ_{em} = 340 nm

Column
Agilent Bio SAX,
50 x 2.1 mm 5 µm

MP A
70 mM bis-tris propane, pH 9.0
2 mM $MgCl_2$

MP B
70 mM bis-tris propane +
1M tetramethyl ammonium
chloride, pH 9.0
2 mM $MgCl_2$

Gradient
0 min, 15% B
Flow Rate: 0.1mL/min

25 min, 27.5% B
Flow Rate: 0.1mL/min

25.1 min, 100% B
Flow Rate: 0.3mL/min

28 min, 100% B
Flow Rate: 0.3mL/min

FLD
λ_{ex} = 280 nm, λ_{em} = 340 nm

Column
AdvanceBio Peptide Mapping,
150 x 2.1 mm 2.7 µm

MP A
0.1% FA in DI Water

MP B
0.1% FA in Acetonitrile

Gradient
0 to 3 min, 3% B
50 min, 35% B
60 min, 97% B
62 min, 97% B
65 min, 3% B

Column Temperature
60 °C

Flow Rate
0.4 mL/min

Column
Zorbax RRHD 300A SB C18,
100 x 2.1 mm 1.8 µm

MP A
0.1% FA +0.1% TFA in DI Water

MP B
90% IPA + 9.8% DI Water +
0.1% FA + 0.1% TFA

Gradient
0 to 5 min, 28% B
23 min, 32.5% B
23.5 min, 80% B
26 min, 80% B

Column Temperature
80 °C

Flow Rate
0.4 mL/min

Variables that can be adjusted to optimize RP chromatography:

Temperature
Increasing MP and column compartment temperature reduces MP viscosity, reduces secondary interactions, and thus can improve peak shape.

Organic MP modifiers
For Reversed Phase separations, the organic solvent is the strong solvent. Adjustments in the percentage of organic modifier in the mobile phase can impact the eluotropic strength. Higher percentages of organic solvent in the mobile phase will reduce the retention of hydrophobic analytes on reversed-phase columns.

Column chemistry
The selectivity of the stationary phase is a variable that can changed to optimize resolution of target analytes. For the analysis of intact capsid proteins the Zorbax RRHD 300-Diphenyl has alternative selectivity of a C18 and offered advantages for different serotypes.

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Glycan and hydrophilic/glycopeptide analysis

Glycans
Mol Wt <5 kDa

Glycopeptides and Hydrophilic Peptides
Mol Wt <10 kDa

Initial bonded phase

AdvanceBio Glycan Mapping, 1.8 µm

For fast separations and high throughput analysis

AdvanceBio Glycan Mapping, 2.7 µm

For high resolution and 250 mm column lengths

Superficially porous for separation efficiency at lower pressure

ZORBAX RRHD 300-HILIC

Also consider: AdvanceBio Glycan Mapping columns

Initial separation conditions

Mobile phase:
A: 100 mM ammonium formate, pH 4.5
B: ACN

Gradient:
25 to 40% A in 12 min

Temperature:
40 °C

Mobile phase:
A: 100 mM ammonium formate, pH 4.5
B: ACN

Gradient:
25 to 60% A in 35 min

Temperature:
40 °C

Mobile phase:
A: ACN
B: 50 mM ammonium formate, pH 4.5

Gradient:
95 to 0% A in 15 min

Temperature:
Ambient

Columns

Dimensions	Application
2.1 x 100 mm 2.1 x 150 mm	High throughput Speed with resolution

Columns

Dimensions	Application
2.1 x 100 mm 2.1 x 150 mm 2.1 x 250 mm 4.6 x 100 mm 4.6 x 150 mm 4.6 x 250 mm	UHPLC speed Robust methods UHPLC resolution HPLC speed Robust methods HPLC resolution

Columns

Dimensions	Application
2.1 x 50 mm 2.1 x 100 mm	Speed Resolution

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Titer determination and cell culture optimization methods

Agilent Bio-Monolith Protein A recommended conditions

Columns: Bio-Monolith Protein A (p/n 5069-3639)

Mobile phase: A: 50 mM phosphate, pH 7.4;
B: 100 mM citric acid, pH 2.8 mM, or 500 mM acetic acid, pH 2.6

Gradient:

Time (min)	%A	%B	
0 to 0.5	100	0	Binding
0.6 to 1.7	0	100	Eluting
1.8 to 3.5	100	0	Re-equilibrating

Temperature: Ambient

Flow rate: 1 mL/min

Injection volume: Variable (50 µL, optimized for CHO cell culture supernatant contains IgG1)

Detection: UV, 280 nm

Sample: IgG1 (1 to 20 mg/mL) and CHO cell supernatant contains IgG1 (up to 20 mg/mL total protein)

Note: Additional salts such as sodium chloride can be added to mobile phases, up 150 mM. Higher salts should be determined experimentally.

Tips and tools

Agilent recognizes that there are many different factors that affect the quality of mAb and protein separations. To enable you to gain the best results, we have developed a series of 'how to' guides. For more information, see:

Keys for Enabling Optimum Peptide Characterizations: A Peptide Mapping "How to" guide (publication **5991-2348EN**)

Ion-exchange Chromatography for Biomolecule Analysis A "How to" guide (publication **5991-3775EN**)

Size Exclusion Chromatography for Biomolecule Analysis A "How to" guide (publication **5991-3651EN**)

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High sensitivity capillary column methods

Mobile phase considerations for reversed-phase methods

For LC/MS methods where the column eluent passes directly from the column to the MS detector the mobile phases must contain only volatile salts, and additives. And for maximum sensitivity there must be no ion suppression or adduct formation.

Low pH

TFA is generally not used for LC/MS separations of proteins and peptides as it suppresses ionization and increases the limit of detection. The first step is normally to replace TFA with 0.1 to 1% formic acid. Acetic acid up to 1% can also be used as an alternative mobile phase modifier. At low pH, the best separation may still be obtained with TFA in the mobile phase but with a reduction in sensitivity. In some cases, the TFA can be displaced postcolumn with an alternative acid, such as propionic acid by the use of a simple online desalting/counter ion-exchange.

Mid and high pH

High pH is less often used for protein characterization but LC/MS can also be done at high pH with 10 to 20 mM NH_4OH as a mobile phase additive.

Agilent Solutions

Put more than 40 years of innovation behind your results

By continually raising the standards for technologies that support your routine analyses, Agilent's R&D efforts have led to breakthroughs, such as:

- **New GC columns** that help you achieve higher levels of inertness and column-to-column reproducibility
- **LC column choices** that deliver the sensitivity and reliability you need for demanding applications
- **Cutting-edge sample preparation products** that promote reliable extraction and concentration
- **Fresh atomic and molecular spectroscopy ideas** for identifying and confirming targets and unknowns

Customers who have been with Agilent for a long time have experienced our commitment firsthand. Now, we look forward to demonstrating how the Agilent approach to relentless innovation can work to your advantage, too.

Chemical Analysis Solutions

Food

From high-volume pesticide screening in food products to rapid identification of pathogens, Agilent understands the analytical needs of food producers, shippers, and regulators. Utilizing our easy-to-use analyzers and up-to-date screening libraries, customers can quickly develop robust and reliable methods. Our leading gas chromatography and mass spectrometry systems are widely regarded as valuable food testing techniques for an array of different analyses.

Environmental

Agilent offers more than 40 years of environmental testing and regulatory expertise. We help government and private labs with a full range of assays, from routine testing of soils for heavy metals to detection of pharmaceuticals in groundwater, in concentrations down to parts per trillion.

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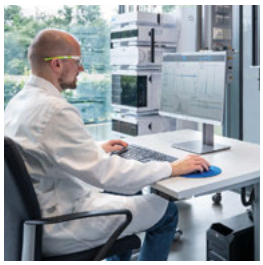
Forensics

Whether testing for poisons in a forensics investigation, screening athletes for performance enhancing drugs, analyzing samples for recreational drugs, or checking a crime scene for explosive residue—lives and professions may be dependent on the accuracy of your equipment. Agilent leads the industry with a comprehensive portfolio of workflow solutions that provide the ability to identify, confirm, and quantify thousands of substances.

Lab informatics

The way a lab captures, analyzes, and shares data profoundly affects its efficiency. Agilent offers a rich, integrated suite of software products built on customer-driven architectural values with the Agilent OpenLab software. OpenLab delivers superior performance and connections across multiple systems, providing open systems integration and investment protection. Our commitment is to deliver value across each step of the life cycle of scientific data—from collection and analysis to interpretation and management.



Energy and chemicals

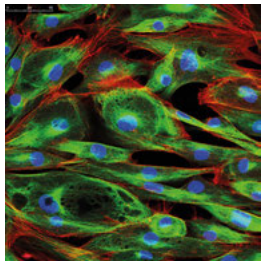
Agilent collaborates closely with process industry customers to offer analytical systems that meet their needs for separation, detection, throughput, and support. We'll even preconfigure custom or standard analyzers so they arrive at the lab ready to go. From crude oil, natural gas, and refining, to specialty chemicals and alternative fuels, Agilent provides the latest technologies and solutions to increase quality, safety, and profitability for energy and chemical labs, while meeting the industry's stringent quality requirements. Agilent leads the way in ASTM collaborations that have evolved—and will continue to evolve—into industry standards.

Materials science

Agilent offers a newly expanded portfolio of instruments used for the research, manufacturing, and testing of advanced materials, from precision optics to pulp and paper. Tools for atomic spectroscopy, molecular spectroscopy, chromatography, and X-ray crystallography all support continuous progress in materials science.

Life Science Solutions

Biopharmaceutical

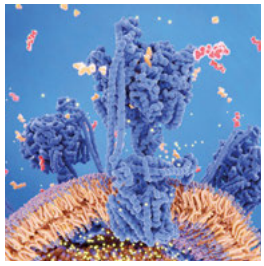


Biotherapeutics have enormous potential to improve human health, with growing numbers of protein and antibody therapeutics to address unmet medical needs. At every development stage, from disease research to QA/QC and manufacturing, Agilent can help you make the right choices for moving therapeutics to market. We understand the biopharmaceutical workflow, so our product families work together seamlessly, as engines of research, discovery, and development. Agilent columns deliver complete characterization of biomolecules using reversed-phase, size exclusion, ion exchange, and affinity chromatography. Our bio-inert supplies ensure that every part of your workflow delivers the performance you need to optimize your bioseparation.

Pharmaceutical

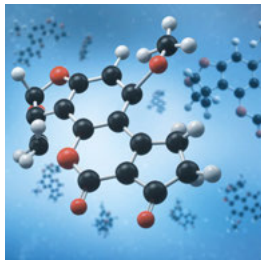
You need the most efficient processes to evaluate drug candidates, determine efficacy, and ensure safety and compliance during development and manufacture. Agilent has worked with pharma companies for many years to ensure reliability and reproducibility for regulatory compliance, from lab-to-lab and around the world. Our pharma solutions provide high-throughput capabilities at every stage of the product life cycle, with automated sample preparation, industry-leading HPLC and UHPLC systems, the largest family of fast LC columns, open access LC/MS, spectroscopy, and automated dissolution. A complete family of LC supplies and lamps help optimize every analysis and take day-to-day lab efficiency one step further.

Proteomics



Research into how large sets of proteins affect the health of an organism requires special sets of analytical tools. Agilent has built a formidable arsenal of liquid chromatograph/mass spectrometers, bio-informatics systems, multiple affinity protein removal columns, and OFFGEL electrophoresis supplies for protein identification and protein biomarker discovery. Accurate-mass mass spectrometry and microfluidic HPLC-Chip/MS are two Agilent innovations speeding the work of proteomics researchers around the globe.

Metabolomics



Collections of small molecules are increasingly being viewed as rich sources of biomarkers, but studying metabolites presents many challenges. The need for speed, accuracy, and powerful interpretation capabilities when looking at chemical profile snapshots is significant because molecules are constantly entering, leaving, or changing within the metabolome. The Agilent GC, LC, and MS portfolios, along with our excellent bio-informatics offerings, user-customizable METLIN metabolite database for LC/MS, and the industry's first commercial GC/MS retention time locked metabolite library align well with the needs of metabolomics researchers.

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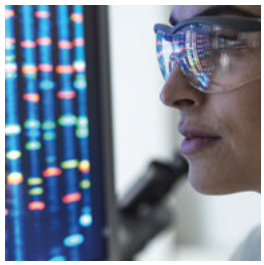
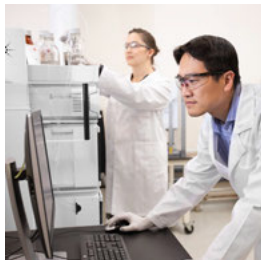
Genomics

Agilent is a global leader in microarrays, scanners, and NGS reagents used in a wide variety of genomic-based disease research experiments. Our SureSelect and HaloPlex target enrichment systems dominate the category, streamlining next-generation sequencing studies. Agilent offers a wide range of catalog CGH and gene expression microarrays and a highly developed capability to produce custom arrays using our free online design tool, SureDesign. All Agilent microarrays feature sensitive, selective 60-mer probes, and, with as many as eight arrays printed on a slide, the cost-per-sample is cost efficient.



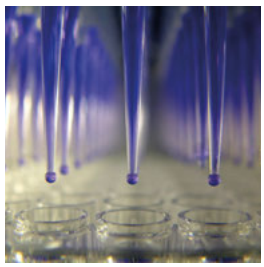
Life science informatics

Mirroring its extensive instrument portfolio, Agilent offers the industry's most extensive suite of bio-informatics software, helping users derive knowledge from complex genomic, proteomic, metabolomic, and other biological data. The SureCall and CytoGenomics software analyzes NGS and aCGH data, and the GeneSpring suite provides multi-omic analysis and visualization capabilities to help compare complex datasets and explore biological questions from multiple perspectives. The GeneSpring suite includes the GX module for microarray-based gene expression and genotyping data, the PA module for pathway analysis and multi-omic analysis, and the MPP software, which analyzes mass spectrometry data from proteomics and metabolomics experiments.



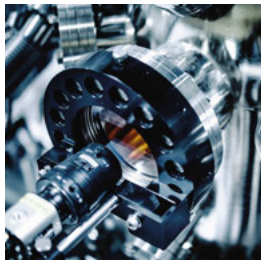
Lab automation

To meet the skyrocketing demand for more throughput and automation, Agilent has substantially expanded its lab automation offerings. The Agilent line of liquid handlers and microplate processors is designed to streamline high-volume life science workflows. Agilent is also continually upgrading its advanced autosamplers for LC, GC, LC/MS, and GC/MS, adding functionality and speed to reflect the performance of its advanced instruments.



Vacuum technology

Agilent works with customers to solve vacuum challenges from experiments in high-energy physics to developing systems for nanotechnology. Agilent manufactures vacuum systems used in its own mass spectrometry instruments, as well as those of other manufacturers. Our vacuum technology has been proven by the most powerful physics experiment ever built, CERN's Big Bang machine, which was used in the discovery of the Higgs boson.



Services and Support

Focus on what you do best

At Agilent, we look for ways to optimize your lab's performance. That is why you can rely on us to provide the tools you need and protect your investment with a broad portfolio of services, backed by a global network of experienced, Agilent-certified service professionals, dedicated to the productivity of your lab.

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The best service available for your Agilent instruments

The Agilent CrossLab services provide laboratories with comprehensive maintenance coverage to suit their specific needs and budget. We offer a range of service plans so that you can choose the level of coverage required.

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Helping you to maintain a compliant laboratory

Agilent offers a comprehensive set of laboratory compliance services, including instrument and software qualifications (IQOQ, OQ, and RQ), based on USP <1058> analytical instrument qualification (AIQ), and custom validation services, such as computer system validation, audits/assessments, custom procedure writing, and more. To help you ensure compliance, the Agilent automated compliance solution is used to support the end-to-end Analytical Instrument Qualification (AIQ) process. The automated compliance engine (ACE) is an electronic, audit-ready qualification solution that addresses data integrity and intended use requirements by:

- Minimizing your audit risk and improving qualification efficiency by harmonizing protocols across platforms
- Standardizing your entire compliance operation with robust test designs that work with all of your instruments and assure adherence to protocol
- Configuring tests and selecting specifications according to your SOP
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Agilent instrument training and method services

Reach optimal scientific and economic outcomes

Transform your lab operations by partnering with Agilent CrossLab to build job-ready skills that you can put to work. Learn the essentials of new techniques or master real-world applications with advanced methodologies.

- Method and application services are available to enhance your workflow – from method development to method maintenance
- Instrument bundled or standalone training and method support is available to transfer and optimize results on new and existing technologies
- We offer flexible training options to accommodate your needs and budget with online or classroom training, or training at your site
- Customers can use our online Cloud Lab solution to learn how to use their software

The Agilent Value Promise—10 years of guaranteed value

In addition to continually evolving products, we offer something else unique to the industry—our 10-year value promise guarantee. The Agilent Value Promise guarantees you at least 10 years of instrument use from the date of your purchase, or we will credit you with the residual value of the system towards a replacement model upgrade. Because we stand behind our systems, our Value Promise maximizes your return on investment by assuring you that your purchase is safe. For more detailed information, please go to **agilent.com/chem/services** or contact your local Agilent Services and Support representative.

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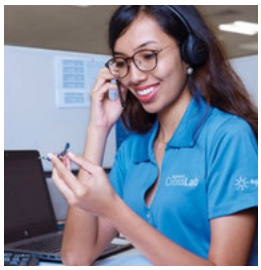

Have a hardware, software, application, instrument repair, or troubleshooting question? Agilent's technical experts are available to answer your questions. With years of laboratory experience, our technical support specialists can provide in-depth knowledge and experience. For questions about supplies found in this catalog, contact your local Agilent office or authorized Agilent distributor, or visit **agilent.com/en/support**.

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