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Consumables Workflow Ordering Guide

Analyzing Monoclonal Antibody (mAbs) and mAb-Derived Therapeutics Using Native SEC-MS

The quantitation of aggregates is of particular concern for protein-based-therapies given their potential effect on efficacy and immunogenicity. Size exclusion chromatography (SEC) combined with UV detection is the gold standard approach for measuring aggregation of therapeutic proteins. However, researchers are increasingly integrating SEC with native-mass spectrometry (Native-MS), a high-resolution MS technique, to gain accurate mass determination.[1] For some experimental goals MS is a more suitable detector than UV. Besides molecular weight confirmation, native-MS detection also measures drug-to-antibody ratios (DAR) of antibody-drug conjugates (ADCs) and provides information on post-translational modifications (PTMs).

While reversed phase separations are commonly paired with MS, there are limitations when analyzing mAbs. The low pH and organic solvent used in reversed phase chromatography denatures mAbs and dissociates fragile non-covalent or acid-labile structures, such as those found in cysteine- or lysine-linked ADCs. SEC offers unique benefits over reverse phase techniques. Since it is performed with buffered mobile phases at neutral pH, it preserves the intact protein's structure and fragile covalent or non-covalent bonds and enables mass measurement of a mAb in its native state. SEC even preserves the cysteine-linked ADC structures that are especially fragile, enabling DAR calculations from the MS data.[2,3]

Challenges associated with SEC-MS include:

- Achieving high-resolution SEC separation of mAb monomers from high molecular weight (HMW) and low molecular weight (LMW) impurities.
- Identifying an SEC column and conditions that are compatible with MS detection so that:
  - The stationary phase surface bonding is stable and does not cause column bleed.
  - The flow rate that supports efficient desolvation of analyte in the MS doesn't cause sample aggregation.
  - The mobile phase is sufficiently volatile and low in salt concentration to accurately detect mass.

Why use Agilent AdvanceBio SEC columns for SEC-MS analysis?

- Particle size and pore characteristics are important factors that improve peak shape, peak sensitivity and resolution. The 1.9 µm particle AdvanceBio SEC columns have optimized pore size and volume for high-resolution separation.
- Secondary interactions with the surface of the SEC resin can prevent free passage through the pores and interfere with size-based separation. The proprietary unique hydrophilic bonding chemistry provides an inert surface to minimize secondary hydrophobic interaction with ADCs and mAbs.
- The AdvanceBio SEC hybrid particles incorporate the best properties of silica and polymer technologies providing best-in-class mechanical robustness that do not bleed, making them ideal for use with MS detectors.
- The AdvanceBio 1.9 µm SEC column works with both denaturing mobile phase conditions (such as acetonitrile/water/TFA), as well as MS compatible native conditions (such as 80 mM ammonium acetate), making it the ideal choice for intact proteins (>2,000 m/z).
- The narrow 2.1 and 4.6 mm id columns are compatible with the low flow rates needed for efficient desolvation/ionization of the analyte. This is required to minimize formation of adducts that interfere with accurate mass measurement.
- The PEEK-lined SS column hardware eliminates metal from the sample flow path. This makes it easier to use lower concentration mobile phase buffers which decrease the formation of adducts that interfere with accurate mass measurement.

Column selection criteria

Selecting the correct pore size for an SEC experiment depends upon the size of the analyte of interest and the goals of the experiment. Pore size selection is based on the molecular weight of proteins, with larger molecular weight proteins requiring larger pore sizes (see Figure 1). AdvanceBio SEC 1.9 µm columns are available in two pore sizes. When performing a desalting experiment where the goal is to separate the protein from a small molecule or buffer solution components, a smaller pore size will maximize separation. In this case, it is beneficial to exclude the protein by selecting a column where the protein molecular weight is greater than the upper size limit of the column.

Choose an SEC column with dimensions that will help you achieve the goal of your SEC-MS experiment and is compatible with your sample constraints.

- Shorter columns of 30 or 50 mm length may be sufficient for simple desalting experiments.[1]
- Longer columns deliver higher resolution, allowing you to separate monomer from dimer, or monomer from fragment.
- Narrower column diameters require smaller injection volumes and are useful when sample availability is limited.
- Narrower columns also require lower flow rates for optimal SEC separation, and are more compatible with optimum flow rates and MS source conditions for efficient desolvation and ionization for Native MS.
- PEEK-lined columns minimize sample contact with metal surfaces, which can improve peak shape by minimizing secondary interactions and enable a relatively dilute volatile mobile phase.[2]

Getting started with Agilent AdvanceBio SEC columns: Tips for ensuring best performance and separation

Column operation and cleaning

- Align flow rates with column id[4] – smaller id columns require lower flow rates for optimal SEC separation to avoid over pressuring the column. The narrower 2.1 and 4.6 mm id columns make them ideal for native MS, which rquires efficient desolvation/ionization of the sample.
  - Working Flow Rate[5]:
    - 4.6 × 150 mm, 0.1 to 0.7 mL/min.
    - 4.6 × 300 mm, 0.1 to 0.5 mL/min.
    - 2.1 mm id columns, 0.05 to 0.10 mL/min.
- Lower the flow ramp rate from the default to 1 mL/min[2] or lower. The gradual increase in flow rate will prolong column lifetime. In Agilent software this setting can be found in the Advanced section of the LC pump controls.
- Set the maximum pressure limit in the LC method to match that of the column (620 bar for AdvanceBio SEC 1.9 µm columns). This is key for any instance in which the maximum pressure capabilities of the LC exceeds that of the column.
- Do not back flush columns. Always flush the column in the direction of the arrow and adjust flow rate to keep pressure below 400 bar.

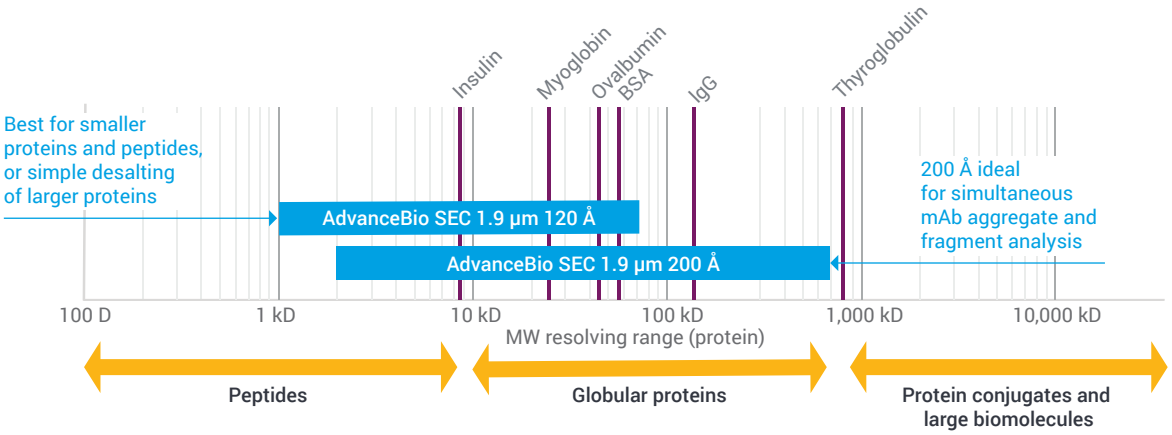

Figure 1. Size of the analyte determines the SEC pore size to be used. Larger proteins require larger pores.

- Rinse with at least five-column volumes of ultrapure water before and after flushing with at least 20-column volumes of the cleaning solution.
- Verify system performance with a suitable SEC standard at regular intervals.

Chromatography optimization

- Filter samples to remove any particulates.
- Use guard columns and/or an in-line filter to extend column lifetime, especially when working with complex or "dirty" samples.
- Ensure that column connections are secure and free of leaks.
- Maximize the resolution of the sub 2 µm SEC particles by minimizing the system dead volume. An Ultra Low Dispersion Kit can be installed on 1290 model LCs to further reduce system volume and avoid band broadening.[6]
- Maximize chromatographic resolution by minimizing sample injection volume. A sample injection volume of 1 to 5 µL is recommended with a maximum injection volume of 1% of the column volume.

MS care and optimization

- Divert the LC stream to waste outside of the retention time(s) of interest, especially around the total permeation point where high amounts of salt may elute to keep the MS source cleaner.
- Use HPLC-grade or higher solvents.
- Use a volatile buffer such as ammonium acetate and optimize SEC mobile phase to lowest buffer concentration that retains chromatographic resolution and preserves protein structure. This keeps the MS source cleaner and minimizes adducts that interfere with mass measurement.
- Wipe down the MS source with a lint-free cloth, daily if possible. Be sure that the source is not too hot to touch!

Column storage

- Short-term storage (less than two weeks) - store the column in the mobile phase used for analysis.
- Extended storage (longer than two weeks) – store the column in filtered 100 mM sodium phosphate, pH ≤ 7.0, with or without 0.02% NaN_3, or 20% methanol in water. Flush the column with a minimum of 10-column volumes. Flushing with water is always recommended prior to introduction of methanol or ethanol. When switching to or from 20% methanol, flushing must be done at low flow rates to avoid over pressuring the column due to high viscosity. Start at a lower flow rate, flush at no more than 0.1 mL/min for 4.6 mm columns and no more than 0.05 mL/min for 2.1 mm columns. Be sure to keep the pressure below 400 bar. Store columns at room temperature.

References

1. Sensitive Native Mass Spectrometry of Macromolecules using Standard Flow LC/MS (agilent.com) – 5994-1739EN
2. Analysis of Antibody Fragment-Drug Conjugates Using an Agilent AdvanceBio SEC 120 Å 1.9 µm PEEK-Lined Column – 5994-3045EN
3. Mass Spectrometric Characterization of Antibody-siRNA Conjugates using the Agilent 6545XT AdvanceBio LC/Q TOF – 5994-2155EN
4. Agilent AdvanceBio SEC 1.9 µm Column User Guide – 5994-0739EN
5. Analysis of Nanobodies Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns – 5994-1869EN
6. Elevate Your mAb Aggregate Analysis – 5994-2709EN

Easy selection and ordering information

To order items listed in the following tables from the Agilent online store, add items to your Favorite Products list by clicking on the MyList* links in the header. Then, enter the quantities for the products you need, Add to Cart and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

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Description	Part No.
MyList Sample Preparation	
Captiva Disposable syringe, 5 mL, 100/pk	9301-6476
Captiva Premium Syringe Filter, PES, 15 mm, 0.2 µm, 100/pk	5190-5096
AdvanceBio Spin columns for desalting or buffer exchange, <100 µL samples, 25/pk, collection tubes included	1980-1103
AdvanceBio Spin 96-sample plate for desalting or buffer exchange, 10 to 50 µL samples, 1/pk	1980-1104
96-well plate, polypropylene, 1.2 mL, 27 mm, round wells, U shape, 25/pk Recommended for wash steps with p/n 1980-1104	5043-9308
96-well plate, polypropylene, 0.33 mL, 14 mm, round wells, V shape, 25/pk Recommended for final collection step with p/n 1980-1104	5043-9312
Sealing mat, 96 wells, round, preslitted, silicone, 50/pk	5042-1389
MyList of Standards	
Agilent NIST mAb, 25 µL	5191-5744
Agilent NISTmAb, 4 x 25 µL	5191-5745
300 Å AdvanceBio SEC calibration standard	5190-9417

Description	Part No.
MyList of AdvanceBio SEC Columns	
120 Å Columns	
AdvanceBio SEC 120 Å, 1.9 µm, 2.1 x 150 mm, PEEK-lined stainless-steel hardware (recommended)	PL1980-3250PK
AdvanceBio SEC 120 Å, 1.9 µm, 2.1 x 50 mm, guard, PEEK-lined stainless-steel hardware (recommended)	PL1980-1250PK
AdvanceBio SEC 120 Å, 1.9 µm, 4.6 x 300 mm	PL1580-5250
AdvanceBio SEC 120 Å, 1.9 µm, 4.6 x 150 mm	PL1580-3250
AdvanceBio SEC 120 Å, 1.9 µm guard, 4.6 x 30 mm	PL1580-1250
200 Å Columns	
AdvanceBio SEC 200 Å, 1.9 µm guard, 2.1 x 50 mm, PEEK-lined SS (recommended)	PL1980-1201PK
AdvanceBio SEC 200 Å, 1.9 µm, 2.1 x 150 mm, PEEK-lined SS (recommended)	PL1980-3201PK
AdvanceBio SEC 200 Å, 1.9 µm guard, 4.6 x 30 mm	PL1580-1201
AdvanceBio SEC 200 Å, 1.9 µm, 4.6 x 300 mm	PL1580-5201
AdvanceBio SEC 200 Å, 1.9 µm, 4.6 x 150 mm	PL1580-3201
MyList of Column Fittings and Connectors	
Agilent InfinityLab Quick Connect Fitting (for connection on column inlet)	5067-5965
Agilent InfinityLab Quick Connect Capillary MP35N 0.12 x 105 mm (for Quick Connect fitting)	5500-1578
Agilent InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Quick Turn Capillary MP35N 0.12 x 280 mm (for Quick Turn fitting)	5500-1596
Mounting tool for QuickTurn fittings	5043-0915
Capillary MP35N 0.17 x 100 mm SL/SL ps/ps (for connecting guard and column)	5500-1278
MyList of Ultra-Low Dispersion Kits	
Ultra-low dispersion tubing kit for Agilent 1290 Infinity II LC	5067-5963
Ultra-low dispersion tubing kit for Agilent 1290 Infinity II Bio*	5004-0007
MyList of Sample Containment	
A-line screw top vial, 2 mL, 12 x 32 mm (12 mm cap) amber, write-on spot, 100/pk	5190-9590
Screw cap, 12 mm, bonded, blue, PTFE/white silicone septa, 100/pk	5190-7021
Vial insert, 250 µL, 5.6 x 30 mm, deactivated glass with polymer feet, 100/pk	5181-8872
InfinityLab Well-plate 96/0.5 mL, 30/pk	5043-9310
InfinityLab microplateWell-plate closing mat, 50/pk	5042-1389

Description	Part No.
MyList of Solvents & Additives	
InfinityLab Ultrapure LC/MS Water, 1L	5191-4498
InfinityLab Ultrapure LC/MS MeOH, 1L (for column storage)	5191-4497
Formic acid, 5 mL	G2453-85060
MyList of Solvent Filtration Supplies‡	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, Regenerated Cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
MyList of Solvent Handling Supplies	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe Purging Bottle, 1L	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (Charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193

** Recommended for the 1290 Infinity II Bio System.*
‡ If using solvents other than those listed in this table, use the InfinityLab Solvent Filtration assembly prior to analysis.

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