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Consumables Workflow Ordering Guide

Analyzing Monoclonal Antibody (mAb) Fragments and Dimers Using Size Exclusion Chromatography (SEC)

Low molecular weight (LMW) fragments and high molecular weight (HMW) dimers and aggregates can be formed from biotherapeutic proteins during drug development, storage, shipment, or delivery. These size variants are a critical quality attribute (CQA) that must be well characterized to prevent an immunogenic response and differences in pharmacokinetics or potency of the drug. Size-based separation using size exclusion chromatography (SEC) is a standard technique used to analyze size variants and monitor the purity level of biotherapeutics such as monoclonal antibodies (mAbs). SEC is based purely on the permeability of proteins through the pores of the column's stationary phase and not due to any kind of interaction with the phase. Thus, globular proteins and peptides separate based on the dynamic radius (size), with larger proteins and aggregates eluting first followed by fragments and small peptides.

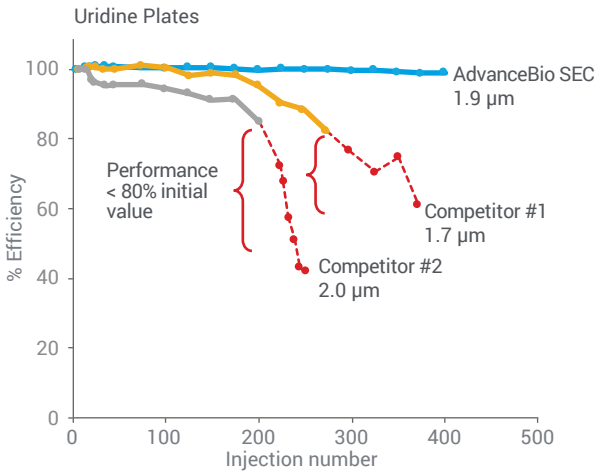
Critical factors that influence mAb fragment and aggregate separation and resolution

Particle size and pore characteristics are important factors that improve peak shape, peak sensitivity, and resolution. The right pore size is required for the effective separation of closely sized aggregates or fragments. Larger pores cause more efficient permeation of biomolecules inside the pores and increase the spread in elution time between compounds. On the other hand, pore volume decreases the strength of the packing material making it more fragile. Finding the right balance between resolution and mechanical strength is critical to achieve the desired separation with SEC columns.

Secondary interactions with the surface of the SEC resin can prevent free passage through the pores and interfere with the size-based separation. When selecting a SEC column, a particle that minimizes secondary interaction is essential.

Why use Agilent AdvanceBio SEC 200 Å 1.9 µm columns for mAb fragment and aggregate separation?

- The proprietary AdvanceBio 1.9 µm monodisperse silica particles are engineered for best-in-class mechanical robustness. This makes them suitable for use with both UHPLC and HPLC instruments, with excellent column lifetime.[1]
- The 1.9 µm particle AdvanceBio SEC columns have pore characteristics ideal for high-resolution fast separation of lower molecular weight protein fragments and mAb aggregates and dimers with a single column.[2]
- The proprietary unique hydrophilic bonding chemistry that provides an inert surface minimizing secondary hydrophobic interaction with ADCs and mAbs.



Column: 4.6 x 300 mm
Mobile phase: 150 mM sodium phosphate, pH 7.0
Flow rate: 0.35 mL/min
Temperature: Ambient
Detector: 220 nm
Sample: Bio-Rad protein mix and uridine (stop flow every 50 injections)

Figure 1. AdvanceBio SEC 200 Å 1.9 µm columns showed less than 2% drop in plate number over 400 injections, confirming excellent mechanical stability.

Recommended starting conditions[3]

Parameter	Value
Column	AdvanceBio SEC 200 Å 4.6 x 300 mm, 1.9 µm (Part No. PL1580-5201)
Instrument	Agilent 1260 Infinity II Bio-inert LC System
Flow Rate	0.35 mL/min
Mobile Phase	150 mM sodium phosphate, pH 7.0
Wavelength	280 nm
Column Temperature	25 °C
Sample	A stressed mAb (1 µg, injected onto column). mAb sample stressed in 100 mM sodium bicarbonate pH 9.0 and incubated overnight at 40 °C

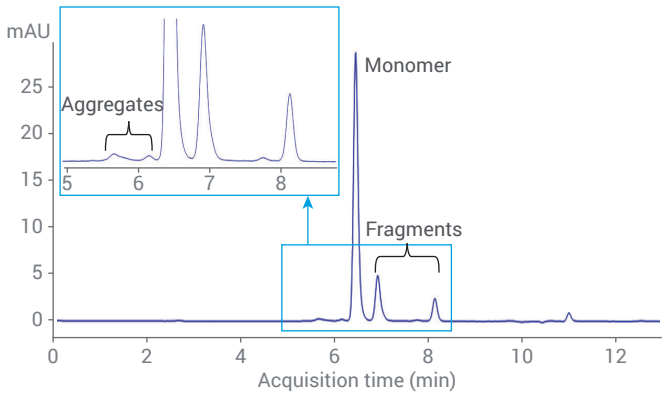


Figure 2. Separation of aggregates and fragments in a stressed mAb sample on an AdvanceBio SEC 200 Å 4.6 x 300 mm, 1.9 µm column under the recommended starting conditions.

How to optimize your chromatographic conditions

Consider adjusting the following to improve separation or maintain protein solubility after viewing the initial chromatogram:

Ionic strength of the mobile phase - Buffers typically used for protocols requiring additional salt include:

- 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 (eg KCl) and guanidine hydrochloride can also be used.

pH - adjust in increments of ± 0.2 units. AdvanceBio SEC columns have a stability range between pH 2.0 to 8.5.

Temperature - SEC separations are typically run between 10 to 30 °C. Higher temperatures may be required to improve the resolution and recovery of hydrophobic peptides. SEC may be run in a cold room to maintain maximum biological activity of temperature sensitive proteins. When running separations at colder temperatures, monitor the pressure to avoid over pressuring and adjust the flow rate as needed. The maximum operating temperature of Agilent AdvanceBio SEC columns is 80 °C.

Note: Higher temperatures can denature proteins.

Organic solvent additives

- 5 to 10% EtOH (or other similar solvents such as MeOH or CH_3CN) in 50 mM sodium phosphate, pH 7.0 can be beneficial for highly hydrophobic proteins.
- 5% DMSO in 50 mM sodium phosphate, pH 7.0 can be added for proteins containing high cysteine residue content and prone to oxidation/aggregation.

Note: It may be necessary to reduce the flow rate to keep below the maximum operating pressure when using higher viscosity mobile phases.

References

1. Fast Separations for Aggregates and Fragments with AdvanceBio SEC columns - 5994-0873EN
2. Size Exclusion Chromatography Analysis of a Monoclonal Antibody and Antibody Drug Conjugate – 5994-0827EN
3. Agilent AdvanceBio SEC 1.9 µm Column user guide - 5994-0739EN
4. Elevate Your mAb Aggregate Analysis - 5994-2709EN
5. Analysis of Antibody Fragment-Drug Conjugates Using an Agilent AdvanceBio SEC 120 Å 1.9 µm PEEK-Lined Column – 5994-3045EN
6. Fast, High-Resolution Size Exclusion Chromatography of Aggregates in Biotherapeutics - 5991-6458EN

Getting started with Agilent AdvanceBio SEC 200 Å columns: Tips to ensure optimal performance and separation

Sample consideration

- 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 Ultralow Dispersion Kit can be installed on 1290 model LCs to further reduce system volume and avoid band broadening.[4]
- Maximize resolution with the smallest possible injection volume. A sample injection volume of 1 to 5 µL is recommended with a maximum injection volume of 1% of the column volume.

Column selection factors

Select the right column for your sample using the following criteria:

- Longer columns result in higher resolution – ideal for separating monomer from dimer, or monomer from fragments.
- Narrower column diameters:
  - require smaller injection volumes – ideal when sample availability is limited.
  - require lower flow rates – ideal for efficient desolvation/ ionization in native MS.[3]
- PEEK-lined columns can improve peak shape by minimizing secondary interaction of the sample with metal surfaces – which also makes these columns ideal when using volatile mobile phase buffers.[5]
- When focusing on higher order aggregate analysis, AdvanceBio SEC 300 Å, 2.7 µm columns provide fast and accurate quantification for mAb aggregates, dimers and monomer with the same reliable performance.[6]

Column operation and cleaning

- Align flow rates with column id[3] – 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 requires efficient desolvation/ ionization of the sample.
  - Working Flow Rate[3]:
    - 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 the flow rate to keep the pressure below 400 bar.
- 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.

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, column 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.

Easy selection and ordering information

To order items listed in the tables below 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.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered online by clicking on the individual part numbers or through your regular sales and distributor channels.

Description	Part No.
MyList of Sample Preparation Supplies	
Captiva disposable syringe, 5 mL, 100/pk	9301-6476
Captiva Premium Syringe Filter, PES, 15 mm, 0.2 µm, 100/pk	5190-5096
MyList of Standards	
Agilent NISTmAb, 4 x 25 µL	5191-5745
300 Å AdvanceBio SEC calibration standard	5190-9417
MyList of AdvanceBio SEC Columns	
AdvanceBio SEC 200 Å, 1.9 µm guard, 4.6 x 30 mm (recommended)	PL1580-1201
AdvanceBio SEC 200 Å, 1.9 µm, 4.6 x 300 mm (recommended)	PL1580-5201
AdvanceBio SEC 200 Å, 1.9 µm, 4.6 x 150 mm	PL1580-3201
AdvanceBio SEC 200 Å, 1.9 µm guard, 2.1 x 50 mm, PEEK-lined SS	PL1980-1201PK
AdvanceBio SEC 200 Å, 1.9 µm, 2.1 x 150 mm, PEEK-lined SS	PL1980-3201PK
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 quick turn fittings	5043-0915
Capillary MP35N 0.17 x 100 mm SL/SL ps/ps (for connecting guard and column)	5500-1278

Description	Part No.
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 Supplies	
A-line screw top vial, 2 mL, 12 x 32 mm (12 mm cap) amber, write-on spot, 100/pk	5190-9590
Screw cap, 12mm, 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 Well-plate closing mat, 50/pk	5042-1389
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.

For additional SEC column solutions for aggregate and fragment analysis, visit:
www.agilent.com/chem/aggregates

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