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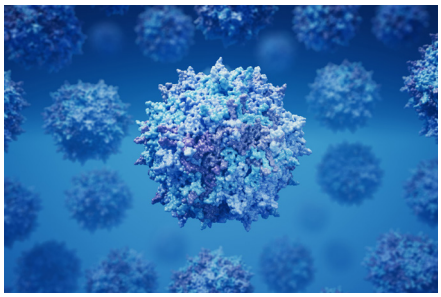
Consumables Workflow Ordering Guide

Aggregate Analysis of Adeno-Associated Viruses and Virus-Like Particles in Biopharma by Liquid Chromatography



Adeno-associated viruses (AAVs) and virus-like particles (VLPs) are emerging biotherapeutic molecules with exciting potential in the vaccine, cell, and gene therapy spaces.

AAVs are noncovalent, self-assembled protein structures that may or may not contain genomic payload in their core. There are several different classes of AAVs that naturally target different organ systems in the body, making them natural delivery vectors for cell and gene therapy. AAV serotypes differ in the structure and chemistry of the proteins that comprise the spherical shell of the AAV. However their size is narrowly defined at 20 to 25 nm in diameter, and ~3.7 MDa when empty or ~5.1 MDa with an oligonucleotide payload.

VLPs are large, self-assembled structures comprised of one or more individual proteins that can serve as vaccines for disease prevention. VLPs have the same structural exterior as a virus but lack the genomic material and replication machinery that render viruses infectious. Thus, they can prompt an immune response without causing an infection.

Like other classes of biotherapeutic molecules, AAVs and VLPs are subject to considerable scrutiny from regulatory agencies worldwide, requiring characterization of the biotherapeutic itself as well as any product- or process-related impurities. Aggregation is a product-related impurity that commonly rises to the level of critical quality attribute, and therefore must be monitored and carefully controlled. The structural similarity between AAVs and VLPs provides the potential to use common approaches for their analysis.

Unlike monoclonal antibodies (mAbs) or adeno-associated viruses (AAVs), different types of VLPs exist in a wider range of sizes, usually between 20 and 150 nm in diameter. This diversity means that a universal method is often impractical and the use of complementary techniques is more important. Size exclusion chromatography (SEC) has long been the gold standard for monitoring protein aggregation and has become a common approach for aggregation analysis of AAVs. Despite the diversity of VLP sizes, SEC still has an important role in aggregation analysis of VLPs, in concert with orthogonal techniques such as analytical ultracentrifugation (AUC), electron microscopy (EM), or field-flow fractionation (FFF).

Overcoming aggregate analysis challenges

Arguably, the first challenge in aggregate analysis of VLPs is choosing the most appropriate primary technique, which depends on the nominal size of the VLP monomer. (Here, "monomer" refers to the nonaggregated single VLP assembly, rather than to an individual protein component of the assembled VLP.) The largest pore size that is currently available in conventional SEC columns is 2000 Å. The conventional rule of thumb for SEC is that the pore size should be about three times the diameter of the analyte in question. For example, a 2000 Å pore would be suitable for an analyte of approximately 670 Å (or 67 nm). In practice, however, many users take 100 nm (1000 Å) as a threshold where SEC is likely to be significantly limited. Smaller VLPs as well as AAVs are comfortably within a suitable size range for SEC, but this leaves a portion of VLPs that are better suited for analysis by another technique. Compared to AUC or EM, SEC is fast and inexpensive, so it remains a preferred approach when possible.

For SEC of either AAVs or VLPs, the two biggest challenges are resolution and sensitivity related to sample scarcity, both in volume and concentration. The Agilent line of Bio SEC-5 columns addresses these difficulties through a stationary phase that enables high resolution and good sample recovery with pore size and column dimension options to suit every situation.

Choosing the right SEC column

Choose an SEC column dimension that will help achieve your separation goals and mitigate your sample constraints. Consider the following when choosing a column dimension:

- Longer columns, such as 300 mm, deliver higher resolution.
- Where resolution permits, shorter, 150 mm columns are recommended where high throughput is a priority.
- While 7.8 mm has long been the classic internal diameter (id) for SEC, narrower column diameters such as 4.6 mm require smaller injection volumes, which is ideal for AAVs and VLPs where sample availability may be limited.

It can be challenging to predict the most suitable pore size for emerging classes of biotherapeutics, simply because their structure in solution is different from the historical targets of biological SEC and GPC. Pore size has often been correlated with molecular weight to define the exclusion limit and total permeation point; however, it is ultimately the hydrodynamic radius of the analyte that determines the optimum pore size. Molecular weight correlations work reasonably well when the analyte has a similar structure in solution to the standards used to establish that correlation. These standards have historically been either globular proteins for biological SEC or relatively linear polymers for GPC. AAVs and VLPs differ in structure from both globular proteins and linear polymers and may differ significantly from one type of VLP to another. Therefore, molecular weight correlations become less reliable and additional information should also be considered. Consider the following when choosing a pore size:

- Pore size recommendations according to molecular weight are listed in Table 1.
- The rule of thumb is to use a pore size that is three times the diameter of the analyte. This is a useful guideline if the approximate sample size is already known.
- Reported examples for similar molecules are another useful reference point.

Column	Exclusion Limit	Total Inclusion Point	Target Analytes
Bio SEC-5, 5 µm, 500 Å	5 MDa	5 kDa	AAVs, small VLPs
Bio SEC-5, 5 µm, 1000 Å	7.5 MDa	50 kDa	AAVs, VLPs, large oligos
Bio SEC-5, 5 µm, 2000 Å	> 10 MDa	150 kDa	VLPs, large oligos

Table 1. Molecular weight ranges for Agilent Bio SEC-5 columns.

AAVs, with their narrowly defined size, illustrate the challenge in choosing a pore size (Figure 1). AAVs have a molecular weight of approximately 5.1 or 3.8 MDa, with or without genomic payload respectively. Table 1 suggests that either the 500 or 1000 Å Bio SEC-5 columns are most suitable. AAVs are 20 to 25 nm in diameter, suggesting a 600 to 750 Å pore would be best according to the rule of thumb. However, in practice, scientists have reported using SEC columns ranging from 450 to 1000 Å from different vendors for AAVs.

The risk of pores being too small is an incomplete analysis of aggregation states, while using pores that are too large can lead to inadequate resolution between monomer and dimer or monomer and fragments. Through empirical study, Agilent recommends the 1000 Å Bio SEC-5 column for aggregate analysis of AAVs. A representative chromatogram and method conditions for AAV aggregate analysis are shown in Figure 2 and Table 2, respectively. More information can be found in the Agilent application brief 5994-4270EN.[1]

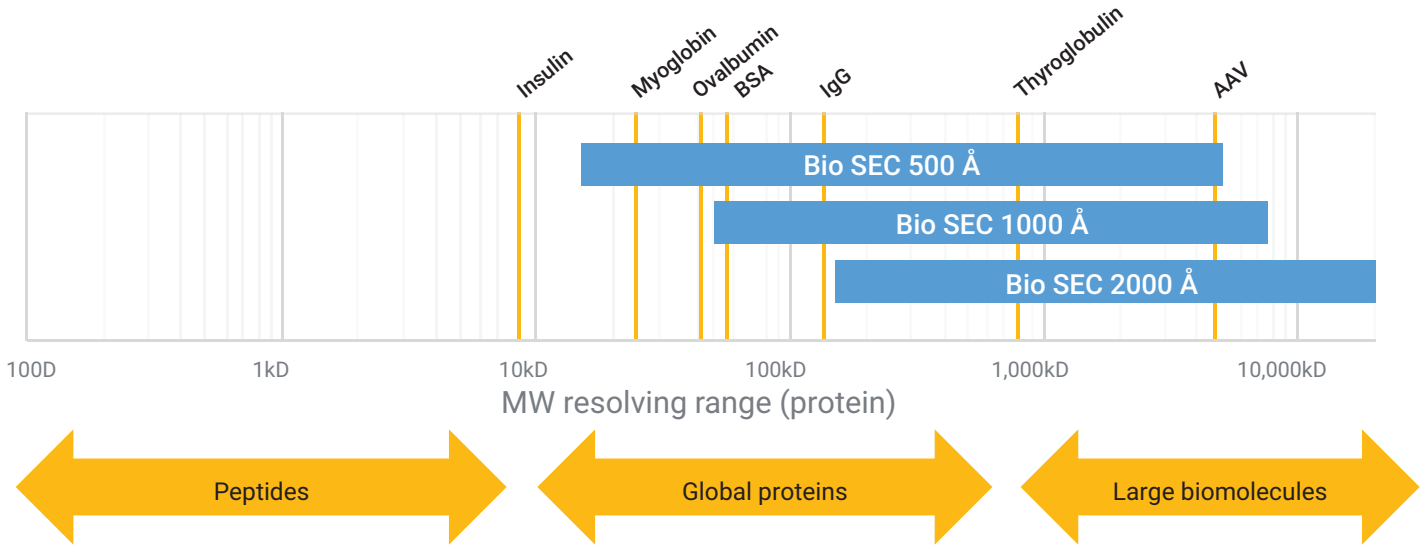


Figure 1. SEC pore size selection based on protein molecular weight.

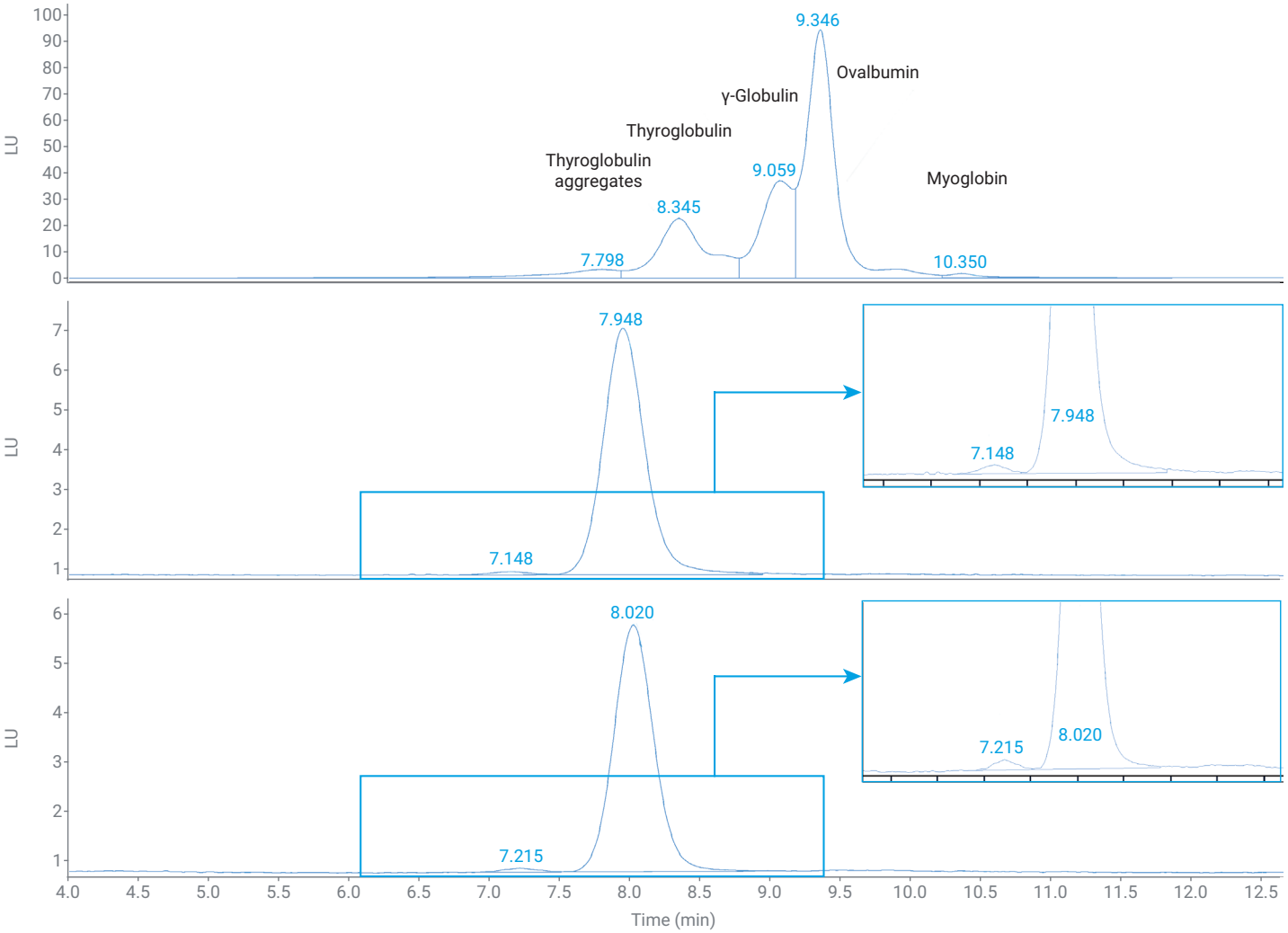


Figure 2. SEC chromatograms for AAV-9 and AAV-2, with an Agilent AdvanceBio SEC 300 Å standard column, using an Agilent Bio SEC-5 1000 Å column.

Parameter	Value
Column	Agilent Bio SEC-5 1000 Å, 4.6 x 300 mm
Flow	0.4 mL/min
Mobile Phase	50 mM phosphate buffer + 400 mM NaCl, pH 7.4
Column Temperature	Ambient
Sample Volume	20 µL
Fluorescence Detection	λex = 280, λem = 340 nm

Table 2. Sample SEC method conditions using an Agilent Bio SEC-5 column for separating AAV aggregates and fragments.

Sometimes, the optimum pore size may be best determined by empirical evaluation.[2] For example, Figure 3 demonstrates the SEC chromatograms for a VLP using 1000 and 2000 Å Bio SEC-5 columns (SEC conditions are displayed in Table 3). In this study, the VLP was reported to be ~40 nm. Following the rule of thumb, an appropriate pore size would be 120 nm, or 1200 Å. Both the 1000 and 2000 Å Bio SEC-5 columns were evaluated, and it was determined that the 2000 Å gave better resolution in this case. For more details, see the Agilent application brief 5994-4227EN.[2]

SEC best practices

The following list comprises some SEC best practices:

- Prepare fresh mobile phase buffer and filter through a 0.2 or 0.45 µm filter to remove particulates and reduce the risk of any microbial growth that could damage the column or LC system.
- Lower the flow ramp rate from the default to 1 mL/min² 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 (240 bar for Bio SEC-5 columns). This is key for any instance in which the LC maximum pressure capabilities exceed that of the column.
- Verify system performance with a suitable SEC standard at regular intervals.
- Maximize chromatographic resolution by minimizing sample injection volume if possible. A sample injection volume of 5 to 10 µL is recommended with a maximum injection volume of 1% of the column volume.
- Should cleaning be necessary, rinse with at least 5 column volumes of ultrapure water before and after flushing with at least 20 column volumes of the cleaning solution to avoid precipitation of buffer salts on the column. Consult the Bio SEC-5 column user guide[3] for additional detail.

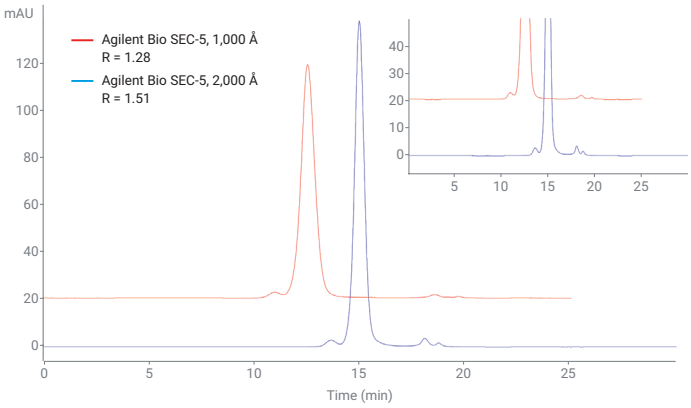


Figure 3. SEC chromatograms for a VLP using 1000 and 2000 Å Agilent Bio SEC-5 columns. The 2000 Å columns gave better resolution between the monomer and aggregate.

Parameter	Value
Column	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)	0.4 mL/min
Flow	0.6 mL/min
Mobile Phase	50 mM phosphate buffer (pH 7.4) with 400 mM sodium chloride
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

Table 3. Sample SEC method conditions for a ~40 nm VLP.

References

1. Liau, B.; Blackwell, A., and Turner, M.L. Resolution of Adeno-Associated Viral Vector Aggregates and Fragments with Agilent Bio SEC-5, *Agilent Technologies application brief*, publication number 5994-4270EN, **2021**
2. Mi, J. Agilent Bio SEC-5 for Analysis of Virus-Like Particles (VLP), *Agilent Technologies application brief*, publication number 5994-4227EN, **2021**
3. Agilent Bio SEC-5 Columns, *Agilent Technologies data sheet*, publication number 5973-1743, **2021**

Easy selection and ordering information

To order items listed in the tables below, add items to your Favorite Products list by clicking the MyList link in the header. Your list will remain under Favorite Products for you 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. If you do not have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled.

Individual items can also be ordered from the Agilent online store by clicking on the part number hyperlinks or through your regular sales and distributor channels.

MyList 1: Agilent Bio SEC-5 Columns

Description	Part Number
500 Å columns	
Bio SEC-5, 5 µm, 500 Å, 4.6 x 300 mm	5190-2533
Bio SEC-5, 5 µm, 500 Å, 4.6 x 150 mm	5190-2534
Bio SEC-5, 5 µm, 500 Å, 4.6 x 50 mm, guard	5190-6860
Bio SEC-5, 5 µm, 500 Å, 7.8 x 300 mm	5190-2531
Bio SEC-5, 5 µm, 500 Å, 7.8 x 150 mm	5190-2532
Bio SEC-5, 5 µm, 500 Å, 7.8 x 50 mm, guard	5190-2535
1000 Å columns	
Bio SEC-5, 5 µm, 1000 Å, 4.6 x 300 mm	5190-2538
Bio SEC-5, 5 µm, 1000 Å, 4.6 x 150 mm	5190-2539
Bio SEC-5, 5 µm, 1000 Å, 4.6 x 50 mm, guard	5190-6861
Bio SEC-5, 5 µm, 1000 Å, 7.8 x 300 mm	5190-2536
Bio SEC-5, 5 µm, 1000 Å, 7.8 x 150 mm	5190-2537
Bio SEC-5, 5 µm, 1000 Å, 7.8 x 50 mm, guard	5190-2540
2000 Å columns	
Agilent Bio SEC-5, 5 µm, 2000 Å, 4.6 x 300 mm	5190-2543
Agilent Bio SEC-5, 5 µm, 2000 Å, 4.6 x 150 mm	5190-2544
Agilent Bio SEC-5, 5 µm, 2000 Å, 4.6 x 50 mm, guard	5190-6862
Agilent Bio SEC-5, 5 µm, 2000 Å, 7.8 x 300 mm	5190-2541
Agilent Bio SEC-5, 5 µm, 2000 Å, 7.8 x 150 mm	5190-2542
Agilent Bio SEC-5, 5 µm, 2000 Å, 7.8 x 50 mm, guard	5190-2545

MyList 2: Supplies and Sample Containment

Description	Part Number
Connectors and tubing	
Mounting tool for quick turn fittings	5043-0915
InfinityLab Quick Connect LC fitting	5067-5965
Quick Connect Capillary MP35N 0.12 x 105mm. for use with Quick Connect Fitting	5500-1578
Quick Turn Capillary MP35N 0.12 x 280mm	5500-1596
Inline filters	
InfinityLab Quick Change inline filter assembly, for UHPLC*	5067-1603
InfinityLab Quick Change filter disc, 2.1 mm id, 0.2 µm pore size, 5/pk	5067-1610
Sample containment	
High recovery vial, screw top, with fixed insert, clear, 300 µL insert volume, Vial size: 12 x 32 mm (12 mm cap), 100/pk.	5188-6591
Cap, screw, blue, PTFE/red silicone septa, 100/pk. Cap size: 12 mm	5182-0717
Vial, crimp/snap top, polypropylene, 250 µL, 1,000/pk. Vial size: 12 x 32 mm (11 mm cap)*	5190-3155
Cap, snap, clear, PTFE/silicone/PTFE septa, 100/pk. Cap size: 11 mm, (for 5190-3155)	5182-0566
InfinityLab Well-plate 96/0.5 mL, 30/pk	5043-9310
InfinityLab Well-plate closing mat, 50/pk	5042-1389

MyList 3: Standards, Solvents and Solvent Supplies

Description	Part Number
Standards and solvents	
300 Å AdvanceBio SEC calibration standard	5190-9417
Agilent NIST mAb, 25 µL	5191-5744
Agilent NIST mAb, 4 x 25 µL	5191-5745
InfinityLab Ultrapure LC/MS Water, 1 L	5191-4498
InfinityLab Water for LC/MS, 6 x 1 L*	5191-5121
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
Solvent handling	
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	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

** Available in select countries.*
‡ If using solvents other than those listed in this table.

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