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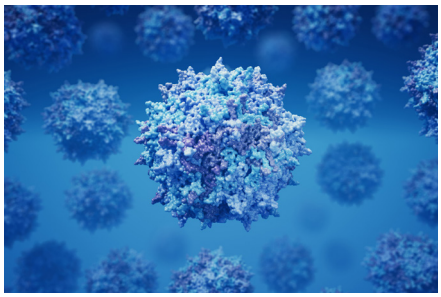
Consumables Workflow Ordering Guide

Aggregate Analysis of Adeno-Associated Viruses and Virus-Like Particles in Biopharma using 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 a genomic payload in their core. There are several different classes of AAVs that naturally target different organ systems in the body, making them logical 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 traditional SEC columns is 2000 Å. The general rule 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 using another technique. Compared to analytical ultracentrifugation (AUC) or electron microscopy (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. Agilent AdvanceBio SEC columns address 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:

- Longer columns, such as 300 mm, deliver higher resolution.
- Where resolution permits, shorter, 150 mm columns are recommended, especially when 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 evaluated. Consider the following when choosing a pore size:

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

Table 1. Molecular weight ranges for wide pore SEC columns.

Column	Exclusion Limit	Total Inclusion Point	Target Analytes
Agilent AdvanceBio SEC 2.7 µm, 500 Å	5 MDa	5 kDa	AAVs, small VLPs
Agilent AdvanceBio SEC 2.7 µm, 1000 Å	10 MDa	15 kDa	VLPs, LNPs, large oligos
Agilent Bio SEC-5, 5 µm, 2000 Å	> 10 MDa	150 kDa	VLPs, large oligos

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 and Figure 1 suggest that 500 Å pores are too small, and that perhaps even 1000 Å pores would be too small to include aggregates, but AAVs, VLPs, and oligonucleotides have very different structures in solution than the globular proteins from which molecular weight guidelines are derived. AAVs are 20 to 25 nm in diameter, suggesting a 600 to 750 Å pore would be best. However, in practice, scientists have reported using SEC columns ranging from 450 to 1000 Å from different vendors for AAVs. If the pores are too small, there is a risk of 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 500 Å AdvanceBio SEC 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 5994-7509EN.[1]

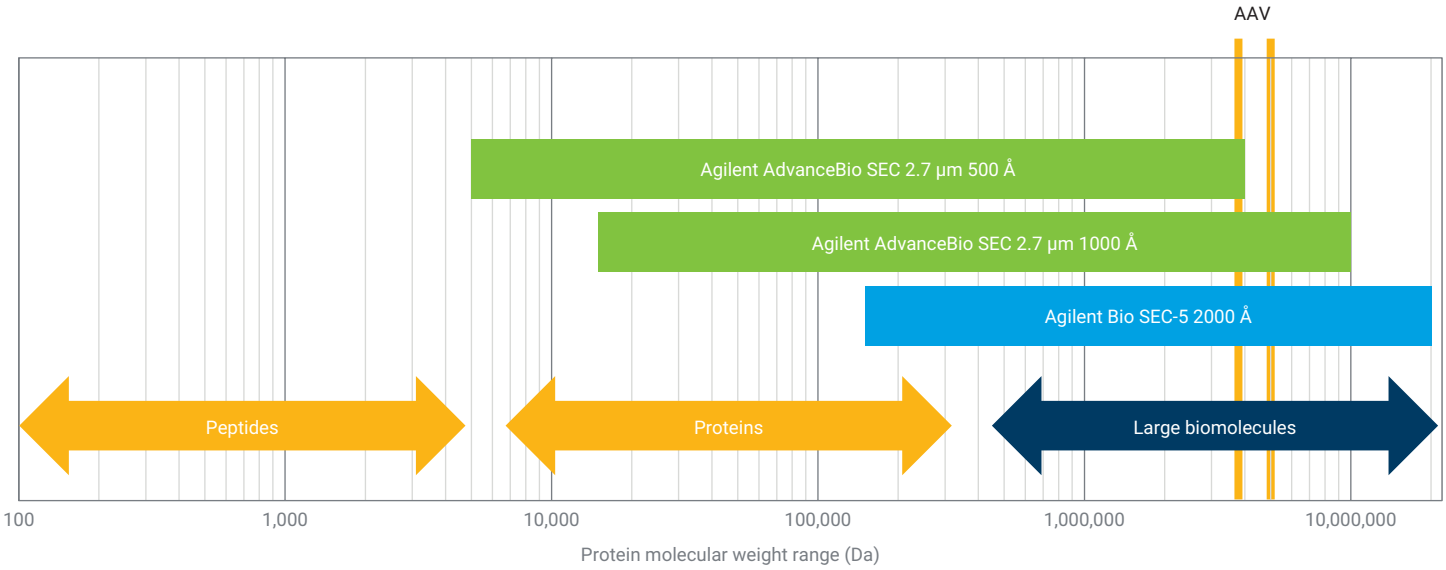


Figure 1. SEC pore size selection based on protein molecular weight.

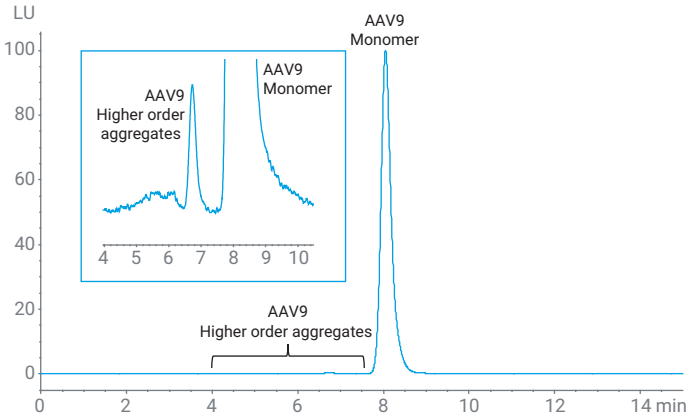


Figure 2. High-resolution separation of AAV9 using an Agilent AdvanceBio SEC 500 Å, 4.6 × 300 mm, 2.7 µm column (part number PL1580-5325).

Table 2. Sample SEC method conditions using an Agilent AdvanceBio SEC 500 Å column for separating AAV aggregates and fragments.

Parameter	Value
Flow Rate	0.35 mL/min
Mobile Phase	50 mM Phosphate buffer, 400 mM NaCl, pH 7.2
Injection Volume	5 µL
Fluorescence Detection	Ex 280 nm, Em 343 nm
Temperature	Ambient
Sample	AAV9

For analytes larger than AAVs, such as VLPs, LNPs, or large oligonucleotides, Agilent has the AdvanceBio SEC 2.7 µm, 1000 Å, and the Agilent Bio SEC-5, 5 µm, 2000 Å columns. Figure 3 illustrates aggregate analysis of a ~50 nm VLP, HPV-16, using AdvanceBio SEC with 1000 Å pores. The 2.7 µm particles offer improved resolution over the previously available 5 µm option, thanks to smaller particles, higher pore volume, and a larger exclusion limit. For more details, see the Agilent application note 5994-7427EN.[2]

Because SEC ultimately separates based on analyte size in solution, empirical determination may be the best way to select the correct pore size for an analyte. This is particularly true for sample types that are not predictable in their folding or three-dimensional structure in solution.

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/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 (400 bar for AdvanceBio SEC 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 five 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 AdvanceBio SEC column user guide[3] for additional details.

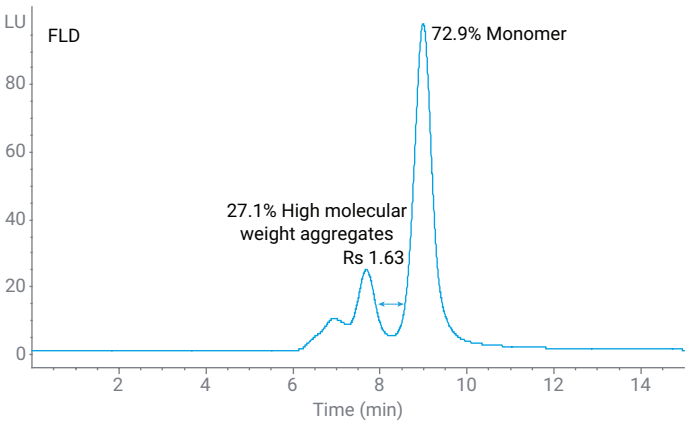


Figure 3. Fluorescence chromatogram of 1 µL injection of HPV-16 VLP sample with excitation at 280 nm and emission at 348 nm.

Table 3. Sample SEC method conditions for a ~50 nm VLP.

HPLC Conditions	
Parameter	**Value**
Column	Agilent AdvanceBio SEC 1000 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5302)
Mobile Phase	50 mM Sodium phosphate + 400 mM NaCl, pH 7.2
Flow Rate	0.35 mL/min
Column Temperature	Room temperature
Injection Volume	1 µL
Detection	FLD excitation 280 nm, emission 348 nm
Run Time	15 min
HPLC System	Agilent 1290 Infinity II Bio LC system with binary high-speed pump

Easy selection and ordering information

To order items listed in the following tables, add the items to your Favorite Products list by clicking the MyList link in the header. Your list will remain under Favorite Products for you to 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 eCommerce-enabled.

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

MyList 1: Size exclusion columns

Description	Part Number
500 Å columns	
AdvanceBio SEC 500 Å, 2.7 µm, 7.8 × 300 mm	PL1180-5325
AdvanceBio SEC 500 Å, 2.7 µm, 7.8 × 50 mm, guard	PL1180-1325
AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 300 mm	PL1580-5325
AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 150 mm	PL1580-3325
AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 50 mm, guard	PL1580-1325
1000 Å columns	
AdvanceBio SEC 1000 Å, 2.7 µm, 7.8 × 300 mm	PL1180-5302
AdvanceBio SEC 1000 Å, 2.7 µm, 7.8 × 50 mm, guard	PL1180-1302
AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 × 300 mm	PL1580-5302
AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 × 150 mm	PL1580-3302
AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 × 50 mm, guard	PL1580-1302
2000 Å columns	
Agilent Bio SEC-5, 2000 Å, 5 µm, 4.6 × 300 mm	5190-2543
Agilent Bio SEC-5, 2000 Å, 5 µm, 4.6 × 150 mm	5190-2544
Agilent Bio SEC-5, 2000 Å, 5 µm, 4.6 × 50 mm, guard	5190-6862
Agilent Bio SEC-5, 2000 Å, 5 µm, 7.8 × 300 mm	5190-2541
Agilent Bio SEC-5, 2000 Å, 5 µm, 7.8 × 150 mm	5190-2542
Agilent Bio SEC-5, 2000 Å, 5 µm, 7.8 × 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 × 105 mm, for use with Quick Connect fitting	5500-1578
Quick Turn Capillary, MP35N, 0.12 × 280 mm	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 × 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 × 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-sample, 0.5 mL, 30/pk	5043-9310
InfinityLab well plate closing mat, 50/pk	5042-1389

** Available in select countries.*

MyList 3: Standards, solvents and solvent supplies

Description	Part Number
Standards and solvents	
AdvanceBio SEC 300 Å protein standard	5190-9417
Agilent NIST mAb, 25 µL	5191-5744
Agilent NIST mAb, 4 × 25 µL	5191-5745
InfinityLab ultrapure LC/MS water, 1 L	5191-4498
InfinityLab water for LC/MS, 6 × 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 inlet filter, glass, 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.

References

1. Coffey, A.; Kumar, C.; Wei T.; Blackwell, A. Improved Wide Pore Size Exclusion Chromatography Columns for AAV Analysis. *Agilent Technologies application brief*, publication number 5994-7509EN, **2024**.
2. Kumar, C.; Wei, T.; Coffey, A.; Blackwell, A. Robust Wide Pore Size Exclusion Columns for Virus-Likes Particle (VLP) Analysis. *Agilent Technologies application note*, publication number 5994-7427EN, **2024**.
3. AdvanceBio SEC Columns, *Agilent Technologies user guide*, publication number 5971-6580EN, **2024**.

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