Agilent

Trusted Answers

Consumables Workflow Ordering Guide

Adeno-Associated Virus Identity Confirmation in Biopharma by LC/MS

While adeno-associated viruses (AAVs) have their own unique set of critical quality attributes, many of the traditional assays used to monitor more established protein-based therapeutics can still be used. AAV capsids comprise ~ 60 copies of three proteins, VP1, VP2, and VP3, in a stoichiometric ratio of approximately 1:1:10, respectively.[1] 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).

The U.S. Food and Drug Administration (FDA) requires AAV products to be unambiguously identified before release, especially in facilities where multiple serotypes or engineered variants are produced.[2] Peptide mapping is an essential method that determines protein sequences and identifies PTMs, and as such is required by the ICH, U.S. FDA, and other regulatory agencies for more established biotherapeutics. While regulations surrounding gene therapies using AAVs are still emerging, peptide mapping may be required in the future.

Previous LC/MS work to confirm the identity and relative abundance of intact capsid proteins has suffered from poor chromatographic resolution. Coeluting proteins make accurate mass measurements more challenging and the similarity of some AAV capsid proteins (i.e. AAV1 and AAV6 differ by six amino acids[3]) makes accurate measurement critical for identity confirmation.

Agilent ZORBAX RRHD Wide Pore columns address this challenge for intact protein analysis, providing good chromatographic separation that enables accurate mass measurements via mass spectrometry (MS) detection.

- Sub 2 µm particle size offers high resolution
- Wide pores enable good mass transfer for efficient separations
- 1200 bar pressure tolerance allows for high efficiency UHPLC methods
- Diphenyl chemistry offers unique selectivity for challenging separations

Agilent AdvanceBio Peptide Mapping columns are designed to provide high resolution peptide maps for protein identification and determination of post translation modifications.

- Superficially porous particles enable high resolution separations at modest backpressures
- Good peak capacity with formic acid mobile phases, which improves MS sensitivity over TFA containing mobile phases

Best practices for effective AAV analysis

Sample preparation

- Many recombinant proteins are formulated in nonvolatile buffers that contain relatively high salt and stabilizing additives such as poloxamers that interfere with MS detection and dirty the instrument quickly. Buffer exchange of the sample before LC/MS analysis can significantly improve spectral quality and allow longer times between MS maintenance. Be aware that buffer exchange may lead to sample instability, so plan to analyze samples directly following the buffer exchange.
- High recovery vials intended for small sample volumes are recommended.

Chromatographic separation

- 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 and help prevent sudden over pressuring. The setting can be found in the Advanced section for LC pump control in Agilent software.
- Set the maximum pressure limit in the LC method to match that of the column (600 bar for AdvanceBio Peptide Mapping, 1200 bar for ZORBAX RRHD columns). This is key for any instance where the maximum pressure capabilities of the LC exceed that of the column.
- Minimize system dead volume to maximize resolution. A low-dead-volume system such as an Agilent 1290 Infinity II outfitted with ultralow dispersion tubing[4] is recommended to minimize dead volume.

Mass spectrometry

- Divert the LC stream to waste outside of the retention time(s) of interest, especially during a high organic rinse at the end of the method and, if possible, as the void volume elutes.
- Use HPLC-grade or higher solvents.
- Establish a regular cleaning routine for the MS source.

Getting started – Intact capsid proteins

Intact capsid protein analysis is described more thoroughly in application note 5994-2434EN, which compares different column chemistries from the ZORBAX RRHD family for separation of VP1, VP2, and VP3 capsid proteins.[5] The workflow is illustrated in Figure 1.

Column selection criteria - Intact capsid proteins

When choosing a reversed phase column, particularly for AAV capsid proteins, it is useful to consider both the detection method that will be used and what is known about the sample. AAV samples are often more dilute than other recombinant protein samples. Choose columns that address sensitivity and resolution, two parameters that have historically been a challenge when separating VP1, VP2, and VP3 capsid proteins.

- **Column diameter:** A 2.1 mm column is recommended over larger id columns both for sensitivity and compatibility with MS detection. The flow rate used with 2.1 mm columns is conducive to efficient electrospray ionization, which also helps sensitivity.
- **Column length:** For reversed phase columns, longer columns can lead to higher resolution, so 100 or 150 mm lengths are recommended.
- **Pore size:** Intact proteins are relatively large in solution, especially under denaturing reversed phase conditions. Large pores are required to ensure efficient mass transfer in and out of stationary phase particles, which in turn improves resolution. We recommend 300 Å pores.
- **Particle size:** Smaller particle sizes increase resolution, so 1.8 µm RRHD columns are recommended.
- **Stationary phase chemistry:** When working to resolve analytes of interest, the selectivity of the stationary phase is another variable that can be changed. Shorter alkyl chain stationary phases such as C4 or C8 are common for intact proteins, but less intuitive options have proved beneficial for VP1, VP2, and VP3. An additional complicating factor is that the differences between each AAV serotype result in different stationary phase requirements. The ZORBAX RRHD SB300-C18 column worked well for AAV2 and AAV7, while the ZORBAX RRHD 300-Diphenyl column worked well for several other serotypes, including AAV9.

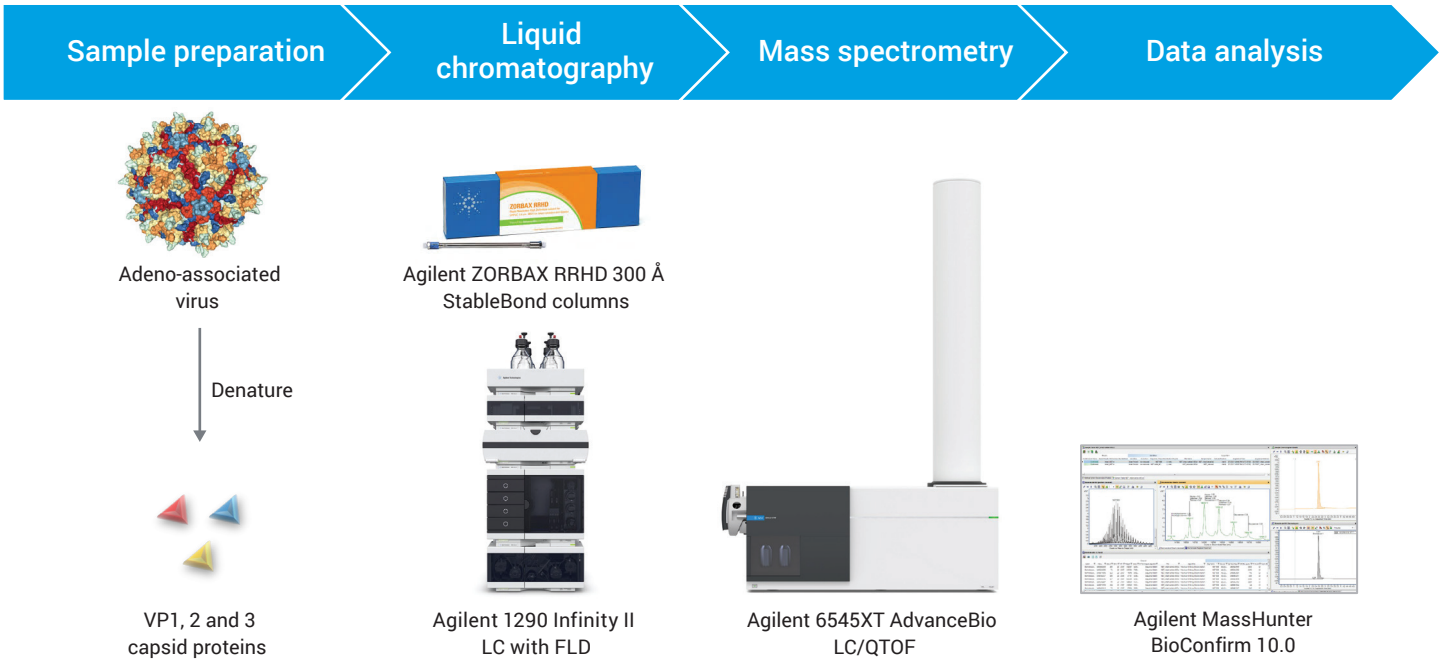

Figure 1. Overview of the process to confirm identity and measure relative quantities of individual capsid proteins comprising the intact AAV capsid.

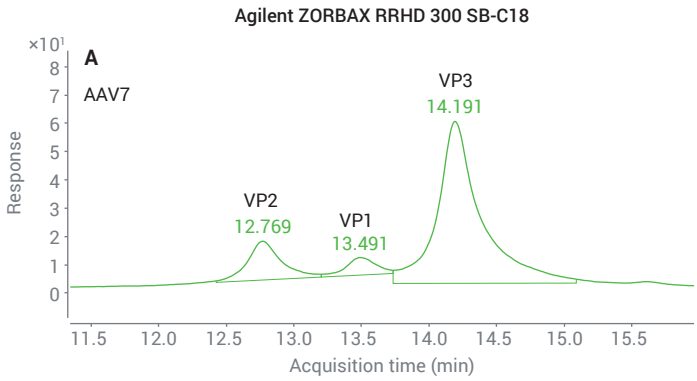

Figure 2. Separation of AAV7 on Agilent ZORBAX RRHD 300 SB-C18 using conditions described in Table 1.

Parameter	Value	
Column	Agilent ZORBAX RRHD 300 Å StableBond C18, 2.1 x 100 mm, 1.8 µm (p/n 858750-902)	
Instrument	Agilent 1290 Infinity II	
Flow Rate	0.4 mL/min	
Mobile Phase A	0.1% formic acid + 0.1% TFA in water	
Mobile Phase B	90% isopropanol, 9.8% water, 0.1% formic acid + 0.1% TFA	
Gradient	Time (min)	%B
	0-5	28%
	23	32.5%
	23.5	80%
	26	80%
Post time	3 minutes	
Column Temperature	80 °C	

Table 1. Starting conditions used for analyzing Intact Capsid Proteins. See reference[5] 5994-2434EN for details.

Getting started – Peptide mapping

Peptide mapping analysis for AAV capsid proteins using the AdvanceBio Peptide Mapping column is described in application notes 5994-1980EN and 5994-2434EN.

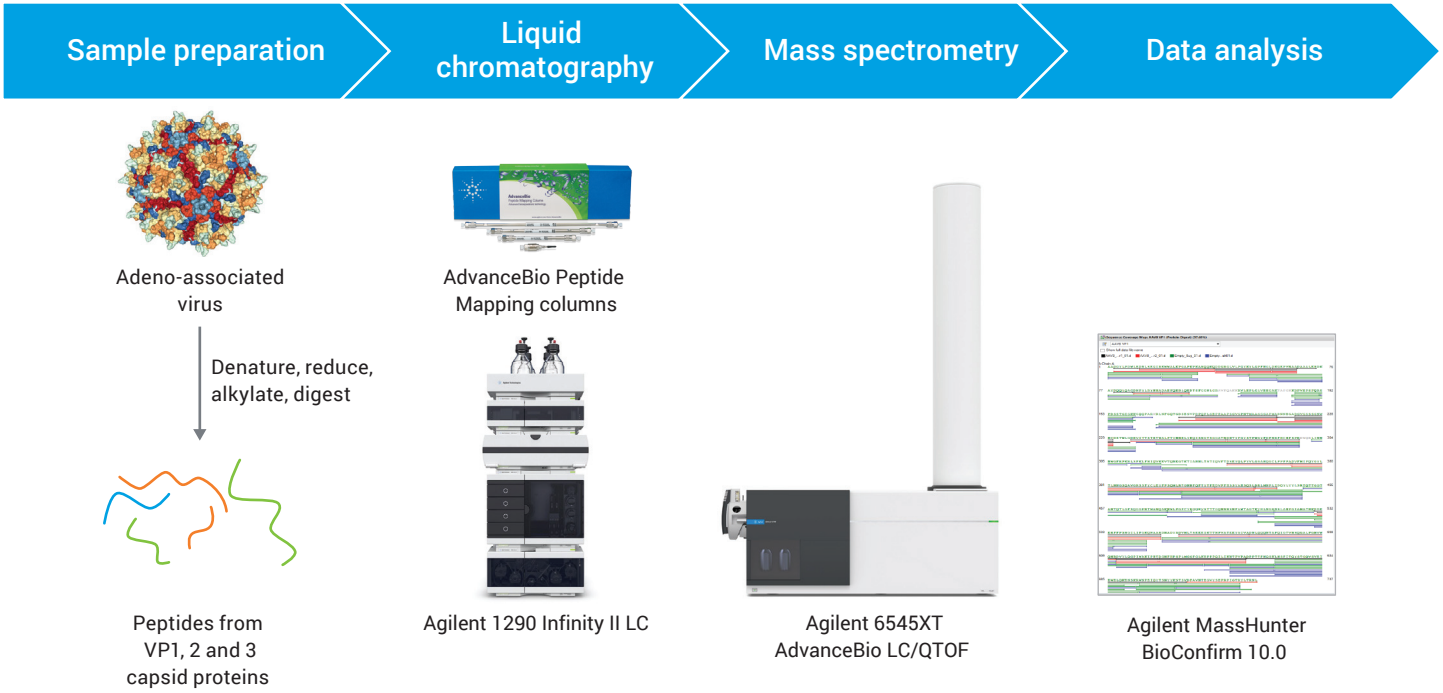

Figure 3. Overview of the process to confirm capsid protein primary structure and identify post-translational modifications.

Column selection criteria – Peptide mapping

Like intact capsid protein analysis, knowledge of the sample and the detection method should guide column selection. The same sensitivity and resolution challenges apply for peptide-mapping separations of digested AAVs. In this case, where the capsid is comprised of three highly related component proteins, the resulting peptide mapping separation is even more complicated than your typical recombinant protein digestion.

- **Column diameter:** A 2.1 mm column is recommended over larger id columns both for sensitivity and compatibility with MS detection. The flow rate used with 2.1 mm columns is conducive to efficient electrospray ionization, which also helps sensitivity.
- **Column length:** For reversed phase columns, longer columns can lead to higher resolution. A 150 mm length column or longer is recommended.
- **Particle size and type:** Smaller particles generally lead to higher resolution; however, slightly larger superficially porous particles offer nearly the same resolution at far lower backpressure. A 2.7 µm superficially porous particle is recommended.
- **Stationary phase chemistry:** C18 stationary phases are the most used option for peptide mapping, though there are a wide variety of C18 column options available. For peptide mapping, you'll want to choose a column and mobile phase system combination that leads to narrow peaks with low tailing to maximize peak capacity. You will also want to balance retention of small, hydrophilic peptides and reasonable elution of long, hydrophobic peptides.

The AdvanceBio Peptide Mapping column recommended here fits these criteria.

Parameter	Value	
Column	Agilent AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm (p/n 653750-902)	
Instrument	Agilent 1290 Infinity II	
Flow Rate	0.4 mL/min	
Mobile Phase A	0.1% formic acid in water	
Mobile Phase B	0.1% formic acid in acetonitrile	
Gradient	Time (min)	%B
	0-3	3%
	50	35%
	60	97%
	62	97%
	62.5	3%
	65	3%
Post time	5 minutes	
Column Temperature	60 °C	

Table 2. Starting conditions used for peptide mapping. See reference[6] 5994-1980EN for details.

References

1. Backovic, A. et al. Capsid Protein Expression and Adeno-Associated Virus like Particles Assembly in Saccharomyces Cerevisiae. Microb. Cell Fact 2012, 11, 124.
2. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) - Guidance for Industry. US Food and Drug Administration 2020.
3. Kuck, D.; Kern, A.; Kleinschmidt, J. A. Development of AAV SerotypeSpecific ELISAs Using Novel Monoclonal Antibodies. Journal of Virological Methods 2007, 140, 17–24.
4. Agilent 1290 Infinity II Ultra Low Dispersion Kit Technical Note
5. LC/MS of Intact Adeno-Associated Virus (AAV) Capsid Proteins for Product Identity (5994-2434EN)
6. Characterization of Viral Vector Particles Using the Agilent 6545XT AdvanceBio LC/Q-TOF (5994-1980EN)

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 # header links. 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 through your regular sales and distributor channels.

MyList 1: Agilent ZORBAX RRHD 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

MyList 2: 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 5 mm, 2.7 µm, 3/pk	851725-911

MyList 3: Standards

Description	Part No.
Agilent NIST mAb, 25 µL	5191-5744
Agilent NIST mAb, 4 x 25 µL	5191-5745
Ten peptide standard, 71 µg, lyophilized	5190-0583
HSA peptide standard	G2455-85001

MyList 4: Sample Preparation

Description	Part No.
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 5: Supplies & Solvents

Description	Part No.
Connections & Tubing	
Agilent InfinityLab Quick Connect Fitting assembly with prefixed 0.12 x 105 mm capillary (for connection on column inlet)	5067-5957
Agilent InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Quick Turn Capillary SST 0.12 x 280 (for Quick Turn fitting)	5500-1191
Mounting tool for quick turn fittings	5043-0915
Inline pressure relief valve kit	
(For use when another detector is used in series after the fluorescence flow cell)	G4212-68001
Ultralow dispersion tubing kit for Agilent 1290 Infinity II	5067-5963
Ultralow dispersion tubing kit for Agilent 1290 Infinity II Bio	5004-0007
Sample Containment	
High recovery vial, screw top, with fixed insert, clear, 300 µL insert volume, 100/pk. Vial size: 12 x 32 mm (12 mm cap)	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 96-well plate, 0.5 mL, 30/pk	5043-9310
InfinityLab 96-well plate closing mat, 50/pk	5042-1389

Description	Part No.
Solvents & Additives	
InfinityLab Ultrapure LC/MS Water, 1 L	5191-4498
InfinityLab Ultrapure LC/MS Acetonitrile, 1 L	5191-4496
Formic acid, 5 mL	G2453-85060
Solvent Filtration	
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	5043-1221
InfinityLab charcoal filter with time strip, 58 g	5043-1193

**Polypropylene vials are chemically resistant and ideal for pH-sensitive samples.*

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info_agilent@agilent.com

Asia Pacific
inquiry_lsca@agilent.com

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