

# AAV8 Reference Standards: revolutionizing empty/full capsid analysis

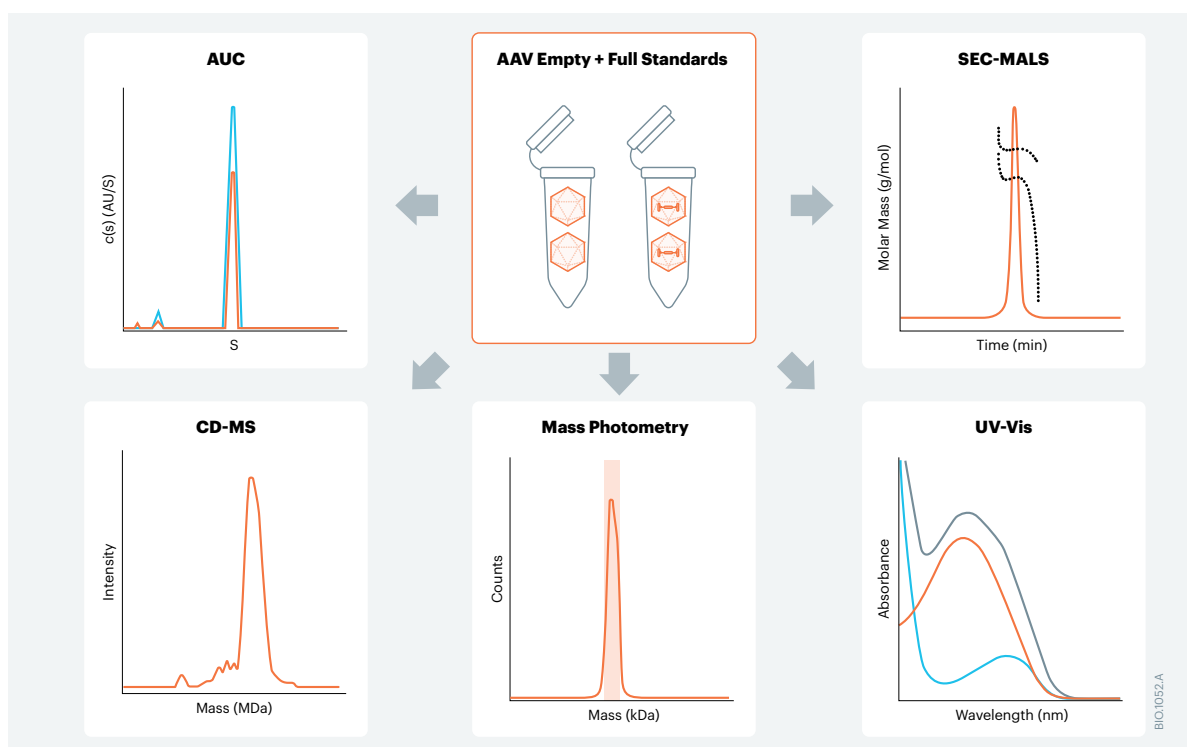


## Introduction

The measurement of the percentage of full capsids is a critical quality attribute for all AAV-based therapeutics. It is essential for manufacturers to maximize the amount of full capsids and limit the number of empty and partial capsids during AAV production. Empty and partial capsids do not only lack efficacy, they also pose safety risks to patients. Although the empty/full capsid analysis has been one of the most well-studied AAV analytical methodologies, the variety of techniques used for analysis, as well as a lack of quality AAV reference standards and method harmonization, have made data comparisons difficult between laboratories and organizations. To help alleviate some of these burdens, USP has developed two AAV Reference Standards, AAV8 (Empty

Capsids, [catalog # 1000301](#)) and AAV8 (Full Capsids, [catalog # 1000302](#)), designed for the assessment of empty/full analysis. These standards have been demonstrated to be of high quality and extremely well characterized, making them suitable for AAV empty/full analysis.

The USP AAV8 Reference Standards were made with a transiently transfected HEK293T system. To limit the percentage of DNA present in the empty standards, the AAV8 (Empty Capsids) was produced in a specialized production that contains no genetic information/plasmid for the transgene. The AAV8 (Full Capsids) were produced with a plasmid encoding a 2.8 kb transgene. The USP Empty and Full Capsids Reference Standards were designed to



**Figure 1.** Analytical methods used to assess the percentages of empty and full capsids.

ensure usability with the most commonly used analytical methods for empty/full analysis of AAV. To do this, USP designed the standards at a high concentration ( $>3.0E+12$  cp/mL) and volume (300  $\mu$ L), so that these standards can be used for most empty/full method, including analytical ultra centrifugation (AUC) which requires a high number of AAV particles. Many commercially available AAV materials lack sufficient volume and/or concentration, which limits their ability to be used in certain methods. The high quality level of the USP AAV Reference Standards was achieved through careful manufacturing to ensure the standards would be suitable for a wide range of analytical methods.

To thoroughly characterize the USP AAV Reference Standards, for accurate and precise measurements, a multi-lab collaborative study was conducted. The study was conducted in five separate labs and included the following empty/full analytical methods: AUC, size-exclusion chromatography with multi-angle light scattering (SEC-MALS), charged detection mass spectroscopy (CD-MS), mass photometry, and uv-spectroscopy. The AAV samples were also analyzed by other biophysical methods, including Peptide Mapping, Next Generation Sequencing (NGS), Digital PCR (dPCR), and Capillary Gel Electrophoresis (CGE), to further characterize these particles.

## Methods

The following methods were used to analyze AAV8 (Empty Capsids) and AAV8 (Full Capsids). Additional information can be found in the corresponding product information sheets.

### SEC-MALS

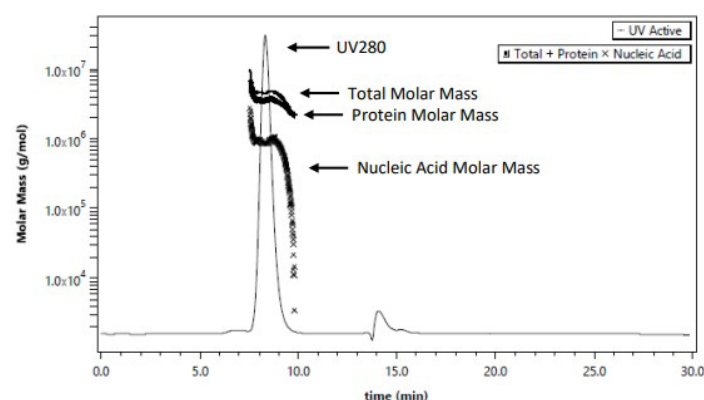
SEC-MALS is a technique that determines the molecular weight and size of molecules in solutions. SEC is first used to separate molecules based on size, and MALS uses light scattering methods to determine the molecular weight. In AAV, these measurements can be used to determine the percentage of full AAV capsids.

An Agilent 1260 Infinity II Bio-inert liquid chromatography system with a DAD detector, Wyatt DAWN MALS/LS detector, and Wyatt Optilab Differential refractive index detector were used. An isocratic gradient was used with the following mobile phase components: 15 mM sodium phosphate, 350 mM sodium chloride, 0.001% Poloxamer 188, pH 7.4. Separation was conducted with a Wyatt silica SEC Protein column (500Å, 5  $\mu$ m, 4.6 mm x 300 mm) at a flow rate of 0.3 mL/min (Figure 2).

SEC-MALS was also used to determine the extinction coefficients for both AAV8 (Empty Capsids) and AAV8 (Full

**Table 1.** Extinction coefficients (L/(g x cm)) determined by SEC-MALS

Sample	$\epsilon_{260}$ (Protein)	$\epsilon_{280}$ (Protein)	$\epsilon_{260}$ (Nucleic Acid)	$\epsilon_{280}$ (Nucleic Acid)
AAV8 (Empty Capsids)	1.24	1.90	N/A	N/A
AAV8 (Full Capsids)	1.24	1.90	24.45	14.05



**Figure 2.** Typical SEC-MALS chromatogram for AAV8 (Full Capsids).

Capsids). This was completed by simultaneously collecting refractive index and UV absorbance data (260 and 280 nm). The extinction coefficients can be seen in Table 1.

### Mass Photometry

Mass photometry is a technique used to determine the molecular weight of biomolecules in solution. This is a single-molecule analysis method that analyzes molecules in their native state by measuring light scattering of single particles. The signal intensity is used to provide mass distribution and relative concentrations of different species within a sample. The mass differences between empty capsids, partial capsids, and full capsids makes mass photometry an ideal method for these measurements (Figure 3).

A SamuxMP mass photometer was used. PBS (phosphate-buffered saline) was used as buffer and MassFference P2 or Empty AAV Capsids were used as calibrants. The samples were diluted to produce 1000 – 3000 counts in the appropriate region.

**CDMS**

CDMS measures the mass-to-charge ratio (m/z) of ions, allowing for the determination of the molecular weight of large molecules, including AAV. Particles are ionized using electrospray ionization and subsequently measured in a mass spectrometer. This is also a single-particle technique, which differs from traditional mass spectrometry. This technique is particularly useful for measuring large heterogenous solutions, such as an AAV sample, which inherently has different species present.

Between 5 – 10 µL were injected into the CDMS instrument with a spray voltage of 2.0 kV. The samples were buffer exchanged to 200 mM ammonium acetate with 0.01% Poloxamer 188.

**SV-AUC**

Sedimentation velocity AUC is a technique that is commonly used to determine the amount of empty, full, and partially filled AAV capsids. In this technique, particles are separated based on their mass and density, which allows for separation and quantification of different AAV species. A specialized centrifuge is required to accommodate the required high speeds used to separate the samples. An in-house AUC method was used for analysis.

**UV Spectroscopy**

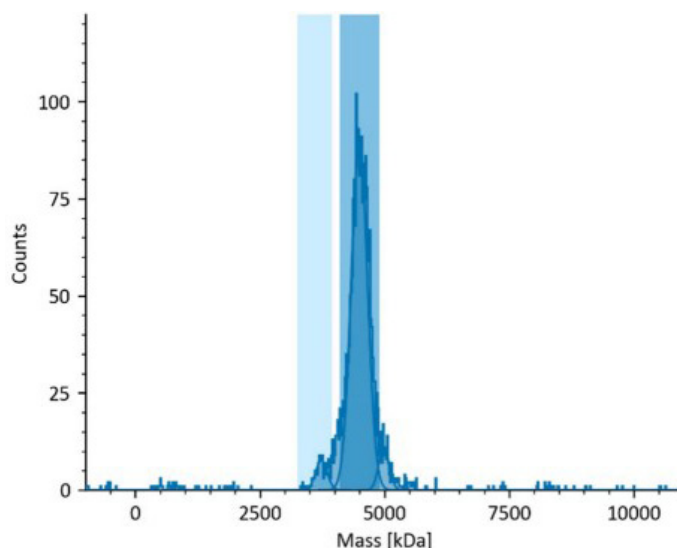
UV spectroscopy is a technique that measures the amount of light absorbed by a sample. By measuring the absorbance, Beer’s Law can then be used to determine the concentration of given samples. In the case of AAV, the empty/full ratio is determined using the A260/A280 ratio. These two wavelengths are used because protein (capsids) have a maximum absorbance at 280 nm, while DNA (transgene) has

a maximum absorbance at 260 nm. A higher A260/A280 ratio indicated a higher percentage of full capsids.

UV absorbance was measured using variable pathlength extension (VPE) spectroscopy. The following method parameters were used: slope mode = quick; target absorbance = 1; data points = 10; search pathlengths = 0.005, 0.0025, 0.05; average time (s) = 0.5; baseline correction = off; scatter correction (nm) = 320, 350.

**Results**

For both AAV8 (Empty Capsids) and AAV8 (Full Capsids), the percentage of empty and full capsids was determined. Data from these measurements can be seen in **Table 2**. Observed differences can be attributed to the sensitivity and



**Figure 3.** Typical chromatogram for AAV8 (Full Capsids).

**Table 2.** Percentages of empty and full capsids in AAV8 reference standards.

Sample	SEC-MALS	Mass Photometry	CDMS	AUC	UV (A260/A280)
<b>Percentage of Empty Particles</b>					
AAV8 (Empty Capsids)	98	95	99	91	100
AAV8 (Full Capsids)	2	5.6	4.3	3.0	5
<b>Percentage of Full Particles</b>					
AAV8 (Empty Capsids)	2	0.5	0.5	1.3	0
AAV8 (Full Capsids)	98	91	80	91	95

**Table 3.** Percentages of partially-filled capsids in AAV8 reference standards.

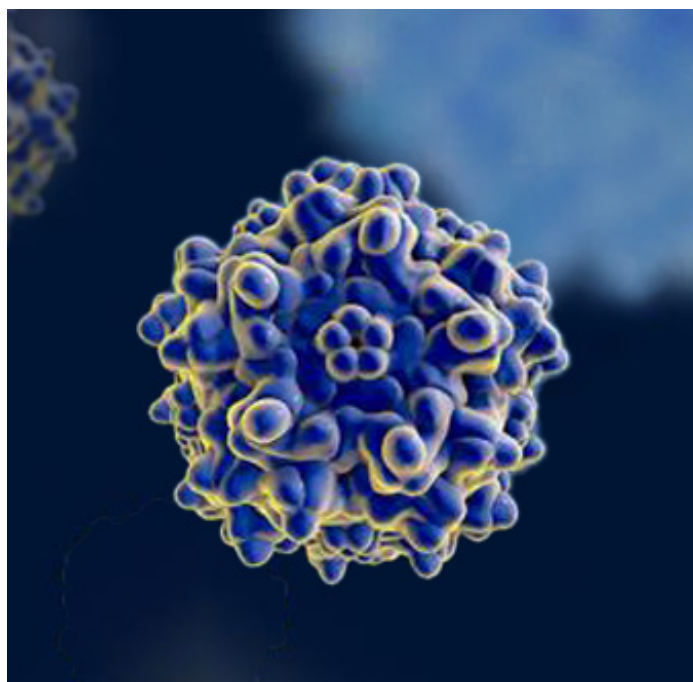
Sample	Mass Photometry	CDMS	AUC
AAV8 (Empty Capsids)	1.4%	1.0%	2.9%
AAV8 (Full Capsids)	3.2%	15.7%	6.4%

specificity of the various analytical methods. Furthermore, these differences further highlight the need for AAV standards, to be analyzed using a variety of approaches. AAV Reference Standards can be used to minimize the variability and promote standardization in analysis using various approaches.

In addition to measuring the percentage of empty and full capsids, some advanced techniques (AUC, mass photometry, and CDMS) are also capable of measuring the amount of intermediate or partially-filled capsids. Partially-filled capsids, like empty capsids, are a form of impurity that can be a cause for safety concerns and reduce efficacy of AAV. Because of the inherent heterogeneity of AAV samples, determining the amount of partially-filled capsids provides important information about the AAV. The amount of partially-filled AAV is shown in **Table 3**.

## Conclusions

The USP AAV8 Empty/Full Reference Standards provide testing laboratories and manufacturers with well-characterized standards that are not available anywhere else. With more than 10 analytical methods currently being used to quantify the percentage of full capsids, these Reference Standards provide users flexibility to use reliable standards with a wide variety of methods. Although alignment between methods can be difficult, a more complete understanding of the particles can be accomplished by using different methods, as each uses unique scientific techniques for analysis. Having a standard that has been characterized by multiple techniques will be advantageous to all labs, as it will either allow for a direct comparison to their in-house method or allow comparison to multiple orthogonal methods. These



standards can also help foster the development of new equipment and methods, by having a reliable standard to use for assessing accuracy of measurements.

In addition to the AAV8 empty/full standards, USP has several other AAV standards and continues to develop new standards to aid AAV manufacturers. Other AAV products currently available in the USP store include:

- Plasmid for Residual DNA Quantification ([Catalog # 1544900](#))
- Quantitative AAV Titration PCR Control ([Catalog # 1012106](#))
- Quantitative HEK-293 Genomic DNA ([Catalog # 1592106](#))
- Quantitative Sf9 Genomic DNA ([Catalog # 1592170](#)).

To keep up with all the latest information, follow USP AAV website at [www.usp.org/biologics/AAV](http://www.usp.org/biologics/AAV).



**More information:** [www.usp.org/biologics/AAV](http://www.usp.org/biologics/AAV)

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