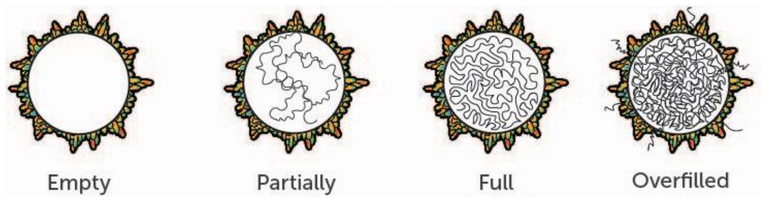
# Comprehensive Size Distribution Analysis of Adeno-Associated Virus Fill-States

Nikki Machalek, Amber Raines & N. Karl Maluf; KBI Biopharma, Louisville, CO USA

## Introduction

Recombinant adeno-associated viruses (AAV) are used extensively as a vector for gene therapy.




Characterization and quantification of viral fill states is necessary to ensure safety and efficacy of gene therapy treatments.

Recently, Maruno et al. (1) published a comprehensive analysis using sedimentation velocity analytical ultracentrifugation (SV-AUC) to characterize AAV size distributions using multiple detection wavelengths. Not only does it allow for adequate separation of the species through ultracentrifugation, this method also enables identification of each viral fill state for well resolved peaks present using multi-wavelength analysis (MWA). In this poster, we compare the results from SV-AUC with MWA with two orthogonal separation methods: sedimentation equilibrium (SE) CsCl density gradient analysis and size exclusion chromatography coupled with multiple angle light scattering (SEC-MALS).

## Methods

Commercial AAV serotype 5 was purchased from Virovek with an expected genome size of ~2.5 kb and a stock concentration of ~2x$10^{13}$ vg/mL. Its neat absorbance was determined using a Nanodrop OneC and was further diluted to the optimal absorbance for each corresponding technique.

For SV-AUC and CsCl density gradient, the experiments were conducted using a Beckman Optima Analytical Ultracentrifuge. Scans were simultaneously collected using five absorbance wavelengths: 230, 255, 260, 270, and 280 nm to carry out the multi-wavelength analysis (MWA).


MWA relies on the fact that the proteinaceous capsids and the ssDNA genome have differing extinction coefficients over a wide range of wavelengths, λ. Consider a solution containing an AAV species. The total absorbance of a species, $A_T$, at a particular λ, is given by Beer's law:

$$A_T = \varepsilon_D D_T l + \varepsilon_C C_T l \qquad [1]$$

where $\varepsilon_D$ and $\varepsilon_C$ are the extinction coefficients for DNA and capsids; $D_T$ and $C_T$ are the total concentrations of DNA and capsids; and *l* is the pathlength. Equation [1] can be linearized as follows:

$$\frac{A_T}{\varepsilon_D l} = D_T + \frac{\varepsilon_C}{\varepsilon_D} C_T \qquad [2]$$

Thus, a plot of $A_T/(\varepsilon_D\, l)$ vs. $\varepsilon_C/\varepsilon_D$ will yield a straight line with a slope of $C_T$ and an intercept of $D_T$. Further, the DNA mass fraction can be calculated from the measured slope and intercept as $D_T/(D_T + C_T)$.

For SEC-MALS, the experiments were conducted using an Agilent 1260 HPLC with in-line UV, Wyatt DAWN HELEOS MALS, and RI detectors in order to perform a protein conjugate analysis.

Protein conjugate analysis, available in the ASTRA data analysis software and described in TN1006 (2), relies on the combination of the three signals (UV, LS, and RI) and the fact that the proteinaceous capsid and ssDNA genome have differing refractive index increments (dn/dc) and extinction coefficients. The mass, and therefore mass fraction, of each component for the overall solution of intact capsids can be calculated due to the conservation of mass principle.


## Size Distribution Results & Conclusions

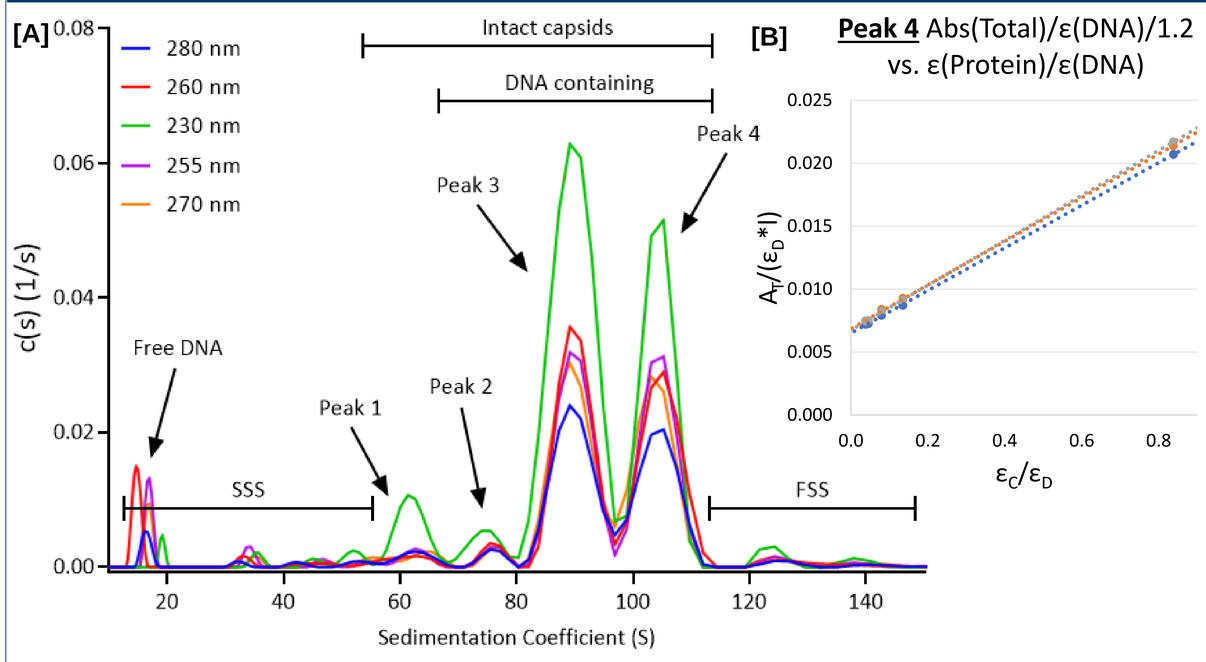



**Figure 1** **[A]** shows overlaid c(s) size distributions for a single replicate analyzed at five wavelengths using SV-AUC. A small fraction of empty capsids (Peak 1) along with three additional resolved species (Peaks 2-4) are present. While it is tempting to conclude that Peak 4 corresponds to the full AAV product (a single packaged 2.5 kb genome), analysis of the DNA mass fraction of these data suggests a different interpretation. **[B]** Shows the data from each replicate (n=3) for Peak 4 fitted to a linear function. From the slope and intercept, the average DNA mass fraction for Peak 4 was calculated as 27.9% while for Peak 3 it was calculated as 16.9%. Note that the expected mass fraction for an AAV with a 2.5 kb genome is 17.5%. This indicates that Peak 3 represents the full virus while Peak 4 represents an over-packaged virus. Small amounts of slower sedimenting species (SSS) including fragments and free-floating DNA, and faster sedimenting species (FSS) such as viral aggregates are also present.

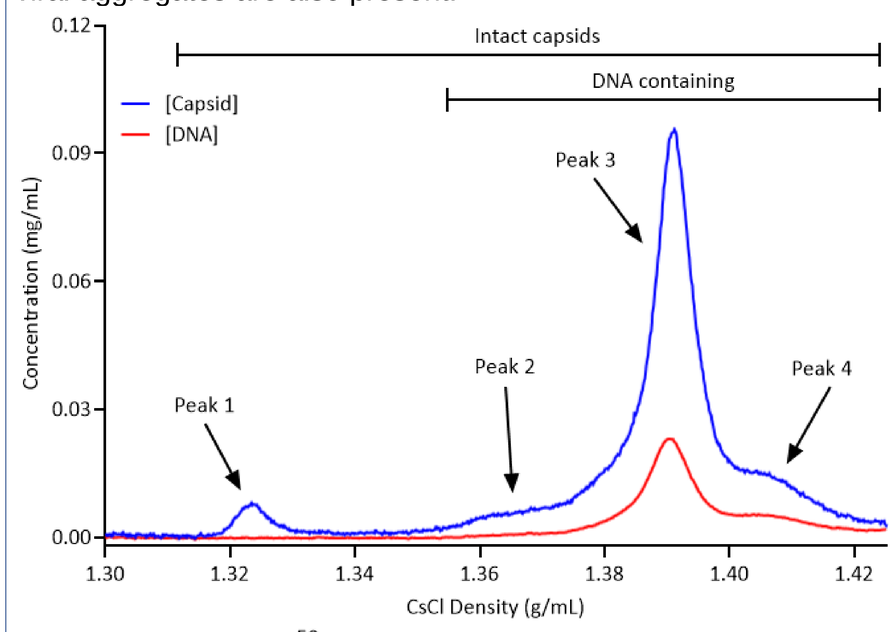



**Figure 2** shows the capsid concentration and DNA concentration vs. the determined CsCl density for a single replicate analyzed using MWA. Note that there are at least four species present with differing densities, which was also observed in the SV-AUC results; however, the resolution for the DNA containing species is substantially decreased.

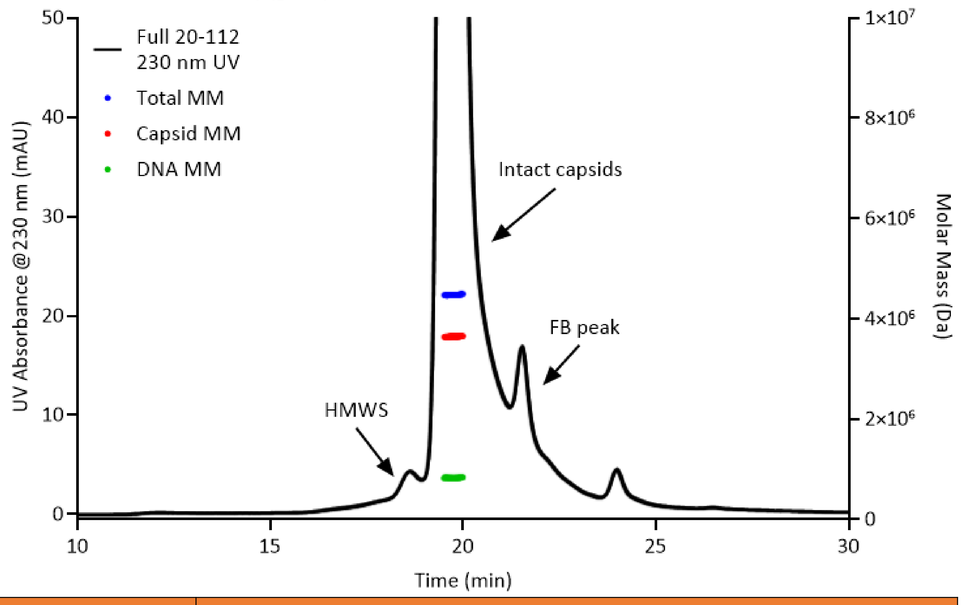



**Figure 3** shows the 230 nm UV chromatogram and molar mass estimates for the capsid and DNA components determined using the protein conjugate analysis for a single replicate tested by SEC-MALS. Note that all intact capsids elute in one peak.

| Technique | Method | Mass fraction of DNA (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Intact capsids | DNA containing | Peak 4 | Peak 3 | Peak 2 | Peak 1 |
| SV-AUC | MWA | 19.8 | 20.8 | 27.9 | 16.9 | 14.6 | 6.4 |
| CsCl density gradient | MWA | 20.0 | 20.8 | 28.3 | 20.1 | 13.3 | 1.4 |
| | Density | N/A | N/A | 22.1 | 16.9 | 10.6 | 0.0 |
| SEC-MALS | Protein conjugate | 18.5 | N/A | N/A | N/A | N/A | N/A |

**Table 1** provides the calculated DNA mass fractions for all capsid species using each method. Note that SEC-MALS cannot resolve individual peak DNA mass fractions, since different fill states are not physically separated. Good agreement is observed across methods. Peak 1 is presumed to represent empty capsids, while Peak 2 represents partially filled capsids, Peak 3 represents the full virus, and Peak 4 represents over-packaged capsids.

**1. Maruno T., Usami K., Ishii K., Torisu T., and Uchiyama S. (2021) Comprehensive Size Distribution and Composition Analysis of Adeno-Associated Virus Vector by Multiwavelength Sedimentation Velocity Analytical Ultracentrifugation. Journal of Pharmaceutical Sciences, 110:3375-3384.**

**2. Wyatt Technology Corporation. Performing a Protein Conjugate Analysis in ASTRA (2018). Technical Note TN1006 Rev D.**