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Advancing AAV Analytics: Post-DGUS Profiling as a Robust Tool for AAV Capsid Characterization

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Introduction

Density gradient ultracentrifugation (DGUC) is a well-established method for separating empty (E) and full (F) AAV capsids based on density differences. However, traditional DGUC is labour-intensive and lacks direct detection capabilities, requiring manual fraction collection and subsequent analysis. These limitations can be overcome by integrating post-DGUC profiling with the PATfix® chromatography system. Compared to analytical ultracentrifugation (AUC), this approach offers a cost-effective and accessible alternative, eliminating the need for an expensive analytical ultracentrifuge while leveraging standard laboratory equipment. Additionally, it simplifies analysis by bypassing the complex data deconvolution and sedimentation velocity calculations required in AUC, instead relying on a stratigraphic readout from the centrifuge tube while maintaining high accuracy.

1. Experimental Approach

Isopycnic ultracentrifugation in self-forming CsCl gradient was performed to separate E and F AAV2/8 capsids. 10 mL of 100 mM TRIS, 3 M CsCl, 0.1 % Poloxamer 188 pH 7.40 was pipetted into the 11.5 mL soft-walled PA ultracentrifuge tube. The corresponding amount of AAV2/8 sample was added and finally, the ultracentrifuge tube was topped with 100 mM TRIS pH 7.5 and mass-balanced with the rest of the tubes to 0.01 g precision. Ultracentrifugation was performed in a fixed angle rotor at 53.500 rpm (average rcf 170000 G) for 23 h at 20 °C in a Sorvall™ WX90+ ultracentrifuge.

After centrifugation, the ultracentrifuge tubes were transferred to the ultracentrifuge tube holder and analysed using the PATfix UC biochromatography system (Sartorius BIA Separations). UV absorbance was monitored at 230 nm, 260 nm, and 280 nm using a flow cell with 50 mm flow path. Tryptophane Intrinsic fluorescence (FL) was monitored at an excitation wavelength of 280 nm and an emission wavelength of 348 nm with a fluorescence detector. Light scattering (LS) was observed at a 90° angle with a MALS 3609 multi-angle light scattering detector. Conductivity is measured as a surrogate indicator of CsCl density. PATfix software was used for instrument control, acquisition, and analysis. All post-DGUC sample profiling experiments were performed at room temperature.

2. Results

AAV2/8 post-DGUC profile - centrifugram

Post-DGUC profiling was performed to separate and characterize AAV subpopulations based on density, followed by multi-detector analysis to quantify E and F capsids. The centrifugram developed with a 5.3E+12 VP load (Figure 1) revealed F capsids as the highest-density subpopulation, eluting at higher CsCl concentrations (higher conductivity), while E capsids eluted at lower densities. The UV absorbance 260/280 ratio (Figure 1b) of 1.33 at 2.8 min confirmed the presence of F capsids, while a ratio of 0.63 for a peak at 5.1 min confirmed E capsids. An additional peak at 2.0 min displayed a 260/280 ratio of 1.33, indicating another population of full capsids with slightly higher intrinsic density. This population, not yet fully characterized, is labelled as high-density (H) AAV.

A comparison of detector responses during post-DGUC profiling highlights that the E/F AAV ratio strongly depends on the detection mode due to the distinct physical and optical properties of E and F AAV capsids. The discrepancy exposes a hidden distortion in UV-based measurements of relative peak size. Specifically, the larger extinction coefficient of DNA compared to capsid proteins inflates the full capsid peak area in UV detection. Light scattering provides a more conservative estimate of full capsid content than UV; however, the greater mass of full capsids can still lead to peak area inflation. In contrast, intrinsic fluorescence offers the most objective representation of relative peak areas, as it remains unaffected by extinction coefficients, capsid mass, or refractive index. This allows for a comparison based solely on protein capsid quantity, regardless of genome content. The FL detector-based results (Figure 1c) from post-DGUC profiling estimated 19.6% F and 80.4% E AAV capsids.

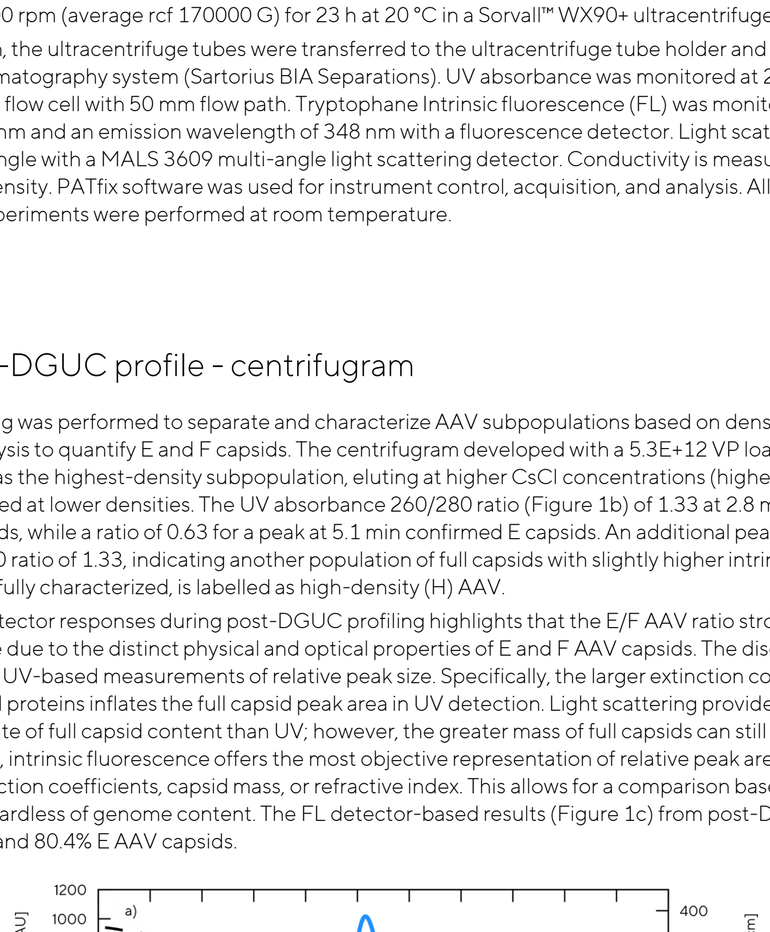

Figure 1: A representative centrifugram of partially purified AAV2/8 virus. Solid lines represent the sample response, while dashed lines indicate the baseline. The dashed black line represents the conductivity of the CsCl solution, serving as a surrogate marker of CsCl density. (a) UV absorbance at 230 nm, (b) UV absorbance at 260 nm (red) and 280 nm (blue), (c) Intrinsic tryptophan fluorescence, and (d) Light scattering at a 90° angle. The increased light scattering signal observed from 7.5 minutes onward is attributed to micelles of Poloxamer 188, which do not sediment in the CsCl density gradient.

References

- Peljhan S, Štokelj M, Prebil SD, Gagnon P, Štrancar A. Multiple-parameter profiling of density gradient ultracentrifugation for characterization of empty and full capsid distribution in AAV preparations. Cell Gene Therapy Insights. 2021;7(2).

Suitability of post-DGUC profiling as an analytical tool

The effective range for E/F ratio evaluation was tested using AAV total VP loads from 1.1E+11 VP to 1.1E+13 VP. Peak areas for F and E AAV capsid peaks were extracted from centrifugrams and plotted as calibration curves for the observed detector signals (Figure 2). Linear regression data for UV 230, 260, and 280 nm, as well as FL and LS signals, are summarized in Table 1, along with the corresponding LOD and LOQ values. These thresholds are also indicated as vertical dashed lines in Figure 2. High coefficients of determination (r^2) and low y-intercept values confirm the linearity of the calibration curves, demonstrating the suitability of post-DGUC profiling as a robust tool for AAV quantification.

The comparison of detector responses highlights that E/F AAV ratio readout is highly detection-mode dependent. The molar extinction coefficient for each component can be derived from the calibration curve slope using Beer-Lambert's law, quantifying how strongly each component absorbs light at a specific wavelength. The ratio of calibration curve slopes for F and E AAV capsids (ξ) for a given detector mode enables normalization of detector responses, improving the accuracy of AAV capsid quantification.

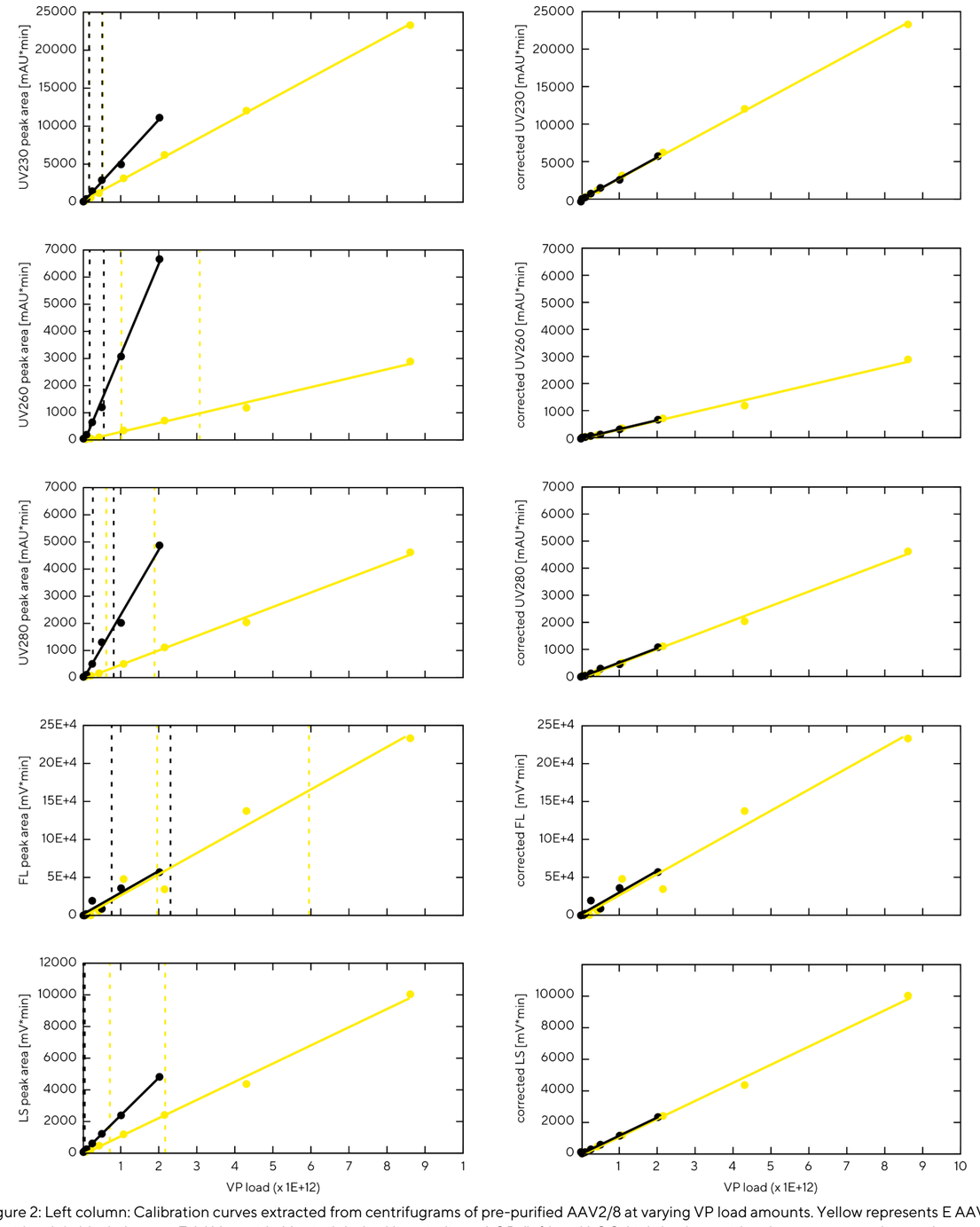

Figure 2: Left column: Calibration curves extracted from centrifugrams of pre-purified AAV2/8 at varying VP load amounts. Yellow represents E AAV capsids, while black denotes F AAV capsids. Vertical dashed lines indicate LOD (left) and LOQ (right) values, with colours corresponding to the respective capsid type. Right column: Calibration curves incorporating the capsid normalization factor, ξ, demonstrating equalized responses for F and E AAV capsids.

Table 2: Summary of calibration curve parameters and detection limits for post-DGUC profiling of AAV2/8. The table includes slope, intercept, and coefficient of determination (r^2) for each detector signal, as well as the limits of detection (LOD) and quantification (LOQ). The capsid normalization factor (ξ) is also provided to account for detection-mode-specific biases in E/F AAV ratio estimation.

Signal		k	n	r^2	LOD	LOQ	ξ
UV 230	F	5,48E-09	-39,08	0,9993	1,04E+11	3,14E+11	1.94
	E	2,83E-09	75,92	0,9999	1,76E+11	5,34E+11	
UV 260	F	3,32E-09	-104,11	0,9988	1,37E+11	4,14E+11	10.1
	E	3,30E-10	-37,86	0,9959	1,02E+12	3,08E+12	
UV 280	F	2,44E-09	-64,52	0,9990	1,25E+11	3,80E+11	4.52
	E	5,40E-10	-52,11	0,9998	2,00E+11	6,05E+11	
FLD	F	2,93E-08	-190,76	0,9820	5,07E+11	1,54E+12	1.05
	E	2,80E-08	-2723,19	0,9870	1,83E+12	5,55E+12	
LS	F	2,37E-09	16,45	1,0000	1,84E+10	5,56E+10	2.06
	E	1,15E-09	-85,85	0,9980	7,15E+11	2,17E+12	

3. Conclusions

Baseline separation of capsid species was achieved in a CsCl density gradient, generating a centrifugram that provides insights beyond those obtained by traditional DGUC or anion exchange chromatography (AEX). Multimodal detection, including UV absorbance, light scattering, and fluorescence, enables precise determination of the empty-to-full (E/F) capsid ratio, capsid integrity, and particle concentration. Due to their nucleic acid content and molecular mass, full AAV capsids exhibit stronger UV absorbance and light scattering, leading to an overestimation of the F AAV fraction. In contrast, fluorescence detection remains independent of capsid content, making it a more balanced approach. However, discrepancies between detection modes necessitate the use of correction factors. To mitigate these biases, a calibration-derived correction factor was introduced, significantly improving E/F ratio accuracy. This optimized approach enhances the specificity and sensitivity of AAV quantification, ensuring robust and reproducible results across a wide concentration range.