A Single Platform for icIEF and CE-SDS Analysis of Adeno-Associated Virus (AAV) for Gene Therapy

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INTRODUCTION

Adeno-associated viruses (AAV) are promising vectors for the delivery of genetic material in gene therapy. During the manufacture of AAV, critical quality attributes like charge heterogeneity and purity must be carefully monitored because they can impact the product's safety and efficacy. Imaged capillary isoelectric focusing (icIEF) and capillary electrophoresis sodium dodecyl sulfate (CE-SDS) are two powerful methods to respectively characterize charge heterogeneity and purity, but traditionally two separate platforms are required to run these methods. Here, we used a single platform to develop icIEF and CE-SDS methods to analyze AAV2 and AAV6 serotypes to monitor product stability, identity and purity. We show that these methods could reproducibly quantify both intact (by icIEF) and denatured AAV (by icIEF and CE-SDS) samples. The CE-SDS method could separate and quantify individual AAV capsid proteins, showed robust repeatability (<5% RSD) while also detecting impurities. The icIEF method was useful for measuring both denatured and intact particles with high repeatability (<4% RSD). Interestingly, preliminary evidence suggests that icIEF can also distinguish between full (1 x 10^{13} GC/mL) and 'empty' (<10^{12} GC/mL) AAV capsids.

METHODS

Both CE-SDS and icIEF were performed on Maurice (**Figure 1**) using the respective cartridges and reagents. All data were analyzed with Compass for iCE software.

CE-SDS method

Maurice CE-SDS PLUS Application Kit (PS-MAK03-S).

Samples in Figures 2-4 were concentrated, and buffer exchanged prior to analysis. In all samples, 20% BioRad SDS solution was added to Maurice CE-SDS PLUS Sample Buffer to a final concentration of 4% SDS. Samples were denatured in the presence of TCEP at 70 °C for 10 minutes, cooled to RT for 5 minutes, and mixed by vortex. Samples were injected for 15 seconds at 4600 V and separated for 30 minutes at 5750 V.

Figure 1. Maurice performs both icIEF and CE-SDS analysis.

icIEF methods - Maurice cIEF Method Development Kit (PS-MDK01-C)

Intact AAVs

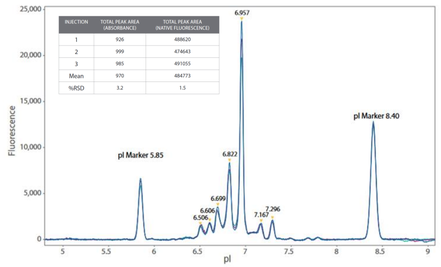
Intact AAV samples contained 50% SimpleSol, 0.35% methylcellulose, 4% 3-10 Pharmalyte, and Maurice pI markers 5.85 and 9.46. Samples were pre-focused for 1 minute at 1500 V then focused at 3000 V for 7 minutes.

Denatured AAVs

Samples were denatured by heating in the presence of 33% DMSO and 16.5 mM DTT for 10 minutes at 70 °C, then cooled to room temperature. Samples were diluted 5-fold, with the final prepared sample containing 0.35% methylcellulose, 2% Pharmalyte 5-8 and 2% Pharmalyte 3-10, 40% formamide, and Maurice pI standards 5.85 and 8.40. Samples were separated using a Maurice cIEF cartridge (PS-MC02-C) for 1 minute at 1500 V followed by 12 minutes at 3000 V.

icIEF RESULTS

Denatured AAV Analysis – Monitor Charge Heterogeneity of AAV Proteins



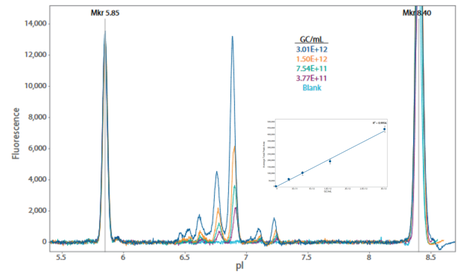
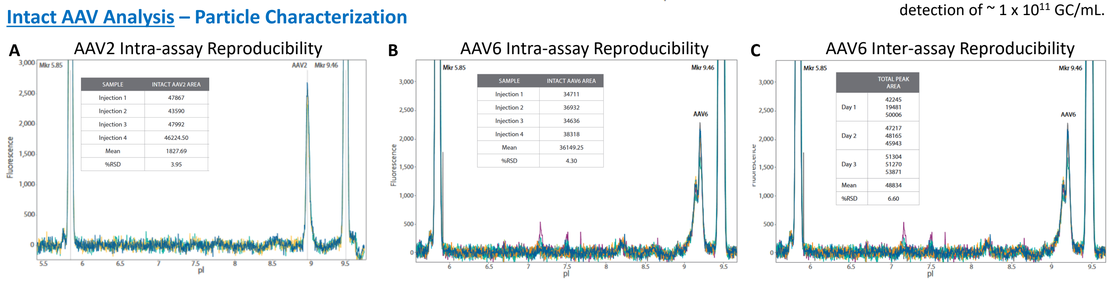


Figure 2. Denatured AAV2 (~1 x 10^{12} GC/mL) was evaluated in triplicate on Maurice using the denatured method (left), revealing 7 distinct peaks between pI 6.5-7.5. Detection of these peaks was repeatable, with RSDs for absorbance (not shown) and native fluorescence under 1.5% (left). Linearity was performed and an R^2 >0.99 was obtained (right), with a limit of detection of ~ 1 x 10^{11} GC/mL.

Intact AAV Analysis – Particle Characterization



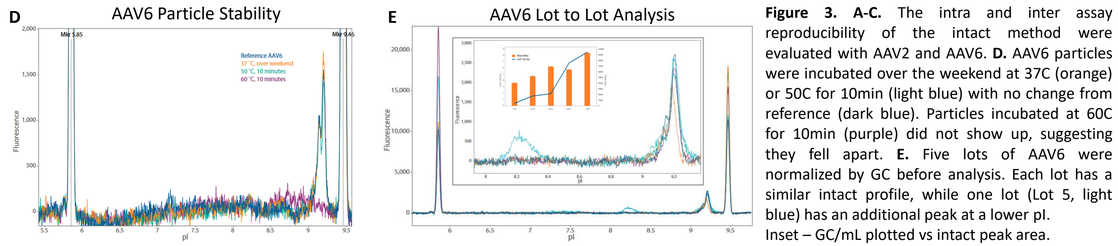


Figure 3. A-C. The intra and inter assay reproducibility of the intact method were evaluated with AAV2 and AAV6. **D.** AAV6 particles were incubated over the weekend at 37C (orange) or 50C for 10min (light blue) with no change from reference (dark blue). Particles incubated at 60C for 10min (purple) did not show up, suggesting they fell apart. **E.** Five lots of AAV6 were normalized by GC before analysis. Each lot has a similar intact profile, while one lot (Lot 5, light blue) has an additional peak at a lower pI.
Inset – GC/mL plotted vs intact peak area.

Empty/Full Analysis

AAVs are packaged with DNA to carry to the host. During the process of making AAVs, empty capsids are formed. Using commercially sourced AAV2 empty (<10^{12} GC/mL) and full (10^{13} GC/mL) particles, we assessed the ability of the intact method to distinguish them (**Figure 4**). Further separation optimization (narrow range ampholytes, e.g.) may help improve the separation of these two populations as the method shown here uses 3-10 ampholytes.

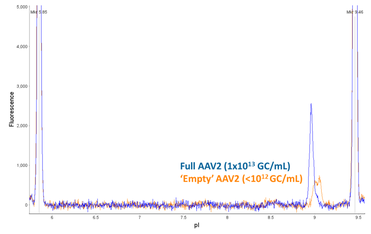


Figure 4. Overlay of intact full and empty AAV2 particles. The full particles contained 1x10^{13} GC/mL, and the 'empty' particles had <10^{12} GC/mL.

CE-SDS RESULTS

CE-SDS Specificity - AAV2 was analyzed on Maurice and compared to a blank sample preparation. The three capsid proteins (VP1, VP2 and VP3) were clearly separated from one another, with a baseline signal between each of them (**Figure 5**).

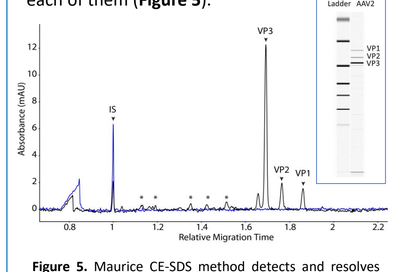


Figure 5. Maurice CE-SDS method detects and resolves AAV capsid proteins. The AAV2 sample is shown in the black trace and the blank sample is shown in the blue trace. The internal standard is labeled as IS and impurities are labeled with an asterisk. Inset = NEW! Lane view for CE-SDS in Compass for iCE software.

CE-SDS Repeatability - The repeatability of 5 replicate injections of AAV2 was determined (**Table 1**). The percent relative standard deviations (%RSDs) of VP3, VP2 and VP1 were 0.5%, 4.0%, and 4.3%, respectively. The average capsid ratios were 7.6:1.3:1 for VP3:VP2:VP1.

	% CORRECTED PEAK AREA			CAPSID PEAK RATIO		
Injection Name	VP3	VP2	VP1	VP3	VP2	VP1
Injection 1	71.6	12.4	8.9	8.0	1.4	1.0
Injection 2	71.0	12.8	9.7	7.3	1.3	1.0
Injection 3	71.3	12.5	9.0	7.9	1.4	1.0
Injection 4	70.8	11.7	9.9	7.2	1.2	1.0
Injection 5	70.6	13.2	9.1	7.8	1.5	1.0
Average	71.1	12.5	9.3	7.6	1.3	1.0
%RSD	0.5	4.0	4.3	4.5	6.9	0.0

Table 1. The AAV2 sample was injected as five replicates and the percent peak area for each capsid protein and their respective ratios were calculated.

CE-SDS Linearity - To test linearity, the sample was diluted from 1.6 x 10^{14} GC/mL to 5.3 x 10^{13} GC/mL (**Figure 6**). An R^2 of 0.9852 for total peak area was obtained.

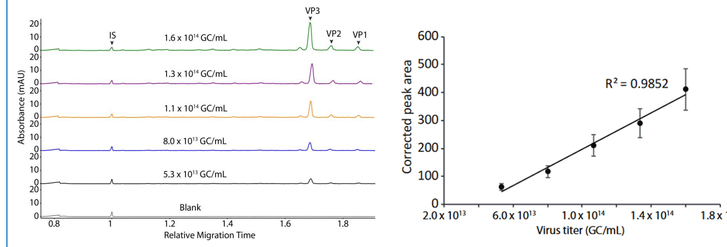


Figure 6. Representative CE-SDS electropherograms from AAV titration. AAV2 was diluted from 1.6 x 10^{14} GC/mL (green trace) to 5.3 x 10^{13} GC/mL (black trace) prior to analysis on Maurice. The bottom (gray) trace is the sample blank.

CONCLUSIONS

- These data show that the CE-SDS method can be used for monitoring AAV purity and identity under denaturing conditions and for quantification of individual viral capsid protein ratios.
- The icIEF method is useful for analysis of both intact and denatured AAV particles, and preliminary evidence suggests it can distinguish between full (1 x 10^{13} GC/mL) and 'empty' (<10^{12} GC/mL) viral particles.

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