Supporting Information

# Microfluidic AAV Purity Characterization: New Insights into Serotype and Sample Treatment Variability

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## Methods for Capsid Protein and ssDNA Characterization

For the characterization of the capsid proteins, two different protein assay protocols, whose key difference was the use of a fluorescent non-covalent dye (Protein Express, Revvity) or a covalent dye (AAV Pico Protein, Revvity), were followed to investigate how different fluorophore types interact with the capsid proteins. The non-covalent dye protocol, as previously described in Coll De Peña et al.,[23] required the mixing of 5 µL of the sample with 7 µL of denaturing reducing buffer with 0.03 M DTT, heated at 100°C for 5 min, and then diluted with 32 µL of nuclease-free water prior to analysis. Since the labeling in this protocol is dynamic and takes place on the chip, the chip is loaded with a gel-dye mixture and a lower marker (Revvity) and analyzed using the Protein Express assay script. In the case of the covalent dye, 4 µL of AAV sample was mixed with 1 µL of 5x Labeling Buffer with 0.17 M DTT (Revvity) and denatured at 75°C for 5 min. The denatures samples were mixed with 5 µL of 20 µM covalent dye (Revvity), shake mixed for 1 min, spun down for 1 min at 1,000 g, and incubated at 35°C for 15 min in the dark. Lastly, 5 µL of the labeled sample were mixed in a 96- or 384-well plate with 5 µL of Stop Solution (Revvity), shake mixed for 1 min, spun down, and transferred to the LabChip platform. The microfluidic chip was loaded with AAV Pico Protein Express gel matrix and lower marker (Revvity) and transferred to the LabChip platform for analysis using the AAV Pico Protein Express assay script.

In contrast, to assess the nucleic acid content of the AAV samples, the capsid was digested via a urea and proteinase K treatment with heat, as described in our previous study,[23] or only heat to extract the NA. In the case of the urea and proteinase K treatment, 10 µL of proteinase K was diluted with 90 µL of 2M urea, and 5 µL of that mixture was added to 5 µL of AAV sample. The samples were heated at 55°C for 60 min, following the deactivation of proteinase K at 95°C for 20 min. The samples are then transferred onto a 384-well plate and are ready for analysis. For the heat treatment samples, 5-10 µL of AAV are heated at 94°C for 10 min, and upon transfer to a 384-well plate, the samples are ready for analysis. For both sample treatment protocols, the microfluidic chip is loaded with AAV DNA gel-dye and a lower marker (Revvity) and analyzed using the AAV DNA assay script.

## Figure S1. Comparison of VP Properties Across the Two Methods

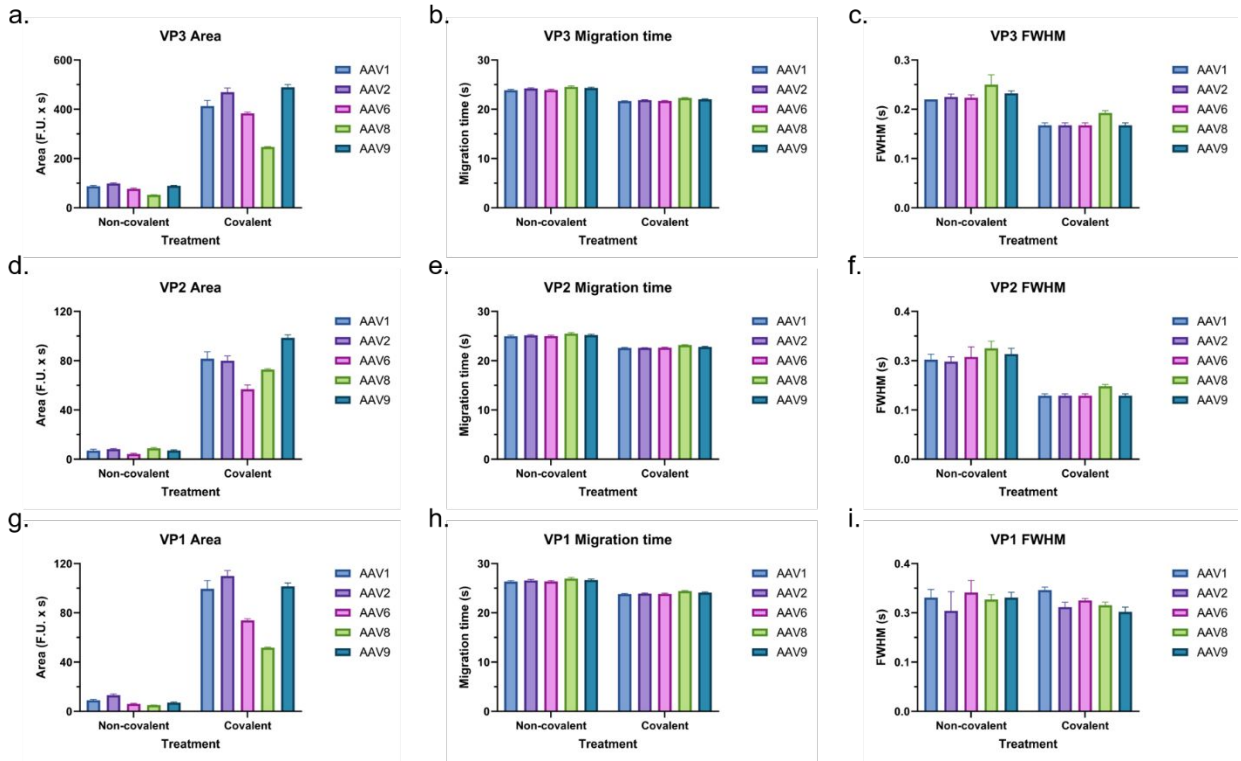


**Figure S1:** Comparison of VP properties across the two methods. (a) Area, (b) migration time, and (c) FWHM of the VP3 peak. (d) Area, (e) migration time, and (f) FWHM of the VP2 peak. (g) Area, (h) migration time, and (i) FWHM of the VP1 peak. Here, we highlight some of the key electrophoresis outcomes of the capsid VPs that we can assess with the proposed method and how they compare between the non-covalent and the covalent dyes. While the covalent peak areas were approximately 6 times larger than the non-covalent, the VP migration time and full-width half-maximum values were similar across the serotypes, suggesting a similar degree of protein denaturation and electrophoresis resolution for each sample.

## Table S1. Sizing Accuracy of VPs using the Non-Covalent and Covalent Methods

**Table S1:** Sizing of the different capsid proteins and sizing accuracy (% CV) using the non-covalent and the covalent methods. The % CV values fall within the ≤ 20% cutoff value.

| Serotype | Non-Covalent | | | | Covalent | | | |
|---|---|---|---|---|---|---|---|---|
| | VP3 Size (kDa) | VP2 Size (kDa) | VP1 Size (kDa) | % CV | VP3 Size (kDa) | VP2 Size (kDa) | VP1 Size (kDa) | % CV |
| **AAV1** | 72.9 ± 0.4 | 87.3 ± 0.5 | 107.3 ± 0.6 | 0.6 | 71.7 ± 0.3 | 85.1 ± 0.7 | 104.3 ± 0.6 | 0.6 |
| **AAV2** | 77.4 ± 1.2 | 89.8 ± 1.3 | 110.6 ± 1.6 | 1.5 | 74.4 ± 0.3 | 85.3 ± 0.5 | 105.9 ± 0.3 | 0.4 |
| **AAV6** | 73.9 ± 0.9 | 88.5 ± 1.1 | 108.4 ± 1.0 | 1.1 | 71.9 ± 0.2 | 85.4 ± 0.3 | 104.5 ± 0.5 | 0.4 |
| **AAV8** | 81.7 ± 1.0 | 94.9 ± 1.1 | 116.1 ± 2.0 | 1.4 | 79.8 ± 0.2 | 92.8 ± 0.2 | 113.1 ± 0.5 | 0.3 |
| **AAV9** | 78.8 ± 1.0 | 90.7 ± 0.9 | 111.9 ± 0.9 | 1.1 | 75.8 ± 0.2 | 87.3 ± 0.2 | 107.9 ± 0.9 | 0.3 |
| | **Average** | | | **1.1** | **Average** | | | **0.4** |

## Table S2. Percentage Full Prediction Accuracy of AAV8 Reference Standard

**Table S2:** Percentage full prediction accuracy of AAV8 reference standard accuracy. The % CV values fall within the ≤ 20% cutoff value except for AAV1 57% full. The UV/Vis percentages of full were determined using UV/Vis (Stunner).

| Serotype | UV/Vis % Full | Predicted % Full | Prediction Error (%) | % CV |
|---|---|---|---|---|
| **AAV1** | 57 | 37.0 ± 14.3 | 20.0 | 38.6 |
| | 29 | 17.5 ± 3.3 | 11.5 | 19.1 |
| **AAV2** | 62 | 61.6 ± 4.7 | 0.4 | 7.6 |
| | 50 | 48.5 ± 3.7 | 1.5 | 7.7 |
| | 25 | 24.7 ± 4.5 | 0.3 | 18.1 |
| **AAV6** | 72 | 73.3 ± 12.6 | 1.3 | 17.2 |
| | 50 | 46.1 ± 4.8 | 3.9 | 10.3 |
| | 25 | 18.5 ± 3.2 | 6.5 | 17.5 |
| **AAV8** | 70 | 58.4 ± 6.5 | 11.6 | 11.2 |
| | 50 | 44.5 ± 3.7 | 5.5 | 8.2 |
| | 25 | 20.5 ± 2.6 | 4.5 | 12.8 |
| | **Average** | | 6.1 | 15.3 |

## Figure S2. Comparison of UV/Vis (Stunner) and LabChip Percentage Full Predictions

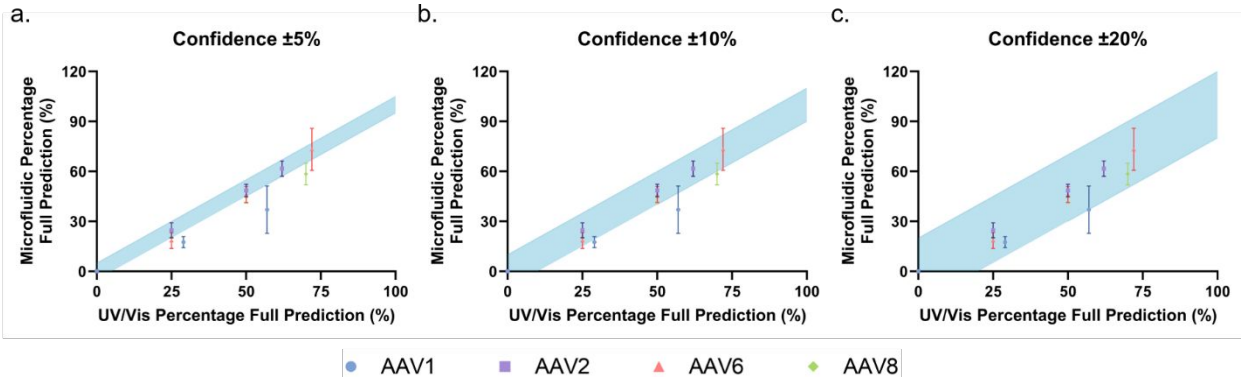


**Figure S2:** Comparison of the proposed method (LabChip) against UV/Vis (Stunner) at (a) ± 5%, (b) ± 10%, and (c) ± 20% confidence intervals with AAV1, 2, 6, and 8. To assess the performance of our microfluidic method against a more adopted method, we looked at how the microfluidic predictions (using AAV8 as the reference standard) compared to the UV/Vis (Stunner) estimations. For most serotypes, the microfluidic predictions were within ± 5-10% of the UV/Vis predictions, suggesting a close agreement between the methods. However, once again, AAV1 was the outlier, with one of the predictions being closer to ± 20%. While we believe this deviation can be attributed to a larger capsid ΔTm, we believe this highlights the potential and limitations of the technique, showing high accuracy for serotypes with lower ΔTm and a higher variation for those closer to the operating capsid ΔTm.