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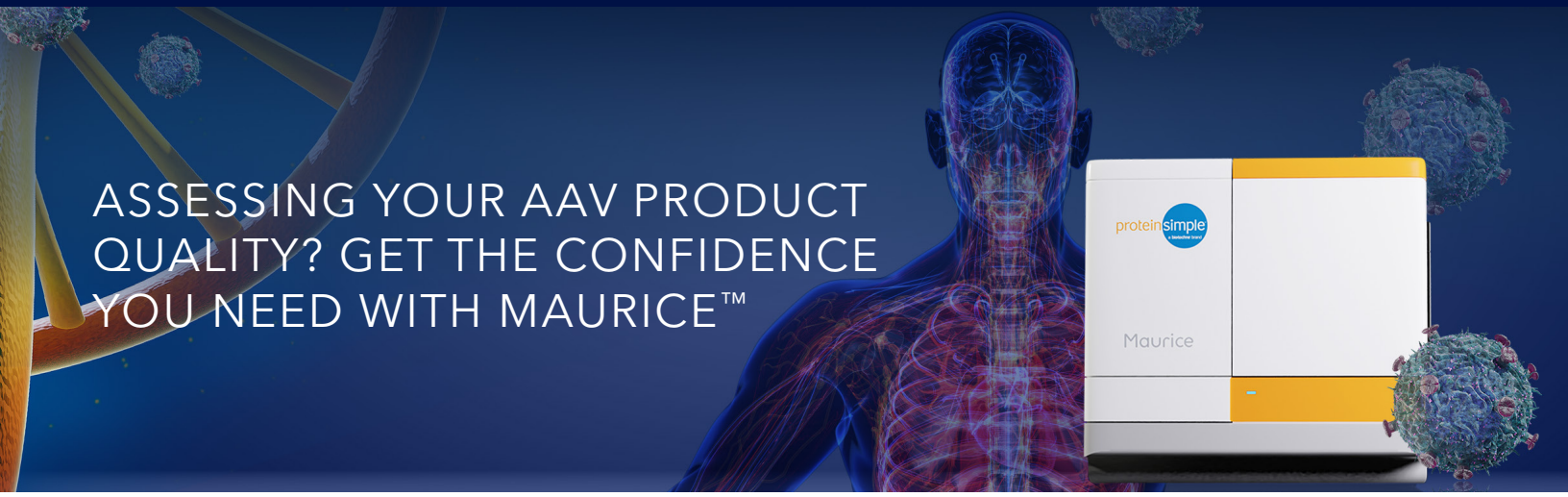
APPLICATION NOTE



ASSESSING YOUR AAV PRODUCT QUALITY? GET THE CONFIDENCE YOU NEED WITH MAURICE™

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INTRODUCTION

Viral capsid content can impact gene therapy product efficacy and is therefore considered a Critical Quality Attribute (CQA) that must be properly evaluated during the development and manufacturing of AAVs. Traditional analytical tools such as transmission electron microscopy (TEM), analytical ultracentrifugation (AUC), and ion-exchange chromatography (IEX) can be used to characterize capsid content but are complex, labor-intensive, and pose challenges in data reproducibility, throughput, and scalability[1-4].

In this application note, we show how imaged-capillary isoelectric focusing (icIEF) technology on Maurice can be used to characterize empty, intermediate and full AAV capsids at native and stability screening conditions, providing robust and reproducible data. With this, Maurice provides crucial data to aid in developing the right formulation for AAV therapeutics.

HOW MAURICE icIEF WORKS

On Maurice icIEF, samples are run using a cIEF cartridge to analyze protein charge heterogeneity and identity while only needing 50 µL of sample. Maurice automates the injection of samples into the capillary in the cartridge where proteins are separated based on their isoelectric point (pI) in the presence of a pH gradient and electric field. Equipped with absorbance and fluorescence detection modes, with absorbance readings taken in real time during separation, high-resolution data is generated rapidly. Additionally, each Maurice cIEF cartridge can be used for up to 100 injections, thus providing ample throughput per cartridge.

Here we use Maurice icIEF to characterize the charge profiles of intact empty and full AAVs. The study also demonstrates how stress and AAV serotypes impact these charge separation profiles. By using Maurice's absorbance and native fluorescence (NF) detection modes, we show how you can gain insight into the assembly of DNA and proteins in the AAV capsids.

MATERIALS AND METHODS

REAGENTS

The following materials were used: Maurice Method Development Kit (PN PS MDK01-C), Maurice cIEF Cartridges (PN PS-MC02-C), Maurice Glass Reagent Vials, 2 mL (PN 046-017), Maurice cIEF Blue Pressure Caps (PN 046-573), Maurice Clear Screw Caps (PN 046-138), Maurice 96-Well Plates (PN 046-021), and Dithiothreitol (DTT), 0.4M (PN 042-251). Additional materials were obtained from Millipore Sigma: Spin-X UF 500 (PN CLS431478), Fluorescent IEF-Marker pI 9.5 (PN 89268-200UL), Fluorescent IEF-Marker pI 5.2 (PN 89149-200UL), Fluorescent IEF-Marker pI 5.5 (PN 77866-200UL), Poloxamer 188 Solution (PN P5556-100ML), Sucrose (PN S7903), Glycine (PN G8898), DMSO (PN PHR1309) and Formamide (PN F7503-250ML). Biolyte 3-10 (PN 1631112) was obtained from Bio-Rad laboratories. AAV8 was provided by Ultragenyx.

AAV SAMPLE PREPARATION

AAV DS1 was diluted to 2.5 x 10^{12} GC/mL and then aliquoted into the ampholyte mix (1 part of AAV sample in 4 parts of ampholyte) in a Maurice 96-well plate. For native fluorescence samples, the ampholyte mix consisted of 0.35% methylcellulose (MC), 2% Pharmalyte 3-10, 2% Biolyte 3-10, and 0.002% pI markers in addition to various solubilizers. The solution was mixed by pipetting and centrifuged for 5 minutes at 2500 x g. The samples were separated at 1500 volts for 1 minute and 3000 volts for 9 minutes and detected by native fluorescence with a 10-second exposure time.

The additive screen was prepared by mixing MC, ampholytes, and pI markers before aliquoting the mixture into centrifuge tubes. The solubilizers were added separately, and deionized (DI) water was used to bring all samples to the same final volume. The ampholyte mixes were vortexed before addition of the sample. In some cases, the powdered solubility additive was added directly to 0.5% MC to obtain higher concentrations.

The denatured samples were prepared 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 3-10 and 2% Biolyte 3-10, 40% formamide and Maurice pI markers.

RESULTS

METHOD DEVELOPMENT FOR INTACT AAVs

The Maurice Method Development Kit provides a wide variety of ampholytes and commonly used solubilizers. The study began by screening different ampholytes using Maurice's native fluorescence (NF) detection mode. Due to the enhanced sensitivity of the fluorescence signal, the NF detection mode allowed the use of a lower amount of sample and yet provided sufficient signals, all of which were suitable for method development. On screening different ampholytes, Pharmalyte 3-10 provided good solubility and reproducibility (n=2) but resulted in poor resolution (**FIGURE 1, Panel A**). Biolyte 3-10 provided good resolution but exhibited some aggregation of the AAV sample and slower focusing of the pI 8.4 marker when detected with absorbance (data not shown). When the two were combined in a 1:1 ratio (4% final), good resolution and decreased aggregation were observed, thus improving reproducibility (**FIGURE 1, Panel C**).

Despite obtaining a better profile by using the 1:1 ampholyte mixture, some unexpected peaks were observed. These peaks were attributed to either partially denatured capsid proteins or aggregates. Therefore, to further enhance separation, various commonly used solubilizing agents including urea, formamide, sucrose, glycine, and SimpleSol were screened, individually or in combination with each other. In another application note, icIEF Analysis of Adeno-Associated Virus Proteins for Gene Therapy, SimpleSol has been shown to help with AAV solubility.

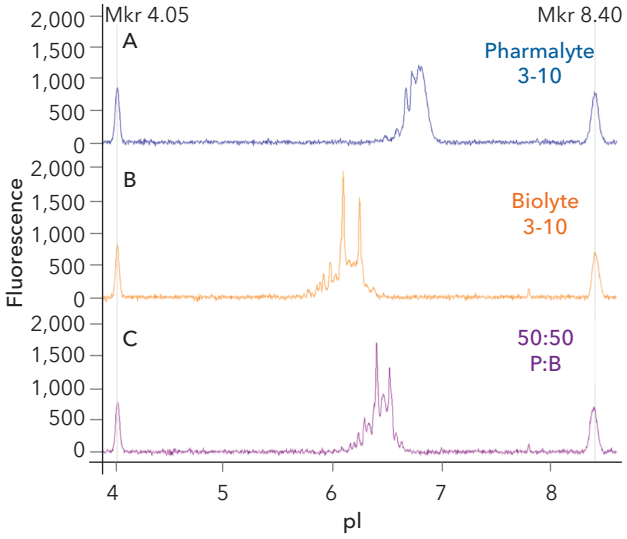


FIGURE 1. Ampholyte screening using AAV8. The AAV8 sample was diluted to 2.5 x 10^{12} GC/mL into a final mixture containing 4% Pharmalyte 3-10 (**Panel A**), 4% Biolyte 3-10 (**Panel B**), or a 50:50 mixture of Pharmalyte:Biolyte, 2% each (**Panel C**). Either ampholyte alone did not provide sufficient separation and solubility; however, when mixed in equal proportions, a better profile was obtained.

Nine different conditions, labeled Mix 1, Mix 2 etc., were created with 5 solubilizing agents and SimpleSol (20-35%), all used in varying proportions. Each of these conditions was tested with AAV DS1 using the 1:1 ampholyte mixture described previously. The absorbance and fluorescence results are shown in Figure 2A and Figure 2B respectively. High resolution signals were observed with Mixes 1, 3, 5, and 7, while the other mixtures produced unsatisfactory results. Interestingly, for Mix 4, the AAV sample showed two peak groups. Of the two groups, the more acidic peak group (pI 6.2-6.6) lacked native fluorescence and appeared to represent aggregates. This was inferred as tryptophan fluorescence can be quenched by the presence of protein aggregation. Upon further inspection of the overlays of absorbance and native fluorescence (data not shown), and the focusing movie, Mixes 1, 3, 5, and 7 were chosen for further testing.

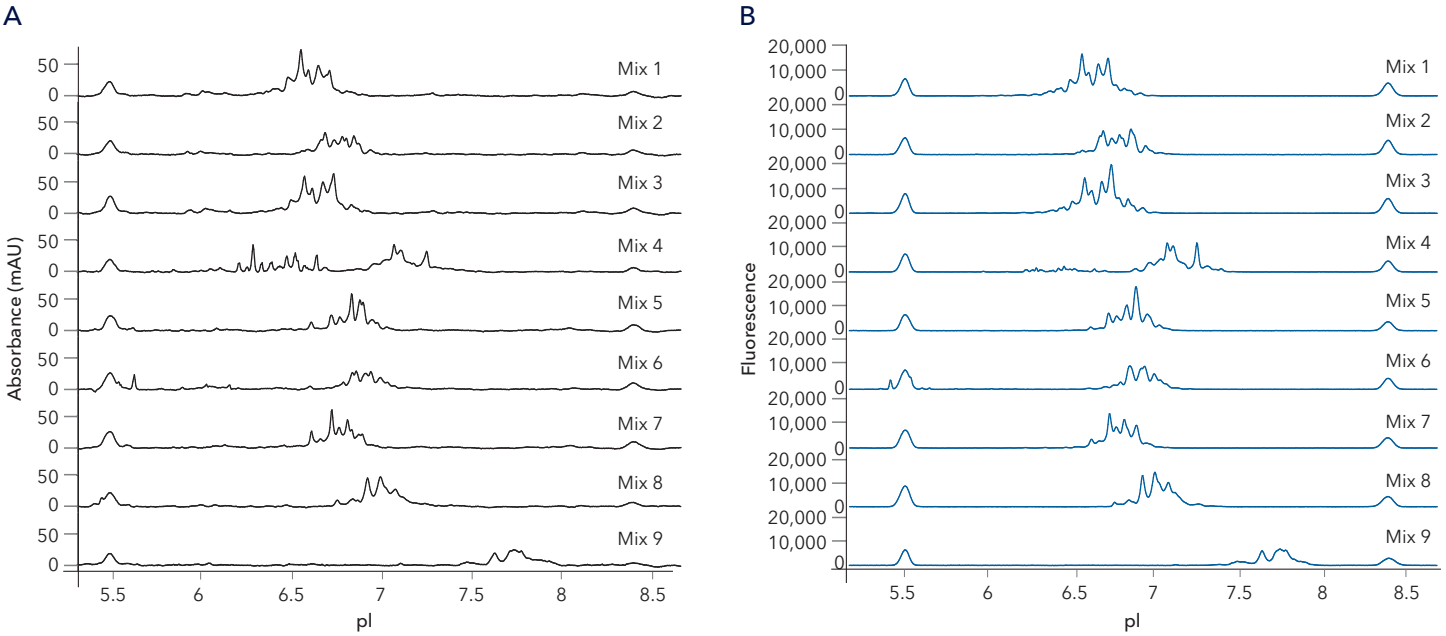


FIGURE 2. Intact AAV method screening. 9 conditions using different solubilizing agents were screened to identify optimal separation. (**A**) Absorbance and (**B**) native fluorescence results are shown for the 9 different conditions tested. Optimal results were found for Mixes 1, 3, 5, and 7, while Mix 4 resulted in two peak groups when detected with absorbance.

REPRODUCIBILITY

Next, Mix 1, Mix 3, Mix 5, and Mix 7 were tested for reproducibility, where replicate injections were evaluated (**FIGURE 3**). Of the four, Mix 5 and Mix 7 (**FIGURES 3C AND 3D**) demonstrated optimal reproducibility and resolution. Additionally, the focusing movies of each mix were examined to ensure the method exhibited minimal aggregation.

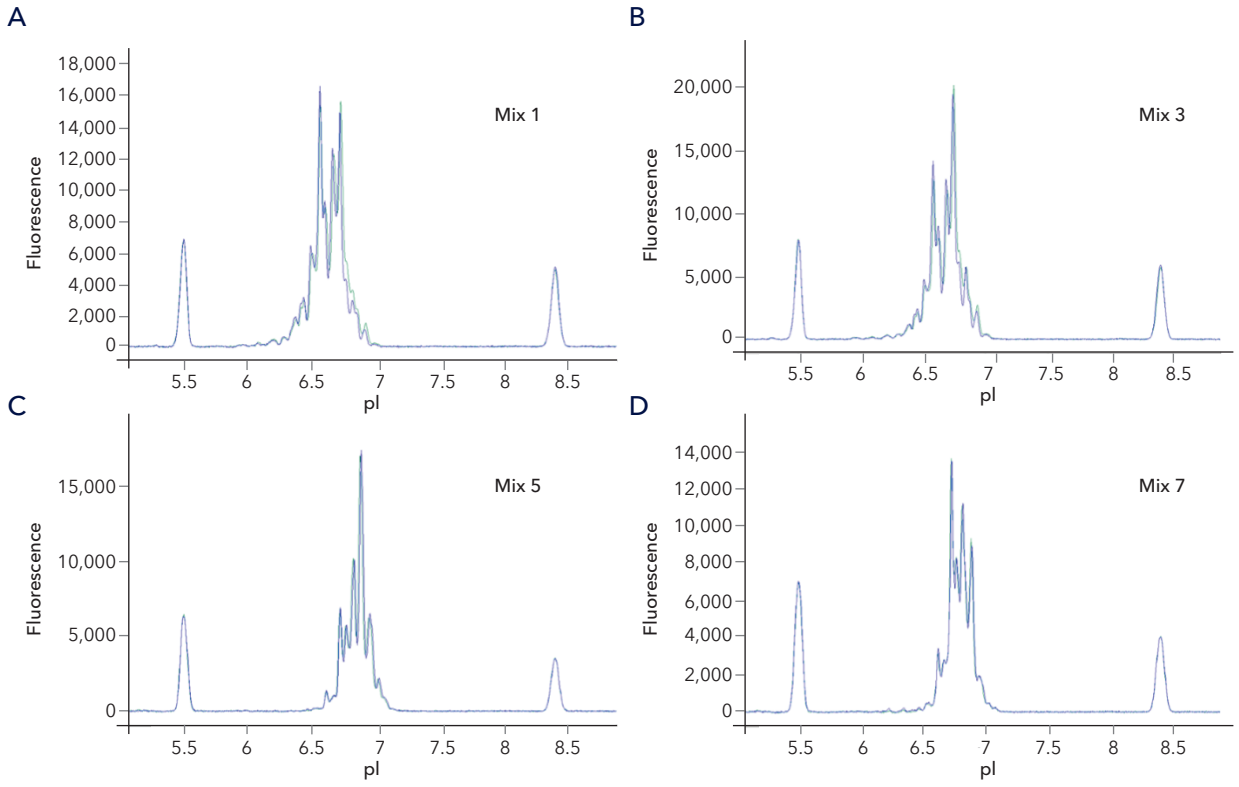


FIGURE 3. Experiments determining the reproducibility of Mixes 1, 3, 5, and 7, shown in (A), (B), (C), and (D) respectively. Overlaying the results from replicate injections for each mix showed that Mix 5 and Mix 7 had the optimal reproducibility.

Mix 5 and Mix 7 were screened further against a series of AAV8 samples, including an AAV8 full, intermediate, and an AAV8 empty sample (**FIGURE 4**). For Mix 7, better alignment of the full, intermediate, and empty signals was observed when compared to Mix 5.

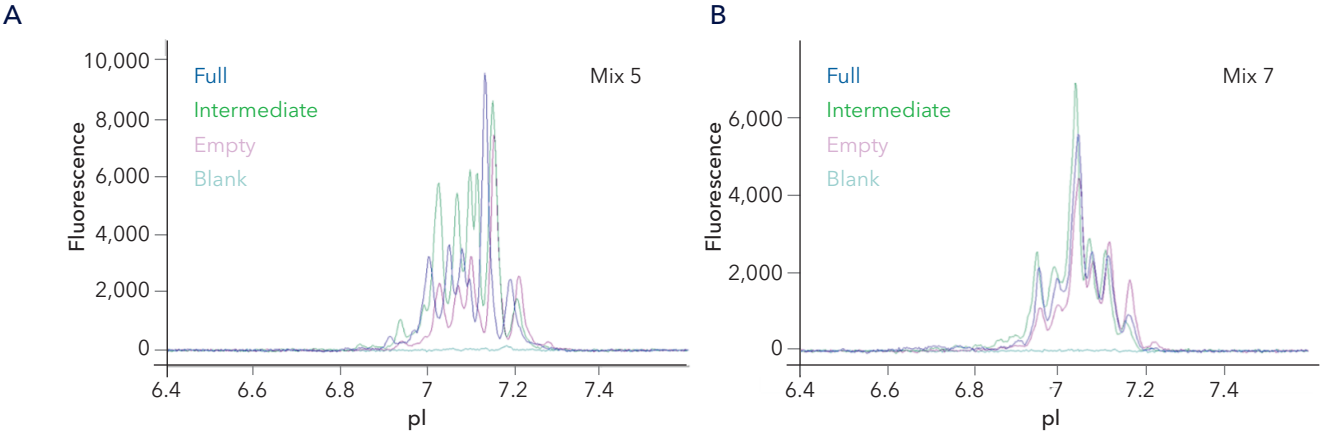


FIGURE 4. Screening of Mix 5 (A) and Mix 7 (B) using full, intermediate, and empty AAV8. Of both mixes, Mix 7 demonstrated a higher degree of alignment between the native fluorescence signals obtained from the three different AAV8 samples.

COMPARISON OF ABSORBANCE AND NATIVE FLUORESCENCE USING THE NEW AAV INTACT METHOD

DNA has a maximum UV absorbance at 260 nm, while proteins have maximum UV absorption at 280 nm[5]. Maurice allows 280 nm absorbance for detection of proteins and has native fluorescence detection to detect intrinsic fluorescence from certain amino acids (tryptophan, and to a lesser extent, tyrosine, and phenylalanine). While not ideal for its detection, DNA does absorb significantly at 280 nm. Therefore, the absorbance signature for intact AAVs contain contributions from proteins and DNA in the sample. In contrast, the native fluorescence does not detect DNA at all. Overlaying the absorbance and native fluorescence profiles of AAV8 samples revealed that the full and intermediate samples had similar absorbance while the empty sample had a very small absorbance signature (FIGURE 5).

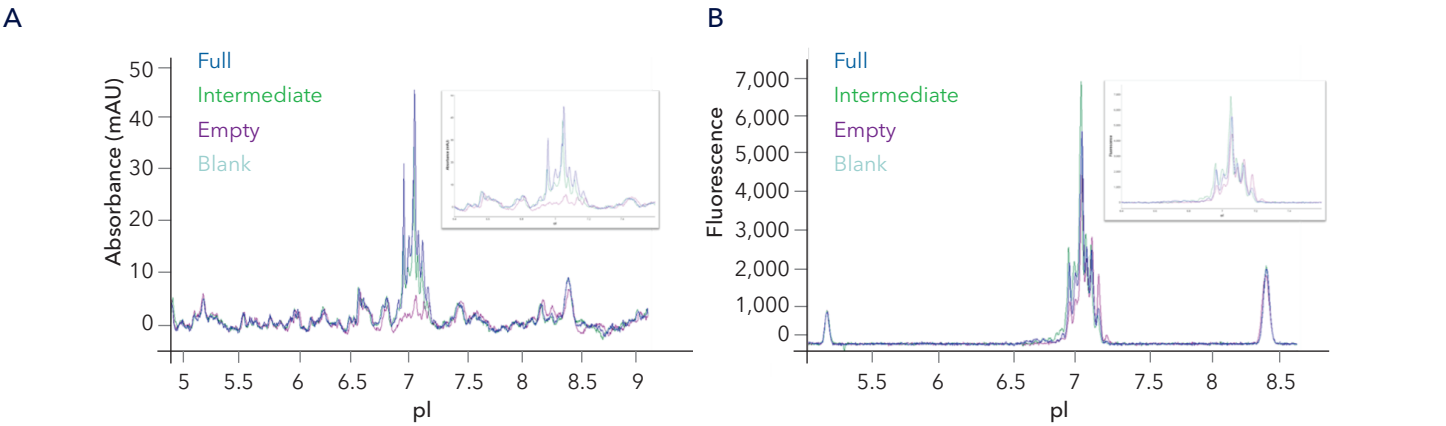


FIGURE 5. Comparison of UV absorbance (A) and native fluorescence (B) for empty, intermediate, and full AAV8 samples. Significantly lower absorbance is observed in the empty AAV sample (purple). All three samples have similar native fluorescence signals.

The AAV8 samples were loaded at equivalent amounts for the analysis, which is confirmed by the native fluorescence images. There were differences in areas of certain peaks in the NF images, attributed to possibly altered protein conformation due to the absence of DNA (FIGURE 6).

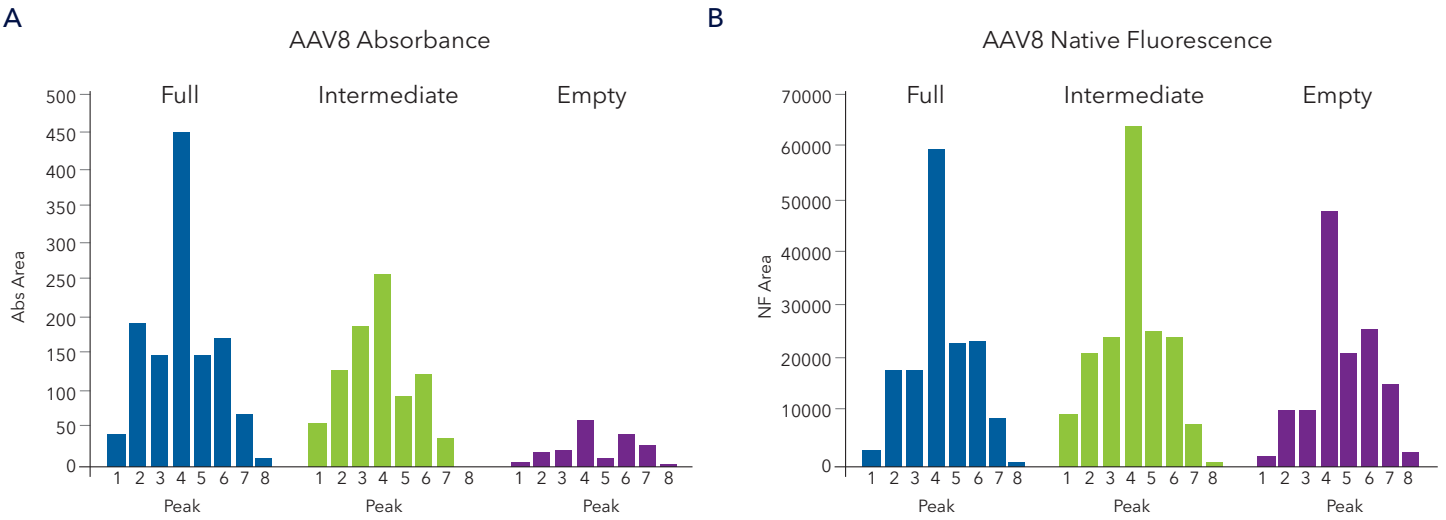


FIGURE 6. Quantitation of absorbance (A) and native fluorescence (B) data from AAV8 panel. The absorbance and native fluorescence were measured, and the data was expressed as peak area.

The total peak area of each sample was analyzed after normalizing the native fluorescence data for the three samples (full, intermediate, and empty). This analysis is useful to understand the true absorbance differences between the samples (data not shown).

From these studies, the apparent pI of empty and full AAV capsids were found to be similar, but the charge heterogeneity was different among capsids containing varying amounts of DNA.

VERIFYING STABILITY INDICATION OF THE INTACT METHOD

HIGH TEMPERATURE STRESS (5 MINUTES)

Each sample was briefly subjected to temperature stress to evaluate the stability-indicating potential of the method established previously. The samples were incubated at 37°C, 50°C, 55°C, and 65°C for 5 minutes before analysis. Compared to the control (4°C), significant increases in the acidic species were observed at 65°C (**FIGURES 7A and 7B**), with associated loss of other, more basic peaks. Different temperatures did not appear to have a significant impact on the empty sample (**FIGURE 7C**). Figure 8 shows the quantitation of the percent peak area with native fluorescence for full, intermediate, and empty samples after they were subjected to temperature stress.

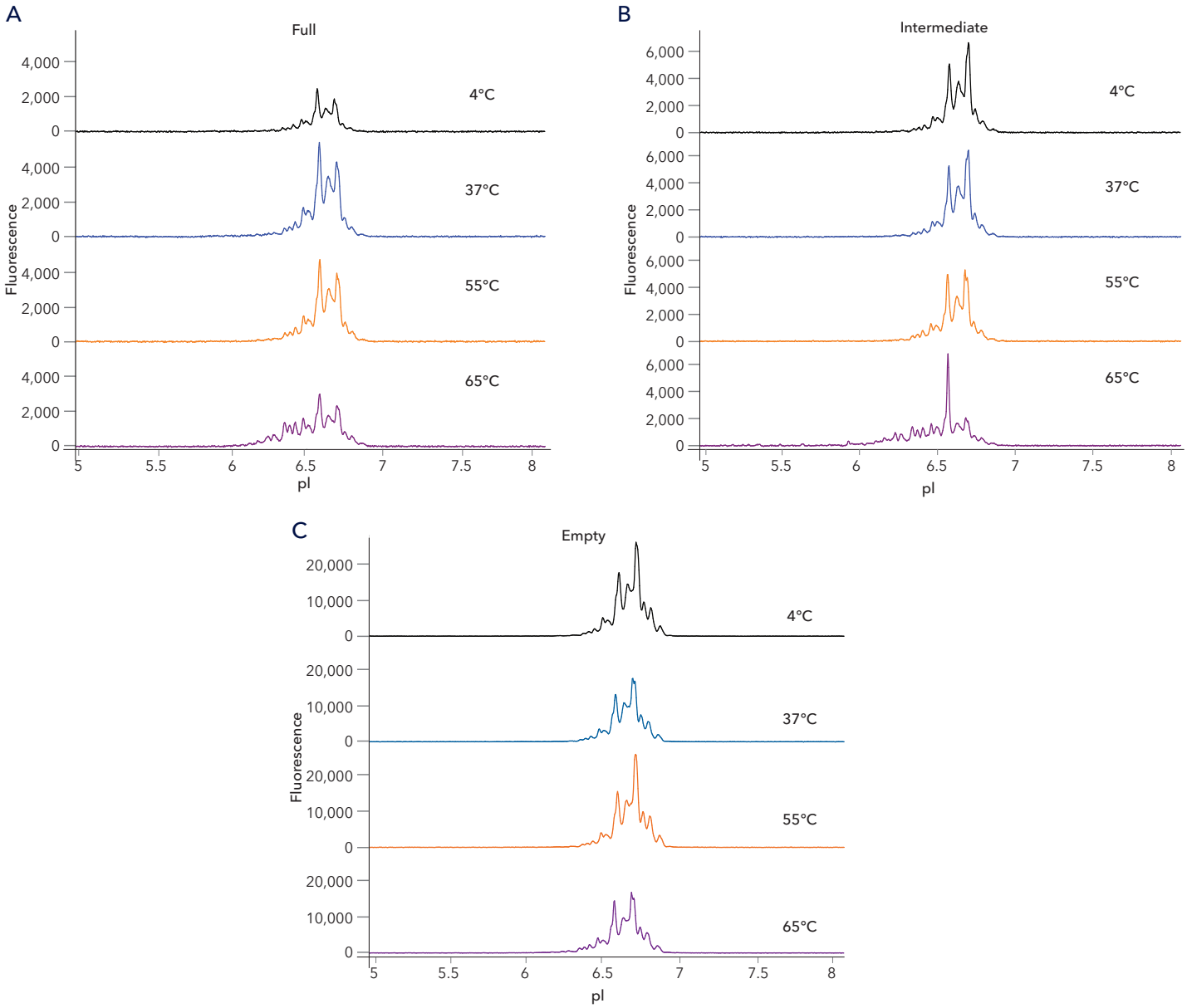
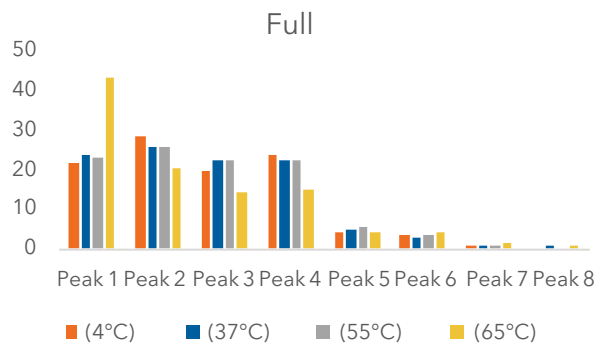


FIGURE 7. Stability-indicating assessment of the intact method. The AAV DS1 samples was incubated at 37, 50, 55, and 65°C for 5 minutes before running on Maurice using the intact method. (**A**) Results of the full AAV sample show an increase in acidic species and denaturation at 65°C. (**B**) The intermediate sample also showed denaturation at 65°C when compared to the control. (**C**) No major changes were observed even at 65°C in the empty AAV DS1 sample.

A



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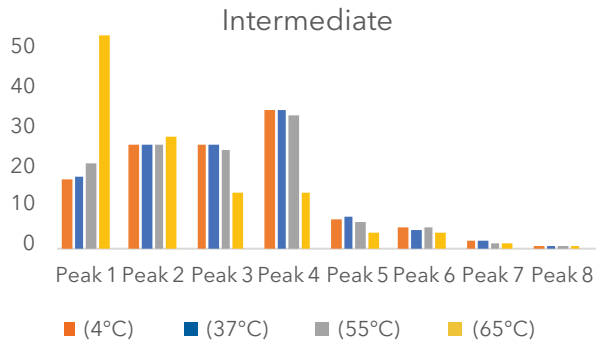

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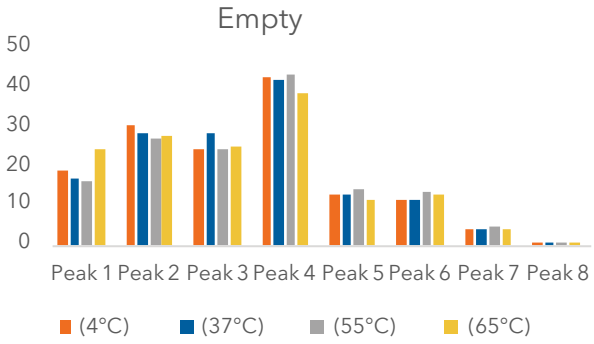

FIGURE 8. Quantitation of % peak area with native fluorescence for the stress series of (A) full, (B) intermediate, and (C) empty samples at four different temperatures is shown.

MILD TEMPERATURE STRESS

To better understand the method's capability to observe small changes in the AAV sample, the same AAV8 samples were used for a longer term, lower temperature study. For this test, AAV8 DS1 was incubated at 37°C for either 3 days, 7 days or 14 days and compared to a control sample (4°C) using the intact method (FIGURE 9). These data show substantial changes in the AAV8 profile in as little as 7 days at 37°C using the intact method. Specifically, there is concordant loss of basic and main peaks, and an increase in the acidic groups. Based on the known post-translational modifications associated with stressed AAVs, the increases in acidic peaks may be the result of deamidation at specific amino acids, similar to what is observed with monoclonal antibodies[6].

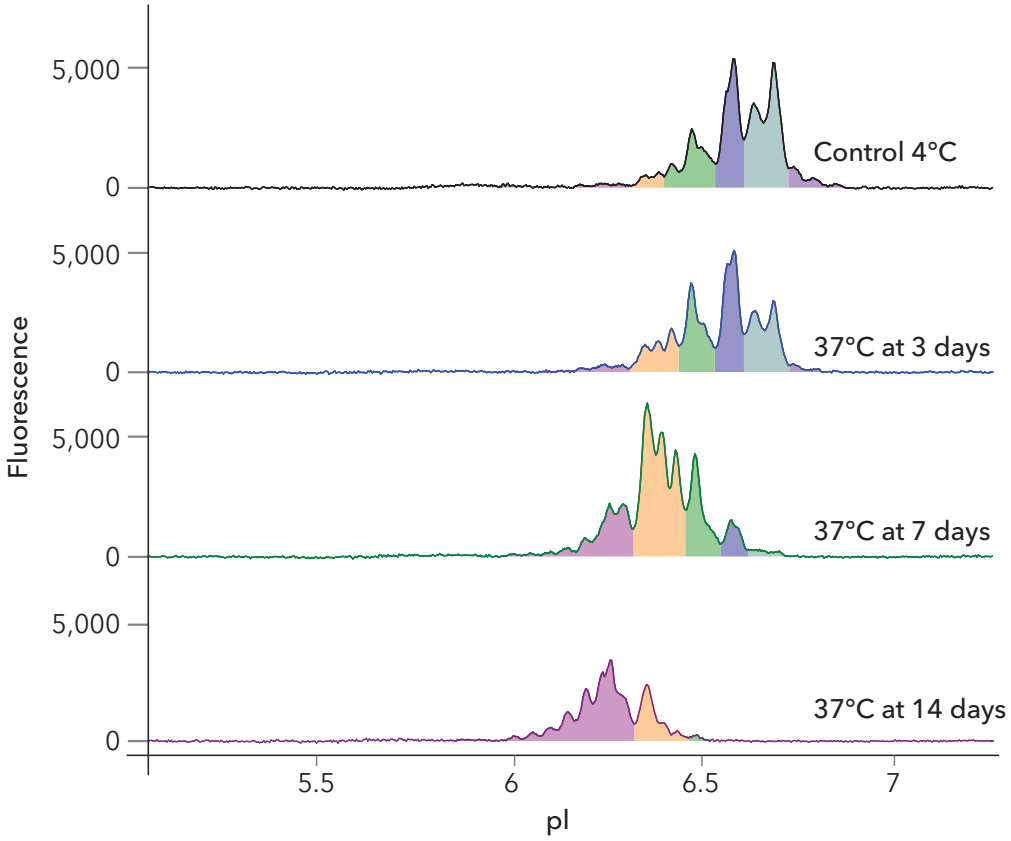

FIGURE 9. Mild temperature stress induces changes in AAV8 DS sample. AAV8 DS was incubated for 3, 7, or 14 days before analysis using the intact method. A control sample was used to compare the changes over time. Substantial profile changes are observed in as little as 7 days with the method.

pH STRESS

Different pH conditions can impact the therapeutic properties of AAVs. These viral particles have been known to require an optimal low pH for timely endosomal escape into the cell[7]. In addition, pH-dependent proteases require an acidic environment to trigger the release of the encapsulated genome[8]. Therefore, pH stress of the AAV DS was also evaluated as a part of this stability study. The AAV8 DS sample was incubated in a pH 10 buffer (20 mM Tris, 200 mM NaCl) for either 4 days or 7 days and compared to a pH 8 control at 4°C control on Maurice using the intact method. Similar to the temperature stress test, the AAV profile shifts to an overall more acidic series of peaks (**FIGURE 10**). The chemical stress may have resulted in a base-catalyzed deamidation reaction.

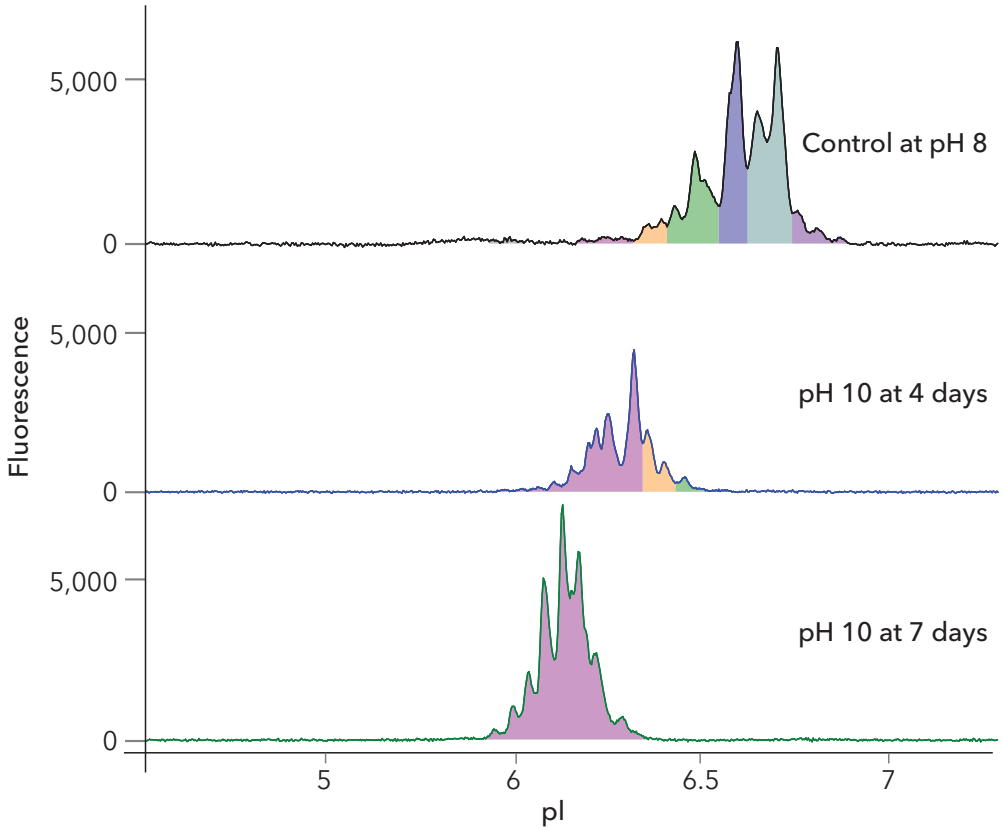

FIGURE 10. Results from chemical stress studies. Incubation at high pH under 37°C induces significant changes in AAV8 DS. The AAV8 DS sample was exchanged into a pH 10 buffer and incubated for either 4 days or 7 days prior to analysis using the intact method, resulting in a dramatic increase in acidic variants.

CONCLUSION

With Maurice's absorbance and native fluorescence detection, AAV capsid content can be assessed with accuracy and reproducibility, while providing ease-of-use, short run times, and low sample volumes. This study demonstrated how a single method for AAV characterization was developed and used to test full, intermediate, and empty AAV8 samples. The absorbance and fluorescence detection modes yielded critical information on DNA content in capsids. Furthermore, the method was found to be stability-indicating and clearly showed the degradation of AAV8 samples under various stress conditions. With Maurice, we were able to quickly develop a method to characterize and assess the stability of empty, intermediate, and full AAVs, providing a critical, high-quality solution for safe, fast, and effective gene therapy development.

To learn more about how to accelerate your gene therapy development with Maurice, visit https://www.proteinsimple.com/cell-and-gene-therapy-vector-characterization.html

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