Charge Characterization of Adeno-Associated Virus (AAV) Capsid Proteins Using icIEF

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INTRODUCTION

Viral vectors like AAVs are becoming a widely used therapeutic approach for the treatment of rare diseases. There are 10 naturally occurring serotypes that infer tissue tropism, with even more being engineered to improve on various functional or physiochemical attributes. These serotypes differ by changes at the amino acid level for the three capsid proteins, VP1, VP2, and VP3 and consequent structural changes or downstream interactions with cellular receptors. Common to all serotypes, these capsid proteins assemble to form a 60-mer that contains a ssDNA genome. A key challenge faced when manufacturing AAVs is maintaining particle stability, and thus efficacy. Certain post-translational modifications to the individual capsid proteins that are often associated with stress (deamidation for example) has been shown to reduce transduction efficiency. While techniques like MS can monitor these changes at a deep level, a rapid and broad view of these changes can enable more efficient decision making for process improvements.

Here we present a rapid charge characterization method for AAV capsid proteins based on icIEF with native fluorescence detection using the Maurice platform (ProteinSimple). The method was used to analyze eight serotypes and was shown to be suitable as a powerful AAV identity assay. The electropherograms of icIEF is also a sensitive capsid protein stability indicator. To analyze upstream samples where the capsid protein concentrations are low and there are more contaminating proteins in the sample, we leveraged our highly specific immunoassay-couped icIEF platform (Simple Western) which uses an amplified chemiluminescence based readout after probing with primary antibodies for the VPs. The chemiluminescence detection has even higher sensitivity. It works well with crude AAV samples in cell lysates. In addition, using VP-specific antibodies, changes to individual VPs can be studied to provide deeper identity information and used as a more sensitive stability indicator.

MATERIALS AND METHODS

Reagent	Vendor	Part Number
Formamide (>99.5%)		47671
Dimethyl sulfoxide (DMSO, >99.7)	SIGMA	D2650
Dithiothreitol (DTT, >99.0%)		D0632
Pharmalyte 3-10	Cytiva	17-0456-01
Pharmalyte 5-8		17-0453-01
Mouse monoclonal antibodies to AAV VP1 and VP1/2/3	PROGEN	61056 (anti-VP1) 61058 (anti-VP1/2/3)
AAVs (2 x 10^13 GC/mL)	Virovek	Custom

Table 1. Materials sourced from outside vendors used in the study.

Reagent	Vendor	Part Number
1% MC		101876
0.5% MC		102505
pI marker 5.85		046-030
pI marker 8.40		046-033
pI ladder 1		040-644
pI marker 8.4 for Peggy Sue		041-036
Maurice electrolytes	ProteinSimple	102506
Maurice cIEF cartridge		090-101
IEF anolyte for Peggy Sue		042-337
IEF catholyte for Peggy Sue		042-338
Antibody diluent 2		042-203
Anti-mouse secondary antibody		042-205
Peggy Sue Charge capillaries		CBS700

Table 2. Materials from ProteinSimple used in the study.

AAV preparation - To 5 μL AAV (2 x 10^13 GC/mL), 1 μL of 160 mM DTT and 7 μL DMSO are added are mixed. The mixture is then heated at 70°C for 10 minutes to denature the sample. The denatured sample is then cooled down to room temperature and kept at room temperature prior to analysis. **Maurice analysis -** The final IEF sample solution contains 50% formamide, 0.35% methylcellulose, 2% Pharmalyte 3-10, 2% Pharmalyte 5-8, and pI markers 5.85 and 8.40. Denatured AAVs were focused at 1.5 kV for 1 minute, then at 3 kV for 12 minutes and imaged using native fluorescence (20 – 80 second exposures). **Peggy Sue analysis -** Samples were focused at 1.5 kV for 1 minute, then at 3 kV for 13 minutes. Focused proteins were captured via UV immobilization (220 seconds), then probed with either anti-VP1/2/3 or anti-VP1 for 60 minutes followed by HRP-conjugated secondary antibody and subsequent chemiluminescence detection.

RESULTS

We first analyzed the method reproducibility and linearity. **Figure 1** shows the reproducibility of the optimized denatured AAV icIEF method, with data of 15 injections over 3 days summarized in **Table 3** and **Table 4**. The method's LOQ was validated as 2.6 X 10^10 VP/mL. The detection is linear from the LOQ to a sample concentration of 5.7 X10^11 VP/mL with R²=0.998, as shown in **Figure 2**. The average pI of the three main peaks (Peaks 3, 4, and 5) across the linear range of detection are shown in **Table 5**.
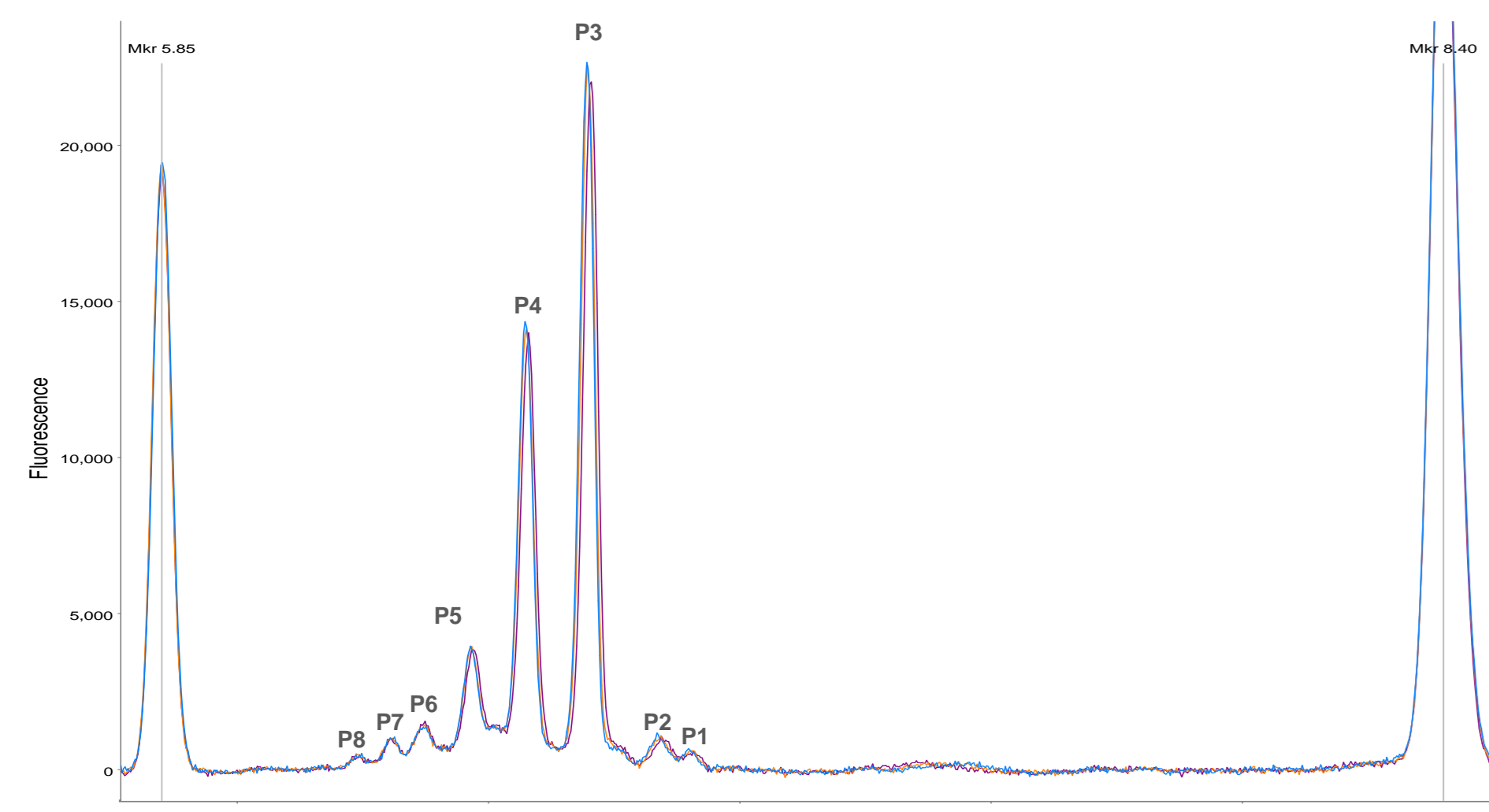
Figure 1. Denatured AAV9 icIEF method reproducibility. AAV9 (XX VP/mL) was denatured and analyzed using the denatured icIEF method. Shown are XX injections overlaid, with eight peaks identified.
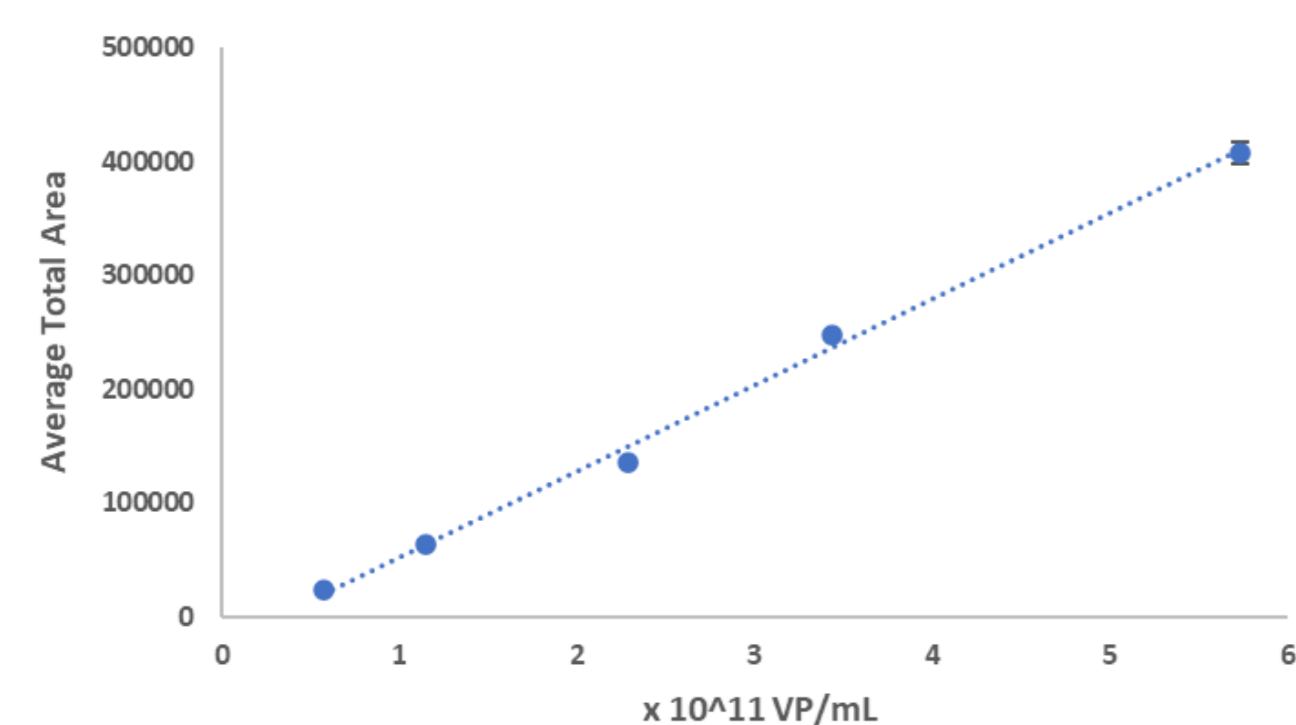
A	Peak 8	Peak 7	Peak 6	Peak 5	Peak 4	Peak 3	Peak 2	Peak 1
Ave	0.9	2.2	4.1	11.4	30.8	46.6	2.7	1.3
RSD%	23.4	7.1	5.8	3.3	1.0	1.2	10.8	10.4

B	Peak 8	Peak 7	Peak 6	Peak 5	Peak 4	Peak 3	Peak 2	Peak 1
Ave	6.34	6.31	6.37	6.47	6.57	6.70	6.84	6.90
STD	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01

Table 3. Precision of capsid protein charge method. Denatured AAV9 was analyzed over 5 days, with 3 injections per day for a total of 15 injections. (A) The %PA was calculated for each of the 8 peaks in the profile and analyzed for %RSD. (B) The apparent pI of each peak was measured and analyzed for standard deviation.

Inject 1	Inject 2	Inject 3	Inject 4	Inject 5	Inject 6	Inject 7	Inject 8	Inject 9	Inject 10	Inject 11	Inject 12	Inject 13	Inject 14	Inject15	Average	3 x STD
6.61	6.62	6.62	6.62	6.62	6.62	6.61	6.61	6.61	6.60	6.62	6.62	6.62	6.62	6.63	6.62	0.02

Table 4. **Averaged pI of the denatured AAV9 from the reproducibility testing.** The 15 injections of denatured AAV9 were analyzed for the average pI value and standard deviation (STD).

Figure 2. Titration of AAV9 for linearity assessment. AAV9 was denatured and then serially diluted two-fold from ~ 6 x 10^11 VP/mL to 6 x 10^10 VP/mL prior to analysis in triplicate on Maurice. A strong linear relationship was observed, with R² > 0.99.

Concentration (VP/mL)	5.7 × 10^10	1.1 × 10^11	2.3 × 10^10	3.4 × 10^11	5.7 × 10^11
pI of Peak 3	6.72	6.72	6.72	6.71	6.70
pI of Peak 4	6.59	6.59	6.58	6.57	6.57
pI of Peak 5	6.49	6.49	6.48	6.48	6.47

Table 5. **Measured pI values of the three major denatured AAV9 peaks at different sample concentrations.** The data show that across the linearity testing range, the main peak pI values only shift by a maximum of 0.2 pH units.

RESULTS

The method was applied to eight different AAV serotypes, which exhibit different peak patterns and isoelectric points (**Figure 3A**). To quantify peak pattern differences, we measured the averaged pI of each sample. In this analysis, each peak's pI is normalized by its peak area percentage, then the normalized peak pI values are averaged to obtain the averaged pI, which reflects all peaks pI values and their peak area percentages (inset right in Figure 3A). The method is powerful enough to detect the difference between AAV1 and AAV6 (as shown in **Figure 3B**) although these two serotypes only have 1% overall amino acid difference (**Figure 3C**).
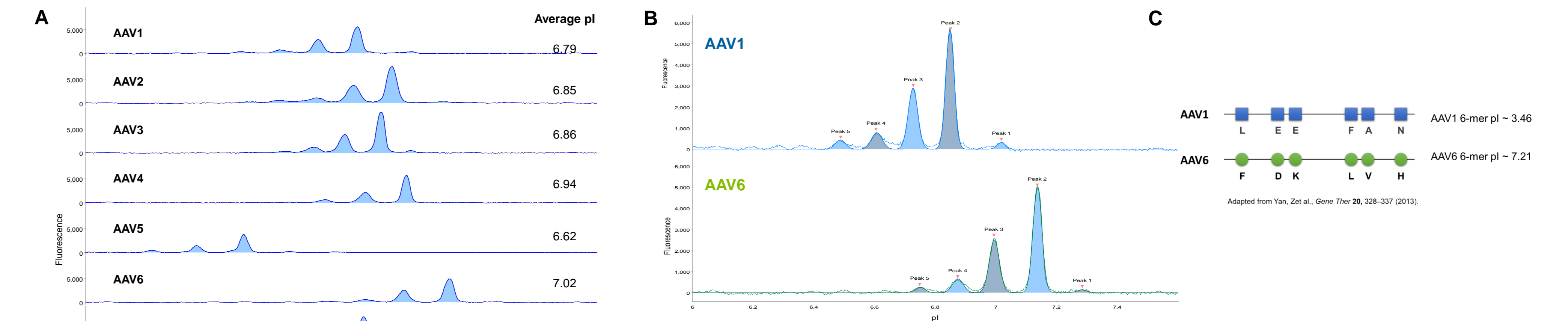
	Average pI
AAV1	6.79
AAV2	6.85
AAV3	6.86
AAV4	6.94
AAV5	6.62
AAV6	7.02
AAV8	6.74
AAV9	6.77

Figure 3. The denatured AAV icIEF method is a platform AAV identity method. (A) AAVs of different serotypes were denatured at 70C for 10min and analyzed at 4 x 10^11 VP/mL (1-9, not 7). Note that AAV5 requires denaturation at 75C, 10min. (B) Comparison of AAV1 and AAV6, which are 99% identical by amino acid sequence. (C) Sequence comparison between AAV1 (top) and AAV6 (bottom), with corresponding calculated 6-mer pI values that support more basic pI profile of AAV6.

AAV1: L E E F A N — AAV1 6-mer pI ~ 3.46
AAV6: F D K L V H — AAV6 6-mer pI ~ 7.21
Adapted from Yan, Zai al., Gene Ther **20**, 328–337 (2013).

The denatured AAV method also is stability indicating. AAV8 was stressed at 37°C for up to 14 days prior to denaturation and analysis on Maurice. The peak patterns show a trend of increasing acidic variants (Figure 4B bar plots). This shifting can be quantitatively described by the averaged pI of the stressed samples, plotted above the bar graphs. As shown in Figure 4B, the averaged pI decreases by ~0.1 units after 14 days of stress.

Reference / 37C 1 Day / 37C 3 Days / 37C 8 Days / 37C 14 Days

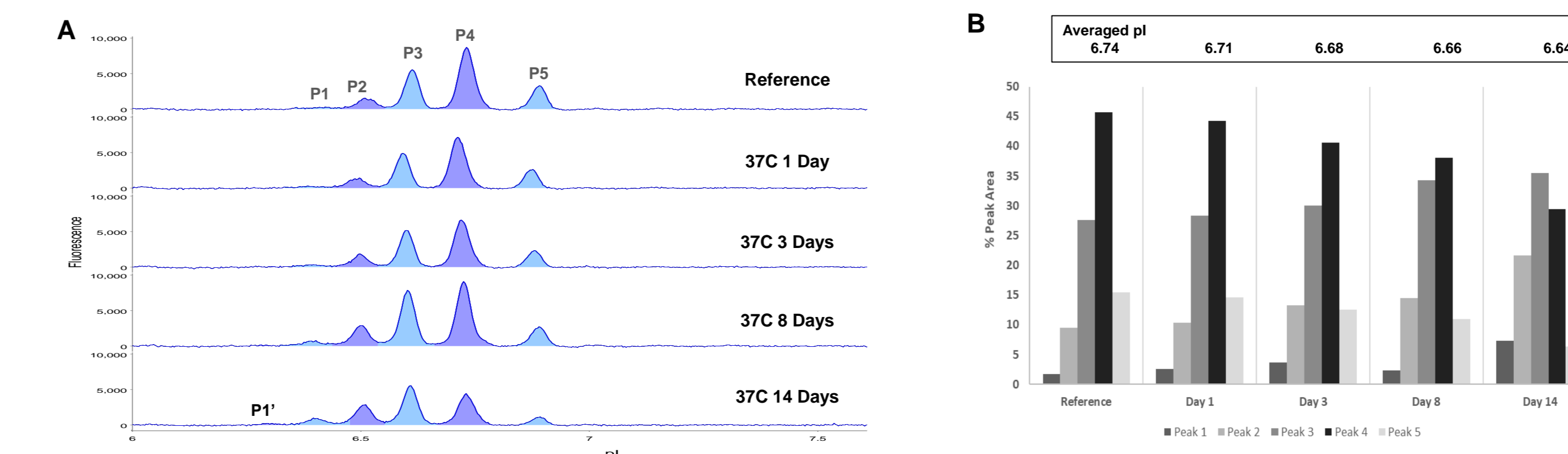| Averaged pI				
6.74	6.71	6.68	6.66	6.64

Figure 4. Denatured AAV icIEF method is stability indicating. AAV8 was stressed at 37C for 1, 3, 8 and 14 days prior to analysis. (A) Denatured AAV8 charge profiles of the stressed samples. (B) Averaged pI and peak area % changes over time of the stressed samples

To study changes to AAV capsid proteins in more complex samples, we moved the Maurice method to the Simple Western Peggy Sue instrument which leverages immunodetection of the denatured AAV proteins after capture the capillary wall. The same denatured method works on Sue, with only minor changes to the separation conditions, even in the presence of HEK293T lysate (**Figure 5A**). The assay exhibits a large dynamic range with LOD/LOQ near 5 x 10^8 VP/mL (**Figure 5B**). Leveraging two AAV antibodies, the complex profile can be deciphered – where VP1 (orange) appears as the acidic series of peaks in the profile when compared to the VP1/2/3 signature (blue, **Figure 5C**). This pair of antibodies can be used to provide deeper information on the changes in the charge profile under stress conditions (**Figure 5D**).
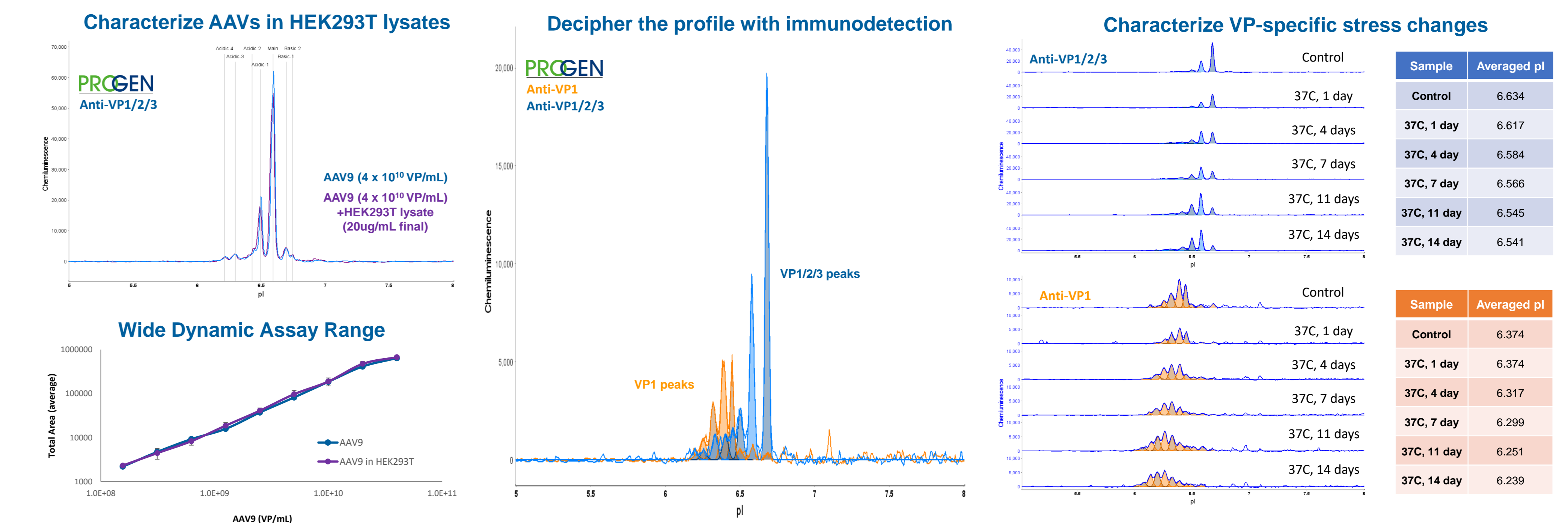
Characterize AAVs in HEK293T lysates

Anti-VP1/2/3
AAV9 (4 x 10^10 VP/mL)
AAV9 (4 x 10^10 VP/mL) +HEK293T lysate (20ug/mL final)

Wide Dynamic Assay Range
AAV9
AAV9 in HEK293T

Decipher the profile with immunodetection
Anti-VP1
Anti-VP1/2/3
VP1/2/3 peaks
VP1 peaks

Characterize VP-specific stress changes

Anti-VP1/2/3: Control / 37C, 1 day / 37C, 4 days / 37C, 7 days / 37C, 11 days / 37C, 14 days

Sample	Averaged pI
Control	6.634
37C, 1 day	6.617
37C, 4 day	6.584
37C, 7 day	6.566
37C, 11 day	6.545
37C, 14 day	6.541

Anti-VP1: Control / 37C, 1 day / 37C, 4 days / 37C, 7 days / 37C, 11 days / 37C, 14 days

Sample	Averaged pI
Control	6.374
37C, 1 day	6.374
37C, 4 day	6.317
37C, 7 day	6.299
37C, 11 day	6.251
37C, 14 day	6.239

Figure 5. Denatured AAV icIEF method with immunodetection on Simple Western. (A) Leveraging immunodetection with an anti-VP1/2/3 antibody from PROGEN, the same denatured profile can be achieved in the presence (purple) or absence (blue) of HEK293T lysate. **(B)** The Simple Western assay has a wide dynamic range of detection that is unimpacted by lysate. **(C)** Using two different PROGEN antibodies, the peak profile of VP1 can be identified (shown in orange) compared to the VP1/2/3 signature (shown in blue). **(D)** AAV8 was stressed at 37C for 1, 3, 8 and 14 days prior to analysis. Averaged pI and peak area % changes over time of the stressed samples are shown in the tables to the right of the electropherograms.

CONCLUSIONS

- The denatured AAV method is platform and can be used to analyze a diverse array of AAVs where it can be used to identify as little as a 1% amino acid difference between serotypes. icIEF with UV fluorescence imaging detection can be used as a serotype identification tool.

- The denatured AAV method is stability indicating, providing a rapid way to detect subtle changes in capsid proteins in pure or complex samples.

- icIEF with chemiluminescence is a useful tool for upstream, low concentration, complex samples. This method performs like the Maurice method with respect to identity and stability indication, suggesting the two platforms can together cover a program from discovery to QC.