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# Assessment of adeno-associated virus purity by capillary electrophoresis-based western

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**A rigorous analytical assessment of recombinant adeno-associated virus (rAAV)-based drug products is critical for their successful development as clinical candidates. It is especially important to ascertain high purity while simultaneously ensuring low levels of impurities in the final drug product. One approach to evaluate the purity of rAAV drug products is to determine the relative stoichiometry of the three viral proteins (VPs) that comprise an rAAV capsid, and the levels of impurities in the final drug product. Here we present two capillary electrophoresis-western (CE-western) assays for quantifying (1) the relative stoichiometry of VP using the anti-AAV B1 antibody, and (2) residual levels of a baculovirus protein impurity, GP64, using the anti-GP64 antibody. In each assay, various purified samples from diverse AAV serotypes were analyzed to determine their VP ratio or GP64 levels. The ratio of VP3/VP1 in rAAV samples was correlated with biological activity, and the clearance of GP64 from the manufacturing process was demonstrated. The results obtained from both assays were further supported by liquid chromatography-mass spectrometry analyses. Overall, we report that CE-western is a high-throughput platform that utilizes low sample volumes for a rapid, sensitive, and robust assessment of the identity, composition, and purity of rAAV drug products.**

## INTRODUCTION

Recombinant adeno-associated viruses (rAAVs) are highly attractive gene delivery vehicles for safe and effective genomic therapies, as evidenced by several approved products in the United States and Europe.[1,2] Additional clinical trials are ongoing for developing rAAV-based therapies for a wide range of indications such as cancer,[3,4] cardiovascular diseases,[5] and rare diseases.[2] Over the years, the adoption of several novel analytical technologies has resulted in a better understanding of the critical quality attributes (CQAs) governing safety, identity, strength, purity, and quality of these products.[6] This has in turn supported the successful clinical development and commercialization of these rAAV-based gene therapy products. The purity of AAV products has a significant impact on overall product quality, as impurities can lead to potential toxicity, immunogenicity, and other safety issues.[6] Typically, the identity and purity of rAAV products is determined by a combination of assays determining the relative stoichiometry of viral proteins (or VP ratio) and quantifying the product-, process-, and host cell-related impurities.[6–8] The determination of the relative stoichiometry of viral proteins involves the separation of the denatured rAAV samples into three distinct component viral proteins, VP1, VP2, and VP3. This is often accomplished by employing separation techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[9] capillary gel electrophoresis (CGE),[10] capillary electrophoresis-sodium dodecyl sulfate (CE-SDS),[11,12] and liquid chromatography (LC).[13] By staining SDS-PAGE gel with fluorescent dyes such as Coomassie blue, silver, or SYPRO Ruby, the separated proteins can be detected and quantified.[14,15] The viral proteins that have been separated by CGE, CE-SDS, and LC are then detected and quantified using UV,[9] laser-induced fluorescence (LIF),[14] and mass spectrometry.[13,16] However, these methods suffer from one or more limitations such as low sensitivity, the need for labeling amino acids with synthetic dyes for detection, and/or, more importantly, require relatively large volumes and high concentrations of rAAV samples.[9,11,16] In this regard, the capillary electrophoresis-based (CE)-western method, offers a solution that requires a minimal sample volume (~3 μL) for analysis, in an automated, and high-throughput system.[9,17,18] The CE-western analysis uses a primary target-specific antibody to detect the protein of interest and a conjugated-secondary antibody against the primary antibody to amplify the chemiluminescence signal for a final readout. Furthermore, it can be used for both purified and unpurified samples, as it is not affected by potential interferences arising from contaminating proteins or DNA impurities present in the sample.[17,19,20] Moreover, antibodies against commonly known protein targets are either commercially available or can be readily manufactured by conventional antibody manufacturing processes. For unknown protein targets, approaches such as amino acid analysis, host cell protein coverage, and LC-mass spectrometry (LC-MS) analysis can be employed to identify a suitable target protein for which an antibody can then be generated. Here we present a case study demonstrating the use of a CE-western platform, Jess®, for a rapid and automated separation of viral capsid proteins in rAAV samples derived from several different serotypes. In order to determine the relative stoichiometry of viral proteins, the separated viral proteins were immunoprobed with a VP-specific antibody, anti-AAV B1. The VP stoichiometry was further correlated with biological activity of samples from AAV serotype 6 (rAAV6). We also explored

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with an orthogonal analysis of viral protein separation and quantification using LC-MS to support the CE-western analysis. In addition to the determination of viral protein stoichiometry of rAAV samples, CE-western was used for the quantification of a baculovirus protein impurity, GP64, in unpurified and purified rAAV samples. Such analyses are often performed by western blot or high-throughput ELISA, which can be highly variable and/or laborious.[17] The analysis of GP64 levels in rAAV samples was equally supported by orthogonal LC-tandem MS (LC-MS/MS) analysis.[13] Furthermore, the GP64 assay was used to demonstrate an efficient clearance of GP64 protein throughout the manufacturing process. In summary, our findings demonstrate that CE-western, owing to its rapid electrophoretic separation and specificity of antibody-based detection, possesses a significant potential in the development of protein-based assays for the analysis of rAAV products.

## RESULTS

### Determination of viral protein stoichiometry by CE-western

#### *Assessment of anti-AAV B1 antibody*

To develop a robust assay for determining the VP ratio of rAAV samples using the CE-western platform, we selected a VP-specific antibody, anti-AAV B1, which has previously been shown to recognize all three viral proteins.[21] VP1, VP2, and VP3, share significant homology, particularly at the C-terminus, which contains the antibody-binding domain against anti-AAV B1.[21] It was thus anticipated that anti-AAV B1 antibody will exhibit a comparable binding affinity to all three viral proteins, thereby enabling a more representative and precise assessment of the VP stoichiometry of rAAV products. Furthermore, this antibody-binding domain is conserved across several serotypes,[22] making it an ideal candidate for developing a serotype-agnostic CE-western assay for determining VP ratio. To verify this, the anti-AAV B1 antibody was initially evaluated against purified AAV6 recombinant VP1, VP2, and VP3 proteins. These proteins were tested individually and in a mixture (1:1:1 ratio) against the anti-AAV B1 antibody. For separation of viral proteins, two different separation modules, 12–230 kDa and 66–440 kDa, were used in the CE-western platform, while the detection was performed using an anti-mouse antibody detection module. The 12–230 kDa module is a low molecular weight, narrow-range separation module more suitable for the separation of smaller proteins, while in comparison, the 66–440 kDa module is a high molecular weight, wide-range separation module most optimal for the separation of relatively larger (e.g., VP1 and VP2) or complex proteins in a mixture. The electrophoretic separation profiles obtained using either separation module showed that peaks for all purified viral proteins (VP1, VP2, and VP3) migrated at ∼107 kDa, ∼92 kDa, and ∼66.5 kDa, respectively, compared with their expected molecular weights of ∼81 kDa, 66 kDa, and 60 kDa, respectively. When tested in the mixture, all peaks were easily separated from one another without any impact on the migration pattern or the apparent molecular weights of any individual viral proteins (Figures 1A and 1B). The observed increase in the apparent molecular weights compared with theoretical molecular weight was partially attributable to the presence of a histidine-tag (his-tag) that was utilized for the purification of these recombinant proteins. A comparable migration pattern has been observed in previous studies.[18] A near baseline separation of all three peaks was accomplished using a 66–440 kDa separation module (Figure 1B), and a partial peak separation, especially between peaks corresponding to VP1 and VP2, was observed when a 12–230 kDa separation module was used (Figure 1A). Consequently, the relative quantification of VP ratio for VP1-3 in the 1:1:1 VP protein mixture revealed a suboptimal recovery (1.0:0.6:1.07) with the 12–230 kDa separation module, whereas a near full recovery (1.0:1.0:0.8) was observed with the 66–440 kDa separation module. Moreover, both separation modules were utilized to evaluate the separation of viral proteins for a representative rAAV6 sample, rAAV-1887. The electropherograms again showed similar separation of viral proteins as observed with purified recombinant viral proteins. The peak migration times for all viral proteins remained consistent as seen with the purified recombinant AAV6 proteins. A significant peak overlap was observed with the 12–230 kDa separation module (Figure 1C), while the 66–440 kDa separation module showed a near baseline separation of all peaks (Figure 1D). Interestingly, regardless of the separation module used, the peak of VP3 in the rAAV6-1887 sample showed a partial peak shoulder that was not observed when purified proteins were evaluated. This may indicate the presence of two closely related molecular species that are migrating under the same peak. Additional efforts, such as including the optimization of loading and separation matrix times or total runtimes, were unsuccessful in achieving significant improvements in the peak separation with the 12–230 kDa separation module (data not shown). Based on these findings, the 66–440 kDa gel system was utilized for further optimization and subsequent analysis of rAAV samples.

#### *Optimization of protein and anti-AAV B1 antibody concentrations*

The anti-AAV B1 antibody and rAAV sample protein concentrations were optimized to achieve a sensitive detection of viral proteins in rAAV samples. To normalize the sample loading amounts, the total protein concentration in samples was determined by the MicroBCA assay. Initially, rAAV-1887 was employed to prepare five different samples with protein concentrations ranging from 20 to 100 μg/mL (corresponds to 3.18E12 capsids/mL – 1.59E13 capsids/mL). As expected, the SDS-PAGE analysis of the undiluted rAAV6-1887 sample showed three distinct viral proteins namely, VP1, VP2, and VP3 (Figure S1A). Subsequently, each of these five samples was titrated against five independent concentrations (1–10 ng/mL) of anti-AAV B1 antibody. The samples were then analyzed by CE-western assay and the peak areas of the separated viral proteins were used to calculate the VP3/VP1 ratio. Based on the results, a consistent VP3/VP1 ratio was observed for samples with a protein concentration of 60–100 μg/mL (corresponds to 9.53E12 capsids/mL–1.59E13 capsids/mL), irrespective of the concentrations of anti-AAV B1 used (Figure S1B). Protein concentrations higher than 100 μg/mL were not tested to avoid the possibility of capsid particle aggregation, while for protein concentrations lower than 60 μg/mL, the VP3/VP1 ratio increased as the protein concentration was lowered. This is because a lower than optimal signal

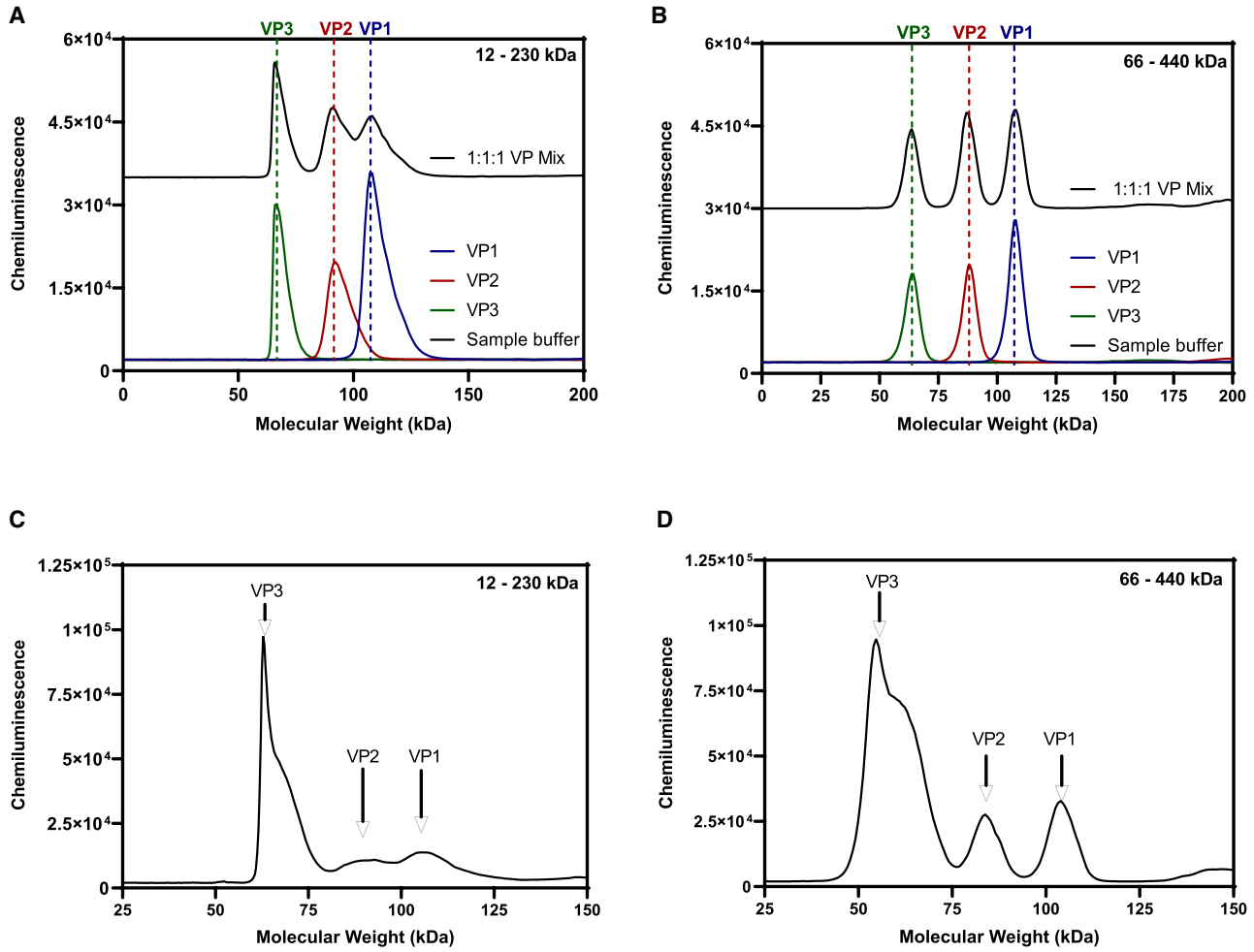


**Figure 1. Separation and relative quantification of viral proteins using CE-western**

The CE-western electropherograms of purified recombinant VP1, VP2, and VP3 proteins, their 1:1:1 mixture (A and B) and a purified rAAV6-1887 (C and D) using two different separation modules (A and C) 12–230 kDa and (B and D) 66–440 kDa are shown. The position and identity of viral protein peaks in the electropherogram are indicated in each figure.

would result in its inaccurate estimation of VP1, thereby enhancing the VP3/VP1 ratio. For protein concentrations between 60 and 100 μg/mL, the VP3/VP1 ratio was optimal with 2.5 ng/mL of anti-AAV B1 antibody. Based on these findings, for subsequent analyses, the concentrations of protein and anti-AAV B1 antibody were set as 80 μg/mL and 2.5 ng/mL, respectively.

### *Analysis of rAAV samples using CE-western and correlation with biological activity*

The optimized conditions of CE-western analysis were employed to analyze six purified rAAV6 samples. These samples were produced using different manufacturing processes, such as differences in manufacturing scale (rAAV-623, rAAV-313, rAAV-705), or alternative capsid design (rAAV-1281, rAAV-1296), and change in the concentration (rAAV-1887), but they shared the same AAV genomic cassette. All six rAAV6 samples showed a near baseline separation of viral protein peaks with apparent molecular weights of ∼110 kDa, ∼88 kDa, and ∼64 kDa, respectively (Figure 2A). Certain samples, specifically rAAV-1281 and rAAV-1296, exhibited a minor shift in the migration times of the viral protein peaks in comparison to the remaining samples. For each sample, the area under each peak was analyzed by the Gaussian peak fitting and the % peak area of each viral protein was determined. The VP stoichiometry for each sample was then determined by normalizing the peak areas of VP2 or VP3 to that of VP1 (Figure 2B). Based on these results, six samples were shown to differ significantly in their normalized VP3 content while the normalized VP2 content remained relatively consistent. Furthermore, the VP ratios obtained for all six samples remained consistent when measurements were performed over several independent experiments (Figure S1C). This suggested that differences in the manufacturing process may result in variations in the VP stoichiometry of the AAV capsid, which may have an impact on its stability and

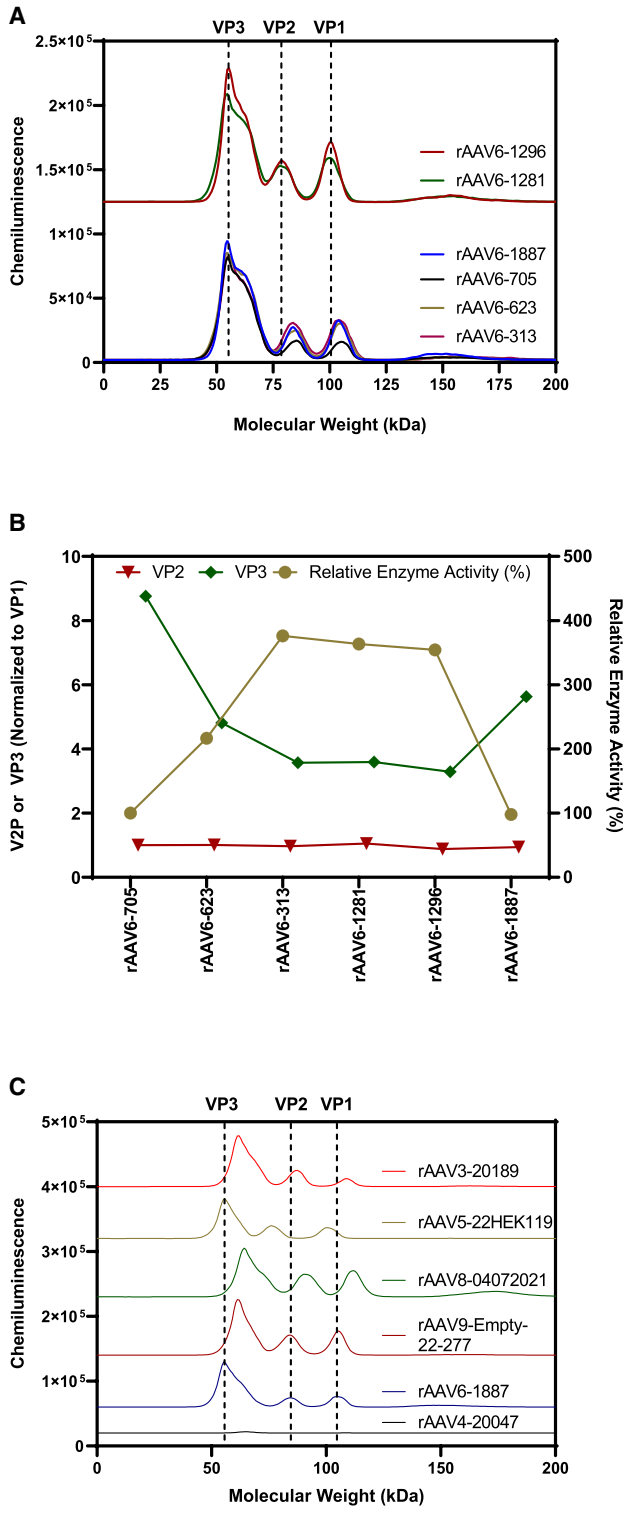


**Figure 2. Determination of VP stoichiometry of rAAV samples**

(A) CE-western electropherograms of six rAAV6 samples showing near baseline separation of viral proteins are presented. (B) The inverse relationship of normalized VP2 and VP3 of six rAAV6 samples and their relative enzyme activity is demonstrated. (C) The application of CE-western assay as a platform assay for the analysis of rAAV samples from different serotypes (AAV3, AAV4, AAV5, AAV6, AAV8, and AAV9) is also presented.

overall biological activity.[23,24] In order to evaluate the impact of VP stoichiometry on biological activity, all six rAAV6 samples were evaluated for their functional enzyme activity in HepG2 cells as previously described.[25] After treatment of HepG2 cells with the samples, the cell culture supernatant was collected and tested for α-galactosidase A enzyme activity. The results were expressed as % of the enzyme activity of the sample relative to that of rAAV6-705 set at 100%. All rAAV6 samples showed an enzymatic activity ranging from 100% to 400% (Figure 2B) relative to rAAV-705. It was evident that the enzyme activity had an inverse relationship with the normalized VP3 content in AAV capsids. However, the impact of the normalized VP2 content in AAV capsids on their biological activity could not be confirmed, as VP2 content across samples did not vary significantly. The impact of reduced VP3 content and thereby an increase in VP1 content has previously been shown to result in an increase in biological activity.[23]

In order to confirm that CE-western can be used as a platform assay for determining VP stoichiometry, we tested several commercially available or in-house produced rAAV samples of different serotypes using the optimized conditions described earlier. The samples selected were composed of various serotypes, including AAV3, AAV4, AAV5, AAV8, and AAV9, and were produced using either HEK293 or Sf9/baculovirus platforms. All test samples, except for rAAV4-20047, showed similar electrophoretic migration patterns and peak separations for all three viral proteins as observed in rAAV6 samples (Figure 2C). Since the amino acid sequence from AAV4 capsid viral proteins do not contain the antibody-recognition sequence against anti-AAV B1 antibody, the lack of chemiluminescent signal and peak separation for rAAV4-20047 was expected.[21,22] Some of the test samples showed a minor shift in migration times for peaks corresponding to the viral proteins compared with those observed for rAAV-1887 and were attributed to differences in molecular sizes of viral proteins in these samples. Furthermore, regardless of the serotype, all samples showed a partial peak shoulder for the VP3 protein peak, as observed earlier. Using the peak areas, the VP ratios (normalized to the peak areas of VP) calculated for each sample ranged from 0.92–2.36 for VP2 and 2.66–10.25 for VP3 (Table 1). These results demonstrate that the anti-AAV B1 antibody-based CE-western assay can be used to determine VP stoichiometry of rAAV samples from different serotypes.

### *Determination of VP stoichiometry by LC-MS*

Liquid chromatography coupled with mass spectrometry (LC-MS) is a commonly used analytical tool for the separation, identification, detection, and quantification of biomolecules. The application of LC-MS to the separation of AAV capsid viral proteins of different serotypes has largely remained underexplored. Recently Zhang et al.[13] and Lam et al.[26] demonstrated the versatility of this approach for the separation and identification of viral proteins from rAAV samples

**Table 1. VP stoichiometry of rAAV samples from different serotypes**

| Sample name | VP1 | VP2/VP1 | VP3/VP1 |
|---|---|---|---|
| rAAV6-1887 | 1.0 | 0.91 | 5.45 |
| rAAV9-2898000 | 1.0 | 1.17 | 3.23 |
| rAAV8-04072021 | 1.0 | 1.17 | 2.66 |
| rAAV5-22HEK119 | 1.0 | 1.27 | 4.19 |
| rAAV3-20189 | 1.0 | 2.36 | 10.25 |

of different serotypes. Nonetheless, LC-MS methods typically require significant optimization for each serotype, and a particular method may not be suitable for evaluating rAAV samples across multiple serotypes. In this regard, a reverse-phase high-performance liquid chromatography coupled with the mass spectrometry (RP-HPLC/MS) assay was developed to separate and quantify the stoichiometry of viral proteins in rAAV6 products. The method for separation of viral proteins was based on the differential hydrophobicity of three viral proteins under denaturing conditions and their subsequent migration onto a reverse-phase column under the influence of a water/acetonitrile gradient (Table S2). The eluted peaks were then detected using a high-sensitivity mass spectrometry detector. For this purpose, rAAV6-1887 was utilized as a representative sample for the development and optimization of the assay. During the method development, a variety of reverse-phase columns, including Waters C4, C8, C18 solid core (300 Å), HALO C4, and diphenyl wide core (1000 Å), were evaluated against the water/acetonitrile gradient. Their performance was compared with respect to the resolution of all three viral protein peaks. The effect of the presence of formic acid or difluoroacetic acid in the mobile phase was also evaluated in order to assess the improvement of the separation profiles of viral proteins. The optimal separation of viral protein was achieved by utilizing the C4 Waters column and 0.1% difluoroacetic acid in the water/acetonitrile mobile phase system. The chromatographic profiles obtained for all six samples using the optimized method (Figure 3A) demonstrated a successful separation of peaks for all viral proteins when the concentration of acetonitrile in the mobile phase was between ∼31% and 35%. However, in comparison with the CE-western assay, the peaks of VP1-3 by LC-MS were not separated at the baseline. As in the case of the CE-western analysis, a minor shift in the retention times for three peaks for some rAAV6 samples was observed. The results indicated comparable separation profiles of viral proteins in all six samples (Figure 3A). Further analysis of the chromatographic profiles showed the presence of peaks for the VP1/2 variant and the VP3 clip for all six samples. VP3 clip (A204-D590) is a truncated viral protein that is formed as a result of cleavage at Asp(D)590-Pro(P)591 and is most likely caused by high temperature and acidic conditions employed during the chromatographic analysis of these samples.[13,16] The VP3 clip (∼4%–7% estimated on deconvoluted mass signal intensity) was integrated and reported with the VP3 peak as a combined peak group. As seen in previous studies, the VP1/2 variant (∼5%–8% estimated on deconvoluted mass signal intensity) and VP3 variant (A212-L736) were also detected,[13,16] but were not successfully resolved as separate peaks. The identification of each major viral protein, VP1, VP2, and VP3, was successfully verified through the deconvolution of the corresponding peak resulting in a molecular mass of ∼81.3 kDa (Figure 3B), ∼66.1 kDa (Figure 3C), and ∼59.5 kDa (Figure 3D), respectively. The intact mass of each major viral protein was found to be within ±5 Da (<10 ppm) of the theoretical mass based on the primary amino acid sequence. Once the peaks for each protein were confirmed, they were manually integrated to determine the area under the peak. VP stoichiometry for each sample was then calculated by normalizing the peak areas of VP2 and VP3 peak against the peak area of VP1 for that sample. The VP stoichiometry determined using LC-MS data for six rAAV6 samples was compared with the data obtained using CE-western analysis. Overall, the comparison of both datasets (Figure 3E) showed that the VP ratios obtained by CE-westerns were quite comparable to those obtained from the LC-MS method. Additionally, the VP ratios of all samples were in close agreement with the predicted stoichiometry of 1:1:10 for an AAV capsid. These results suggested that the VP stoichiometry determined by the CE-western-based assay was well supported by the orthogonal analysis using an LC-MS-based assay.

### Quantification of residual baculovirus protein, GP64, by CE-western

The residual host cell protein impurities arising from expression systems such as HEK293 and Sf9/baculovirus are typically measured using a commercial or target-specific ELISA,[27] while the residual baculovirus protein impurities in rAAVs samples produced in Sf9/baculovirus expression systems have been measured using western blots. *Autographa californica nucleopolyhedrovirus* (AcMNPV) is the most commonly used baculovirus to produce recombinant biologics including AAV using the baculovirus expression vector system (BEVS).[28] The budded virus consists of 34 viral proteins and 11 host cell proteins, including three major proteins: GP64 protein, the capsid protein VP39, and the DNA binding protein P6.9 (AC100).[29] Among these, GP64 is a major component of the viral protein envelope and is an essential viral protein required for the entry and cell-to-cell transmission of baculovirus (AcMNPV) *in vitro* and *in vivo*. It allows for the envelope-phosphoglycoprotein-mediated fusion of viral and host endosomal membranes leading to virus entry into the host cell for infection.[29] Due to its high relative abundance, GP64 protein is an ideal representative baculovirus protein to monitor impurity levels in the rAAV products produced by the baculovirus/Sf9 cell production system.[29,30]

#### *Selection of anti-GP64 and rGP64 standard for CE-western*

In order to develop a CE-western assay for the baculovirus protein GP64, a mouse monoclonal anti-GP64 antibody, AcV5, which targets the GP64 envelope protein of the AcMNPV baculovirus, was employed. In order to verify if anti-GP64 antibody specifically binds to GP64, the enrichment of GP64 using anti-GP64-agarose beads from a clarified harvest from an AAV production process and purified drug product was attempted, but the efforts were unsuccessful due to overall low amounts of the GP64 present in these samples. Alternatively, GP64 was enriched from a clarified harvest sample using ion-exchange chromatography columns, namely Sartobind and

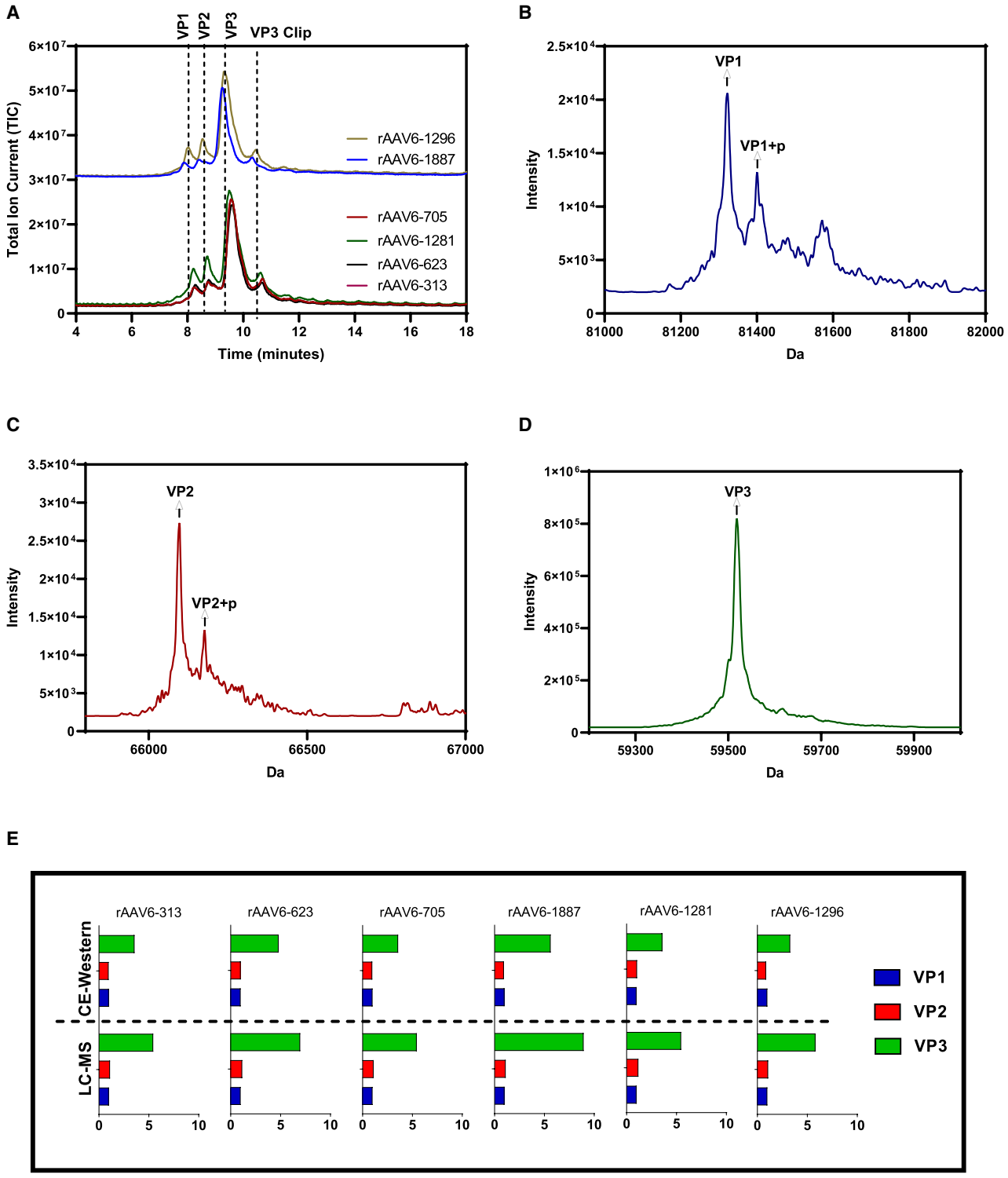


**Figure 3. Determination of by VP stoichiometry of rAAV6 samples by LC-MS and comparison to CE-western**

(A) LC-MS chromatograms of six rAAV6 samples demonstrating a successful separation of individual viral proteins. The deconvoluted total ion chromatograms of (B) VP1, (C) VP2, and (D) VP3 of rAAV6-1887 indicating both main peaks and their major phosphorylated peaks are shown. (E) Consolidated comparison of relative quantitation of viral protein ratios across all samples tested using CE-western and LC-MS is presented.

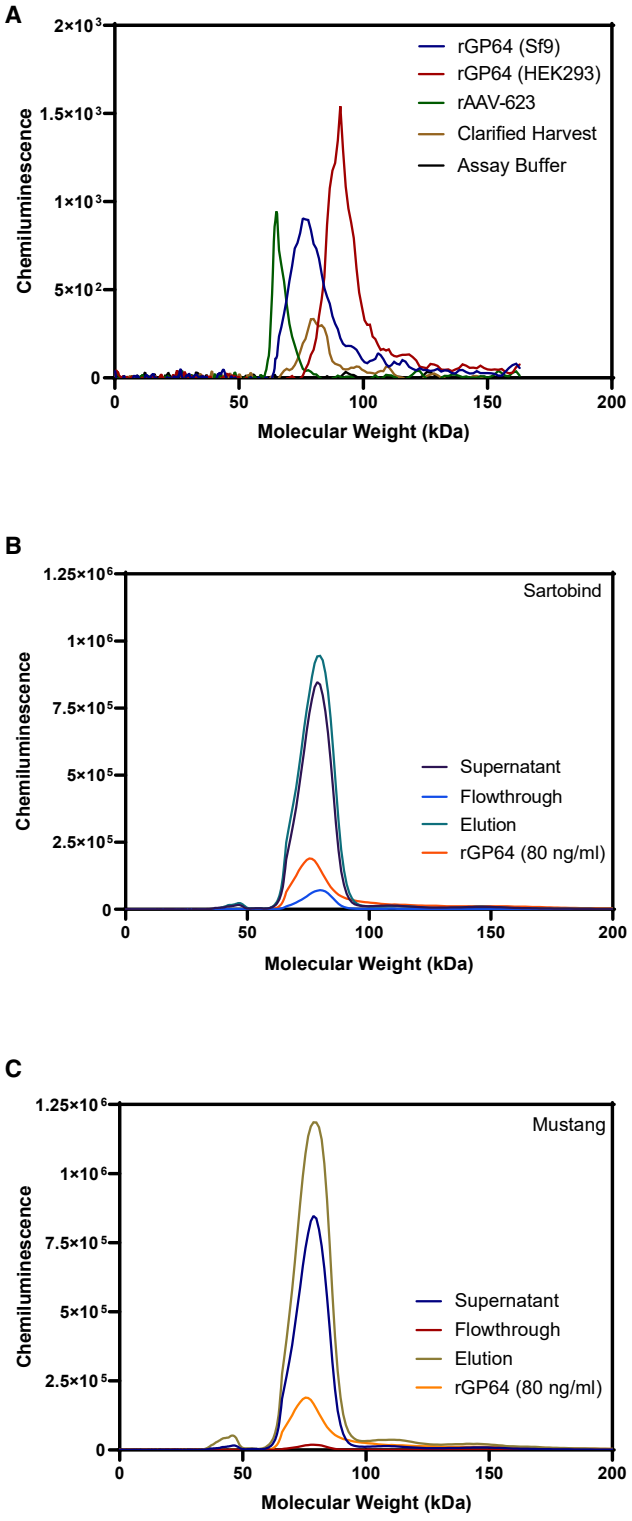


**Figure 4. CE-western analysis for GP64 protein using anti-GP64 AcV5 antibody**

(A) A commercially available purified rGP64 protein produced in Sf9 cells and HEK293 were run along with a clarified harvest and purified final rAAV6 drug product from an AAV production. In addition, GP64 was enriched from a clarified harvest material using (B) Sartorius and (C) Mustang ion-exchange columns.

Mustang, using the procedure as described in the literature.[31] The binding affinity of GP64 from clarified harvest to these columns was found to be weak, therefore no stringent wash steps were included. The lane-view (virtual blots) images of in-process and enriched GP64 samples obtained during CE-western analysis are presented in Figures S2A and S2B, respectively. In addition, the elution fractions obtained from the Sartobind and Mustang columns were analyzed either for total protein by SDS-PAGE (Figures S3A and S3C) or by western blot (Figures S3B and S3D) using anti-GP64 antibody. The enriched fractions obtained from both columns revealed the presence of GP64 as a single distinct band at ~70 kDa, thereby confirming the specific binding of the anti-GP64 antibody against its target protein, GP64. Based on these results, anti-GP64 antibody can be used in the CE-western assay to test residual levels of GP64 in purified and unpurified AAV samples.

It was imperative to establish a suitable rGP64 protein standard for development of a sensitive assay for quantification of residual levels of GP64 in AAV samples. In this regard, two commercially available purified rGP64 proteins expressed in HEK293 and Sf9/baculovirus cells were used. GP64 exists as a trimer protein (~175 kDa) in the native form. However, under denaturing conditions (during the CE-western assay), it was predicted to migrate with a mass of ~58.6 kDa as a monomeric protein. However, electropherograms obtained for rGP64 proteins showed that both HEK293-produced and Sf9/baculovirus-produced rGP64 migrated at a much higher apparent molecular weight of ~91 kDa and ~75 kDa, respectively (Figure 4A). The presence of an affinity-tag in each recombinant protein along with any post-translational modifications could have resulted in the migration at a high apparent molecular weight in the CE-western assay. Furthermore, a clarified harvest and a purified vector product (rAAV6-623) produced in the Sf9/baculovirus system, were also tested along with these two recombinant rGP64s. It was observed that the GP64 migrated at ~65 kDa and ~75 kDa in the purified AAV product and the unpurified clarified harvest sample, respectively (Figure 4A). This suggested that the same antibody could be used to detect GP64 in different in-process or purified samples. Furthermore, this indicated that there could be a truncation or loss of the post-translational modifications on the rGP64 protein during the purification of purified AAV product from the clarified harvest. These results further indicate that the source of rGP64 had a significant impact on the migration and apparent molecular weight in the CE-western assay. Moreover, the unpurified harvest, enriched GP64 from the unpurified harvest using Sartobind (Figure 4B) or Mustang (Figure 4C), and purified rGP64 from the Sf9 expression system were tested in the CE-western assay. All samples showed a single peak at ~70 kDa, indicating that the antibody detects GP64 regardless of

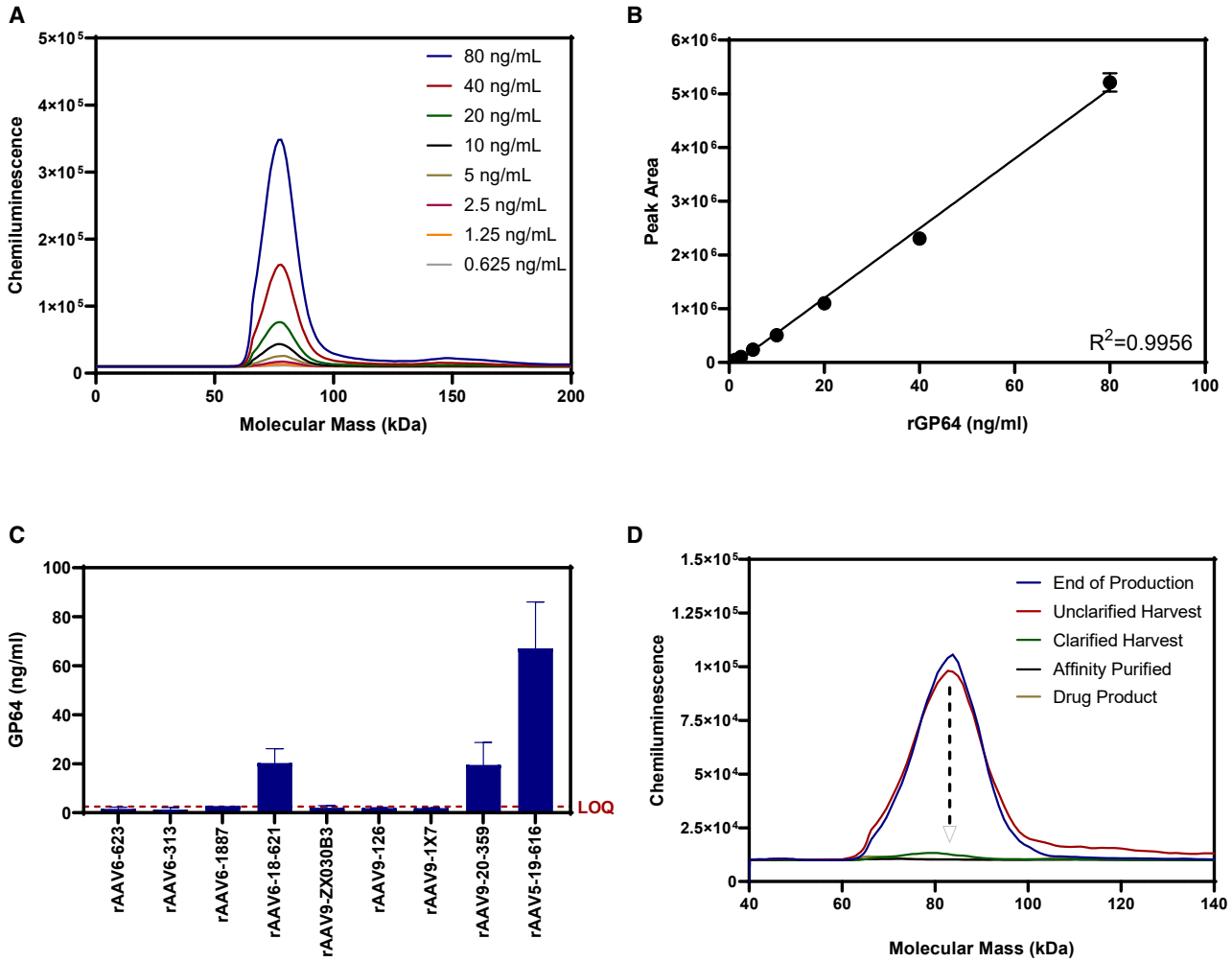


**Figure 5. Quantification of residual levels of GP64 in rAAV samples and demonstration of GP64 clearance using CE-western assay**

The electropherograms (A) and subsequent linear regression analysis (B) of area under the peak obtained for different concentrations of rGP64 (0.625–80 ng/mL) are presented. The error bars correspond to mean ± SD obtained from duplicate points ($N = 2$). (C) Residual GP64 levels in several rAAV products from different serotypes (produced in both Sf9 and HEK293 cells, see Table S1) were quantified using the CE-western assay. The red dotted line in the graph corresponds to LOQ (2.5 ng/mL) of the assay while the error bars correspond to mean ± SD obtained from independent experiments with duplicate injections in each run (rAAV6-623, $N = 15$; rAAV6-313, $N = 6$; rAAV6-1887, $N = 1$; rAAV6-18-621, $N = 2$; rAAV9-2X030, $N = 2$; rAAV9-136, $N = 2$; rAAV9-1X7, $N = 2$; rAAV9-20-359, $N = 1$; and rAAV5-19-616, $N = 2$). (D) The clearance of GP64 protein impurity levels from the manufacturing process was demonstrated by measuring GP64 levels for samples from different steps within the manufacturing process. The in-process samples including clarified harvest, affinity-purified, and drug products were run alongside end of production cell harvest and the reduction of GP64 levels was demonstrated (indicated by the arrow).

the source from which it was enriched or purified. This further suggests that the differences in migration and apparent molecular weights seen for purified and unpurified AAV earlier may be primarily due to post-processing of samples during the production process.

### *Quantification of residual GP64 levels in rAAV samples using CE-western*

A standard curve ranging from 0.625 ng/mL to 80 ng/mL was prepared by a 2-fold serial dilution of a stock solution of the insect cell-produced rGP64. The standards were analyzed by CE-western using a 12–230 kDa separation module as described in the methods section. In all rGP64 standard electropherograms, a single major peak was observed corresponding to GP64 (Figure 5A). The area under the peak corresponding to GP64 increased with increasing concentration of the rGP64 standards (Figure 5A). The electrophoretic data for all standards was analyzed by Gaussian curve fitting and the area under the peak for rGP64 was plotted against the concentration of rGP64. The standard curve fit showed a linear response of the peak area vs. rGP64 concentration with a coefficient of determination ($R^2$) >0.99 (Figure 5B). The reproducibility of the standard curve was evaluated over several independent experiments using different commercial batches of the rGP64 standard, independent sample

replicates, multiple analysts, and multiple instruments over several days ($N$ = 17). The peak area responses of all rGP64 standards remained consistent with a low variance (CV < 35%) across all data points. As GP64 concentration further reduced, the GP64 peak in the electropherogram was still detectable above the background noise (up to 0.05 ng/mL). The peak area, however, did not decrease in a linear fashion, and significantly high variability (CV 35%–95%) was observed in the peak areas at these concentrations. Based on these results, the limit of quantitation (LOQ) for this assay was set to 2.5 ng/mL (CV <25% and signal/noise ratio >800). Any results below this limit were obtained by extrapolation of the standard curve and considered as estimates only as long as the GP64 peak could be detected and easily distinguished from the baseline noise. To ascertain whether the GP64-based CE-western assay is capable of quantifying residual levels of GP64 in purified samples, a variety of purified rAAV samples from diverse serotypes (AAV5, AAV6, and AAV9) were tested. The analysis of these samples by CE-western revealed that the residual GP64 levels in the purified samples ranged from ∼1 ng/mL to 80 ng/mL (Figure 5C). The assay demonstrated the ability to detect and quantify GP64 levels as low as 2.5 ng/mL with high precision and accuracy. With the western blot-based assay, such low detection and quantification limits were not achieved (see Figure S4) further supporting superiority of CE-western analysis over the traditional western blot analysis. Three samples (rAAV6-18-621, rAAV9-20-359, rAAV5-19-616) showed relatively high GP64 levels ranging from ∼10 to 80 ng/mL, while all other samples (excluding rAAV9-ZX030B3) showed GP64 levels below the quantification limit (estimated ∼1–2 ng/mL) as the GP64 peak was still detectable. Since GP64 is not expressed by HEK293 cells, the sample, rAAV9-HEK-ZX030B3, did not show the peak corresponding to GP64 that was distinctly separate from the baseline noise. For this sample, a significant amount of baseline noise with multiple minor peaks (<< 0.625 ng/mL) was observed. This further confirmed that the anti-GP64 antibody did not reliably detect GP64 in HEK293 samples and was highly specific for detection of GP64 produced from baculovirus origin only. These findings further validated the utilization of GP64-based CE-western assay as a platform assay for detecting and quantifying residual baculovirus impurity levels in rAAV samples from diverse serotypes.

#### *Clearance of residual baculovirus protein GP64 by CE-western assay*

In order to assess the performance of a manufacturing process with regard to clearance of the baculovirus protein GP64, several samples were collected at different stages of the manufacturing of the product rAAV9-JRP028 including clarified harvest, affinity-purified, eluted final product, and end of production cells. These samples were evaluated for their GP64 levels in the CE-western assay using optimized conditions as described above. As expected, earlier stages of the manufacturing process (clarified harvest) or end of production sample, showed relatively higher concentrations of GP64 levels, while later stages of purification, affinity-purified and final purified product, contained substantially lower amounts (<0.6 ng/mL) of GP64 (Figure 5D). During the manufacturing process, an overall >6-log clearance of GP64 (Table S3) from unclarified harvest to purified drug product was achieved. As estimated from CE-western data, GP64 migrated at progressively lower apparent molecular mass in samples throughout the process steps (∼79 kDa for clarified harvest, ∼71 kDa for affinity-purified, and ∼66 kDa for the final drug product). This suggests that during the initial stages of production, GP64 may be expressed as a significantly larger protein complex or may be associated with other proteins, thereby leading to its migration at a higher molecular weight than expected. Subsequent processing steps may result in truncation of the protein complex or removal of post-translation modification resulting in reduced apparent molecular weights. Overall, the results presented here demonstrate the effectiveness of the CE-western-based GP64 assay for demonstrating the clearance of residual baculovirus impurities in the final product.

#### *Analysis of GP64 levels by LC-MS/MS analysis*

In order to verify the levels of GP64 or other baculovirus proteins, a select group of purified rAAV6 samples, namely rAAV-623, rAAV6-313, and rAAV6-1887, were evaluated by LC-MS/MS analysis. The samples were proteolytically digested and analyzed using the SWATH LC-MS/MS approach. The MS data were searched against a database containing AAV6 viral proteins, *Spodoptera frugiperda* and *Autographa californica* nuclear polyhedrosis virus (AcMNPV) proteins. None of the tested samples showed the presence of any baculovirus proteins including GP64 above the detection limit of the assay (1 ng/mL). These results further confirmed the residual GP64 levels determined by the CE-western assay.

## DISCUSSION

The clinical advancement of rAAV-based gene therapies requires meticulous analysis and monitoring of the purity of drug products to ensure product quality and patient safety. In this regard, VP stoichiometry of AAV capsids along with an overall assessment of product-, process-, and host cell-related impurities play an important role in determining overall purity of the rAAV product as well as ensuring consistency of capsid particles produced during manufacturing.[6] AAV capsids are composed of 60 individual protein subunits of three viral proteins, VP1, VP2, and VP3, with a theoretical ratio of 1:1:10.[24] Capillary-electrophoresis-based techniques have proven to be particularly advantageous in fast and efficient separation of viral proteins. However, they are frequently constrained by the need for higher sample concentrations and test sample volumes. To overcome these challenges, a CE-western platform was developed that employs an anti-AAV B1 antibody capable of detecting all three viral proteins. Purified rAAV6 proteins and rAAV samples were tested to confirm that a 66–440 kDa separation module in the CE-western assay was optimal for efficient separation of viral proteins in AAV samples. A serotype-agnostic protein quantitation by MicroBCA was used to normalize the sample concentration in the assay. Furthermore, the assay conditions were standardized for protein and antibody concentrations to obtain consistent VP ratio measurements. In this assay, rAAV samples from multiple serotypes were assessed and their VP stoichiometry was determined. Additionally, an orthogonal LC-MS assay was developed to determine VP stoichiometry for rAAV6 samples.

Despite the lack of a near baseline separation, the LC-MS assay was able to determine the relative ratio of viral proteins in rAAV6 samples. The assay was used to confirm the identity of all three major AAV6 viral proteins, but it was also capable of detecting additional VP variants previously seen by other groups.[13,16] In general, the ratios of VP1, VP2, and VP3 determined for rAAV samples by either CE-western or LC-MS methods were in close agreement with the theoretical ratio of 1:1:10 in an AAV capsid. The variation in VP3 (and hence VP1) content in AAV capsid was shown to significantly impact the biological activity of these AAV samples, further bolstering the earlier reports where VP stoichiometry was shown to impact the infectivity and biological activity of rAAV samples.[23,24]

In addition to determining VP stoichiometry, an assessment of the purity of AAV products was performed by measuring residual levels of a baculovirus protein GP64. The clearance of these protein impurities from rAAV products arising from the host cell expression system is imperative to alleviate concerns regarding the immunogenicity arising from impurities present in such products.[32] In contrast to commonly used western blot for determining levels of protein impurities, the CE-western assay described in this work provides a faster and more quantitative determination of GP64 levels in rAAV samples of diverse serotypes.[33] During the assay development, an Sf9-produced recombinant GP64 protein was used to generate a standard curve, and an anti-GP64 monoclonal antibody was used for the detection and quantification of GP64 in test samples. After initial optimization efforts, several rAAV samples from different serotypes were evaluated in this assay. Commercially available purified samples of rAAV5-19-616, rAAV6-18-621, and rAAV9-20-369 exhibited significantly higher levels of GP64, as these samples were produced by a CsCl-based one-step purification system. On the other hand, remaining samples showed much lower levels of GP64 (<5 ng/mL), as these samples were produced via multiple steps of purification, including affinity purification and polishing steps, which would further remove impurities such as GP64 from the purified product. Additionally, several in-process rAAV samples were tested for their GP64 levels in the CE-western assay in order to demonstrate the clearance of GP64 protein impurity from the process. Although manufacturing controls such as column purification, polishing, and viral filtration are in place, analytical tools such as CE-western are critical for quantitative assessment and subsequent verification of the clearance of such impurities from the final product.[7,34,35]

Overall, our studies demonstrate that the CE-western platform combines capillary-electrophoresis-based robust separation with antibody-specific detection to achieve accurate quantification of protein-based CQAs of rAAV products. It is imperative to conduct a comprehensive analysis of purity of rAAV products by determining viral protein stoichiometry and quantifying residual levels of impurities in the quality assessment of rAAV products. More importantly, the adoption of novel technologies, such as CE with western detection, facilitates the rapid development of safer, high-quality, and more efficacious rAAV gene therapies.

## MATERIALS AND METHODS

### Materials

The commercially purchased rAAV samples from Virovek (Hayward, CA, USA) and those produced in Sangamo Therapeutics[36] are presented in Table S1. Purified recombinant His-tagged AAV6 viral proteins (VP1, VP2, VP3 recombinant protein set catalog #72006) and anti-AAV VP1/VP2/VP3 mouse monoclonal antibody (B1, supernatant) from Progen (Heidelberg, Germany) were used per the manufacturer's instructions. Unless otherwise specified, all samples were used as neat. When needed, samples were concentrated using Amicon Ultra centrifugal filters (10 kDa MWCO; Sigma Aldrich, St. Louis, MO, USA), or diluted in the relevant formulation buffer to achieve a desired concentration. AcMNPV Envelope glycoprotein rGP64 (Met1-Thr481, His-tag) produced in Sf9 and HEK293 cells was purchased from SinoBiological Inc. (Waye, PA, USA). The precast Bolt 4%–12%, Bis-Tris gels (1 mm) mini gels, Bolt 4X LDS sample buffer, Bolt 10X sample reducing agent, Bolt MOPS SDS running buffer, PVDF membrane (0.45 μm), Pageruler Prestained Protein Ladder (10–180 kDa), and SuperSignal West Pico Peroxide Solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-GP64 primary mouse monoclonal antibody (AcV5) and Donkey Anti-Mouse IgG H&L secondary antibody (HRP) from Abcam (Cambridge, UK), were used per manufacturer's instructions. MS-grade difluoroacetic acid (DFA) and ACQUITY UPLC Protein BEH C4 column (300 Å, 1.7 μm, 2.1 mm × 100 mm) were obtained from Waters Corporation (Milford, MA, USA), while acetic acid, water, and acetonitrile were Optima LC/MS grade and purchased from Fisher Scientific (Hampton, NH, USA).

### Methods

#### *SDS-PAGE and western blots*

Precast Bolt 4%–12% Bis-Tris, 1.0 mm, mini protein gels from Thermo Fisher were used in the Bolt MOPS SDS running buffer (10 and 12 wells). The denatured samples (rGP64 standards and rAAV) were prepared by heating them in Eppendorf tubes for 5 min at 95°C. Next, 20 μL of heat-denatured sample was loaded into each lane of a precast gel. The gels were run at 200 V until the dye-front reached the bottom of the gel and washed with deionized water for 5 min. The gels were either stained with SimplyBlue SafeStain for a duration of 20 min and de-stained with water, or they were utilized to transfer to a PVDF membrane using iBlot 2 transfer stacks in iBlot 2 transfer system as per manufacturer's instructions. The primary antibody (anti-GP64, 1:2,000 dilution), secondary antibody (Donkey Anti-Mouse IgG H&L [HRP], 1:5,000 dilution), and SuperSignal West Pico Peroxide substrate were used to detect GP64 protein in test samples. The gels were imaged using Odyssey DLx Imaging System (LI-COR Biotechnology, Lincoln, NE, USA) while the membranes were imaged using a Chemidoc Imaging System (Bio-Rad, Hercules, CA, USA).

#### *Biological activity of rAAV6 samples*

The biological activity elucidated by rAAV6 samples was measured in terms of the induction of the expression and subsequent activity of

alpha-galactosidase A in the human hepatocellular carcinoma (HepG2) cell line by a procedure similar to previously described.[25] After the infection of the cells with rAAV6 samples, the cells were incubated and the supernatant was analyzed for the activity of the expressed enzyme against a fluorescence substrate. The fluorescence signal produced from the enzymatic reaction was measured using a SpectraMax i3x (Molecular Devices, San Jose, CA). Then the dose-response curves were analyzed and fit into a 4-parameter logistic regression analysis using SoftMax-Pro Software (Molecular Devices). The biological activity was expressed as a % relative enzyme activity against a reference sample, rAAV6-705, which was set at 100%.

#### *Enrichment of rGP64 from an Sf9 cell culture clarified harvest*

An enrichment of GP64 from an Sf9 cell culture clarified harvest was performed as described in the literature.[31] In short, Sartobind Lab IEX S15 (Sartorius, Göttingen, Germany) or Mustang S XT Acrodisc (Pall Life Sciences, Ann Harbor, MI, USA) was equilibrated with 5 mL of 0.2 N NaOH and 10 mM Tris, pH7.4, 1 mM EDTA. A clarified harvest of an rAAV production run was bound to the column and washed with 10 mM Tris, pH7.4 buffer containing increasing concentrations of NaCl (20–100 mM), followed by elution with 10 mM Tris, pH 7.4, 1 mM EDTA, and 150 mM NaCl. The elution fractions were then analyzed by SDS-PAGE, stained for total protein content, and immunoblotted using anti-GP64.

#### *CE-western analysis of rAAV samples for the determination of VP stoichiometry and GP64 protein*

All CE-western assays were performed using capillary electrophoresis-based separation modules and associated reagents on an automated CE-western system, Jess (BioTechne, Minneapolis, MN, USA). For the determination of the VP stoichiometry, if needed, rAAV samples were concentrated ∼4–5x and their protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific). The concentrated samples were then adjusted to the desired concentration using the relevant formulation buffer or 0.1X sample buffer. The separation of rAAV viral proteins, namely VP1, VP2, and VP3, was performed using a 12–230 kDa or 66–440 kDa separation module (containing 25 capillaries each). In each experiment, all samples were tested in duplicate or triplicate (one replicate per capillary). For analysis using the 12–230 kDa separation module, peak separation was optimized to set stacking matrix load time (12.0 s) and sample load time (6.0 s), while for 66–440 kDa separation cartridge, standard assay run conditions were used. Then rAAV samples were mixed with a 5x Fluorescent (FL) Master Mix and heat denatured for 5 min at 95°C and cooled to room temperature before loading in the separation module. Anti-AAV VP1/VP2/VP3 mouse monoclonal antibody (B1, supernatant) and anti-mouse detection module were used for the binding and detection of viral proteins. After the completion of a run, the electropherograms were annotated for each of viral protein peaks and then analyzed using Compass Software using Gaussian peak fitting. The peak areas of each peak were used for the determination of VP stoichiometry and were represented as a ratio normalized to the peak area of VP1.

For the quantification of residual GP64 levels in rAAV samples, a GP64 standard curve was prepared initially. For that, the lyophilized rGP64 was first reconstituted to 250 μg/mL as recommended by manufacturer, and then it was serially diluted (2-fold) using 0.1X sample buffer to obtain rGP64 standards of different concentrations ranging from 0.625 ng/mL to 80 ng/mL. Then the rGP64 standards and rAAV samples, both in-process and purified, were heat denatured as described earlier. The separation was performed using a 12–230 kDa separation cartridge (25 capillaries) using the standard assay conditions as per the manufacturer's instructions. For the immuno-detection of GP64, anti-GP64(AcV5), a primary mouse monoclonal antibody at a 1:50 dilution was used along with an anti-mouse detection module. The peak annotations and analysis of electropherograms was performed using Compass Software using Gaussian peak fitting. The area under the peak of the GP64 for each standard was used to generate a linear regression curve fit. The residual GP64 levels in rAAV samples were then calculated from the area under the peak of the electropherogram and interpolation it against the GP64 standard curve.

#### *LC-MS analysis for determination of VP stoichiometry of rAAV6 samples*

The LC-MS assay for the determination of VP stoichiometry of rAVAV6 samples was performed by reverse-phase liquid chromatography using a Bruker Elute UHPLC coupled to a Bruker ESI-QTOF Impact II MS (Bruker, Billerica, MA, USA). Previously published protocol[13] was further optimized to obtain efficient separation of VP proteins in rAAV6 samples. All samples ($7.00 \times 10^{11}$ vg) were buffer exchanged against the formulation buffer using Amicon Ultra centrifugal filters, acidified with acetic acid, and centrifuged to remove any particulates. The resulting samples (∼50 μL) were then transferred to independent HPLC vials and placed in an autosampler set at a constant temperature of 6°C. The chromatographic separation was achieved using a Waters C4 column (set at 80°C) under a linear gradient of mobile phase A (100% water, 0.1%DFA) and B (100% acetonitrile, 0.1% DFA) mobile phase (Table S1). The samples were tested as two independent preparations and 10 μL of each preparation was injected onto the column connected to the Bruker HPLC and Impact II MS system. The MS acquisition parameters included endplate offset (500 V), capillary voltage (4.5 kV), nebulizer gas pressure (2.5 bar), gas flow rate (6 L/min), source temperature (220°C), collision-induced dissociation (CID) energy (50 eV), and scan range (300–3000 m/z). All test articles showed distinct separation of the three AAV VPs, ionized by electrospray ionization (ESI), and finally detected using MS analysis. The data were further analyzed by deconvolution of m/z between 40 and 90 kDa (mass accuracy +/− 5 Da for 40–90 kDa, or <100 ppm) to molecular weight of individual viral protein subunits. The intact mass of each viral protein was confirmed against the theoretical value calculated from the primary amino acid sequence including any subsequent post-translational modifications (PTM) observed for each protein. For each viral protein and variants, the peak identification was performed on Bruker Compass Data Analysis v6.0 and

peak AUC (area under the curve) integration was performed with a manual drop-line mode using Protein Metrics Byos software (v5.1.1).

#### *LC-MS/MS analysis for the quantification of residual GP64 levels in rAAV samples*

The analysis of rAAV samples by LC-MS/MS was conducted at Alphalyse Inc. (Odense M, Denmark). The purified samples were enzymatically digested, and the peptides were separated using an RP-nanoLC system. The MS data were acquired on a TripleTOF6600 (Sciex) in information dependent acquisition (IDA) mode, then the SWATH analysis was used for the protein quantification. A set of known proteins was used to generate a standard curve for quantification purposes. To confirm the identity of a protein, a minimum of two peptides was required. The lowest limit of quantification and limit of detection of the assay were 10 ng/mL and 1 ng/mL, respectively.

## DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request and receipt of appropriate legal approvals.

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## AUTHOR CONTRIBUTIONS

J.A. and S.L.K. conceived the overall study and experimental designs. J.A. and J.G. performed experiments and corresponding data analysis for CE-western assays. J.A. performed biological activity assays and analyzed data. Y.B. conducted experiments and performed data analysis for LC-MS assays. All authors contributed to the writing, editing, and review of the manuscript.

## DECLARATION OF INTERESTS

All authors are current or former full-time employees and own stocks of Sangamo Therapeutics, Inc.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101321.

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