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APPLICATION NOTE

DO YOUR AAVs CONTAIN DNA?

RAPID AND SENSITIVE EMPTY/FULL CAPSID QUANTIFICATION WITH **SIMPLE WESTERN**

EMPTY AAV PARTICLES ARE UNWANTED BIOPROCESS IMPURITIES FOR GENE THERAPIES

Adeno-associated viruses (AAVs) are frequently used as viral vectors in gene therapies to address human diseases, with over 200 studies around the world conducting active clinical Phase 1-3 trials. As gene delivery systems, AAVs include a gene of interest encoded in plasmid DNA that can be up to 5kb in length. AAVs can exist as a heterogeneous population, giving a final sample that is, for example, 30% full (including the desired plasmid DNA) and 70% empty or partially empty (devoid of the desired plasmid DNA). These empty or partially empty AAV particles can impact potency and immunogenicity and thus are unwanted byproducts of the AAV manufacturing bioprocess. 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] Therefore, there exists a need for better methods and systems for the efficient and sensitive quantification of Empty/Full status of AAV samples to be used for gene delivery.

A SENSITIVE AND REPRODUCIBLE MULTI-ATTRIBUTE TOOL FOR AAV EMPTY/FULL STATUS THAT SCALES WITH MANUFACTURING WORKFLOWS

In this Application Note, we developed a novel method for quantifying the DNA content of AAV particles with **Simple Western™, a next-generation biomolecular analytical tool that seamlessly combines capillary electrophoresis and immunodetection with conventional Western blot antibodies**. Here, the Simple Western method automatically separates AAV samples by Size or Charge followed by specific and sensitive detection using anti-DNA and anti-VP1/2/3 antibodies directly in the capillary for quantitative and reproducible measurement of these central AAV components. For each antibody, we identify the range in which the signal intensity is linearly related to the amount of sample loaded, resulting in a rapid and sensitive assay for accurately quantifying the ratio of % full AAVs to total AAVs in a sample (also known as the Content Ratio).

AAVs are challenging and expensive to manufacture. Therefore, a critical advantage of this assay for AAV analysis is the ability to analyze low abundance samples – this assay requires as little as 3 µL of starting material and can detect as few as 7.2 x 10^7 viral particles (VP) per well. With Simple Western, **24 samples may be analyzed in just 5 hours, or 96 samples overnight**, providing the high throughput you need to scale with your AAV manufacturing workflows. Simple Western also provides flexible multiplex chemiluminescence and NIR/IR fluorescence detection with industry-leading sensitivity, which enables simultaneous Total Protein Detection for multi-attribute analysis of process-related impurities combined with an identity assay.

In addition to demonstration of a CE-SDS-based assay for Empty/Full status, we also show a proof-of-concept that AAV Empty/Full status can be characterized on Simple Western Charge for analysis of intact AAVs by isoelectric focusing (IEF) and immunodetection as an orthogonal assay.

Given its advantages in detection sensitivity and specificity over other CE-based approaches and the rapid time to results, reproducibility, and ease of use, Simple Western is poised to solve a critical need for accurate, sensitive, and rapid quantification of Empty/Full status in AAV manufacturing workflows.

MATERIALS AND METHODS TO EVALUATE AAV CAPSID CONTENT

All size experiments in this study were performed on Jess™ and all charge experiments were performed on Peggy Sue™. Materials for Simple Western Size assays are listed in TABLE 1 and materials for Simple Western Charge assays are listed in TABLE 2.

SAMPLE PREPARATION AND RUNNING CONDITIONS FOR SIMPLE WESTERN SIZE

To prepare AAV samples for analysis, 2 µL of AAV sample was mixed with 48 µL of Sample Buffer (1X final concentration), Fluorescent Master Mix (1X final concentration), and deionized water. Then, the sample mixture was heated at 95 °C for 10 minutes and cooled to room temperature.

NAME	VENDOR	P/N
10X Sample Buffer	Bio-Techne	042-195
EZ Standard Pack		PS-ST01EZ-8
Wash Buffer		042-202
Antibody Diluent 2		042-203
Streptavidin-HRP		042-414
Anti-rabbit secondary antibody		042-206
20X anti-mouse secondary antibody NIR		043-821
RePlex™ Module		RP-001
Luminol-S		043-311
Peroxide		043-379
12-230 kDa Pre-filled Plates		PS-PP03
Capillary cartridges, 25 capillaries		PS-CC01
Rabbit anti-dsDNA antibody	Novus Biologicals	NBP3-07302
Mouse anti-VP1/2/3 antibody	PROGEN	65158
Nuclease (Benzonase)	Sigma	E1014
AAV1, 2, 3, 5 6, 9 Full (~2 x 10^{12} VP/mL) and Empty	Virovek	N/A

TABLE 1A. Materials used for Simple Western Size experiments.

% FULL	AAV # (VP/mL)	GENOME COPY # (GC/mL)
5	2.18 x 10^{13}	9.64 x 10^{11}
25	2.41 x 10^{13}	6.12 x 10^{12}
34	1.69 x 10^{13}	5.81 x 10^{12}
56	1.49 x 10^{13}	8.41 x 10^{12}
81	1.26 x 10^{13}	1.02 x 10^{13}
92	1.21 x 10^{13}	1.11 x 10^{13}

TABLE 1B. AAV9 series from Virovek with the % full / total AAVs, AAV particl number (VP/mL), and genome copy number (GC/mL) used in Simple Western Size experiments.

To analyze the samples on Jess, the RePlex™ assay was used, which removes antibodies from the first round of probing for a sequential second round of immunoassay with fresh antibodies. RePlex was chosen in place of multiplex due to cross-reactivity between the antibodies. For Probe 1 of RePlex, samples were probed with the primary anti-dsDNA monoclonal antibody diluted 1:50 in Antibody Diluent 2 and a secondary anti-rabbit HRP detection antibody. For Probe 2 of RePlex, samples were probed with the primary anti-VP1/2/3 monoclonal antibody diluted 1:50 dilution in Antibody Diluent 2 and a secondary anti-mouse NIR detection antibody diluted 1X in Antibody Diluent 2.

To treat samples with nuclease, benzonase was diluted 1:10 in the vendor-recommended buffer, and a buffer-only sample was included as a negative control. Each benzonase treatment was performed with a 30-minute incubation directly in the capillary.

SAMPLE PREPARATION AND RUNNING CONDITIONS FOR SIMPLE WESTERN CHARGE

AAV samples were diluted stepwise in SimpleSol before final mixing into IEF master mixture. The final sample solutions contained: 50% SimpleSol, 1% Biolyte 5-8 and 1% Biolyte 8-10, 0.35% methylcellulose, 10 mM arginine, p*I* marker ladder 3 (p*I* 4.9 – 7.3), p*I* markers 8.4 and 9.7. The focusing conditions were set to 35 minutes at a constant power of 21000 microwatts and the immobilization time was 220 seconds.

NAME	VENDOR	PART NUMBER
Amplified Mouse Secondary Antibody Detection Kit	Bio-Techne	041-127
SimpleSol		046-575
500 mM Arginine		042-691
p*I* Standard Ladder 3		040-646
p*I* Standard 8.4		041-036
p*I* Standard 9.7		040-790
Mouse anti-ssDNA antibody		NBP2-29849
1% Methyl cellulose		101876
Mouse anti-VP1/2/3 antibody	PROGEN	61058-647
Nuclease (Benzonase)	Sigma	E1014
Biolyte 5-8	Bio-Rad	1631192
Biolyte 8-10	Bio-Rad	1631182
AAV9-CMV-GFP	Virovek	N/A

TABLE 2. Materials used for Simple Western Charge experiments.

RESULTS OF EMPTY/FULL AAV ASSAY USING SIMPLE WESTERN SIZE

First, we developed an Empty/Full AAV immunoassay on Simple Western Size that simultaneously measures VP1/2/3 and DNA content of AAV samples with anti-VP1/2/3 and anti-DNA antibodies, respectively. To prove this concept, a series of AAV9 samples with a range of % full DNA capsid content from 5% full to 92% full was analyzed on the Simple Western Jess instrument using the RePlex assay. RePlex removes the antibodies from the first probing cycle for a second probing cycle with fresh antibodies. In this RePlex assay, the anti-DNA antibody was used in Probe 1 with chemiluminescence detection, and the anti-VP1/2/3 antibody was used in Probe 2 with NIR detection, resulting in sequential immunodetection of both AAV proteins and DNA content in a single run. Unlike the strip and re-probe method of the traditional Western blot, RePlex does not suffer from signal loss between probing cycles because the sample is covalently bound to the capillary wall.

This analysis revealed three signals corresponding to the VP1/2/3 proteins at the expected molecular weight (MW) range between 66 and 100 kDa with baseline separation from the signal corresponding to the DNA content, which appeared in the upper MW range of >300 kDa (**FIGURE 1A, B**). In general, the DNA peak area increased with increasing % full AAV9 sample, while the VP1/2/3 signal remained relatively stable (**FIGURE 1C**). These results suggest that Simple Western can measure the % of full AAVs to total AAVs in a sample with antibodies individually targeting DNA and VP1/2/3 molecules, respectively.

From these results, we generated a calibration curve to calculate the % full AAVs in a given sample using the Simple Western assay. To do so, each VP1/2/3 sum peak area was divided by the VP1/2/3 sum peak area from the lowest % full AAV9 sample from the vendor, such that the VP1/2/3 sum peak area from the 5% full AAV9 sample had a relative value of 1. Then, we normalized the DNA peak areas by dividing each absolute DNA peak area by its corresponding relative VP1/2/3 sum peak areas and these normalized DNA areas were plotted by the % full value of each AAV9 sample. With these values plotted, we performed a linear regression analysis to create a calibration curve (**FIGURE 1D**). Finally, this calibration curve was used to calculate the observed % full to empty or partially empty ratio in the sample that is expected to be 25% full as specified by the vendor. In agreement with this expected value, we observed a 25% full to 75% empty or partially empty ratio (**FIGURE 1E**). Collectively, these results show that the Simple Western assay can accurately quantify the Content Ratio or the ratio of full to total AAV particles in a sample.

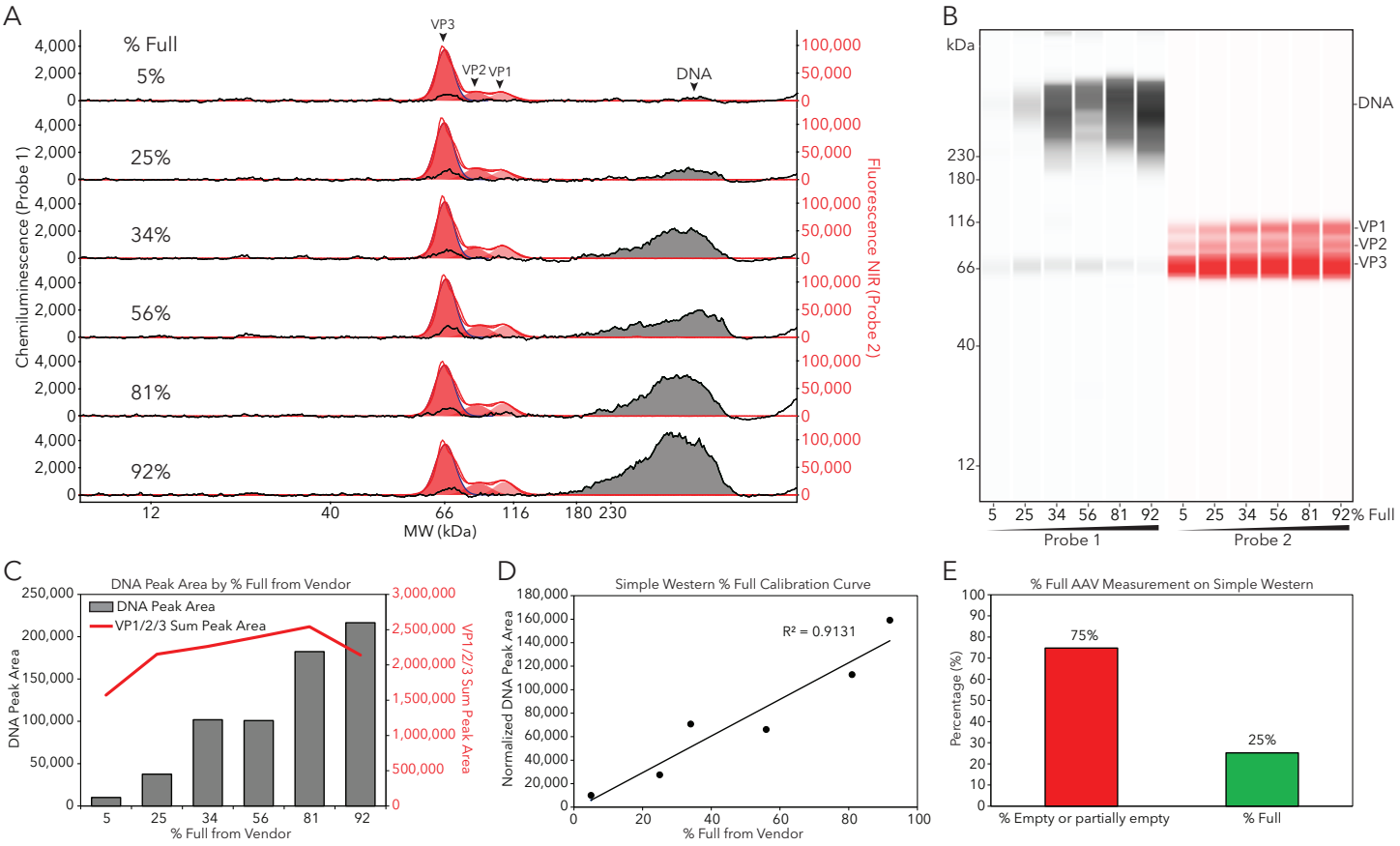

FIGURE 1. The Simple Western Empty/Full AAV Assay. (**A**) Electropherogram view and (**B**) lane view of AAV9 samples with a range of % full values were probed with the anti-DNA antibody in Probe 1 of RePlex with chemiluminescence detection and the anti-VP1/2/3 antibody in Probe 2 of RePlex with NIR detection. The % full AAV value in each sample is indicated by "% Full." (**C**) DNA and VP1/2/3 peak areas were plotted by the % full AAV9 sample. (**D**) DNA peak areas were normalized by relative VP1/2/3 peak areas and plotted by the theoretical % full AAV9 sample to generate a Simple Western % full calibration curve. (**E**) The Simple Western % full calibration curve was used to calculate the observed % full to empty (or partially empty) ratio in the expected 25% full sample provided by the vendor.

HIGHLY SPECIFIC DNA DETECTION

To provide additional proof that the signal in the upper MW region is the result of DNA detection with the anti-DNA antibody, we tested if treatment of AAV viral particles with nuclease results in a decrease in DNA as indicated by a diminished signal from the anti-DNA antibody. When we compared untreated AAV viral particles with viral particles treated with nuclease buffer alone and particles treated with nuclease, only the sample containing nuclease resulted in a signal from the anti-DNA antibody that was virtually undetectable (**FIGURE 2**, bottom panel). Meanwhile, the signal from the anti-VP1/2/3 antibody remained relatively stable across all three sample treatments (**FIGURE 2**). These results indicate that nuclease can dissolve the nucleic acid content of AAV particles and are consistent with the conclusion that empty and full AAVs can be identified by Simple Western with the anti-DNA and anti-VP1/2/3 antibodies.

LIMIT OF DETECTION OF 7.2 X 10^7 VP PER WELL

The results thus far indicate that the Simple Western assay can quantify Content Ratio or the ratio of full to total AAV particles in a sample. With this proof of concept established, we sought to determine the dynamic and linear range of DNA and VP1/2/3 detection. To do so, we prepared a serial dilution series of the 92% full AAV9 sample, and each sample dilution was loaded for analysis on Jess using RePlex, as described above. As expected, both DNA and VP1/2/3 signals decreased with decreasing AAV9 sample concentration (**FIGURE 3A**). Then, we plotted the DNA and VP1/2/3 peak areas by AAV9 sample concentration to determine the dynamic (**FIGURE 3B**) and linear (**FIGURE 3C**) range of detection. A linear relation was revealed for both DNA and VP1/2/3 detection between 4.8 x 10^{11} VP/mL and 2.4 x 10^{10} VP/mL. Because only 3 µL of sample are loaded per well, this corresponds to as little as 7.2 x 10^7 VP loaded per well.

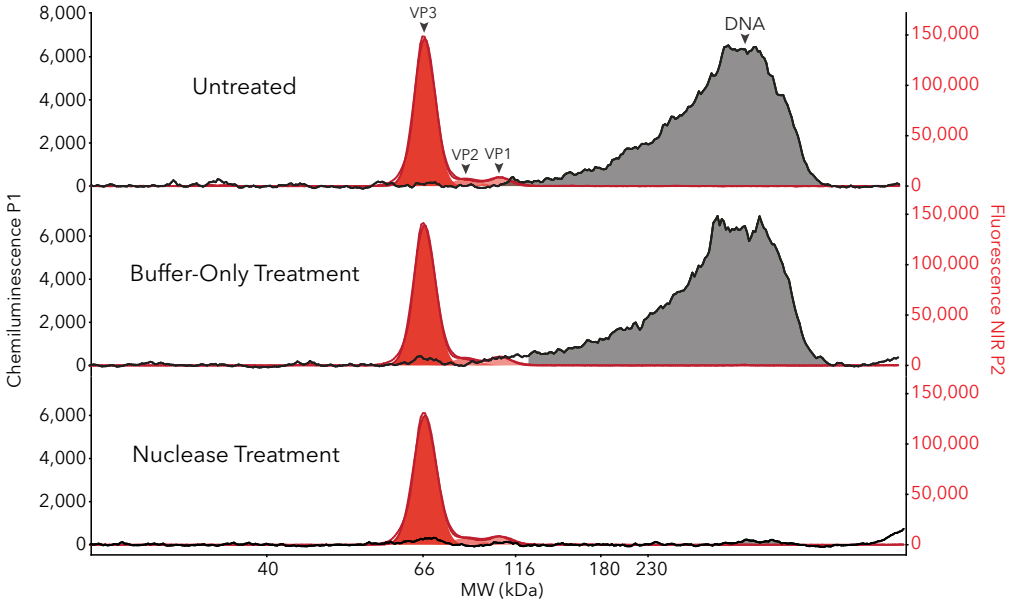

FIGURE 2. Simple Western analysis of 92% full AA9 samples that were untreated (top panel), treated with buffer only (middle panel), or treated with buffer containing nuclease (bottom panel). Shown here is the electropherogram view of AAV9 samples probed with the anti-DNA antibody in Probe 1 of RePlex with chemiluminescence detection and the anti-VP1/2/3 antibody in Probe 2 of RePlex with NIR detection.

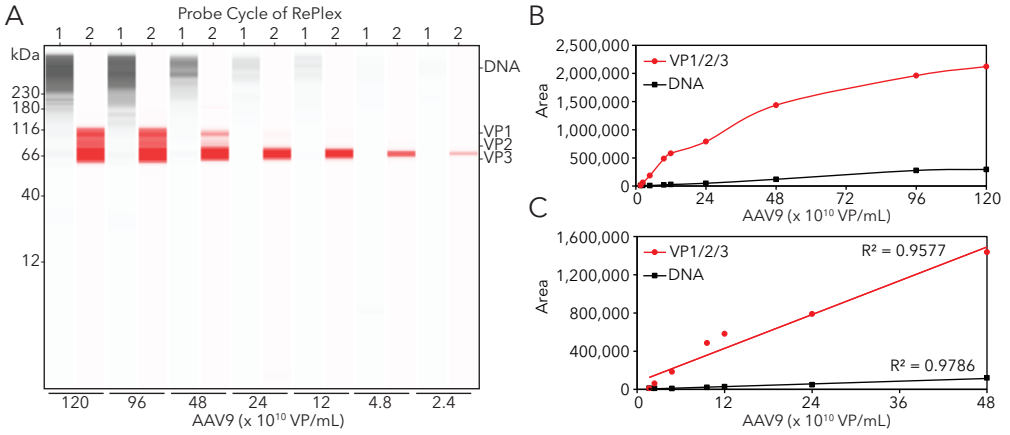

FIGURE 3. Simple Western analysis of a serial dilution series of the 92% full AAV9 sample. (**A**) Lane view of AAV9 samples probed with the anti-DNA antibody in Probe 1 of RePlex with chemiluminescence detection and the anti-VP1/2/3 antibody in Probe 2 of RePlex with NIR detection. DNA and VP1/2/3 peak area were plotted against AAV9 sample concentration to determine the (**B**) dynamic range and (**C**) linear range of detection.

APPLICABILITY TO NUMEROUS AAV SEROTYPES

Next, we asked if the Simple Western Empty/Full AAV assay could be extended to other serotypes besides AAV9. Thus, we analyzed empty and full samples of AAV1, 2, 3, 5, and 6 serotypes on Jess using RePlex, as described above. These results showed that DNA was only detected in full AAV samples whereas little to no DNA was detected in empty AAV samples (**FIGURE 4**). Thus, these results suggest that the Simple Western Empty/Full AAV assay can be extended to other AAV serotypes in addition to AAV9.

CONSISTENT RESULTS: DAY-TO-DAY REPRODUCIBILITY

Other immunoanalytical methods typically used in AAV workflows, like for example the traditional Western blot and ELISA, are laborious and have many hands-on steps that negatively impact reproducibility. By contrast, Simple Western performs all sample separation, immobilization, washing, and detection steps automatically in the capillary, producing reproducible results even across different days. Thus, we measured the inter-day reproducibility of the Simple Western Empty/Full AAV assay. To do so, we analyzed empty and full samples of AAV1, AAV6, and AAV9 serotypes in triplicate once per day for 3 days on Jess using RePlex, as described above. Then, we calculated the average DNA and VP1/2/3 peak areas (**FIGURE 5**) and CV percentages (**TABLE 3**) across all replicates and time points (n=9). It is important to note that AAV1/6 serotype samples are at least 90% full according to the manufacturer's specifications, which matches the relative DNA signal intensities in our assay (**FIGURE 5B**). Under the conditions tested here, the reproducibility was highest for VP3 detection, with all CVs of ≤10.0%. With AAV6 as the only exception, higher CVs were observed for DNA detection, likely due to its wide separation range and broad peak area in the upper MW region. Nevertheless, this analysis indicates that it is reasonable to expect consistent results when the Simple Western Empty/Full AAV assay is applied to the same sample across multiple days.

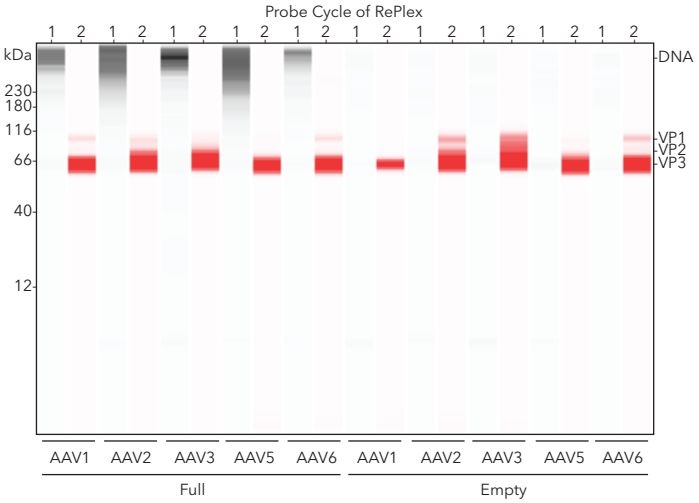

FIGURE 4. The Simple Western Empty/Full assay applied to different AAV serotypes. Lane view of AAV samples probed with the anti-DNA antibody in Probe 1 of RePlex with chemiluminescence detection and the anti-VP1/2/3 antibody in Probe 2 of RePlex with NIR detection.

SAMPLE	DNA	VP3	VP2	VP1
AAV1 Full	22.8	5.3	11.6	7.7
AAV1 Empty	38.7	8.5	28.5	27.2
AAV6 Full	5.4	2.2	8.0	5.3
AAV6 Empty	5.6	3.7	11.1	10.5
AAV9 92% Full	15.5	10.0	18.6	22.0
AAV9 5% Full	46.5	7.2	19.0	12.7

TABLE 3. CV percentages of DNA and VP1/2/3 detection resulting from inter-day reproducibility analysis of the Simple Western Empty/Full AAV assay (n=9).

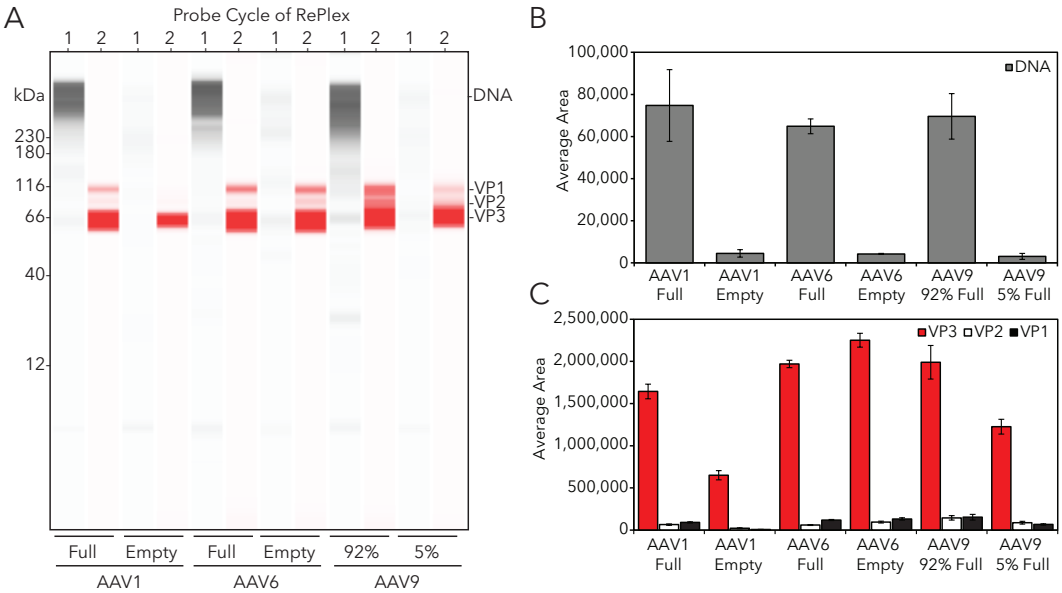

FIGURE 5. Inter-day reproducibility of the Simple Western Empty/Full AAV assay. (**A**) Lane view of AAV9 samples probed with the anti-DNA antibody in Probe 1 of RePlex with chemiluminescence detection and the anti-VP1/2/3 antibody in Probe 2 of RePlex with NIR detection. Average peak areas of (**B**) DNA and (**C**) VP1/2/3 detection were plotted from each serotype with error bars representing the standard deviations from the means (n=9).

RESULTS OF EMPTY/FULL AAV ASSAY USING SIMPLE WESTERN CHARGE

With the Empty/Full AAV assay established on Simple Western Size, we lastly tested if this Empty/Full assay could be extended to Simple Western Charge, which separates non-denatured (intact) particles by IEF followed seamlessly by immunodetection with conventional Western blot antibodies. As described in the Materials and Methods above, samples were prepared and analyzed on the Simple Western Peggy Sue instrument and probed with anti-DNA and anti-VP1/2/3 antibodies.

From this experimental analysis, strong signals corresponding to intact AAV9 particles were observed in the 77% full AAV9 sample with both antibodies (**FIGURE 6A**, top panel). In contrast, only a robust signal was detected with the anti-VP1/2/3 antibody in the 9% full AA9 sample, whereas little to no signal was observed with the anti-DNA antibody (**FIGURE 6A**, bottom panel). Interestingly, the charge separation profile of the empty AAV9 particles detected with the anti-VP1/2/3 antibody was differently distributed compared to full particles, indicating that the nucleic acid content of the capsid may have an impact on the stability of the viral particle. Nonetheless, these results demonstrate that the Simple Western Empty/Full AAV assay can be performed on Simple Western Charge in addition to Simple Western Size. The Simple Western Charge assay could serve as an orthogonal method and may provide new insights for AAV analysis, like for example the impact of DNA capsid content on the structural stability and isoelectric point of the overall viral particle.

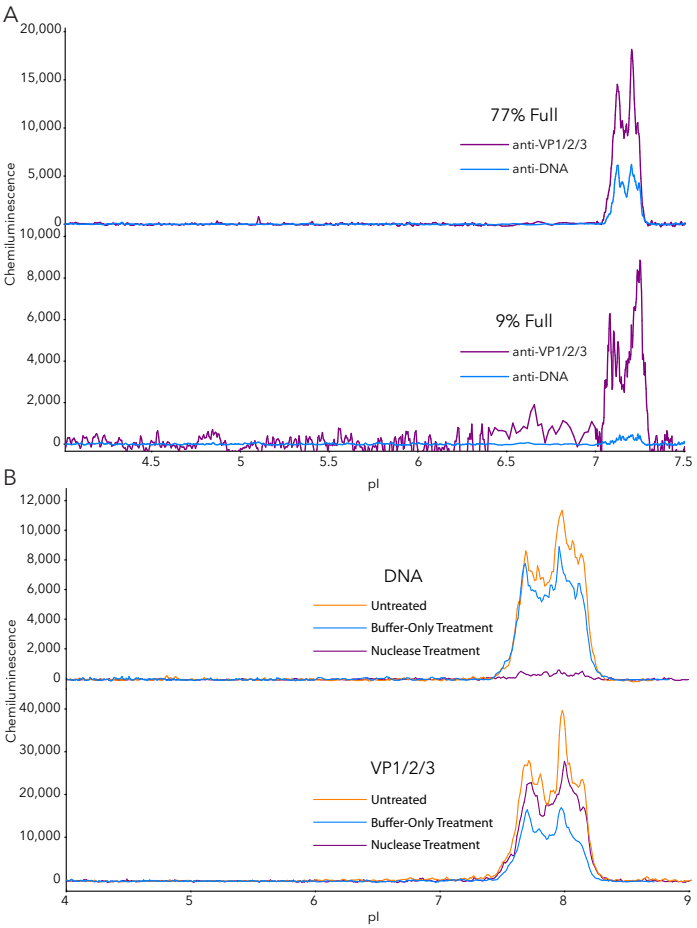

FIGURE 6. Simple Western Charge Empty/Full Assay of AAV9 samples. (**A**) Signals were detected using anti-DNA and anti-VP1/23 antibodies on 77% and 9% full AAV9 samples. (**B**) Nuclease treatment of 77% full AAV9 particles probed with anti-DNA and anti-VP1/2/3 antibodies.

Like with Simple Western Size, we tested if treatment of AAV9 viral particles with nuclease resulted in a decrease in DNA as indicated by a diminished signal from the anti-DNA antibody. When we compared untreated AAV viral particles with viral particles treated with nuclease buffer alone or particles treated with nuclease, only the sample containing nuclease resulted in a virtually undetectable anti-DNA signal (**FIGURE 6B**, top panel). Meanwhile, the signal from the anti-VP1/2/3 antibody remained relatively stable across all three sample treatments (**FIGURE 6B**, bottom panel). These data indicate that nuclease can dissolve the DNA content of AAV9 particles and they confirm that the signal resulting from the anti-DNA antibody is the result of DNA present in the viral capsid.

We compared the peak areas of VP1/2/3 and DNA by overlaying their respective electropherograms from the 77% and 9% full AAV9 samples (**FIGURE 7A**). When we quantified the VP1/2/3 and DNA peak areas from this comparison, we found that the fold change in VP1/2/3 was 1.8X while the fold change in DNA was 15.8X (**FIGURE 7B**). Therefore, the fold increase in DNA content when normalized to VP1/2/3 was 8.9X, which closely matches the manufacturer's specifications of these AAV9 samples (9% and 77% full).

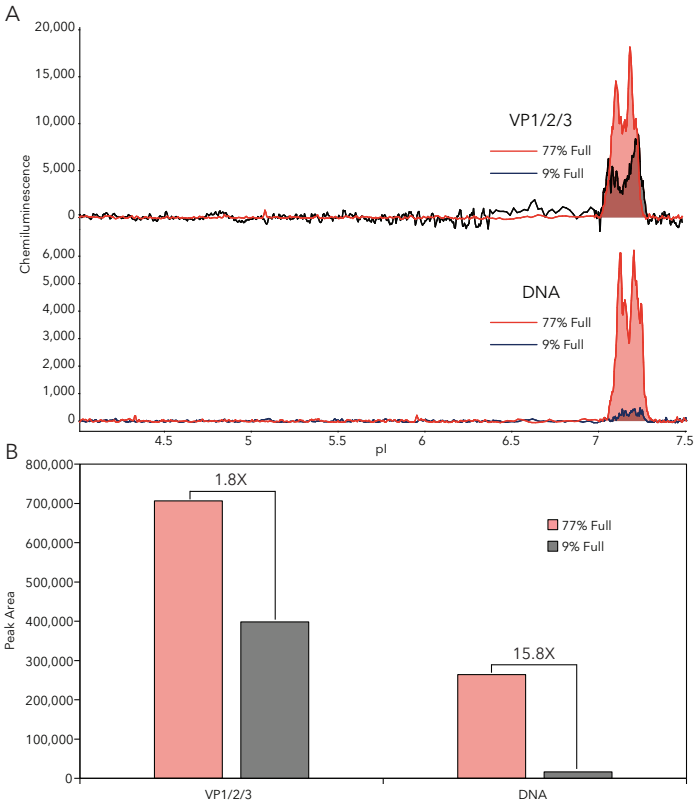

FIGURE 7. Comparison of VP1/2/3 and DNA signals resulting from Simple Western Charge Empty/Full Assay of AAV9 samples. (**A**) Signals were detected using anti-VP1/2/3 (top panel) and anti-DNA (bottom panel) antibodies on 77% full (red) and 9% full (black) AAV9 samples. (**B**) Quantification of VP1/2/3 and DNA peak areas of 77% full and 9% full AAV9 samples.

AUTOMATED EMPTY/FULL AAV QUANTIFICATION WITH HIGH SENSITIVITY, REPRODUCIBILITY, AND SCALABILITY

In cell and gene therapy applications, the purity of the viral vector is critical for the efficacy and safety of treatment. Empty viral particles resulting from bioprocess manufacturing can only reduce efficacy and contribute to toxicity. Methods for the rapid determination of the percentage of empty viral particles in a sample have historically been lacking. Here, we developed new methods for the quantitative characterization of the full and empty viral capsid content in a sample using Simple Western Size and Charge assays with antibodies that target the viral capsid and the nucleic acid content individually. The results obtained by this method showed a linear correlation between the signal targeting the nucleic acid content and the percentage of full AAV capsids in each sample, enabling a quantitative assessment of the percentage of empty or partially empty viral capsids in a sample.

A major advantage of this method is that Simple Western is a fully automated immunodetection platform with a throughput of up to 96 samples that can be processed overnight. This automation not only decreases labor costs but also enables faster iterations during in-process development. Equally advantageous is the tiny sample volume requirement of Simple Western (as little as 3 µL sample) that minimizes impact on final product titer and allows for the analysis of as few as 7.2 x 10^7 VP per well.

SIMPLE WESTERN IS A MULTI-ATTRIBUTE METHOD THAT CAN BE USED ON COMPLEX SAMPLES

Simple Western is an immunoassay that is capable of specifically detecting proteins even in highly complex samples like cell lysates. This contrasts with other methods for assessing capsid content like CE-SDS, cIEF, SEC-MALS, and HPLC, which rely on direct UV or fluorometric detection, limiting their use on complex sample types.[5-6] While ELISA can provide AAV identity information in cell lysates, it cannot provide purity information in the same assay. In this regard, ELISA is a single-attribute method. By contrast, Simple Western is a multi-attribute method because it can deliver identity, capsid protein ratio, empty/full assessment, viral titer, protein expression potency, and purity in the same assay with industry-leading 5X Total Protein Detection that is comparable to the most sensitive SDS-PAGE gel staining techniques like SYPRO Ruby.[7] The 5X Total Protein Detection assay on Simple Western can be used for the detection of process-related impurities in AAV manufacturing.[7] Therefore, we anticipate that the multi-attribute Simple Western assay can be integrated into upstream workflows where crude or partially purified samples are processed as well as downstream workflows for the detection of residual product-related impurities and Empty/Full AAV capsid content ratios.

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