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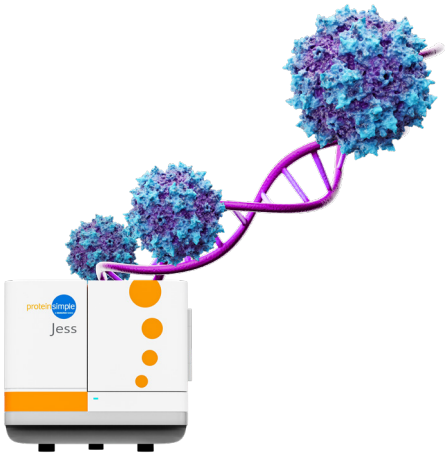
proteinsimple

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.

### All-In-One Assays for Titer, Capsid Protein Ratio, and Empty/Full Ratio

Simple Western™ is the only platform that provides the complete picture of your AAVs in complex lysates to guide downstream process decisions and optimization. With the separative performance of capillary electrophoresis by size (SW-Size) or charge (SW-Charge) and the specificity of an immunoassay, Simple Western resolves individual capsid proteins and DNA for high-specificity multi-attribute analysis of titer, capsid protein ratio, and empty/full status. With hands-free automation, you get quantitative and reproducible measurements in your sample from any process step without laborious purification.

Here, we describe straightforward assay development to measure titer and capsid protein ratio with 2 Simple Western runs and empty/full ratio* with 2 additional runs. Once the titer and DNA assay standards are optimized for a given serotype, it is possible to get multi-attribute measurements with 2 runs, one titer and one DNA, for any sample in that given program. These assays provide reproducible quantification with CVs of 15% or less for titer and capsid protein ratio and 15% or less for empty/full ratio measurements. In addition, the assays described here apply to other AAV serotypes and require 3 µL of diluted unprocessed or processed sample. We also show 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.

As a result, Simple Western is meeting industry needs for robust protein characterization with advantages critical to gene therapy manufacturing, including speed, simple assay development, and scalable automation. And you don't have to take our word for it – scientists at Merck separately developed Simple Western assays for titer, capsid protein ratio, and empty/full ratio analysis of coxsackievirus, stating:

*"In short, the Simple Western method was an invaluable analytical tool providing timely information to help guide downstream process decisions and optimization."*[5]

## Materials and Methods

All size Simple Western Size experiments (SW-Size) in this study were performed on Jess™ and all Charge experiments (SW-Charge) were performed on Peggy Sue™.

*Methods for determining viral empty/full ratios are protected by patents, including U.S. Pat. Nos. 11,535,900 and 11,827,946.

## Titer and Capsid Protein Ratio by SW-Size

Materials required for measuring titer and capsid protein ratio using SW-Size are provided in TABLE 1. If you are also running the empty/full assay, the Anti-Rabbit Detection Module includes Luminol-S, Peroxide, Antibody Diluent 2, and Streptavidin HRP (TABLE 2). They may be purchased a la carte (TABLE 1) if not.

Additional materials required but not provided by Bio-Techne:

- Anti-AAV VP1/VP2/VP3 mouse monoclonal, B1, liquid, purified (PROGEN, 690058)
- Pluronic F-68
- 1X PBS
- Fully characterized AAV standards with known titer from a matching cell line, e.g. HEK293.

The standards and unknown samples are diluted to 1.25X final concentration in 1X PBS containing 0.001% Pluronic-F68, then diluted 1:5 in 5X Master Mix to create 1X Master Mix and sample concentrations. The standards and samples are then denatured at 95 °C for 5 minutes. The anti-AAV VP1/2/3 antibody is diluted to 20 µg/mL in Antibody Diluent 2, and the Anti-Mouse Secondary HRP is diluted to 1X in Antibody Diluent 2. Note that the Anti-Mouse Secondary Antibody (040-655) is used for VP detection despite being specified for Charge assays on Peggy Sue™ and NanoPro™ 1000 (TABLE 1). The AAV standards and samples are loaded on the Jess plate according to the 66-440 kDa Separation Module Product Insert (PL3-0005) with default instrument settings, except the sample load time was set to 6 seconds.

| Item | Vendor | Part Number |
|---|---|---|
| 66-440 kDa Separation Module | ProteinSimple, a Bio-Techne Brand | SM-W005 |
| Goat-Anti-Mouse-HRP Secondary Antibody | | 040-655 |
| Luminol-S | | 043-311 |
| Peroxide | | 043-379 |
| Antibody Diluent 2 | | 042-203 |
| Streptavidin HRP | | 042-414 |

TABLE 1. Materials needed for titer and capsid protein ratio assays.

## Empty/Full Ratio by SW-Size

| Item | Vendor | Part Number |
|---|---|---|
| 66-440 kDa Separation Module | ProteinSimple, a Bio-Techne Brand | SM-W005 |
| Anti-Rabbit Detection Module | | DM-001 |
| Milk-free Antibody Diluent | | 043-524 |
| dsDNA Antibody | Novus Biologicals, a Bio-Techne Brand | NBP3-07302 |

TABLE 2. Materials for empty/full assays using SW-Size.

Additional materials required but not provided by Bio-Techne:

- Pluronic F-68
- 1X PBS
- AAV standards with known empty/full ratios from a matching cell line, e.g. HEK293. The same standard for the titer analysis may be used.

The 5X Master Mix is denatured at 95 °C for 5 minutes, cooled on ice, centrifuged, vortexed, and stored on ice. The samples are diluted to 1.25X final concentration in PBS containing 0.001% Pluronic F-68 and mixed with the denatured 5X Master Mix to create a 1X Master Mix and sample concentration. It is important not to heat denature the samples.

The anti-DNA antibody is diluted to 2 µg/mL in Milk-Free Antibody Diluent and the Anti-Rabbit Secondary Antibody is used at ready-to-use (RTU) stock concentration. The samples and antibodies are loaded on the Jess plate according to the 66-440 kDa Separation Module Product Insert (PL3-0005), except Milk-Free Antibody is used in place of Antibody Diluent 2 for blocking. Instrument settings are default, except the stacking load time is set to 36 seconds.

## Empty/Full Ratio by SW-Charge

Materials required for measuring empty/full ratio using SW-Charge are provided in TABLE 3. Additional materials required but not provided by Bio-Techne:

- 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)

| Item | Vendor | Part Number |
|---|---|---|
| Amplified Mouse Secondary Antibody Detection Kit | ProteinSimple, a Bio-Techne Brand | 041-127 |
| SimpleSol | | 046-575 |
| 500 mM Arginine | | 042-691 |
| pI Standard Ladder 3 | | 040-646 |
| pI Standard 8.4 | | 041-036 |
| pI Standard 9.7 | | 040-790 |
| 1% Methyl cellulose | | 101876 |
| Mouse anti-ssDNA antibody | Novus Biologicals, a Bio-Techne Brand | NBP2-29849 |

TABLE 3. Materials for empty/full assays using SW-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, pI marker ladder 3 (pI 4.9 – 7.3), pI 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.

# Results

## Titer and Capsid Protein Ratio by SW-Size

The standard curve should be built with an empty or full reference sample with a matching serotype and cell line and with a known titer specified by the vendor or determined by an orthogonal method. If you are running the empty/full assay after the titer assay, include the reference sample (either empty or full) that will **not** be used in the standard curve of the titer assay as an unknown so that all titers are determined on the same run. For example, if you are measuring titer with full reference particles, treat empty reference particles as unknown samples.

In the initial run, build a 5-point, 5-fold serial dilution series from $10^{11}$ VP/mL to 1.6 x $10^{8}$ VP/mL that will guide the optimization of a standard curve for titer quantification in the second run. The reference sample (empty or full) not in the standard curve that will be treated as an unknown should be diluted to an initial dilution equal to approximately the 2nd standard point (approximately top half of the curve).

Depending on the number of samples and the process step (in process, purified, etc.), the samples should be diluted to a starting dilution and titrated 3-fold if 3 dilutions per sample are required or 5-fold if 2 dilutions per sample are required. General guidance for starting sample dilutions is provided in TABLE 4.

Overlaid electropherograms using the All Exposures option resulting from an acceptable standard curve and a standard curve with signals that are too high are provided in FIGURE 1. The top point of the curve should have 4 short exposures overlaid (1-8s).

For the second run, build an optimized 5-point, 3- or 4-fold standard curve, using the bottom of the curve from

| Sample Type | Recommended Starting Dilution |
|---|---|
| In process | 1:1.25 |
| Purified | 1:10 |
| Purified/Concentrated | 1:100 |
| Drug Substance | 1:1000 |

TABLE 4. Recommended starting dilution by common AAV manufacturing sample types.

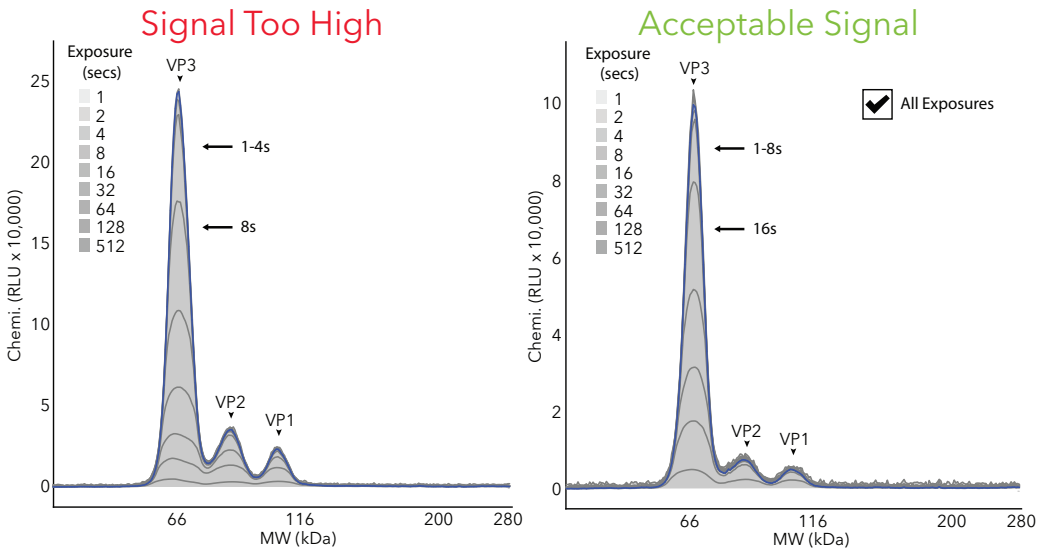



FIGURE 1. Optimizing standard dilution to calculate titer. The top standard point should have at least 4 short exposures (1-8s) overlaid, as shown on the **right**. When the signal is too high, only 1-4s (or less) are overlaid, as shown on the **left**.

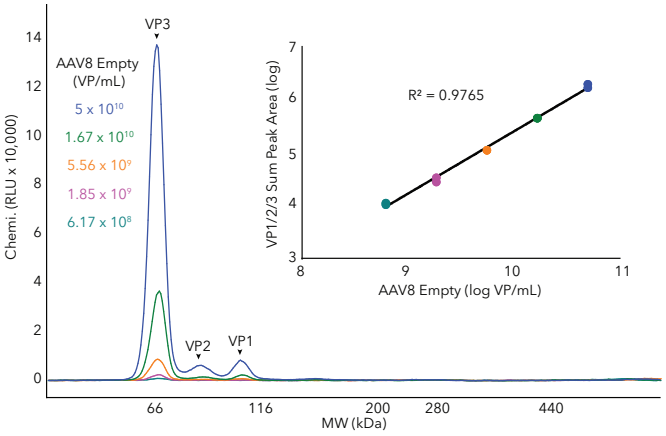



FIGURE 2. Example standard curve to measure titer.

| Standards (VP/mL) | Rep 1 | Rep 2 | Average |
|---|---|---|---|
| $5 \times 10^{10}$ | 100.6% | 115.8% | 108.2% |
| $1.67 \times 10^{10}$ | 98.4% | 98.7% | 98.6% |
| $5.56 \times 10^{9}$ | 90.3% | 90.4% | 90.3% |
| $1.85 \times 10^{9}$ | 83.7% | 97.4% | 90.5% |
| $6.17 \times 10^{8}$ | 108.7% | 115.2% | 112.0% |

TABLE 5. Recovery percentages from each point of the standard curve to measure titer and capsid protein ratios.

| Sample | VP/mL | % CV |
|---|---|---|
| 1 | $1.52 \times 10^{13}$ | 14.0 |
| 2 | $5.90 \times 10^{12}$ | 5.1 |
| 3 | $4.73 \times 10^{12}$ | 0.1 |
| 4 | $5.14 \times 10^{12}$ | 2.5 |

TABLE 6. Average titer measurements and CV percentages of unknown samples were interpolated using the standard curve.

the first run to choose between a 3- or 4-fold standard curve resulting in an identical or similar top calibrator as determined in the first run. Sometimes, a 2.5-fold standard curve is necessary. Run the unknowns in 2-fold dilutions based on the initial run, aiming to fall in the middle range of the standard curve with 3 dilutions per sample, if possible, and a minimum of 2 dilutions.

Create the standard curve from the optimized 2nd run by plotting the Total VP area (VP1+VP2+VP3) against the known concentration (VP/mL) with a log-log $1/y^2$ weighted fit. An example standard curve is provided in FIGURE 2. You may choose to use only the VP3 peak area if the standards and unknowns are from the same program. Interpolate the standard curve values against the standard curve fit to ensure you have a valid standard curve; the average % recovery of the standard curve points should be +/- 20% (example provided in TABLE 5). Interpolate the titer of the unknown total VP peak area or VP3-only peak area from the standard curve and correct for dilution to determine titer. If multiple unknown dilutions fall within the standard curve, then average their corrected titers to establish the final titer (example provided in TABLE 6).

Capsid ratios should be determined from the 2nd optimized run. The capsid ratio should not be determined using a signal where the VP3 signal has less than 4 exposures overlaid (FIGURE 3, top panels). Conversely, the capsid ratio should not be determined from VP1 and VP2 peaks that are not clearly defined and above the background noise (FIGURE 3, bottom panels).

If the above criteria are met, then the capsid ratio is determined by simply dividing the VP3 and VP2 area by the VP1 area; the VP1 ratio will always be 1.0, so dilution correction is not necessary. If multiple sample dilutions meet the criteria, determine the capsid ratio at each dilution, then average the individual capsid ratios to establish the final capsid ratio (TABLE 7).

## Empty/Full Ratio by SW-Size

Ideally, assay development is performed as described in "Setting Up Your Assay" before running the empty/full assay below. The typical titer range for a well-developed empty/full assay using Simple Western is between $10^{10}$ and $10^{9}$ VP/mL. It is important to note that all standards and samples need to be run at the same titer for successful

| Sample | Concentration or Dilution | VP2 | VP3 |
|---|---|---|---|
| Std. | $5.00 \times 10^{10}$ (VP/mL) | 1.21 | 14.67 |
| | $1.67 \times 10^{10}$ (VP/mL) | 1.00 | 14.91 |
| | $5.56 \times 10^{9}$ (VP/mL) | 1.03 | 11.93 |
| | Average | 1.1 | 13.8 |
| 1 | 1:500 | 0.79 | 13.48 |
| | 1:1000 | 0.84 | 12.13 |
| | 1:2000 | 0.78 | 9.24 |
| | Average | 0.8 | 11.6 |
| 2 | 1:250 | 0.59 | 11.28 |
| | 1:500 | 0.57 | 8.88 |
| | 1:1000 | 0.61 | 8.94 |
| | Avg. | 0.6 | 9.7 |
| 3 | 1:250 | 0.61 | 9.31 |
| | 1:500 | 0.63 | 8.07 |
| | 1:1000 | 0.89 | 8.01 |
| | Avg. | 0.7 | 8.5 |
| 4 | 1:250 | 0.71 | 8.84 |
| | 1:500 | 0.71 | 7.35 |
| | 1:1000 | 0.70 | 6.45 |
| | Average | 0.7 | 7.5 |

TABLE 7. VP1:2:3 ratio measurements generated from the optimized 2nd run of the titer assay. Measurements are reported relative to VP1, so VP1 is equal to 1 in all samples.

empty/full determination of unknown samples with equal loading, so the only variable should be the % DNA. If an initial DNA assay, to determine what VP/mL to run the standards and samples, cannot be run before the complete empty/full assay, we recommend using a titer of 3 x $10^9$ VP/mL based on the titers of various serotypes we have evaluated by Simple Western.

A 5-point % empty/full standard curve should be built as follows: full only, 75% full/25% empty, 50% full/50%

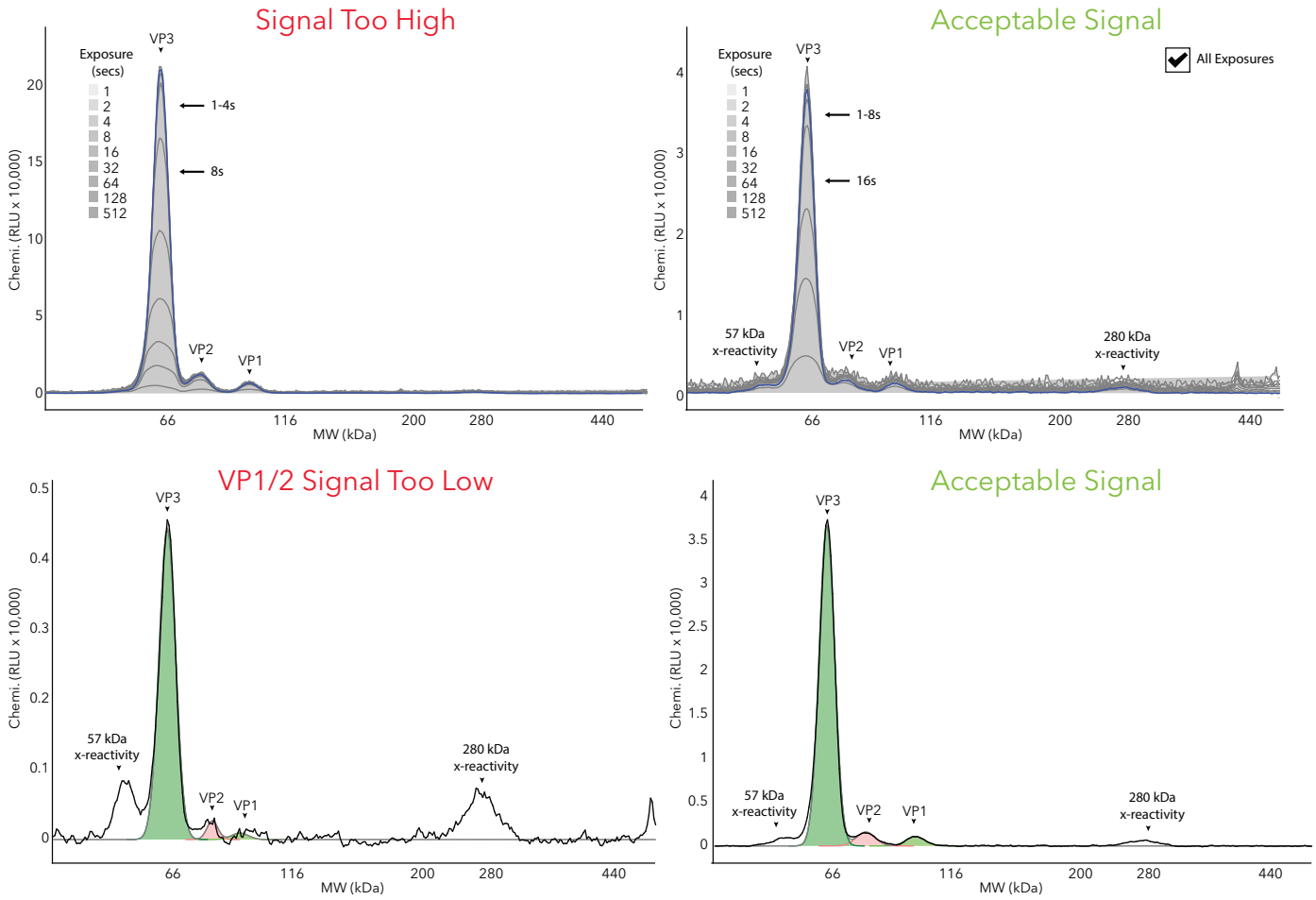



**FIGURE 3. Examples of standards or samples from which capsid ratio can be determined.** The guidance applies to standards and samples. VP3 should have at least 4 short exposures (1-8s) overlaid (**top right**). Do not calculate VP3 peak area from too high signal where only 1-4s or less exposures are overlaid (**top left**). The VP1/2 peaks should be clearly defined above background noise (**bottom right**). Note that cross-reactivity may occur at 280 kDa and 57 kDa.

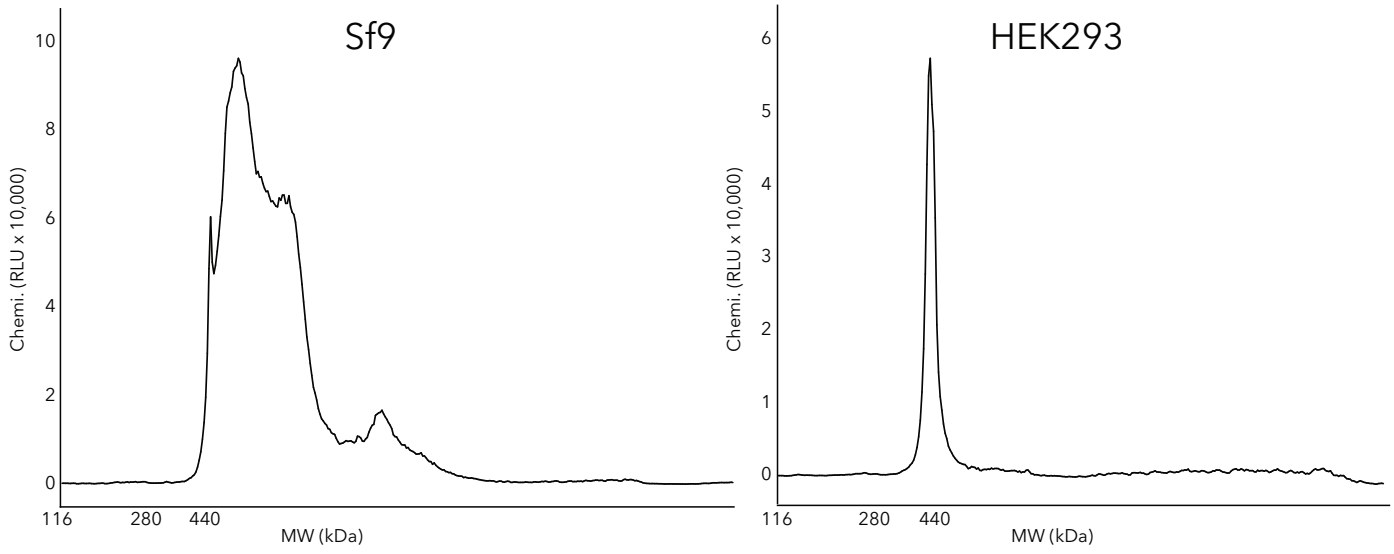



**FIGURE 4. Example** DNA profiles from AAVs produced in Sf9 (**left**) and HEK293 (**right**) cells.

empty, 25% empty/75% full, and empty only. Each standard's final % empty/full will be determined based on the known % empty/full of the empty and full Standards.

Note that the DNA peak profile can vary, as shown by DNA detected in AAVs produced in Sf9 and HEK293 (FIGURE 4). It is, therefore, important to use standards produced from the same cell line as the unknown samples. The same standard may be used for the titer analysis.

As with the titer standards, the top standard point should have at least 4 short exposures overlay (FIGURE 5). Run the unknowns at the same VP/mL as the % empty/full standard curve (FIGURE 6). The only variable should be the % DNA that is to be determined from the standard curve.

Plot the total DNA area by the % empty/full of the standard curve and apply a simple linear regression (FIGURE 6). See the Appendix for recommended peak fit settings. Interpolate the standard curve values against the standard curve fit to ensure you have a valid standard curve; the average % recovery of the standard curve points should be +/- 20% (example provided in TABLE 8).

| % Full Std | Rep 1 | Rep 2 | Rep 3 | Average |
|---|---|---|---|---|
| 88.46% | 99.2% | 108.9% | 98.4% | 102.1% |
| 68.14% | 99.3% | 106.2% | 102.3% | 102.6% |
| 47.81% | 93.7% | 91.5% | 104.2% | 96.4% |
| 27.49% | 88.5% | 104.4% | 97.4% | 96.8% |
| 7.16% | 98.1% | 101.1% | 107.1% | 102.1% |

TABLE 8. Recovery percentages from each point of the % full standard curve.

| Sample | % Empty/Full | % CV |
|---|---|---|
| 1 | 90.7% | 3.7 |
| 2 | 55.8% | 10.0 |
| 3 | 0.8% | 3.9 |

TABLE 9. Average empty/full measurements and CV percentages of unknown samples were interpolated using the standard curve.

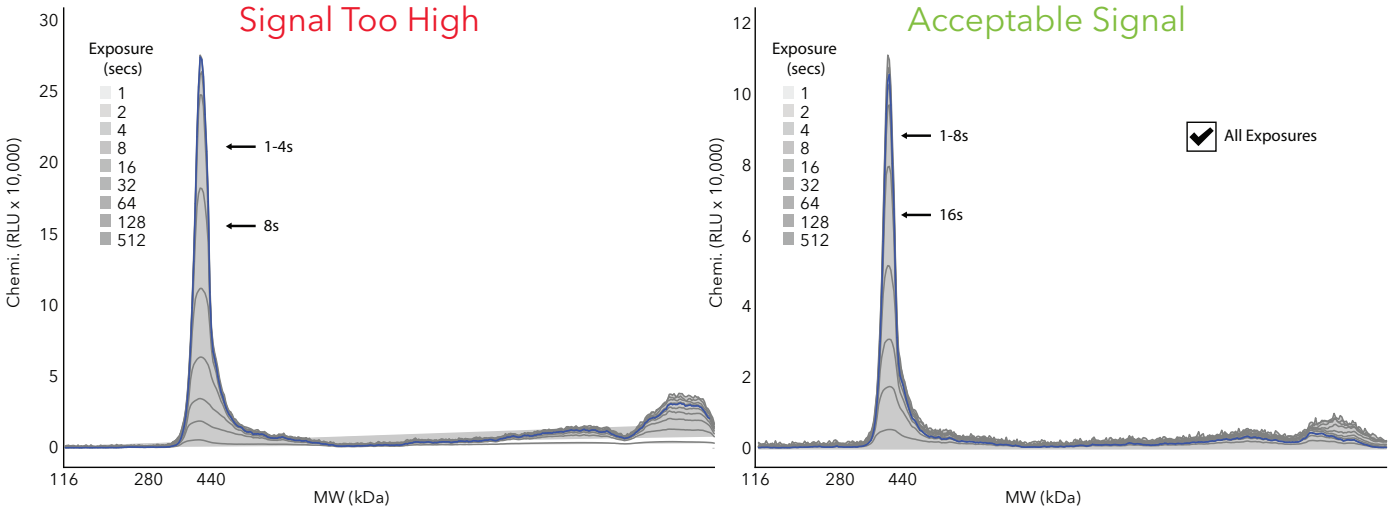



FIGURE 5. Optimizing standard dilution to calculate empty/full ratios. The top standard point should have at least 4 short exposures (1-8s) overlaid, as shown on the **right**. When the signal is too high, only 1-4s (or less) are overlaid, as shown on the **left**.

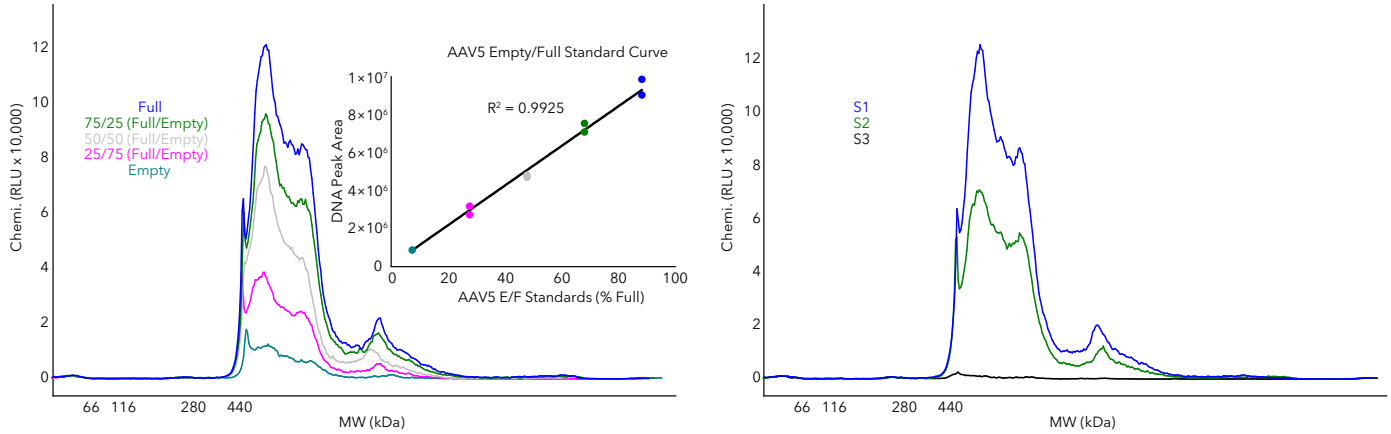



**FIGURE 6.** Example standard curve (**left**) to measure empty/full ratios and unknown samples S1-3 (**right**).

Interpolate the % empty/full of unknown total DNA area from the standard curve for each replicate and determine the average % empty/full of each unknown (example provided in TABLE 9).

## Empty/Full Ratio by SW-Charge

With the Empty/Full AAV assay established on Simple Western Size, we lastly tested if this Empty/Full assay could be extended to SW-Charge, which separates non-denatured (intact) particles by IEF followed seamlessly by immunodetection with conventional Western blot antibodies. From this experimental analysis, strong signals corresponding to intact AAV9 particles were observed in the 77% full AAV9 sample with both antibodies (FIGURE 7A, 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 7A, 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 SW-Charge in addition to SW-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 7B, top panel). Meanwhile, the signal from the anti-VP1/2/3 antibody remained relatively stable across all three sample treatments (FIGURE 7B, 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 8A). 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 8B). 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).

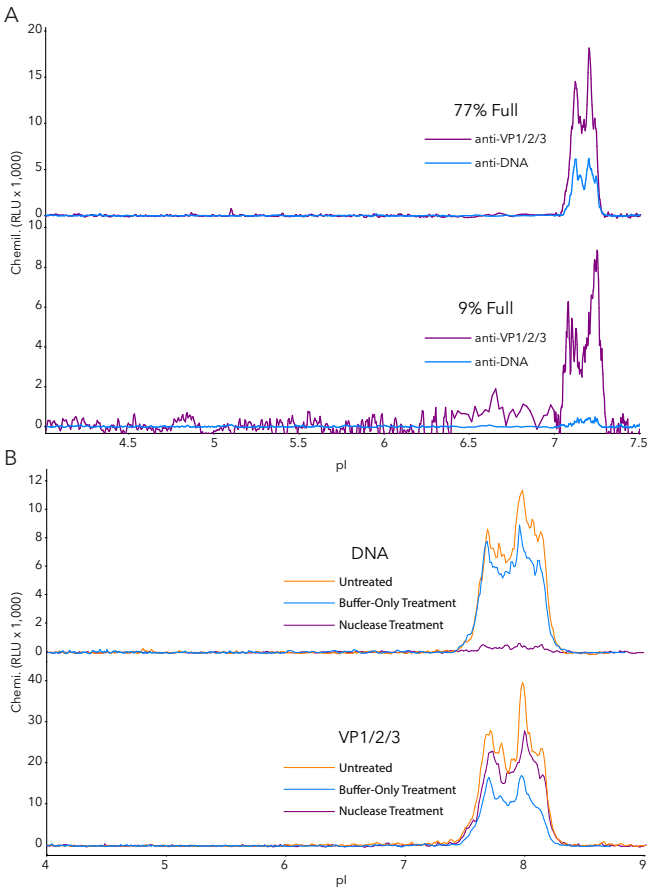



**FIGURE 7. SW-Charge Empty/Full Assay of AAV9 samples.** (**A**) Signals were detected using anti-DNA and anti-VP1/23 antibodies on 77% and 9% full AAV9 samples. (**B**) Nuclease treatment of 77% full AAV9 particles probed with anti-DNA and anti-VP1/2/3 antibodies.

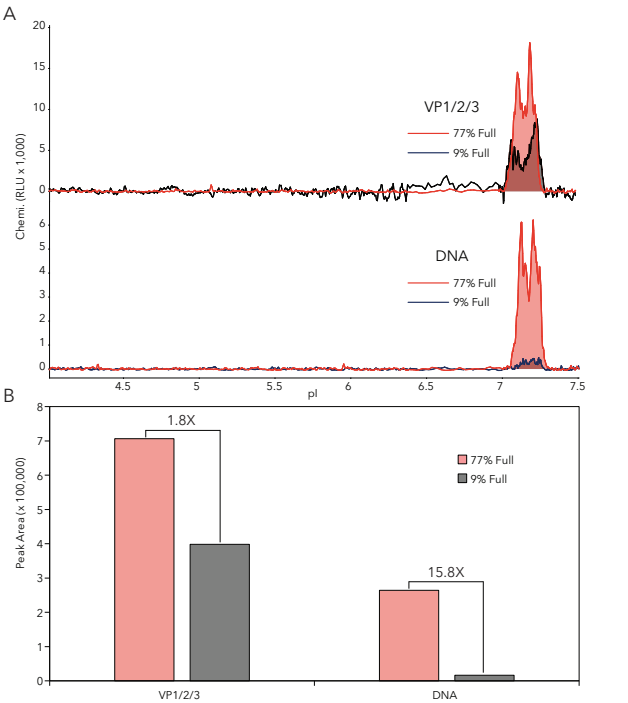



**FIGURE 8. Comparison of VP1/2/3 and DNA signals resulting from SW-Charge.** (**A**) Signals were detected using anti-VP1/2/3 (top panel) and anti-DNA (bottom panel) antibodies on 77% full (red) and 9% full (black) AAV9 samples. (**B**) Quantification of VP1/2/3 and DNA peak areas.

## Sensitive, Reproducible, & Scalable Empty/Full Ratio Measurements

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. Plus, 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 fluorimetric detection, limiting their use on complex sample types.

## References

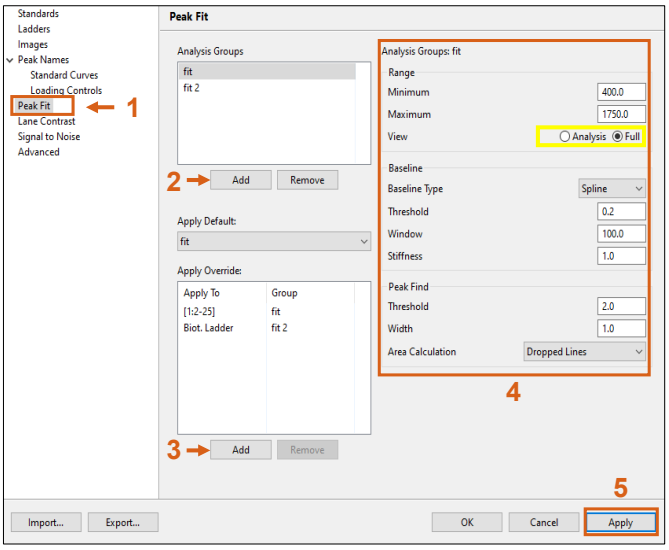

1. Fu et al. (2019) Hum Gene Ther Methods **30** 144–52
2. Lock et al. (2012) Hum Gene Ther Methods **23** 56–64
3. Yang et al. Waters Applications Note
4. Wang et al. (2019) Mol Ther Methods Clin Dev **15** 257–263
5. Gillespie et al. (2023) Hum Gene Ther **34** 68–77


## Appendix

### Recommended Total DNA Assay Settings

1. Under **Edit -> Analysis**, select **Peak Fit**
2. In the **Analysis Groups** section, add a **fit 2** group
3. Under **Apply Override**, add two groups and update as shown below
   a. **fit** settings for Total DNA
   b. **fit 2** settings for the Biotin Ladder (default settings)
4. Update the analysis parameters for **fit** as shown below for **Range**, **Baseline** and **Peak Find**, respectively
   a. It is important to select **Full View**, this will allow the user to appreciate the length of the capillary for proper baseline fitting
   b. Note: These are recommended analysis settings some adjustment may be necessary
5. Select **Apply**

Based on how the recommended settings work, it is best to fine tune the settings by adjusting parameters in the **Baseline** section, like **Threshold** and **Window.** You may need to adjust the **Maximum Range** slightly depending on where the cap end bump appears.








6004517633_LG_AN_Simple-Western-AAV-Empty-Full_CHH