Unchained Labs

APPLICATION NOTE

Take a peek inside your AAV capsid with Stunner

Introduction

Effectively quantifying adeno-associated virus (AAV) titers and determining empty/full ratios is a major challenge for developing and manufacturing gene therapy vectors. The list of currently available tools includes AUC, TEM, ELISA, PCR-based methods and more, but none of them deliver results quickly, easily, and from low sample volumes. TEM and AUC are gold-standard measurements, but require a lot of time, sample, and specialized equipment. ELISA and qPCR are solid workhorse techniques but take hours and require development and optimization for each AAV vector. In addition, qPCR accuracy can leave something to be desired.[1] Digital droplet PCR (ddPCR) is more precise than qPCR but has a smaller dynamic range, requiring exact sample dilutions.

Stunner is the first platform that gives researchers a full read-out on AAV capsid titer, empty/full ratio, and aggregation – in about a minute using only 2 µL (Figure 1). Stunner combines high-speed UV/Vis spectroscopy with static and dynamic light scattering (SLS & DLS) to measure titers down to 1E12 vg/mL and check size and aggregation of 96 samples in just 1 hour. For even higher throughput, Stunner is automation-friendly with its micro-volume SBS format plates. For regulatory environments, Stunner's performance can be verified for US and European Pharmacopeia compliance and souped-up with 21 CFR Part 11 tools.

Stunner makes sample titer determination faster and easier, making it possible to check capsid titer and empty/full ratios way more often. A read on Stunner before a PCR method takes the guesswork out of choosing a dilution factor. For those last-minute experiments, a quick check of sample titer can also spot aggregates that mess up your results. Using Stunner to judge capsid titers gives a faster, more precise and fuller picture of your sample that avoids the slow assay development needed to optimize an ELISA method for every capsid type.

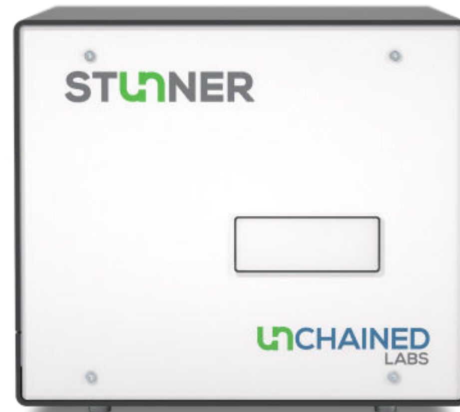


Figure 1: Stunner: the one-of-a-kind combination of UV/Vis, dynamic light scattering (DLS), and static light scattering (SLS).

This app note describes how Stunner's AAV Quant application uses UV/Vis spectroscopy, SLS, and DLS to quantify AAV empty/full ratios, full capsid titer, empty capsid titer, free & aggregated DNA, and free & aggregated proteins.

Methods

AAV2-CMV-GFP, AAV5-CMV-LacZ, empty AAV5, AAV9-CMV-GFP, and empty AAV9 (Virovek) were diluted to 2E12–2E13 cp/mL in PBS, pH 7.0, with 0.001% Pluronic F68 or citrate-phosphate buffer, 150 mM NaCl, pH 4.0. Capsid titers of stock solutions were determined with the appropriate serotype AAV titration ELISA (PROGEN).

Encapsidated ssDNA titers were determined by a SYBR Gold fluorescence assay. AAV stock solutions were prepared in Tris buffer with 0.25 U/mL DNaseI and 0.5 U/mL benzonase (Thermo Fisher) and incubated at 37 °C for 30 minutes. SDS, SYBR Gold, and EDTA were added to final concentrations of 0.05%, 1x, and 1.5 mM, respectively. Half of each

sample was heated to 75 or 90 °C for 10 minutes then cooled to room temperature. DNA concentrations were determined using a fluorescent plate reader and a DNA standard curve (NoLimits DNA Fragments, 2500–5000 bp, Thermo Fisher). Encapsidated ssDNA mass concentrations were calculated by subtracting the concentration of the unheated sample from the concentration of the heated sample. The mass concentration was converted to vg/mL equivalents with the molecular weight of the viral genome.

2 µL of each sample were loaded in quadruplicate in a Stunner plate. The AAV Quant application was selected in Stunner Client using 4 DLS acquisitions of 5 seconds each with a water blank. Stunner measures the full UV/Vis absorbance spectrum and determines protein and ssDNA titers based on molar extinction coefficients and differentiating the absorbance contributions of protein, ssDNA, and common impurities in AAV samples. Molar extinction coefficients for each AAV serotype were calculated based on published amino acid sequences. Molar extinction coefficients for each viral genome were calculated using the nucleic acid sequence provided by the manufacturer. Total capsid titer, full capsid titer, ssDNA titers, protein titers, % empty, and % full were determined by Stunner Analysis.

Results

AAV Quant on Stunner combines DLS, SLS, and UV/Vis techniques to determine capsid titers and simultaneously gather insight on total protein, total DNA, and empty/full ratio (Figure 2). DLS is a classic light scattering technique to identify size and size distribution for particles in solution. The DLS intensity distribution shows the relative intensities of light scattered by capsids and the aggregates commonly found in AAV samples. Gathered during a DLS experiment, SLS intensity is directly proportional to the particle concentration. SLS intensity could therefore be used to determine capsid titer, but full and empty capsids scatter different amounts of light at the same concentration. So, for a given intensity of light scattered from a sample, you need more than just DLS and SLS to get an accurate particle concentration – you need UV/Vis data too.

UV/Vis spectroscopy determines the total amount of protein and DNA present in a sample, but by itself cannot tell how much of that protein and DNA is packaged into AAV capsids.[2] For a purified sample, the ratio of protein and DNA present is also related to the empty/full ratio. When combined with DLS information, Stunner has enough info to calculate total capsid titer, full capsid titer, and the empty/full ratio of an AAV sample.

Stunner differentiated the protein titer of a sample of AAV5 at approximately 2E13 cp/mL into

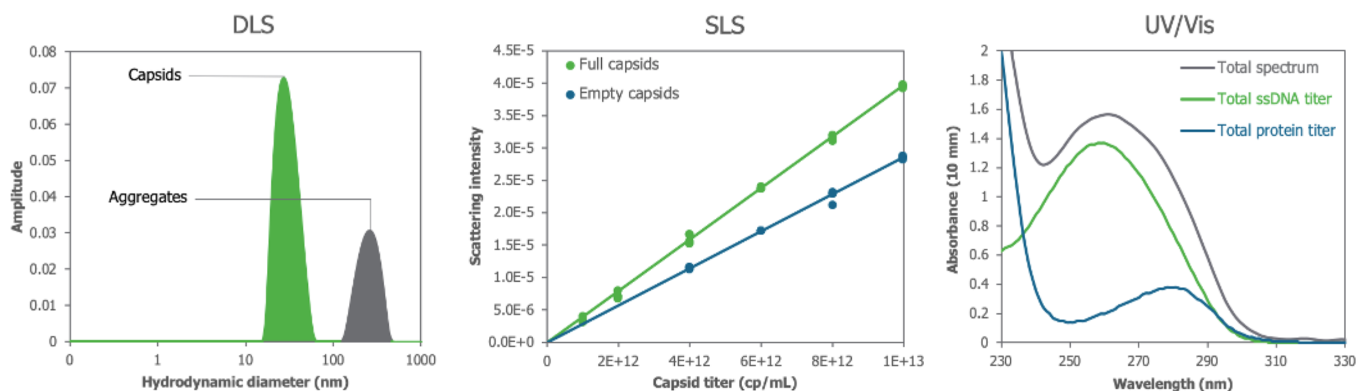


Figure 2: The DLS intensity distribution shows the relative intensity of light scattered by intact and aggregated capsids. SLS scattering intensity, collected at the same time as DLS, is directly proportional to capsid titer, but full and empty capsids scatter different intensities of light at the same titer. UV/Vis spectroscopy can quantify the amount of ssDNA and protein present, which in a purified AAV sample is related to the empty/full ratio. Stunner combines all 3 detection methods to determine capsid titer, full titer, and empty/full ratio, but can uncover finer details too.

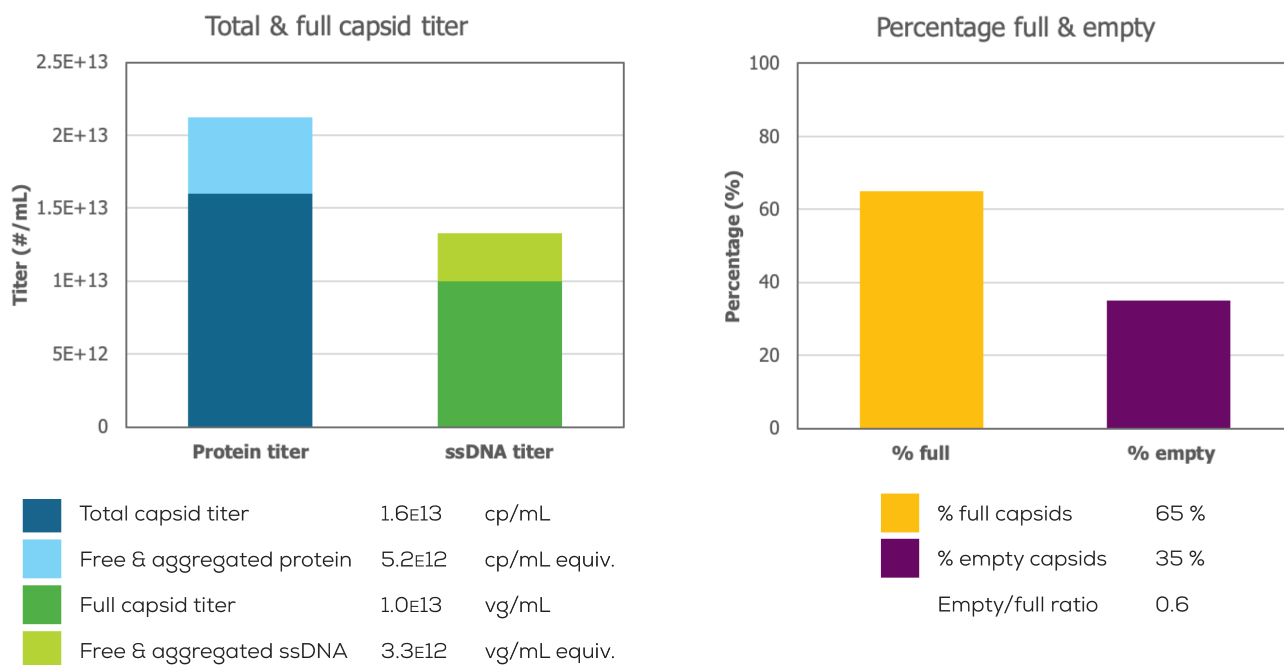


Figure 3: By combining UV/Vis absorbance, DLS, and SLS, Stunner can report AAV total capsid titer, full capsid titer, free & aggregated protein, free & aggregated ssDNA, % full, % empty, and empty/full ratio.

two components: free & aggregated proteins (light blue) and intact total capsid titer (dark blue), which includes full and empty capsids (Figure 3). Protein titer is the maximum number of capsids that could possibly be assembled from the proteins in a sample, as measured by UV/Vis absorbance. Stunner represents protein titers as cp/mL equivalents. The ssDNA titer is the maximum number of viral genomes possible if all the DNA measured by UV/Vis is ssDNA genomes. Stunner separates this value into free & aggregated ssDNA (light green) reported in vg/mL equivalents, and full capsid titer (dark green) reported in vg/mL.

Values for % full (yellow) are determined by dividing full capsid titer (dark green) by the total capsid titer (dark blue), while % empty (purple) is the difference between % full and 100%. Stunner also reports empty/full ratios, so you can use whichever metric is most convenient to you.

Capsid titers, ssDNA titers, and % full

Sandwich ELISAs can determine AAV capsid titers but require antibodies targeted to the AAV serotype of interest and high-quality standards, which are not always available. Stunner's capsid titers were compared against ELISA for a dilution series of empty and full AAV9 from 2E12–2E13 cp/mL (Figure 4A). Linear regressions between the Stunner and ELISA capsid titers had R^2 values > 0.98 and slopes near 1, showing a high level of agreement between these two methods of determining AAV capsid titers.

Along with the total capsid titer, Stunner calculated the full capsid titer of the AAV9 dilution series by combining the ratio of ssDNA and protein, determined by UV/Vis absorbance, with the number of intact capsids, determined by DLS and SLS (Figure 4B). This full capsid titer is equivalent to the amount of encapsidated ssDNA in a sample. The total encapsidated ssDNA was also quantified by an increase in SYBR Gold fluorescence after the AAV capsids were disrupted, as described above. Both Stunner and the SYBR Gold assay quantify total encapsidated ssDNA, contrasting with PCR methods that are sequence-specific and will not quantify any DNA not targeted by a primer.

Stunner also reported % full of the empty and full AAV9 dilution series. The results agree closely with % full values determined by combining encapsi-

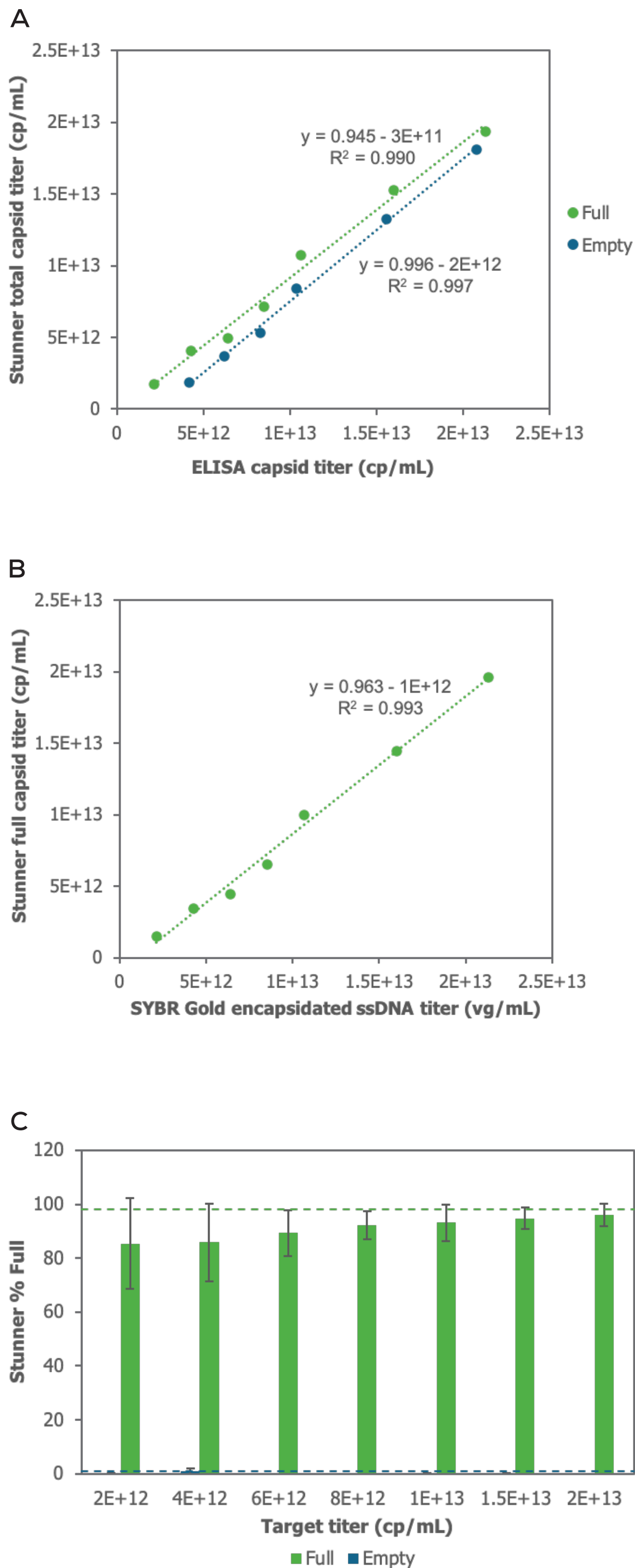


Figure 4: Titers determined by Stunner for a dilution series of full and empty AAV9 from 2E12–2E13 cp/mL compared to known ELISA titer (**A**) or encapsidated ssDNA titer determined by SYBR Gold assay (**B**). % full by Stunner for both dilution series (**C**) compared to % full by SYBR Gold assay and ELISA for full (green dashed line) and empty (blue dashed line) stocks. Error bars are ± 1 standard deviation (SD).

dated ssDNA from the SYBR Gold assay and total titer from ELISA (Figure 4C). Stunner does not use standards, labels or additional reagents, instead relying on the well-known biophysical properties of proteins and nucleic acids. Stunner is a single-shot quantification tool that requires no sample prep and gets you the results you need, fast.

One of the most difficult problems in AAV production is low yield since most of the AAV capsids produced in a single lot contain no DNA. Quantifying the empty/full ratio in mixtures of capsids requires either two separate assays (a PCR-based method and ELISA) or choosing between time-consuming or volume-intensive assays (TEM or AUC). Stunner used just 2 µL of sample and a few minutes to determine the % full and % empty of known mixes of empty and full AAV9 at 1E13 cp/mL and 2E13 cp/mL (Figure 5). The precision of this assay was quite impressive with standard deviations of less than 5% in most cases.

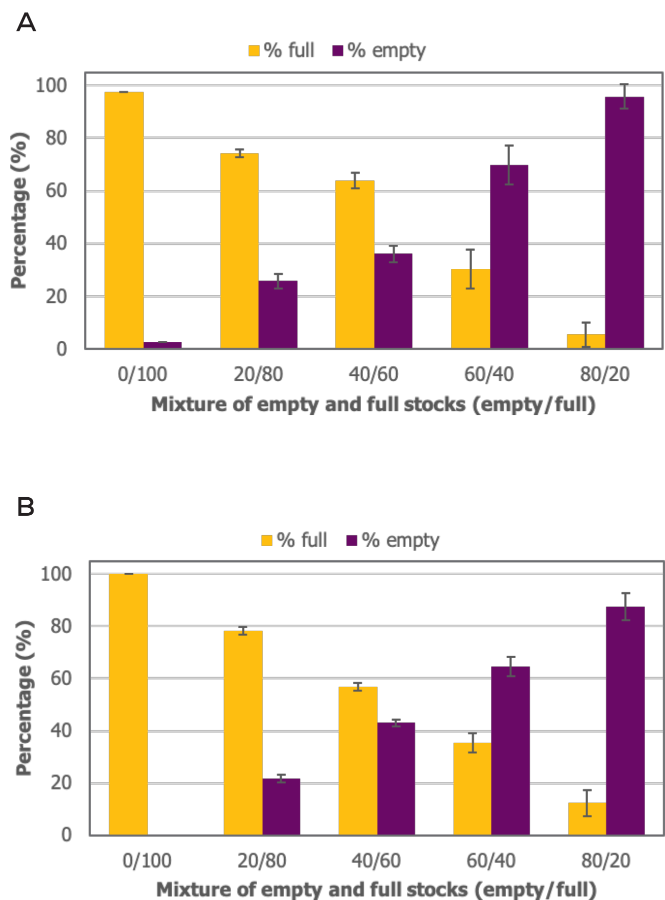


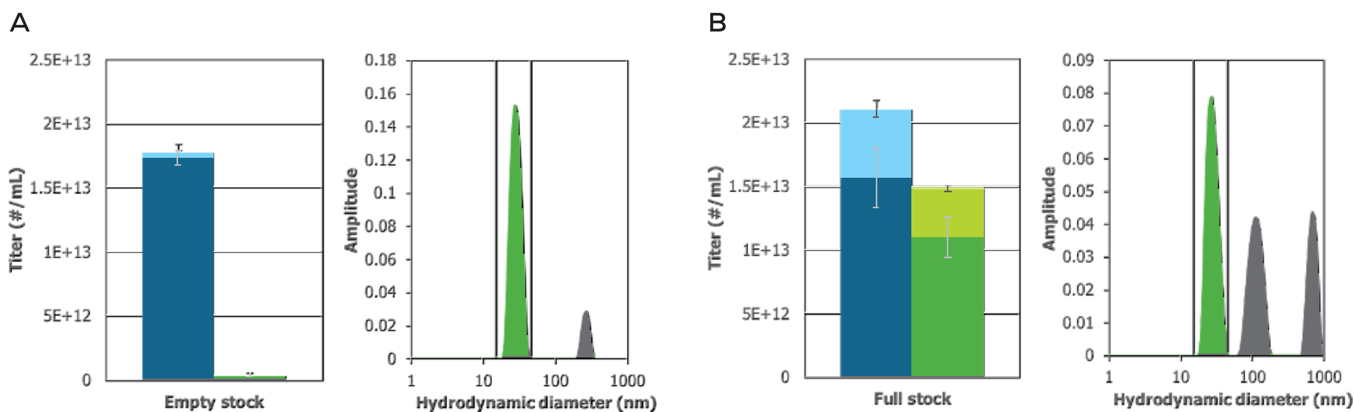
Figure 5: Percent full (yellow) and empty (purple) of known mixes of AAV9 in quadruplicate at 1E13 (**A**) and 2E13 (**B**) cp/mL. Error bars are ± 1 SD.

Aggregation and titers

AAV samples often contain a mix of intact virus, free protein, free DNA, and aggregated viruses. Identifying and quantifying these impurities can be vital to understanding exactly what's in your sample and making decisions on optimizing your AAV manufacturing processes. Only with UV/Vis, DLS and SLS combined can you get these insights. In one example, Stunner found that a stock of empty AAV5 contained mostly intact capsids with a precision of 3.4% CV and the sample included a small amount of aggregated protein impurities (Figure 6A). However, a stock of refined "full" AAV5 at a comparable capsid titer contained significantly more aggregated protein and DNA – and contained only 70% full capsids (Figure 6B). Even in the aggregated sample, Stunner showed precision between 1.6% and 15% CV. Regardless of how clean a sample is or how many full capsids it has, Stunner helps you figure out exactly what you've got.

Assessing AAV storage conditions is a slow process, made even slower when the only analytical tools you have are functional assays. Storage experiments are made more complicated because some AAV serotypes are more prone to aggregation than others.[2] Functional assays might tell you that you have lost infectious particles but won't tell you why. Stunner's DLS shows if a sample has aggregated so you can tell when a sample of AAV has gone bad without wasting time on cell-based assays.

Stunner DLS intensity distributions of AAV2 and AAV9 stored at 4 °C for 2 weeks showed AAV2 had peaks at much larger hydrodynamic diameters than AAV9, indicating significant aggregation (Figure 7). The large light blue bar of the AAV2 samples shows the extent of storage-induced aggregation while the dark blue and green bars dominating the graph for AAV9 show that most of the sample is intact, full capsids. Quickly reject-



Results	Empty stock average
Total capsid titer	1.7E13 ± 3.4% cp/mL
Full capsid titer	<LLOQ
Protein titer	1.8E13 ± 3.7% cp/mL
ssDNA titer	<LLOQ
% full	1.9 ± 1.1%

Results	Empty stock average
Total capsid titer	1.6E13 ± 15% cp/mL
Full capsid titer	1.1E13 ± 14% vg/mL
Protein titer	2.1E13 ± 3.1% cp/mL
ssDNA titer	1.5E13 ± 1.6% vg/mL equiv.
% full	70 ± 2.5%

Figure 6: Stunner determined the total & full capsid, protein and ssDNA titers, as well as aggregation and % full of stocks of empty (**A**) and full (**B**) AAV5 in quadruplicate at 2E13 cp/mL. The green regions of the intensity distributions show the scattering intensity due to intact viral capsids while the gray regions show the scattering and size of aggregates. Vertical grey lines show the upper and lower size limits of intact capsids.

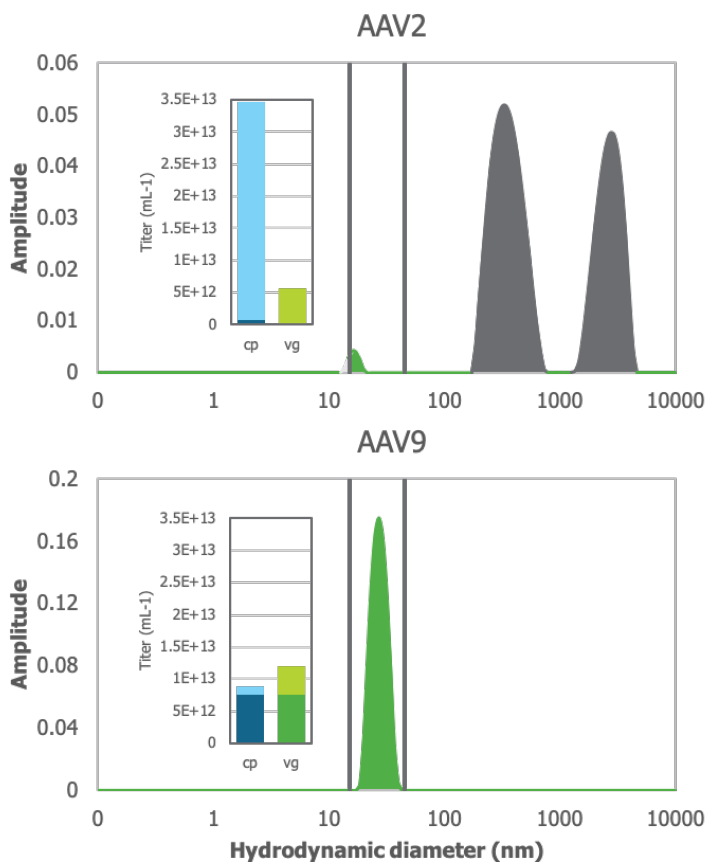


Figure 7: After storing both samples at 4 °C for 2 weeks, DLS intensity distributions show AAV2 had more aggregation than AAV9 while AAV9 had more full, intact capsids.

ing samples you know won't work in a cell-based assay saves time and means you can focus on the samples you know will work.

Choosing the right buffer in formulation studies of AAV vectors is absolutely necessary, especially when it comes to pH.[3] If the tools you use to characterize your AAV are too slow or take too much sample, you might miss out on the optimal buffer. Stunner uses 2 µL to screen up to 96 samples for aggregation after stress in just 1 hour, so you can identify the most protective buffer.

Heating full AAV9 to 45 °C for 25 minutes in a neutral (pH 7) buffer caused some aggregation, but heating it in an acidic (pH 4) buffer caused nearly complete aggregation (Figure 8). With its lightning-fast DLS reads, Stunner makes it easy to evaluate more buffers and stress conditions than traditional methods while also providing titer results.

Conclusion

Quantifying AAV titers and empty/full ratios is an arduous task. Other methods are time-consuming and require significant sample amounts. Stunner speeds things up with high-throughput determination of capsid titer, ssDNA titer, and empty/full ratio from tiny sample volumes. In addition to accurate titers, Stunner's combo of UV/Vis and DLS makes it easy to look deeper into AAV quality – identifying aggregates, problem samples, or testing stress conditions in just minutes. Regardless of whether a vector is in research, development or manufacturing, Stunner gives you the data you need and helps you know your capsid inside out.

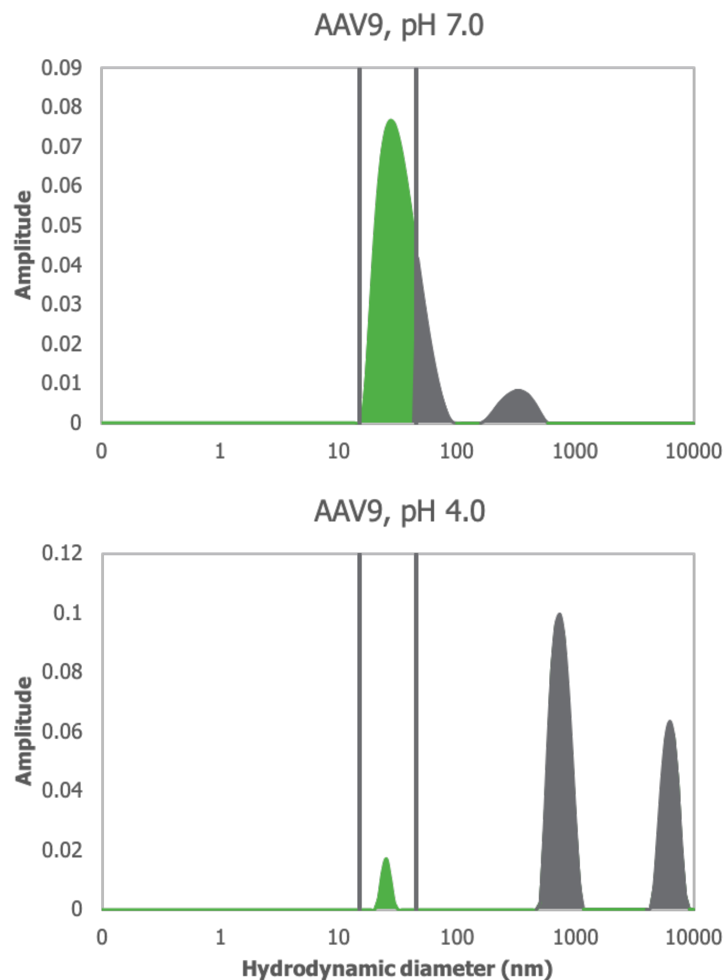


Figure 8: 1E13 vg/mL full AAV9 in pH 7.0 and 4.0 buffers were heated to 45 °C for 25 minutes then measured on Stunner.

References

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