DNA leaks before capsids pop: AAV thermal stability on Uncle

Introduction

Characterizing viral stability for gene therapies using adeno-associated virus (AAV) vectors is a complex task and requires understanding behaviors of whole viral particles, viral genomes, and capsid proteins. Serotypes, formulations, manufacturing methods, and storage conditions all need to be evaluated for their impact on stability. Functional assays, like transduction or infectivity assays, take days and require highly skilled users, while electron microscopy is low throughput and expensive. Even routine assays like AAV ELISAs, which can be used to measure intact capsid titers, or qPCR, which looks at genome quantification, can take several hours. Uncle is ready to help by combining thermal ramp studies with versatile analytical tools to give stability insights that look at capsid, protein and DNA behavior all on the same instrument and in the same experiment. With Uncle, results come in under 2 hours so you can screen more candidates, formulations, or conditions and get to the right answers quickly.

Uncle is a multi-modal stability platform with 3 detection methods: full-spectrum fluorescence, static light scattering (SLS), and dynamic light scattering (DLS) to quickly profile viral thermal stability (Figure 1). Temperature control (15–95 °C) gives you flexibility in your experimental design, and sealed sample chambers minimize evaporation and keep hazardous samples contained. Multiple measurements such as fluorescence, aggregation, sizing, and size distribution can be performed in just one experiment, allowing you to obtain orthogonal stability metrics from the same samples. The low volume, multi-well quartz cuvette chambers require only 9 µL of sample and Uncle can measure up to 48 samples simultaneously. AAV concentrations as low as 5E11 vg/mL can be studied with dye-based fluorescence, enabling stability insights earlier in

Figure 1: Uncle: a one-stop protein stability platform.

workflows. For regulated labs, Uncle has you covered with 21 CFR part 11 software tools, providing full sample tracking, data authenticity, and user accountability.

Thermal denaturation of AAV involves two pathways: genome ejection and capsid disruption (Figure 2).[1] Genome ejection from AAV capsids occurs at relatively lower temperatures as DNA starts to escape from intact capsids. Uncle can track genome ejection with DNA-binding fluorescent dyes, like SYBR Gold, to determine a melting temperature (T_m) based on DNA release.[2]

AAV capsid disruption occurs when protein unfolding causes the viral capsid to lose structural integrity and break apart. The AAV-ID assay is a traditional example of a capsid disruption assay that uses differential scanning fluorimetry (DSF) with SYPRO Orange dye to identify protein unfolding and measure thermal stability of different serotypes and in various formulations.[3,4] However, extrinsic dyes are known to interact with hydrophobic regions of proteins to alter unfolding and aggregation behavior, and dyes are not compatible with some excipients. Capsid

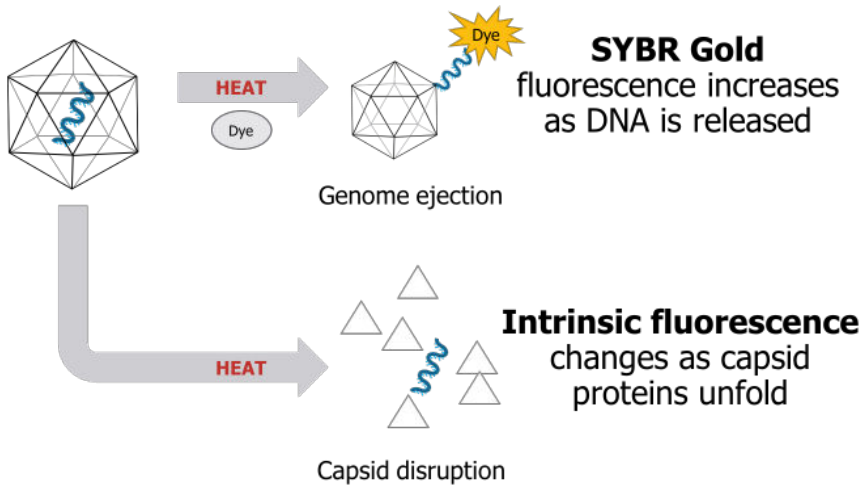


Figure 2: Heat-induced degradation of AAV involves two pathways: genome ejection and capsid disruption. In genome ejection the capsid remains intact, but the viral genome is released. Capsid disruption occurs when capsid proteins denature and capsid structure is lost. Uncle can track both degradation pathways with full-spectrum fluorescence detection.

disruption of AAV can be studied on Uncle without a dye by monitoring the intrinsic protein fluorescence of capsid proteins to determine their unfolding behavior. Uncle's full-spectrum fluorescence detection and two lasers equip it for studying both genome ejection and capsid disruption of AAV.

As a part of the Capsid Stability & DLS application, Uncle reports the fluorescence intensity from DNA present at the start of an experiment and the signal from total DNA present at the end of a thermal ramp. These results can help determine the amount of DNA released from viral particles ruptured during a thermal ramp.

For AAV samples, aggregation is a significant problem and can happen to intact capsids or as a result of protein unfolding. Capsid aggregation is more common in some serotypes and at high concentrations.[5,6] On Uncle, SLS monitors aggregation during a thermal ramp and reports a T_{agg} metric where severe aggregation begins. DLS quantifies aggregation by reporting particle size and size distribution. DLS intensity also correlates with capsid particle concentration for non-aggregated samples.

This application note illustrates Uncle's ability to determine the thermal stability of AAV vectors by monitoring genome ejection, capsid unfolding, and aggregation at different concentrations, serotypes, and formulations. In addition, Uncle determines the size of AAV particles with DLS both before and after thermal ramps. It also shows how to use Uncle to detect free DNA before an experiment, total DNA released by a thermal ramp, and how to measure a viral particle count. This huge data set is available with Uncle's three detection methods and Capsid Stability & DLS application (available on Uncles shipped after April 1, 2020).

Methods

10,000x SYBR Gold in DMSO (Thermo Fisher Scientific) was diluted to a 400x working stock in phosphate-buffered saline (PBS), pH 7.4 (MilliporeSigma). AAV vectors expressing CMV-GFP (Virovek) were used between 5E11 and 1E14 vg/mL in a formulation buffer of PBS with 0.001% Pluronic F68 in the case of AAV9, or the same buffer with 100 mM sodium citrate in the case of AAV1 and 2. When used as an excipient, arginine was spiked into the formulation buffers to the final concentration of 300 mM. DNA standards were extracted from AAV vectors using the PureLink viral DNA/RNA extraction kit (Thermo Fisher), quantified by UV/Vis spectrometry with the Lunatic (Unchained Labs), then diluted to 0.1, 2, 8, or 16 ng/µL in PBS with 20x SYBR Gold.

For all experiments, samples were centrifuged in a benchtop centrifuge for 30 seconds at 14,000 rpm to spin down any large particles. 9 µL of each AAV sample were run in triplicate. T_{agg} 266 and T_{agg} 473

were determined based on the SLS signals of their respective lasers.

Genome ejection

AAV vectors were tested with 20x SYBR Gold, 1 set of standards, and 1 well of buffer alone with the Capsid Stability & DLS application on Uncle. SYBR Gold was excited with a 473 nm laser. DLS reads were 4 acquisitions of 5 seconds each. Initial fluorescence and DLS were detected at 25 °C. The samples were heated from 25–95 °C at a rate of 0.5 °C/minute while monitoring fluorescence. Final DLS was read at 95 °C and the samples were cooled to 25 °C for a final fluorescence measurement. Uncle Analysis software determined the T_m based on the area under the fluorescence intensity curves from 500–650 nm (Figure 3).

Capsid protein unfolding

Capsid protein unfolding was tested with the T_m & T_{agg} with Optional DLS application on Uncle. Intrinsic protein fluorescence was excited with a 266 nm laser. DLS reads were 4 acquisitions of 5 seconds each. Samples were heated from 25–95 °C at a rate of 0.5 °C/minute while monitoring fluorescence. DLS measurements were taken at the beginning and end of the thermal ramp. Uncle Analysis software determined the T_m and T_{onset} based on the barycentric mean (BCM) of the fluorescence intensity curves from 300–430 nm (Figure 4).

DLS particle intensity

AAV9 was diluted to 5, 24, 43, 68, and 100 x 10^{11} vg/mL in formulation buffer. Particle intensities were determined for each sample using the Capsid Stability & DLS application to construct a standard curve. Particle intensities for seven AAV9 samples diluted to 8.2, 21, 34, 46, 59, 72, and 84 x 10^{11} vg/mL in formulation buffer were measured and the standard curve was used to determine the particle count of the samples.

Results

AAV stability

Genome ejection from AAV9 can be detected with Uncle's Capsid Stability application and SYBR Gold fluorescent dye (Figure 5). In an experiment run, a thermal ramp is performed and the resulting SYBR Gold fluorescence increases as DNA concentration increases. The behavior of the dye can be analyzed in several phases. First, any free viral DNA or residual cell DNA in the sample will bind to SYBR Gold and result in

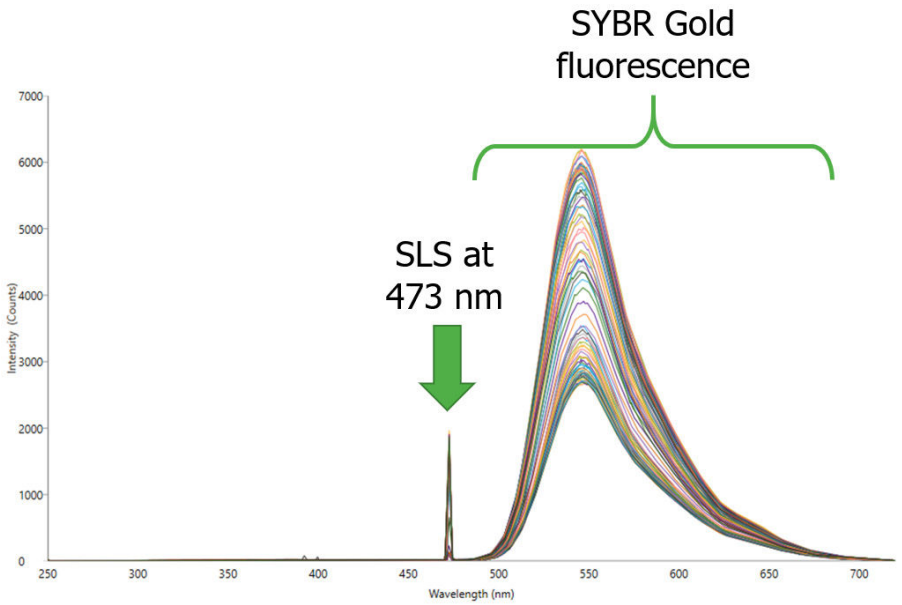


Figure 3: The Capsid Stability app on Uncle uses a 473 nm laser to excite SYBR Gold bound to DNA and measures SLS. The viral vector is heated causing the genomes to eject and bind to SYBR Gold, increasing fluorescence intensity from approximately 500–650 nm. Uncle Analysis uses the increase to determine T_m and T_{onset} based on the area under the fluorescence intensity curve.

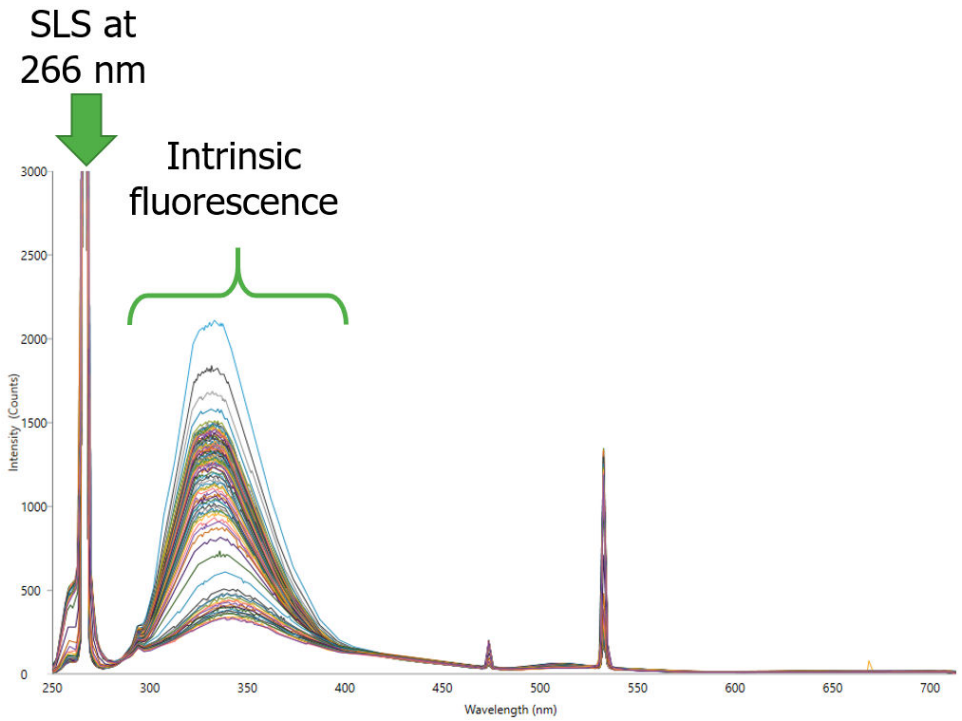


Figure 4: Uncle uses a 266 nm laser to excite intrinsic protein fluorescence and measures SLS. As the viral capsid proteins unfold their intrinsic fluorescence decreases and shifts to the right. This shift is tracked by monitoring the spectral central of mass, or BCM.

an initial fluorescence signal. Second, since the dye's signal is temperature dependent, signal will decrease as temperature increases. Third, DNA release from capsids into the buffer starts at approximately 50 °C. The newly released viral genomes bind to SYBR Gold and increase the fluorescence intensity. Uncle uses this change in intensity to determine a T_m of about 57 °C for this sample.

Uncle uses the SLS signal of the 473 nm laser to monitor AAV9 aggregation during the thermal ramp as part of the Capsid Stability application. Aggregation of AAV9 begins significantly after genome ejection, with a T_{agg} 473 of about 75 °C (Figure 5).

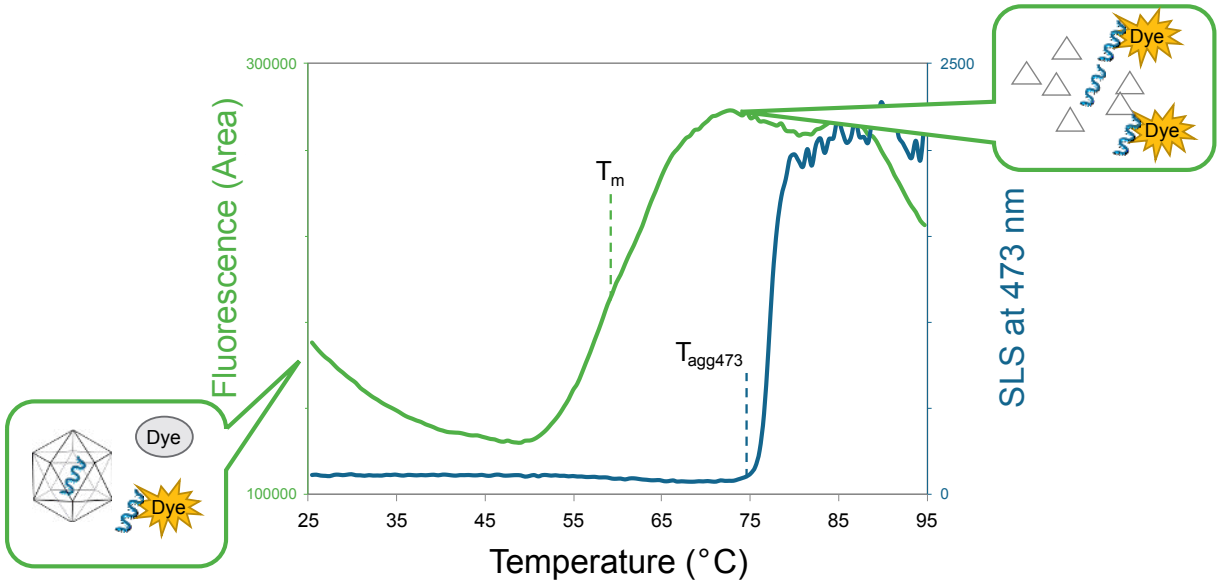


Figure 5: Genome ejection melting curve (**green line**) based on fluorescent intensity of SYBR Gold bound to DNA and SLS 473 intensity curve (**blue line**) of 1E13 vg/mL AAV9.

To test for a relationship between genome ejection and concentration, a range of 5E11 to 1E13 vg/mL AAV9 was tested. The resulting T_ms of genome ejection were between 57.2 and 57.8 °C, suggesting any concentration-dependent effect is minimal (Figure 6). As expected, fluorescence intensity is lower for the lower concentration samples as less DNA is present.

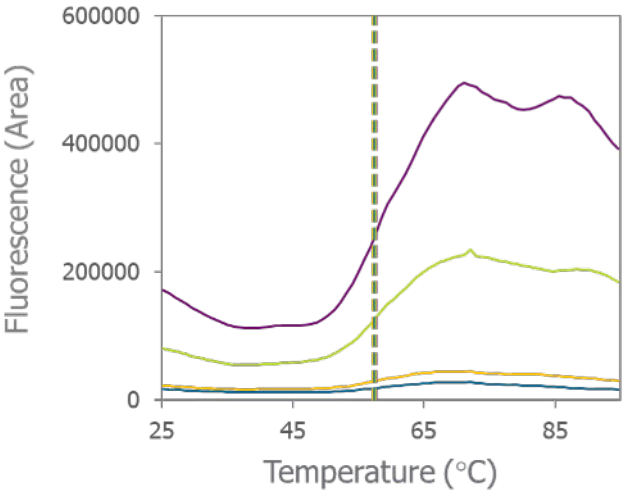


Figure 6: Genome ejection melting curves of 5E11 (**blue**), 1E12 (**yellow**), 5E12 (**light green**), and 1E13 (**purple**) vg/mL AAV9. Dashed lines indicate the T_m's of all samples as determined by Uncle Analysis software.

Capsid protein thermal stability can be tested by monitoring changes in the intrinsic fluorescence signal of the protein. As capsid proteins are heated and unfold, the local environment of fluorescent amino acids change and as a result the protein's total fluorescence behavior changes. When applied to AAV9, Uncle's DSF using intrinsic fluorescence found the T_m of the unfolding capsid proteins was 78.6 °C (Figure 7). The T_{onset} indicating the beginning of protein unfolding at 74.2 °C aligns closely with the aggregation temperatures by SLS at 266 nm (74.5 °C) and 473 nm (75.2 °C). This simultaneous behavior suggests that aggregation is driven by protein unfolding.

Since the capsid unfolding T_m was over 20 °C hotter than the genome ejection T_m for AAV9, genome ejection during thermal stress precedes capsid unfolding and suggests the two pathways may be independent. Uncle makes it possible to quantify AAV thermal stability based on DNA ejection or capsid disruption with a single instrument.

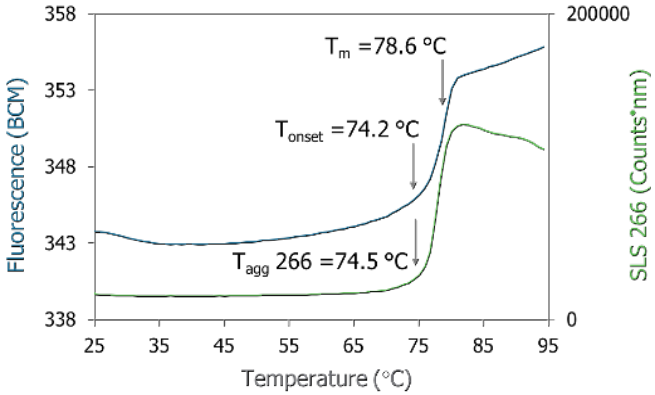


Figure 7: Average capsid unfolding curve by intrinsic fluorescence and average SLS 266 curve of 1E13 vg/mL AAV9 in triplicate with average T_m, T_{onset}, and T_{agg} 266.

Using DLS to determine viral particle size and size distribution is another way to detect aggregation or quickly check a sample's quality. Uncle can measure DLS of a sample before and after heating in both the T_m & T_{agg} and the Capsid Stability applications. Size and polydispersity before a thermal ramp are indicators of sample quality at the outset of an experiment and DLS after heating confirms the extent of aggregation.

Initial DLS results show AAV9 is monodisperse with a Z-average diameter of approximately 29 nm, as expected for non-aggregated AAV (Figure 8, green lines). After a thermal ramp the sample aggregated significantly with a Z-average diameter well over 1000 nm (Figure 8, blue lines).

Different serotypes of AAV have different thermal stabilities for both genome ejection and capsid disruption. Understanding these differences can be helpful in selecting a gene therapy vector or when optimizing for capsid stability through mutagenesis. Notably, the T_ms of capsid protein unfolding for AAV serotypes 1, 2, and 9 determined on Uncle agree closely with literature T_m values determined by DSF with SYPRO Orange dye (Figure 9).[4]

Differences are again seen when examining the genome ejection T_m for different serotypes. AAV2 with T_m = 49.6 °C shows lower stability than AAV9 with T_m = 57.2 °C (Figure 10), consistent with the pattern seen by capsid protein unfolding behavior. These data indicate AAV9

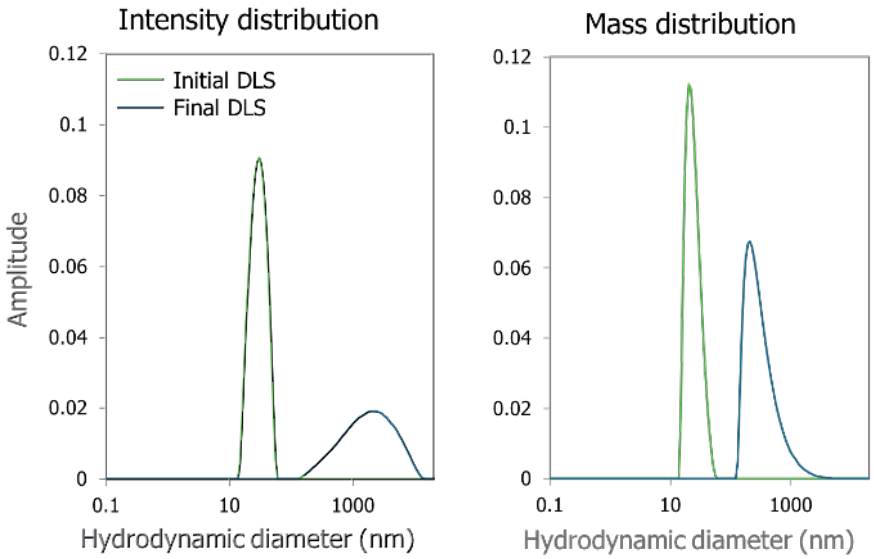
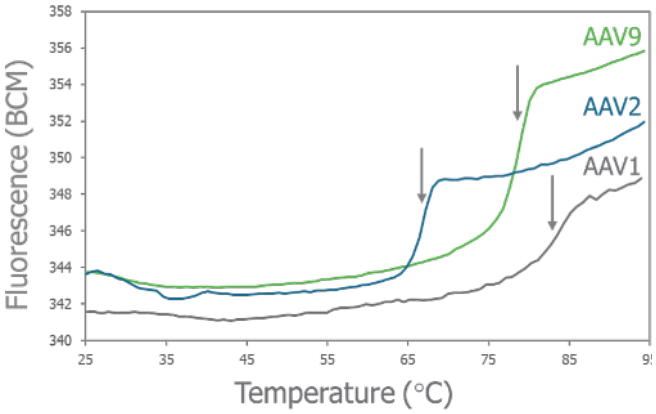


Figure 8: Intensity and mass distributions before (**green lines**) and after (**blue lines**) thermal ramp of 5E12 vg/mL AAV9 in PBS.

A



B

	By SYPRO Orange (Bennett)	Intrinsic fluorescence on Uncle
AAV1	83.5	83.2
AAV2	68.2	66.7
AAV9	77.0	78.6
Concentration	1E14 vg/mL	5-10E12 vg/mL

Figure 9: Capsid unfolding curves of AAV1 (**gray**), AAV2 (**blue**), and AAV9 (**green**) by intrinsic fluorescence and a table of the published T_m of each serotype determined by DSF with SYPRO Orange or Uncle intrinsic fluorescence.

is more thermally stable than AAV2 via multiple methods easily performed on Uncle.

The effect of excipients on AAV stability by genome ejection and capsid disruption can also be studied on Uncle. Based on intrinsic fluorescence, AAV9 samples in buffers with added arginine show capsid protein unfolding at a lower temperature than those in PBS alone (Figure 11). Meanwhile, SYBR Gold fluorescence results for AAV9 in both buffers show that arginine impacts genome ejection by approximately 12 °C, greater than arginine's impact on protein unfolding. These results indicate arginine at this concentration has a negative impact on the conformational stability of this AAV vector, and that the impact is most significant on genome ejection.

Initial and final DNA

DNA present before a thermal ramp and DNA released during heating can be quantified on Uncle with the Capsid Stability application. DNA present prior to a thermal ramp can be indicative of residual cell DNA or viral DNA released during storage. SYBR Gold fluorescence measured before and after a thermal ramp correlate strongly with AAV9 titer (Figure 12). The higher fluorescence measured after heating indicates the amount of DNA released from the AAV particles. By comparing fluorescence intensity to standard

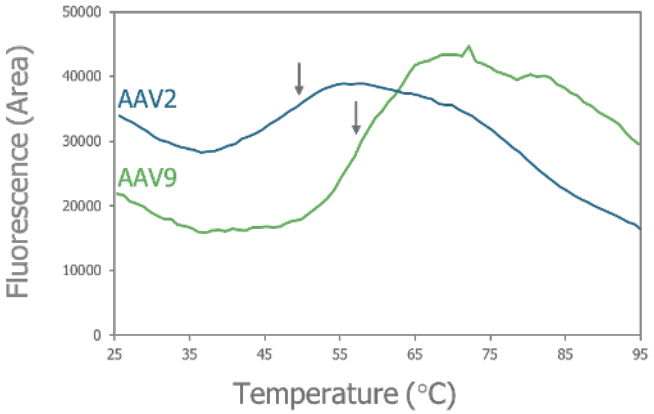


Figure 10: Genome ejection curves of 5E12 vg/mL AAV2 (**blue line**) and 1E12 vg/mL AAV9 (**green line**) in their formulation buffer with arrows indicating the T_ms.

curves, the amount of encapsulated DNA can be determined. To check if all capsids were ruptured by the thermal ramp, the final DNA concentration was compared to the expected final DNA concentration if all viral DNA was released, based on the manufacturer's supplied genome titer and sequence.

Particle counts

Determining capsid particle counts for AAV is a pain and requires a lot of time and effort. Thankfully, Uncle's DLS can be used to quickly check out AAV particle count, provided there is not significant aggregation. The particle intensity metric delivered by the Capsid Stability & DLS application measures the scattering intensity of the sample and applies a few calibration adjustments to enable the largest possible linear range. Particle intensity is linear with concentration and has a lower limit of approximately 5E11 AAV9 vg/mL (Figure 13A). With the particle intensity from a known dilution series of AAV you can create a standard curve to help quantify the particle count in unknown samples. When testing a verification set of AAV concentrations, the slope of known vs. calculated particle concentration was nearly 1 with an $R^2 > 0.995$, indicating highly accurate and precise measurements (Figure 13B).

Conclusion

Optimizing every aspect of an AAV gene therapy vector from serotype to formulation to production process is hard work. Stability studies are a huge, time-consuming piece of that puzzle, especially if you're relying on functional assays. Uncle can help with its impressive array of analytical tools for assessing genome ejection, capsid unfolding, and vector aggregation. Even better, Uncle also delivers results on a sample's free DNA, particle count, and encapsulated DNA. With all this data from a single instrument, after a few experiments you'll wonder why you didn't add Uncle to your viral toolbox sooner.

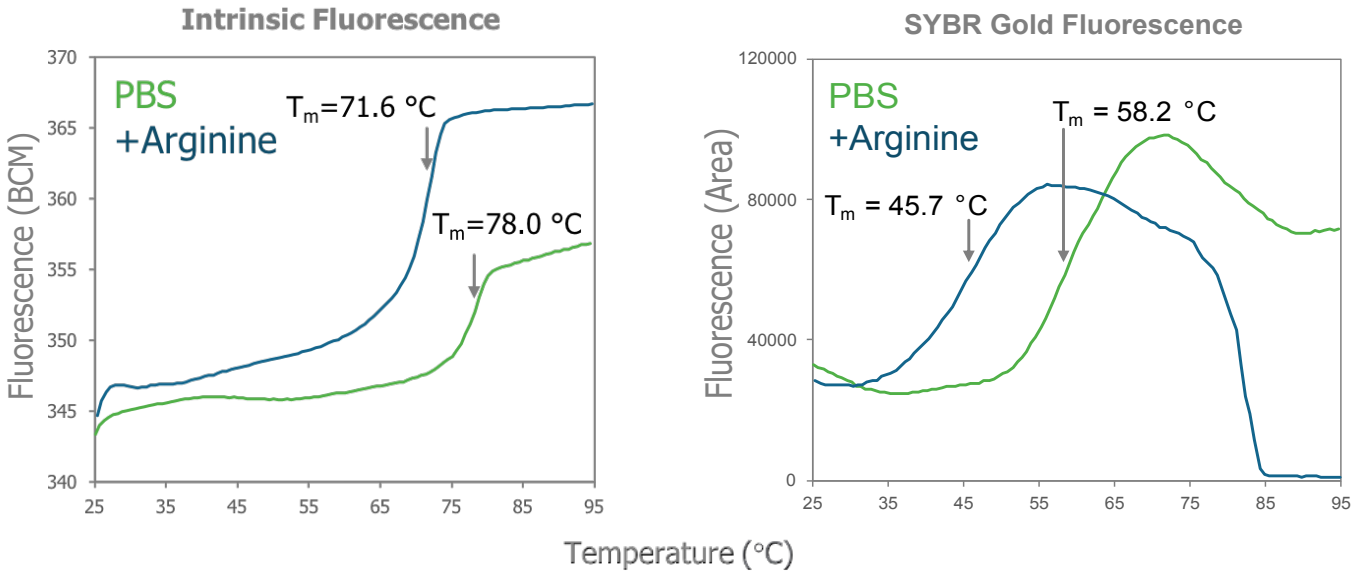
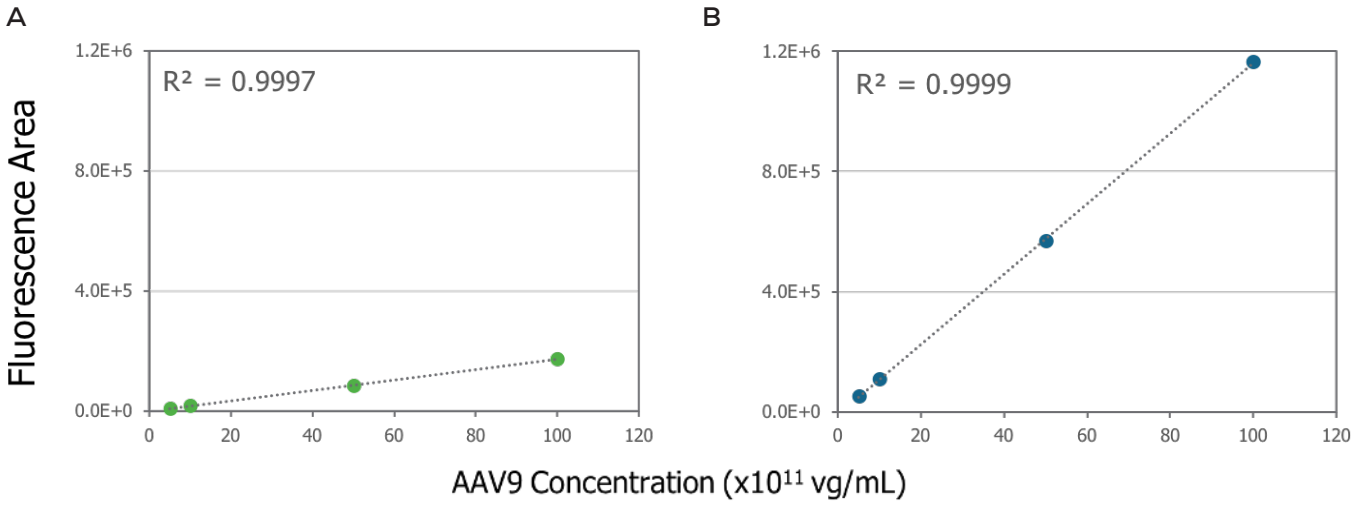


Figure 11: Capsid unfolding curves by intrinsic fluorescence and genome ejection curves by SYBR Gold fluorescence of 4E12 vg/mL AAV9 in PBS (**green curves**) and PBS with 300 mM arginine (**blue curves**). Arrows indicate T_ms.



AAV Concentration (vg/mL)	Initial DNA (ng/µL)	Final DNA (ng/µL)	Expected Final DNA (ng/µL)
5×10^{11}	0.05	0.66	0.64
1×10^{12}	0.95	1.28	1.28
5×10^{12}	0.53	6.89	6.42
1×10^{13}	1.21	13.18	12.83

Figure 12: SYBR Gold fluorescence intensity before (**A**) and after (**B**) thermal ramp at several AAV9 concentrations in PBS. Average DNA concentration values at each point (**table**) and the expected concentration of the encapsulated DNA.

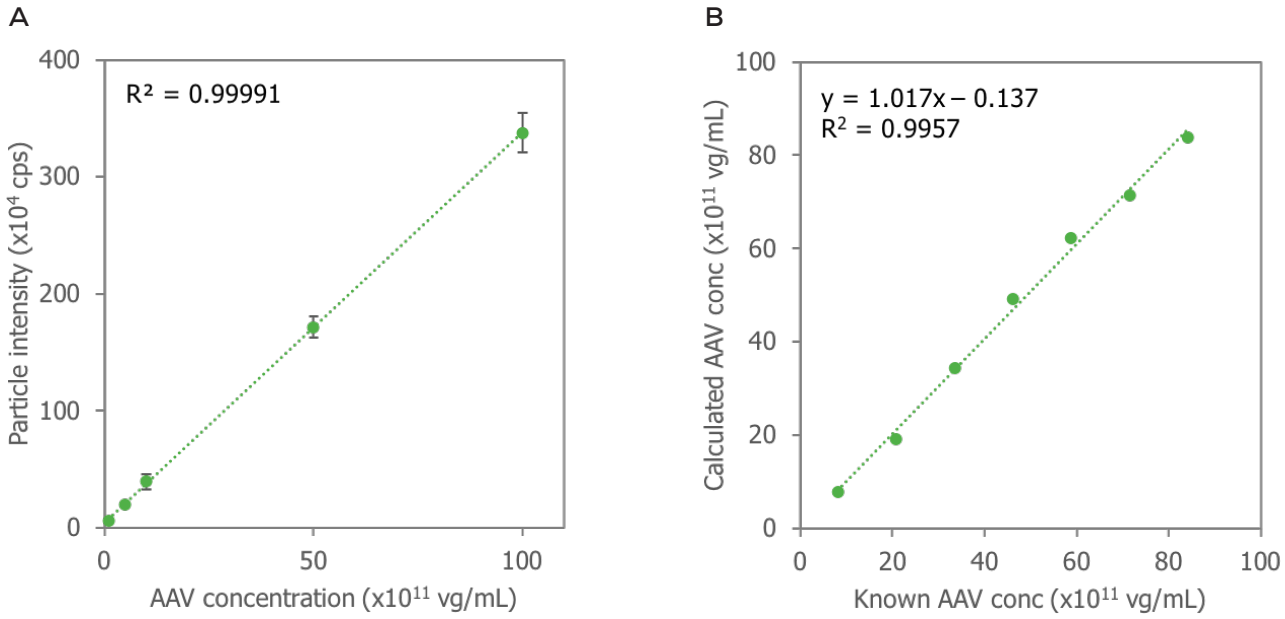


Figure 13: Standard curve of AAV9 concentration vs. particle intensity (**A**) and calculated vs. known AAV9 concentration with linear regression (**B**).

References

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Note: SYPRO is a registered trademark of Molecular Probes, Inc. SYBR is a registered trademark of Thermo Fisher Scientific. The Capsid Stability & DLS application is only available to Uncles shipped after April 1, 2020.

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