Explore AAV buffers and stability with Big Tuna and Uncle

Introduction

Adeno-associated virus (AAV) vectors endure a lot of stress when exploring the unknowns of a purification process: buffer salts, pH and ionic strength all impact stability and yield, and can decrease AAV infectivity.[1] Proper buffer selection and stability characterization are critical to determine the best conditions, but most workflows are a hassle due to high sample volume requirements and time-consuming buffer exchange.

Conventional exchange methods used for buffer exchange, such as dialysis and centrifugal devices, are labor-intensive, prone to inconsistency, and difficult to manage in larger numbers. Big Tuna was developed as a high-throughput automated buffer exchange system to enable uniform sample handling and a degree of process control that can't be achieved by manual methods (Figure 1A).

AAV stability should be characterized in each new process buffer. However, these studies are rarely performed due to the difficulty of the assays involved. Functional assays, such as transduction or infectivity assays, take days with expensive and sensitive setups. Electron microscopy and other structural characterization tools are low-throughput and require highly skilled operators. None are sufficient to effectively screen stability for all buffers used during process development.

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 1B). Multiple measurements such as fluorescence, aggregation, sizing, and size distribution can be performed in just one experiment. Uncle can measure up to 48 samples simultaneously and requires only 9 µL of sample in its multi-well quartz cuvette chambers.

A

B

Figure 1: **(A)** Big Tuna automates buffer exchange and concentration of up to 96 unique samples. **(B)** Uncle: the one-stop AAV stability platform.

Thermal denaturation of AAV involves two pathways: genome ejection and capsid disruption.[2] Uncle's Viral Toolbox can track genome ejection with DNA-binding fluorescent dyes, like SYBR Gold, to determine a melting temperature (T_m). Capsid disruption occurs when the viral capsid loses structural integrity, while unfolding behavior can be studied by monitoring the intrinsic fluorescence of capsid proteins. For more info on how Uncle characterizes AAV thermal stability, check out our application note: DNA leaks before capsids pop: AAV thermal stability on Uncle.

In this application note, Big Tuna and Uncle were used in combination to perform an AAV stability screen in different buffers. Big Tuna buffer exchanged AAV9 into 5 buffers and concentrated the resulting samples 2-fold. Uncle determined the thermal stability of the AAV vector by monitoring genome ejection, capsid unfolding, and aggregation in the different buffers. In addition, Uncle determined the size of AAV particles with DLS both before and after thermal ramps.

Methods

AAV and buffer preparation

AAV9-CMV-GFP (Virovek) in phosphate-buffered saline (PBS), pH 7.4 with 0.001% Pluronic F68 was prepared to 7E11 cp/mL, as determined by AAV9 Titration ELISA (Progen) according to the manufacturer's protocol. The 5 buffers (Table 1) were prepared and passed through 0.2 µm filters. PBS, pH 7.4 with 0.001% Pluronic F68 was used as a process control where the initial and final buffer would be the same. The other 4 buffers are published chromatography buffers. Citrate-phosphate buffer, pH 4.0 has been shown to negatively impact AAV9 stability and here was used as a positive control.[3] The buffers were placed on the deck of Big Tuna prior to buffer exchange. AAV9 was manually pipetted into 10 wells of a 10 kDa Unfilter 96 (380 µL/well).

Buffer exchange

Big Tuna uses a pressure-based ultrafiltration/diafiltration (UF/DF) method to remove and replace buffer. The plate is gently mixed, ensuring that AAV cannot accumulate at the membrane surface, while keeping flow more uniform and faster than dead-end filtration methods. The setup is adaptable and allows for buffer exchange of up to 96 unique vectors and formulations in a single experiment with as little as 100 µL per sample.

The buffer exchange protocol was set to 96% total exchange per pool with a target volume removal per cycle of 33%. The orbital mixing duty cycle was set at 100%. Each exchange was performed in duplicate. To concentrate each sample 2-fold after buffer exchange, final well volume was targeted to 190 µL. Capsid titers were determined in triplicate by ELISA after buffer exchange and concentration. Expected titers were determined using the initial volume, final volume, and the starting capsid titer.

Capsid disruption

For all Uncle experiments, 9 µL of each AAV sample was run in triplicate and heated from 15–95 °C at a rate of 0.4 °C/minute while monitoring fluorescence. 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 and were taken at the beginning and end of the thermal ramp. Uncle Analysis software determined the T_m based on the barycentric mean (BCM) of the fluorescence intensity curves from 315–430 nm.

Genome ejection

10,000x SYBR Gold in DMSO (Thermo Fisher Scientific) was diluted to a 400x working stock in each of the buffers. AAV9 samples and buffer controls were tested with 20x SYBR Gold with the Capsid Stability application on Uncle, part of the Viral Toolbox. SYBR Gold was excited with a 473 nm laser. Uncle Analysis software determined the genome ejection T_m from

Treatment	Buffers
Concentrated only	PBS, pH 7.4, 0.001% Pluronic F68
Buffer exchanged & concentrated	25 mM Na_2HPO_4, 100 mM NaCl, 2 mM $MgCl_2$, pH 6.5[4]
Buffer exchanged & concentrated	20 mM Tris, 500 mM NaCl, pH 8.0[5]
Buffer exchanged & concentrated	100 mM NaAcetate, 500 mM NaCl, pH 5.0[5]
Buffer exchanged & concentrated; Positive control for AAV stability	61 mM citric acid, 78 mM Na_2HPO_4, 150 mM NaCl, pH 4.0[3]

Table 1: Big Tuna buffer exchanged 7E11 cp/mL AAV9 in PBS, pH 7.4 with 0.001% Pluronic F68 into these 5 buffers followed by 2-fold concentration.

Buffer	Expected titer (cp/mL)	Measured titer (cp/mL)	% Recovery
PBS, pH 7.4	1.62E12	1.63E12	101 ± 0.8
Na_2HPO_4, pH 6.5	1.59E12	1.54E12	96 ± 2.5
Tris, pH 8.0	1.64E12	1.61E12	98 ± 2.0
NaAcetate, pH 5.0	1.57E12	1.43E12	91 ± 1.3
Citrate-phosphate, pH 4.0	1.58E12	1.12E12	71 ± 7.4

Table 2: Expected capsid titer, measured capsid titer and % recovery of AAV9 after buffer exchange as determined by ELISA.

the area under the fluorescence intensity curves from 500–650 nm. T_{agg} 473 was determined based on the SLS intensity.

Results

Buffer exchange

Big Tuna completed the buffer exchange and concentration of all 10 wells in less than 2 hours. Expected and measured AAV9 capsid titers after buffer exchange and concentration were determined along with % recovery (Table 2). The PBS, pH 7.4 process control and three of the buffers showed recoveries between 90-100%. The ELISA measured noticeably lower than expected capsid titer in the citrate-phosphate buffer control which warranted further characterization.

Aggregation can occur on intact capsids or because of capsid protein unfolding and is strongly impacted by buffer conditions. On Uncle, SLS monitors aggregation during a thermal ramp and is used to identify aggregation temperatures (T_{agg}s) while DLS detects aggregation by reporting particle size and size distribution.

Uncle DLS measurements of AAV9 in PBS before exchange, after exchange, and three of the chromatography buffers showed a single, well-defined intensity peak at approximately 25 nm (Figure 2). This indicates these samples consisted of intact, non-aggregated capsids. AAV9 in the citrate-phosphate buffer positive control (Figure 2, red line) had a second peak at ~120 nm, indicating the presence of aggregates. This result is consistent with the lower measured recovery in the ELISA, as AAV aggregates are not efficiently captured nor quantified by this method. Uncle's initial DLS reads can immediately

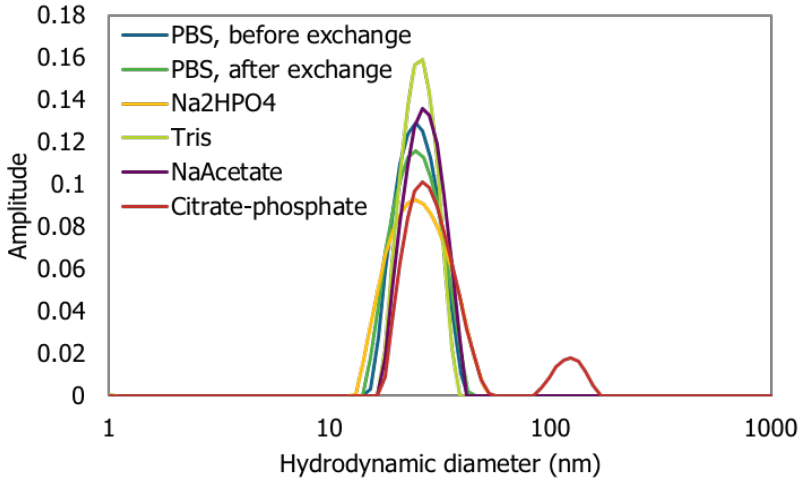

Figure 2: Representative initial DLS intensity distributions of AAV9 before exchange (blue) and all 5 concentrated samples.

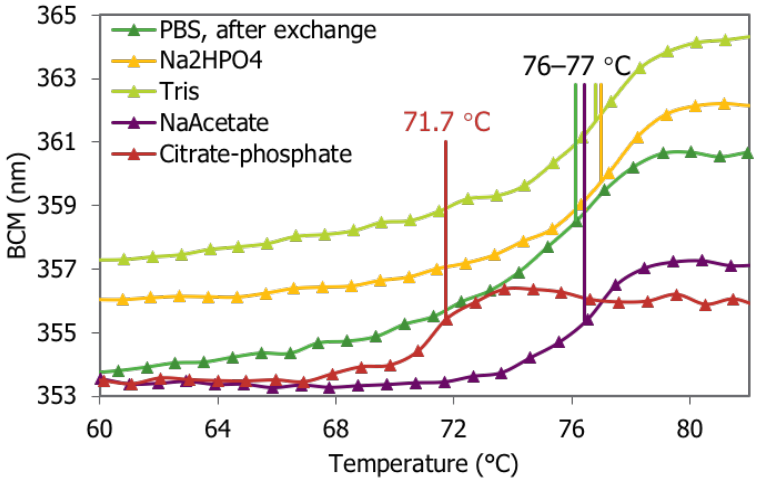

Figure 3: Capsid disruption melting curves of the 5 concentrated samples with their capsid unfolding T_ms (solid vertical lines).

assess AAV aggregation and sample quality as part of process development.

Thermal stability

The average capsid unfolding T_ms of 4 of the 5 concentrated samples were between 76–77 °C (Figure 3). AAV9 in citrate-phosphate buffer had a significantly lower capsid unfolding T_m of 71.7 °C. These results indicate AAV9 capsids have similar thermal stability in 4 of the 5 tested conditions and significantly worse stability in citrate-phosphate pH 4.0 buffer.

AAV9 in the PBS process control and citrate-phosphate buffer positive control showed the largest differences in thermal stability (Figure 4). The difference in capsid unfolding T_m between these 2 samples was 4.4 °C while the difference in genome ejection T_m was 11.9 °C. While DLS showed AAV9 aggregated somewhat in citrate-phosphate buffer at room temperature, it suffered significant aggregation at T_{agg} = 43.5 °C, coinciding closely with genome ejection. When the vector was in PBS, T_{agg} was closer to the capsid unfolding T_m. Formulations can have wildly different impacts on AAV genome ejection, capsid stability and aggregation. Uncle provides the tools to investigate all three of these events in a single, easy-to-use platform.

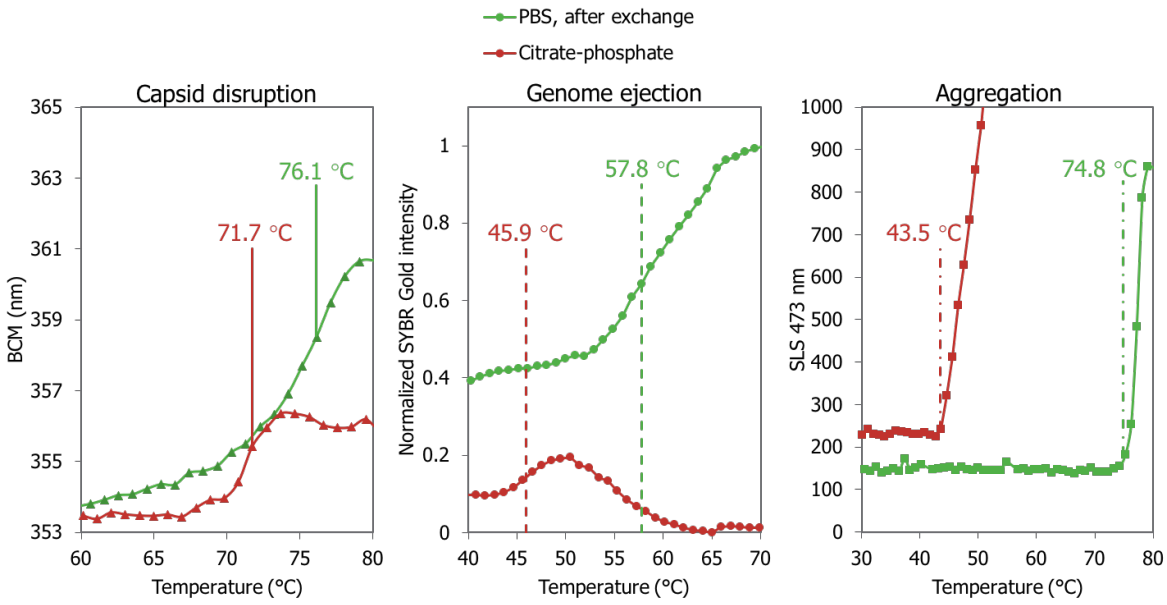

Figure 4: Capsid disruption (left), genome ejection (middle) and SLS 473 intensity (right) curves of AAV9 in PBS (green) and citrate-phosphate (red) after concentration by Big Tuna. Uncle determined capsid unfolding T_ms (solid line), genome ejection T_ms (dashed line) and T_{agg}s (dash dot line).

Conclusion

AAV process development and stability characterization is an underexplored space due to low assay throughput and high sample volume requirements. To push the frontier in these areas, Big Tuna automates buffer exchange at low volumes, and quickly concentrates AAV samples in parallel and into different buffers or formulations. Uncle's Viral Toolbox and intrinsic fluorescence detection studies AAV stability and aggregation. The combination of buffer exchange and concentration with multi-modal sample characterization makes the duo of Big Tuna and Uncle the perfect guides for mapping out AAV stability.

References

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