

# Identification of AAV Serotypes and Characterisation of their Stability by nano-DSF for use in a Gene Therapy Program

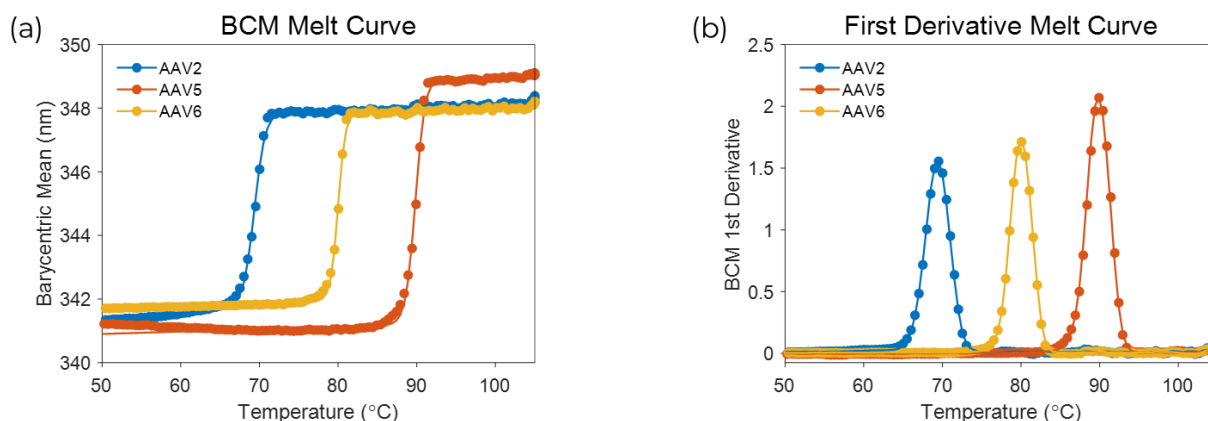
## Introduction

Gene therapy is a therapeutic technique that modifies a person's genes to treat or cure a disease. This modification can work by replacing a disease-causing gene with a healthy copy of the gene, inactivating a disease-causing gene, or introducing a new gene into the body to help treat a disease. There are a wide variety of gene therapy treatments, but a common technique is to use viral vectors, such as adeno-associated viruses (AAVs), to deliver therapeutic genes into human cells for replication.[2]

AAV's have many favourable features including their lack of pathogenicity, replication incompetence, ability to infect non-dividing cells, and their ability to integrate into the host cell genome at a specific site.[3] These promising features led to their advancement with early research focusing on a single AAV variant (serotype) AAV2. Since then, new AAV serotypes have been discovered to provide higher transduction efficiencies in certain cells or tissues compared to AAV2. These unique cell tropisms have led to the development of a larger number of AAV serotypes to package different therapeutic genes to treat specific disease.[4] The different AAV serotypes not only have different cell tropisms, but they also differ in their structural properties and stability due to their varying amino acid sequences and capsid structures. These differences give each AAV serotype a unique melting temperature.[4,5]

To develop efficient AAV therapeutics, it is crucial to characterise key attributes of AAV's during the development process. One key attribute is the thermal stability of the AAV capsid, which can be used for serotype identification, optimising capsid formulation, and for comparison purposes during any process changes.[6] Capsid stability can be measured with differential scanning fluorimetry (DSF) by monitoring the change of intrinsic fluorescence from the tryptophan residues within the AAV during a thermal ramp.

In this application note we used the SUPR-DSF to measure the thermal stability of three AAV serotypes: AAV2, AAV5, and AAV6. The SUPR-DSF measured the intrinsic fluorescence of each serotype during a thermal ramp to generate melt curves. In addition, we performed a concentration dilution series on AAV2 and AAV6 to determine the minimal sample requirements. All samples were performed at a well volume of 10 $\mu$ L in triplicate on a single 384-well microplate emphasising the high-throughput possibilities of the instrument.



**Figure 1** – (a) Barycentric mean (BCM) melt curves for 3 AAV serotypes: AAV2, AAV5, and AAV6. (b) First derivative representation of BCM melt curve where the peaks correspond to the melting temperatures.

**Table 1** – Melting temperatures for AAV serotypes determined by fitting a gaussian to the first derivative of the barycentric mean (BCM) melt curve. Standard deviation (SD) values are for the  $T_m$ . The errors were found to be the same or less for onset temperatures ( $T_{onset}$ ).

Samples	$T_{onset}$ (°C)	$T_m$ (°C)	SD (°C)
AAV2	65.8	69.4	0.04
AAV5	86.5	89.9	0.07
AAV6	76.8	80.0	0.01

## Results

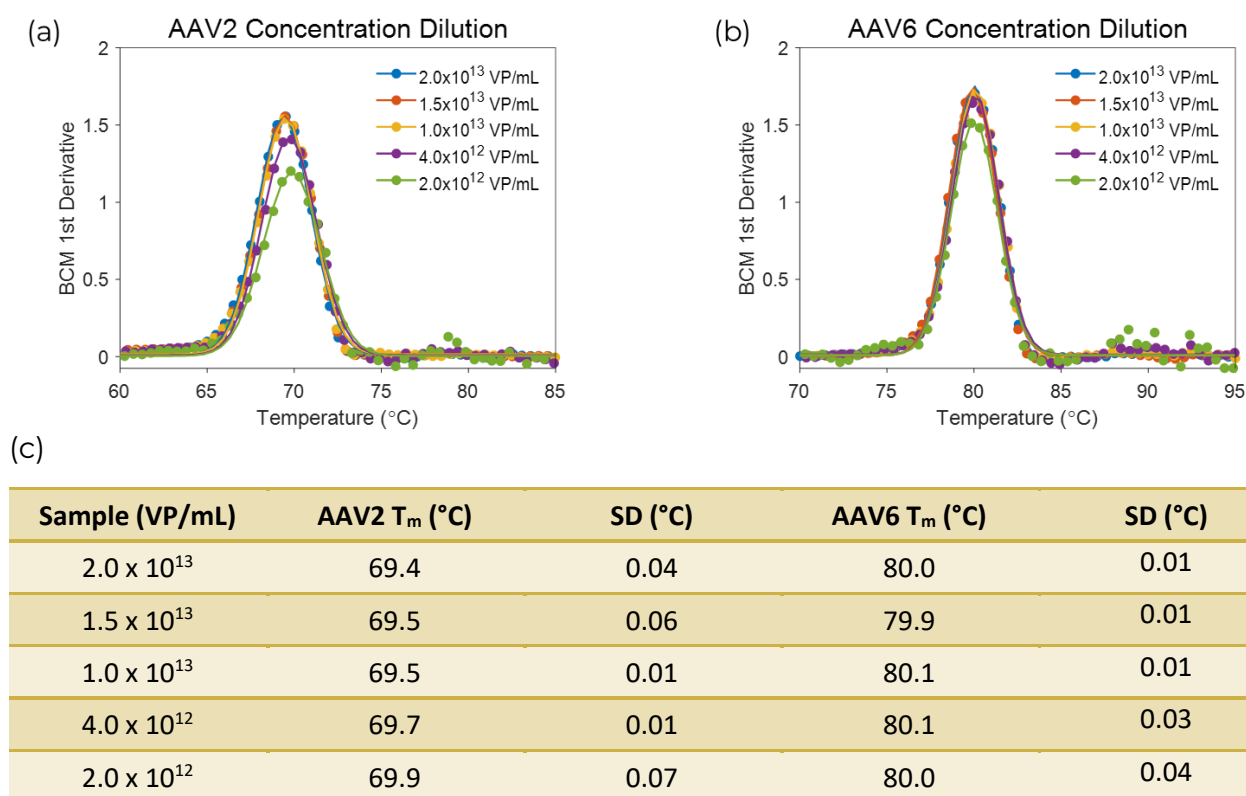
The barycentric mean (BCM) melt curve for AAV2, AAV5, and AAV6 are shown in **Figure 1(a)**. The AAV serotypes have a very flat pre- and post-transition baselines with a steep inflection point between the baselines. All three AAV serotypes shift to longer wavelength during unfolding due to the tryptophan becoming exposed to the aqueous environment after their capsids break. The steep transition, i.e. temperature range it takes to move from the folded to unfolded baseline, implies this happens very quickly. The fast transition is also evident by looking at the onset temperature ( $T_{onset}$ ), which on average is only 3.4°C before the melting temperature ( $T_m$ ) for the three AAVs. The fast unfolding is expected for AAVs since we are measuring a capsid breaking compared to a slower unfolding typically observed with most proteins or antibodies.

**Figure 1(b)** illustrates the first derivative of the BCM melt curve where the peaks correspond to the apparent melting temperatures of the AAV capsids. The first derivative melt curves were fit to a gaussian to obtain the melting temperatures ( $T_m$ ), which are shown in **Table 1**. The replicates for each AAV agreed very well with  $SD < 0.1^\circ\text{C}$  and the melting temperature values agreed with other reported values.<sup>[4]</sup> The three AAVs had a wide variation in melting temperatures due to the differences in their amino acid sequences and capsid structures. AAV2 had the lowest melting temperature of 69.4°C, followed by AAV6 at 80.0°C, and the most stable serotype was AAV5 at 89.9°C. The variation of melting temperatures highlights the importance of measuring thermal stability of your AAVs during the entire development process since a small change can lead to a large impact on product stability.

Next, we looked at a concentration dilution series of AAV2 and AAV6 to see what the minimal amount of sample is required to accurately measure their stability. The BCM first derivative melt curve for AAV2 **Figure 2(a)** and AAV6 **Figure (2b)** are shown for five different concentrations. The fitted melting temperatures for both AAVs are displayed in the **Figure (2c)** with the replicates for both serotypes agreeing very well with  $SD < 0.1^\circ\text{C}$  for all concentrations. Both AAV2 and AAV6 produced excellent melt curves for all concentrations tested showing we can measure capsid stability for these two AAV serotypes at  $2 \times 10^{12}$  VP/mL.

Looking at the results, we can determine the melting temperatures for AAV2 begin to shift to slightly higher temperatures for the two lowest concentration samples versus the higher concentration samples. This is evident in the **Figure 2(a)** where the two lowest concentration samples have a slightly lower magnitude, and their peaks are shifted slightly to the right. On the other hand, AAV6 showed nearly identical melting temperatures for all concentrations tested. The lowest concentration had a small magnitude change, but this didn't affect the melting temperature.

The differences in stability between AAV2 and AAV6 at lower concentrations could be due to their slight differences in their amino acid compositions. AAV2 also has a slightly lower BCM 1<sup>st</sup> derivative amplitude making it more susceptible to effects from background at lower concentrations. These characteristics can explain the stability differences we are seeing from the serotypes and is a key attribute that can be used while developing your product. Therefore, it is recommended to always use the same concentration of AAV when you are performing any comparison studies or formulation screens.



**Figure 2** – BCM first derivative melt curves for a concentration dilution series for (a) AAV2 and (b) AAV6. (c) Table of melting temperatures with errors for AAV2 and AAV6 concentration dilution series.

## Conclusion

The SUPR-DSF from Protein Stable demonstrates high data quality, capable of quantifying stability of AAV capsids in a 384-well microplate at a well volume of 10µL. Three different AAV serotypes were characterised (AAV2, AAV5, and AAV6), and the melting temperatures obtained from the BCM first derivative melt curves agreed very well with reported values.<sup>[4]</sup> The melt curves were observed to have very fast transitions, which aligns well with the expected behaviour of AAV capsids breaking during a thermal ramp. The melting temperatures varied by 20°C, highlighting the large differences in stability among the various AAV serotypes. These large differences in stability enable scientists to use DSF measurements to identify serotypes and adds to the importance of measuring AAV product stability all along the development process.

The concentration dilution series of AAV2 and AAV6 exhibited concentration variations between the serotypes. AAV2 has a small concentration effect, with lower concentration samples having a slightly higher melting temperature compared to the control. On the other hand, all concentrations for AAV6 had nearly identical melting temperatures. The differences in AAV2 stability demonstrate why it is important to use the same concentration of sample when characterising AAV samples during development. The results from the concentration studies indicate a concentration of 2x10<sup>12</sup> VP/mL is sufficient to measure the stability of your AAV capsids safely and accurately.

The experiments demonstrated here show how the SUPR-DSF can be a valuable tool in characterising AAVs during the development process in a gene therapy program. The SUPR-DSF can determine the AAVs' stability by measuring their intrinsic fluorescence during a thermal ramp, eliminating the need to contaminate your samples with extrinsic dyes. Pairing this with the ease of use of 384-well microplates enables easy workflow scaling up to larger scale experiments that require increased number of samples.

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## Methodology

### Sample Preparation

The AAVs were sourced from Virovek. AAV5 and AAV6 were formulated in PBS with 0.001% Poloxamer 188 (P188). AAV2 was formulated in PBS with 100 mM citrate and 0.001% P188. Samples were manually pipetted in triplicate into a 384-well plate at a 10 $\mu$ L well volume. After dispensing, the plate was sealed with an optically clear adhesive film. The plate was then immediately transferred to the SUPR-DSF for measurement.<sup>[1]</sup>

### Thermal Denaturation Measurement

The SUPR-DSF was set-up to measure the fluorescence spectra of the AAV samples from 20°C to 105°C with a 1°C per minute ramp rate. Quantification of the spectral shift, that is indicative of protein denaturation, was determined via the barycentric mean (BCM) calculation. Background subtraction was done on all samples by subtracting the PBS control wells from the sample data. The BCM data was analysed as a function of temperature and the first derivative was determined from the BCM melt curve. Melting temperatures were calculated from the peaks of the derivative.

Methods and results for all samples are available on request.

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## References

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