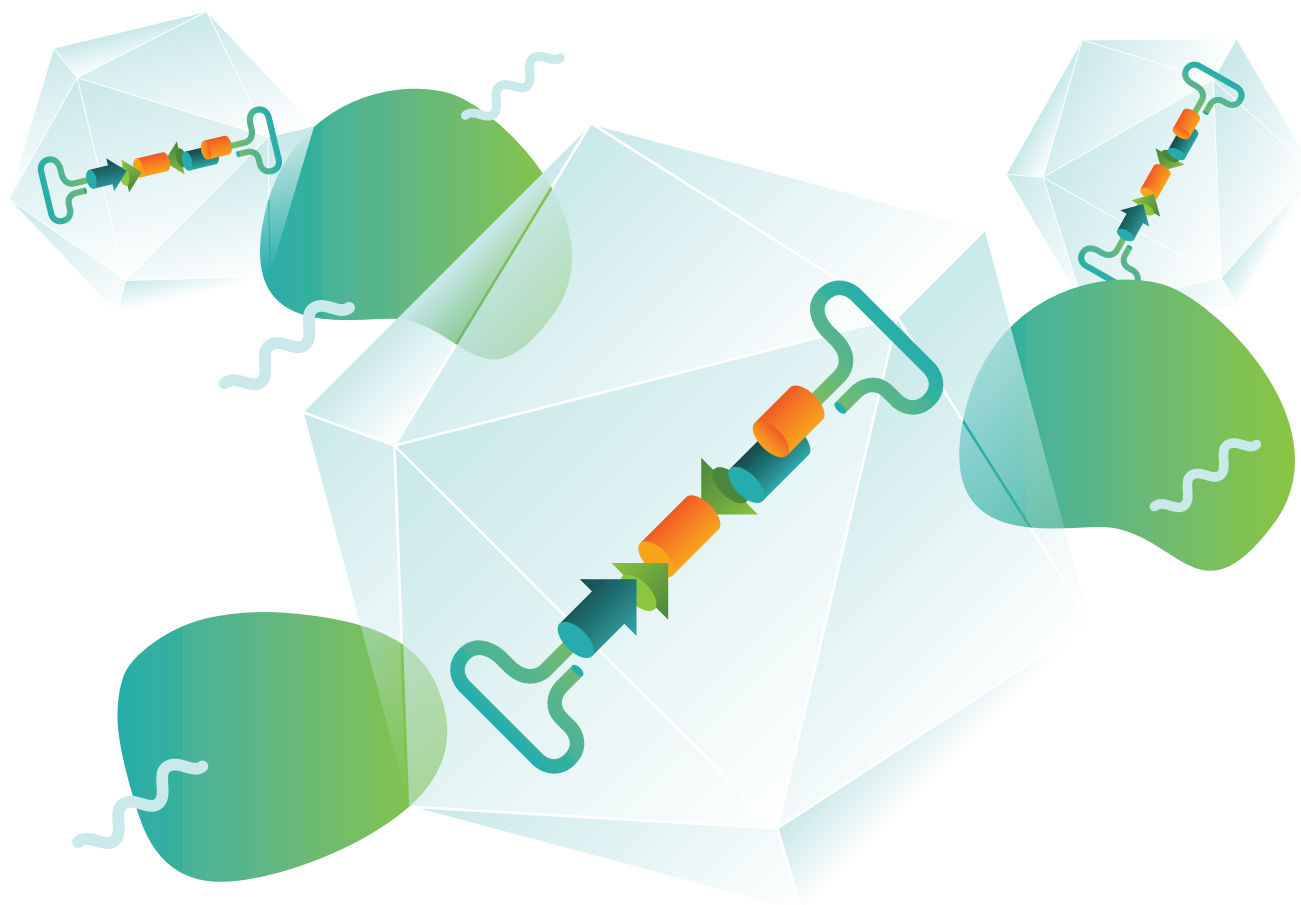




An Effective and Efficient Approach to Monitor AAV Packaging Efficiency During Research and Production



Scope

This White Paper outlines the use of a novel biolayer interferometry (BLI) assay for the evaluation of % full AAV capsid in test samples representing different stages of process development.

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Introduction

Despite the success of AAV for gene therapies and vaccines, many challenges still exist within AAV manufacturing.

The manufacturing process involves several steps, including production of the AAV vector in host cells, purification of the vector, and characterization to ensure quality and safety. Scientists struggle with poor transfection efficiency, low yields, and low vector concentrations, among many other potential problems. Because of safety and efficacy concerns, one of the most critical evaluations to be made is the determination of the percentage of full and empty capsids.

Current Status of E/F Measurement

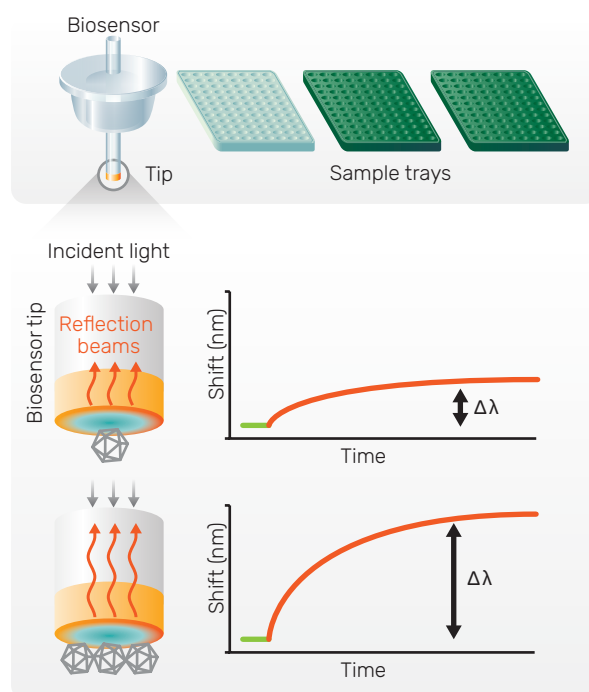
Many techniques have been used for evaluating the percentage of full and empty capsids¹. For example, ultraviolet absorbance studies comparing absorbance between 260 and 280 nm (UV A260/A280) and size exclusion chromatography with multiangle light scattering (SEC-MALS) are fairly straightforward to implement using tools most laboratories already possess. Alternatively, techniques such as cryo transmission electron microscopy (cryo-TEM) and charge detection mass spectrometry (CDMS) can provide additional details such as capsid size and shape or the mass and charge of capsid populations, but at a higher cost and difficulty level for instrumentation and implementation. Anion exchange can also be used to determine the ratio of empty and full AAV particles in purified AAV samples but requires extensive optimization for each serotype. Analytical ultracentrifugation, another method for empty and full ratio determination, is limited by its requirement for large sample volumes, very low throughput – around 7 samples in 6 hours – and very expensive equipment.

While the techniques mentioned above can provide fairly accurate results in purified buffers, all of them suffer from an inability to quantitatively measure samples in more complex matrices. In fact, most assays for % full determination are not crude sample compatible and are therefore not suitable for integration into the AAV manufacturing process. Thus, the complexity of the matrix in early development can make accurate measurements of % full versus % empty extremely difficult. Additionally, most above-mentioned techniques require high sample volume and high capsid concentrations in the range of $1\text{E}+12$ vp/mL. This can be challenging for in-process samples that generally have a lower titer.

Biolayer Interferometry

Biolayer interferometry (BLI) is a biophysical technique that measures the binding interactions between two molecules because of change of refractive index. With BLI, a probe is coated with a specific molecule that binds to another molecule of interest. Upon binding of the second molecule, a change in the interference pattern of light is observed. The accumulation of biomolecules binding to the surface of the probe causes changes in interference that can be monitored in real time to calculate concentrations, binding constants, and kinetics, making BLI a powerful tool for a wide range of applications (Figure 1).

Figure 1. The BLI technology. Biolayer interferometry uses specially coated probes that bind compounds of interest from samples in 96-well and 384-well plates. As binding occurs, a shift in wavelength is observed that is proportional to the number of bound molecules. Binding is observed in real time and is used to determine concentration.



AAV Ratio Kit

Gator Bio offers the AAV Ratio Kit for empty/full ratio determination based on BLI. The AAV Ratio Kit is uniquely suited for integration into the manufacturing process because it is compatible with crude samples such as clarified cell lysate, culture media, and many in-process buffers. The AAV Ratio Kit, uses the AAVX probe (sensor) coated with a highly specific anti AAV nanobody that binds to AAV capsids even in complex matrices (Fig 2A). The captured AAV capsids are then lysed to release the single stranded DNA cargo, which is quantified using the ssDNA probe (another sensor) that is coated with a protein highly specific to ssDNA. The signal obtained from binding of single stranded DNA to the ssDNA probe is normalized to the signal obtained from binding of AAV capsid to the AAVX probe. This allows calculation of the percent full (Figure 2B). The kit is compatible with a wide range of sample matrices and enables the determination of 96 samples in one assay. The kit only requires 100 μ L of sample at a titer of $5.00\text{E}+10\text{vp/mL}$ making it well suited for in process use. Finally, this assay can be run in a high throughput format using 96-well and 384-well plates, making it amenable to process monitoring and testing to enable real time decision making at any process step.

Case Study

In this study, the Gator Bio's AAV Ratio Kit was used to quantitate capsid content in five test samples provided by a partner company developing AAV gene therapy solutions. These samples were well-characterized reference materials spiked into several in-process buffers. The five test samples were prepared across the empty-to-full range in eight different buffers from different stages of the manufacturing process (Buffer 1 through Buffer 8). The level of purity ranged from purified capsids in formulation buffer (Buffer 1) to clarified cell lysates (Buffer 7). The expected percentage of full capsids for the 5 test spike ratio samples were 0%, 25%, 50%, 75%, and 100% full. Standards to generate calibration curves were prepared in all buffer formulations at 10%, 30%, 45%, 60%, 75%, and 90% full.

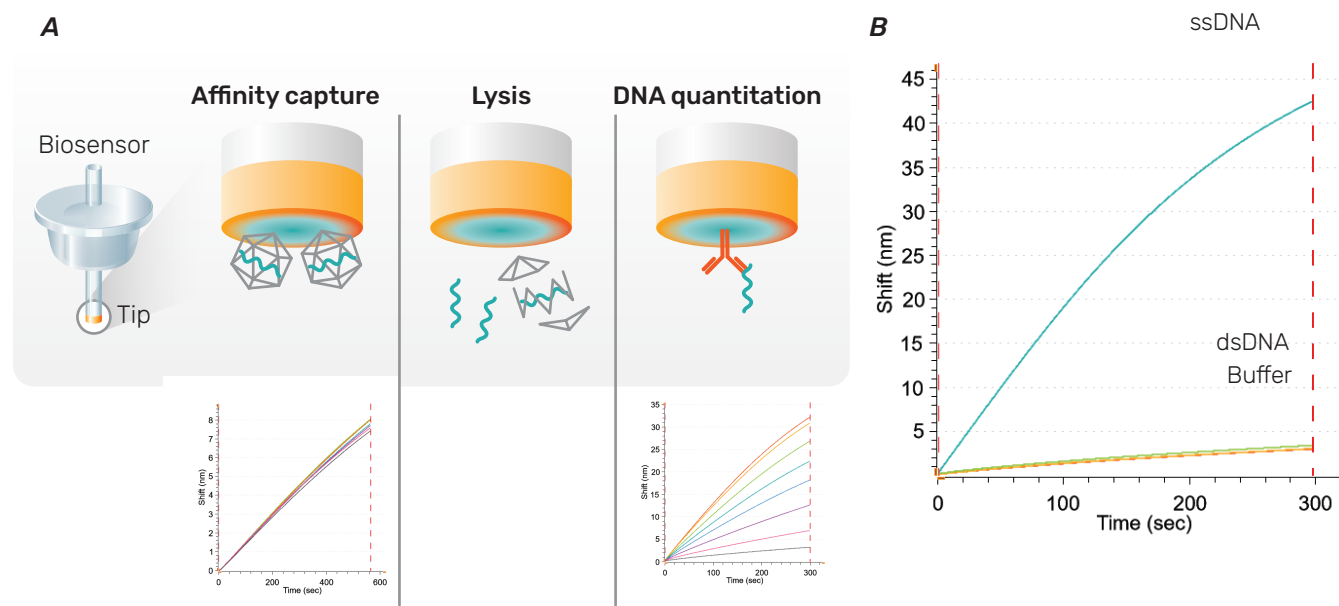


Figure 2. Determination of % full AAV. (A) The AAV Ratio Kit uses two different probes to determine the % full AAV. The first probe, the AAVX probe, binds AAV particles. The probe is then moved to a solution where the AAV particles are lysed and DNA is released. The amount of DNA is then determined using a second ssDNA probe. The % full AAV is then calculated by comparing the concentration of the AAV bound in the first step to the amount of ssDNA released in the second step. **(B)** The ssDNA probe is specific to ssDNA and does not detect dsDNA as evident from the signal with dsDNA being similar to Q buffer.

Dilutional Accuracy of the Gator Ratio Kit

As a first test of the BLI assay, dilutional accuracy was assessed to determine if the BLI %full measurement is semi-quantitative in Buffers 1 – 8. To assess dilutional accuracy, standards were prepared at eight theoretical %full levels in each buffer. Normalized shift was measured by BLI to fit a standard curve for each buffer, and %full measurements were calculated from the standard curve. These % full measurements were compared to expected % full values. Table 1 shows the % difference between the measured and the expected % full values for the prepared standards in the eight different buffers. As shown, most values lie within 20% of the expected % full value, which demonstrates suitable dilutional accuracy for the AAV Ratio Kit to be considered a semi-quantitative measurement of % full at all in process steps, even in upstream samples.

% Full	Buffer							
	1	2	3	4	5	6	7	8
90%	9	4	2	14	13	18	6	10
75%	12	13	3	24	18	17	8	19
60%	2	1	15	28	4	3	13	17
45%	5	12	6	19	10	0	19	19
30%	2	22	9	12	8	8	11	12
10%	27	33	40	5	9	11	38	3

Difference from expected values:

<20%

20-30%

>30%

Table 1: Dilutional Accuracy of % full determinations for AAV standards. The % difference between the expected % full values for the AAV standards (listed in the left column) and the calculated % full values using the AAV Ratio Kit are shown in each cell for the eight different buffers (Buffer 1-8 listed along the top). Most of the calculated results are within 20% of the expected values. Even in upstream buffers (Buffer 7 and 8) most values fall within 20% of the expected values.

Formulation Buffer (Buffer 1)

As a second test of the BLI assay, the AAV Ratio Kit was compared with orthogonal techniques for the five test samples prepared in formulation buffer (i.e., Buffer 1). 7 standards with spike concentrations of 0, 30, 45, 60, 75, 90 and 100 % full were prepared in the same buffer (Buffer 1) to generate a standard curve to determine the % full for the test samples. Figure 3 shows the nanometer shift signal for AAV capture on AAVX probes (A), single stranded DNA capture on single stranded DNA binding probes (B), and the single stranded DNA standard curve after normalization (C). Excellent linearity is observed with $R^2 = 0.98$ and the % full for the test samples were determined from the standard curve.

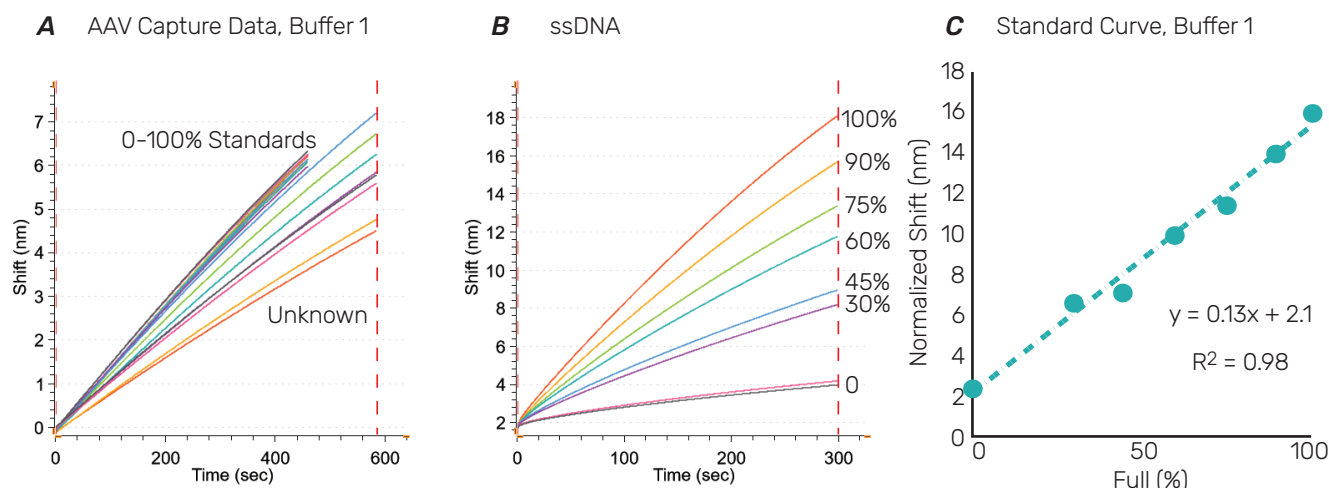


Figure 3. Determination of % full for standards and samples prepared in formulation buffer (Buffer 1).

(A) Response for AAV capture on the AAVX probe. Test samples were captured for a longer time to ensure that a nm threshold of about 4–6 nm could be achieved for all the wells. This ensures that roughly the same number of capsids are captured onto each probe. (B) Response for the ssDNA probe after AAV lysis. (C) Standard curve generated for determining the % full for test samples.

The results obtained for the five test samples were then compared to orthogonal techniques typically used for % full evaluations. As shown in Figure 4, the Gator Bio AAV Ratio Kit displays the same rank ordering of the % full samples as orthogonal techniques and is comparable for samples prepared in formulation buffer. Note that similar comparisons were not possible for samples prepared in more complex buffers because most orthogonal techniques are not compatible with complex buffers.

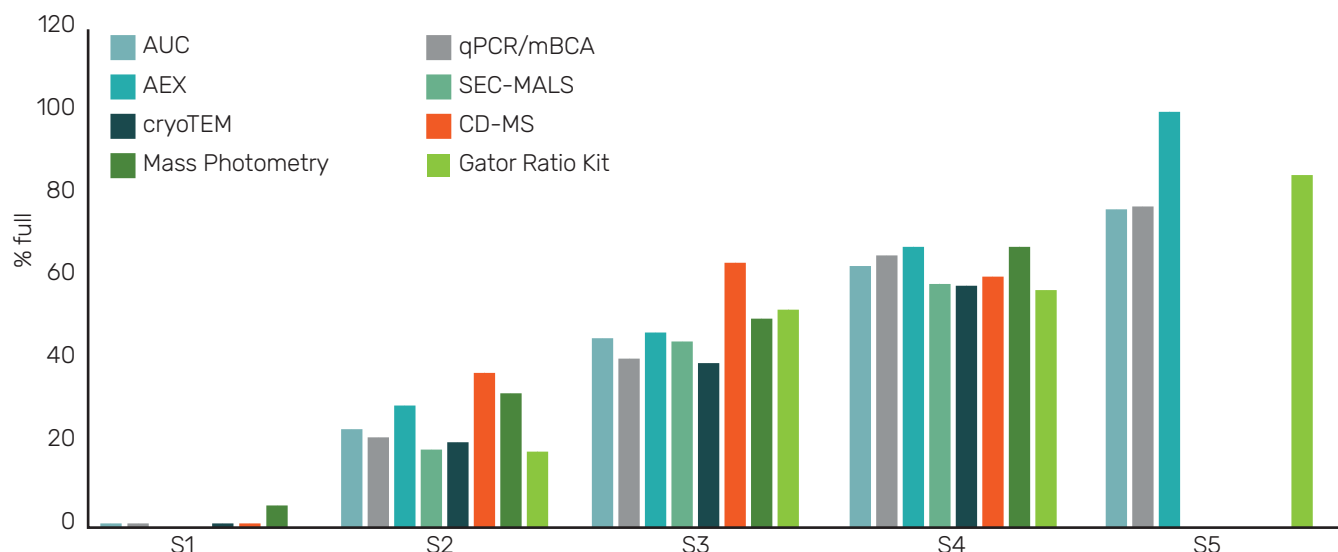


Figure 4: Comparison of techniques for % full determination in formulation buffer. The BLI result using the AAV Ratio Kit is shown for 5 test samples prepared in formulation buffer (Buffer 1) and compared with orthogonal techniques. The rank ordering is in agreement with the other techniques and shows a progression of increasingly higher % full, as expected.

Upstream Buffers (Buffer 7 and Buffer 8)

Because the BLI assay response is very specific for the molecules of interest, the AAV Ratio Kit is capable of measuring concentrations of AAV and ssDNA even in very crude matrices. As a final test of the BLI assay, test samples were tested in several in-process buffers from various stages of the manufacturing process.

The data in Figure 5 show the nanometer shift signal for AAV capture on AAVX probes (A, D), single stranded DNA capture on single stranded DNA binding probes (B, E) and single stranded DNA standard curve after normalization (C, F) for samples prepared in upstream buffers – Buffer 7 and Buffer 8, respectively. The results were then used to calculate the % full values for the test samples. Despite the complex buffer conditions, excellent linearity is observed in both buffers for the standards with $R^2 = 0.99$ and 0.98 for Buffer 7 and 8, respectively, indicating the high quality of the linear response and providing more confidence in the determination of the unknown values.

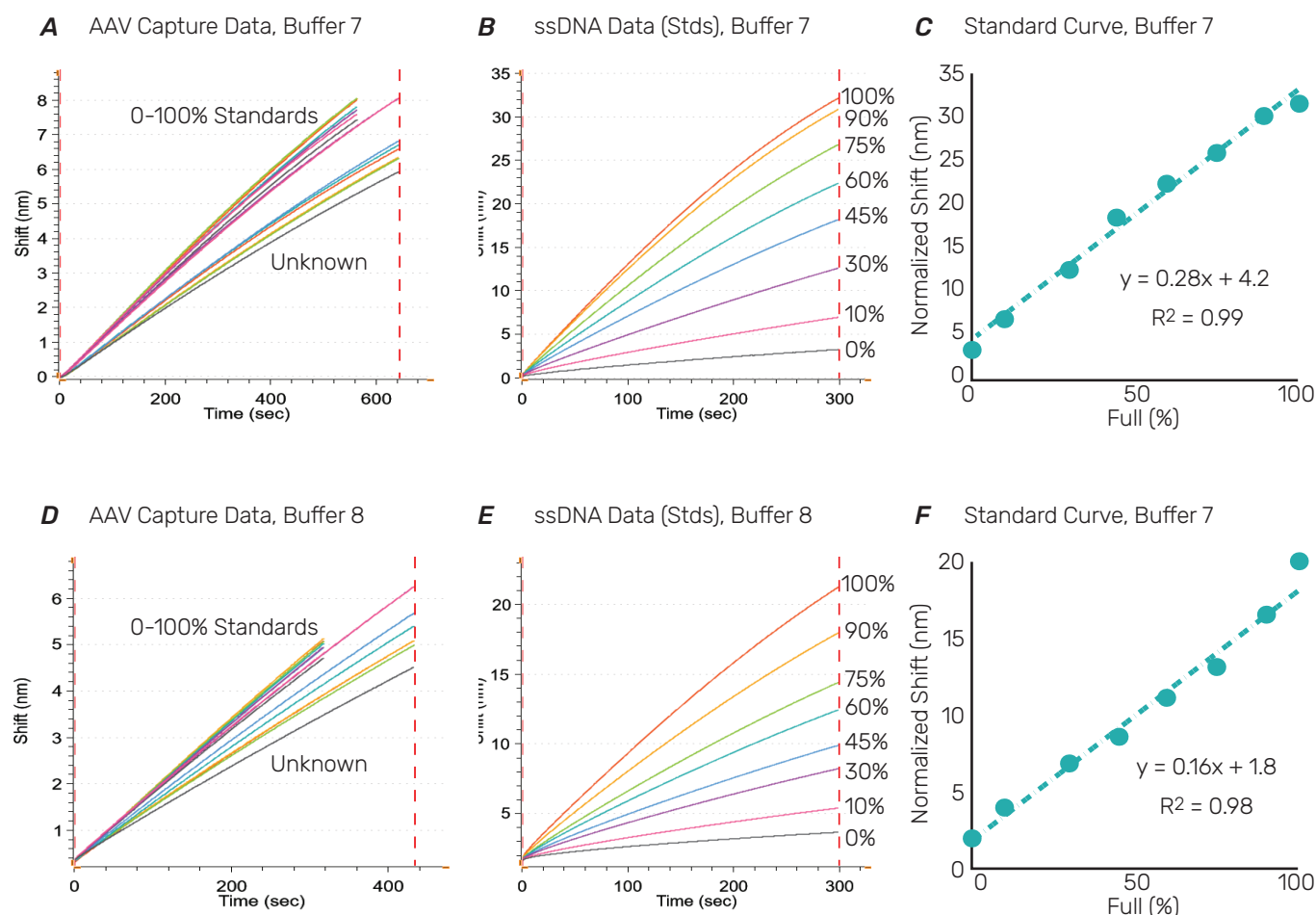


Figure 5. Determination of % full for standards and samples in upstream buffers (Buffer 7 and 8).

Response for the AAVX probe in standards and samples in (A) Buffer 7 and (D) Buffer 8. Test samples were captured for a longer time to ensure that a nm threshold of about 5-6 nm could be achieved for all the wells. Response for the ssDNA probe in standards after AAV lysis in (B) Buffer 7 and (E) Buffer 8. Standard curve generated for determining the % full for test samples in (C) Buffer 7 and (F) Buffer 8.

Comparison of Results

The results from all 5 test samples in Buffers 1, 7, and 8 are summarized in Table 2. In all buffers, the measured % full increases proportional to the expected values. These results indicate that the AAV Ratio Kit is an effective tool for determining the % full in all manufacturing stages. Additionally, the kit provides a method for evaluating the success of the manufacturing and purification process by observing the continual increase in % full as the sample becomes more concentrated and purified.

Expected % Full Value	Values Obtained Using Gator AAV Ratio Kit		
	Buffer 1	Buffer 7	Buffer 8
S1 (0%)	0	0	0
S2 (25%)	19	25	32
S3 (50%)	53	63	70
S4 (75%)	58	81	88
S5 (100%)	86	93	92

Table 2: Summary of % full for test samples.

The expected % full values are compared with the calculated values for the test samples (S1 to S5) prepared in formulation buffer (Buffer 1) and in upstream buffers (Buffer 7 and 8). Even in complex buffer formulations, the Gator Bio AAV Ratio Kit provides excellent results with rank ordering of increasing % full and calculated values comparable to expected values.

Conclusion

One of the most important measurements to be made during AAV production is determining the percentage of capsids that carry the gene of interest. This is particularly difficult in the earlier manufacturing stages where capsid titers are low, and sample volume is limited. Moreover, complex matrices can interfere with traditional measurement methods. Using biolayer interferometry and probes coated with AAV specific and ssDNA specific proteins, the Gator Bio AAV Ratio Kit enables the determination of the % full AAV by measuring the encapsidated ssDNA that is released upon denaturation, and normalizing it to the capsid titer measured by BLI in the sample. Because the method is highly specific for both AAV capsids and ssDNA analysis in crude as well as clean matrix, it can be used to monitor progression of % full enrichment from harvesting to formulation.

Gator Bio provides a full suite of solutions to support the development of effective and safe AAV-based therapeutics (Figure 8). With instruments, probes and kits to determine AAV concentrations and capsid packaging these solutions support early-stage discovery to end-stage manufacturing and QC. The methods are non-destructive, require minimal sample preparation, and provide real-time measurements, making them an efficient and practical approach for assessing AAV quality.

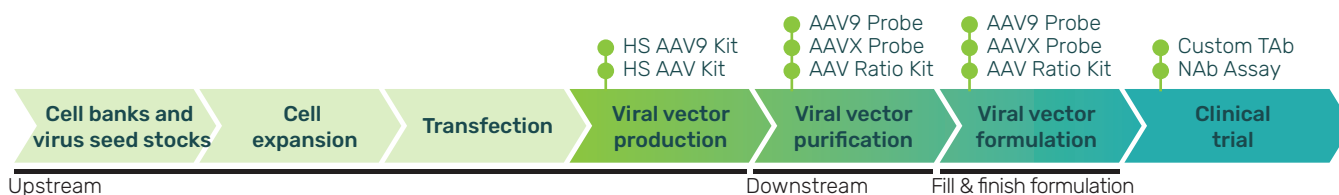


Figure 6: Gator Bio solutions for AAV manufacturing. Gator Bio provides rapid and high sensitivity solutions that are serotype independent for AAV titer determination and % full determinations during all stages of AAV development and manufacturing.

References

1) Werle, A.K., et. al., Mol Ther Methods Clin Dev. 2021 Dec 10; 23: 254–262.