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Application Note

April 23, 2021

Keywords or phrases:
Octet®, AAV2 capsid quantification, ELISA, ddPCR

Rapid, Automated, At-Line AAV2 Virus Quantitation Advances Bioprocessing in Gene Therapy

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Abstract

Rapid and accurate methods for the quantitation of Adeno-associated virus (AAV) particles are an unmet need for advancing bioprocessing in gene therapy. Viral capsid titer is commonly measured by ELISA, empty vs. full capsid titer differentiation and ratio is obtained by Analytical Ultracentrifugation (AUC), and viral genome titer is measured increasingly by ddPCR. These methods are generally time consuming and labor-intensive, and are hence not practical for at-line, rapid measurement of viral titer during bioprocessing and manufacturing. We have developed a quick, high-throughput and robust AAV2 capsid quantification method using Octet® Bio-Layer Interferometry system, capable of virus titer determination in samples along the purification process. The method accelerates assay of multiple 96-well or 384-well plates in less than 1 hour, saving significant time and labor compared to ELISA and ddPCR assays.

The Octet® AAV2 titer assay demonstrated excellent precision and reliability, with very minimal matrix effects relative to ELISA and ddPCR. The method could be extended as a generic viral titer assay for at-line testing of any AAV serotype in a bioprocess setting to offer near real-time feedback on the bioprocess, enhancing efficiency and productivity in virus manufacturing.

Introduction

Recombinant adeno-associated virus (rAAV) is one of the most promising viral vectors in the field of gene therapy for genetic disorders, as demonstrated by the increasing number of clinical trials and promising results. Two products have received marketing approval, Glybera (uniQure) in EU and Luxturna (Spark Therapeutics) in the US. Yet, the analytical methods for AAV bioprocess and manufacturing are still in early stages of development and require continuous enhancement[1].

Rapid, at-line, accurate methods for the quantitation of AAV virus particles are essential for bioprocessing in gene therapy. Capsid titer is commonly measured by ELISA, while empty vs. full capsid titer differentiation and ratio are obtained with analytical ultracentrifuge (AUC), and viral genome titer is measured increasingly by ddPCR[2]. Current methods are time consuming and labor intensive, and hence are not practical for at-line, rapid measurement of viral titer to monitor development of bioprocess and manufacturing. In this article, we report the development of a rapid, high-throughput capsid assay for AAV2 on Sartorius' Octet® platform that demonstrates excellent precision, reliability in comparison to ELISA and ddPCR that can be applied as a generic assay method for viral titer at-line in a bioprocess setting.

Octet® System and Bio-Layer Interferometry

The principles of concentration measurement with an Octet® system are similar to established immunoassays such as ELISA. However, quantitation protocols on the Octet® platform provide several advantages. The Octet® platform monitors binding of analyte from solution to a biosensor surface in real time, without need for labels, secondary binders or other detection reagents. This real-time monitoring of binding interactions enables clear discrimination between specific and non-specific binding signals, which can shorten assay development times dramatically. Octet® quantitation assays are also much faster: quantitation of a 96-well plate of samples can be performed in 5–60 minutes, depending on the instrument model.

Octet® systems are routinely used for protein characterization across various stages of biotherapeutics R&D, measuring binding affinity and kinetics of drug-target interactions as well as measuring protein concentration in bioprocess samples. The same principle in use for quantitation of protein therapeutics is applied here for the analysis of AAV virus particles. The Octet® platform uses a simple Dip and Read approach for rapid analysis of samples in 96- and 384-well microplate formats. The concentration of the target virus particles in a sample is determined via a direct binding assay. Biosensors coated with a capture molecule, called the ligand, are dipped into solutions containing the analyte in a highly parallel, automated method to measure binding interactions. In a typical quantitation assay, a standard curve is generated using known amounts of the analyte, and unknown sample concentrations are interpolated from the standard curve. Concentrations can be calculated from the initial binding rate of the interaction which is based on the initial slope of binding or from the point at which binding reaches an equilibrium.

In this application note, we describe the development of an assay for quantifying AAV2 virus particles. The assay was constructed by capturing AAV2 virus using heparin immobilized onto Streptavidin Biosensors. We show that the working assay can be used to quantify AAV2 particles from purified as well as complex bioprocess matrices with a dynamic range of $4.15 \times 10^8 - 2.66 \times 10^{10}$ gc/mL (genome copy/mL). Depending on the Octet® instrument used, the quantitation assay can be completed in as fast as 30 minutes, significantly accelerating assay timelines compared to ELISA and ddPCR based methods. The Octet® assay can be extended to any AAV serotype by using an appropriate capture molecule and following the assay development steps described herein.

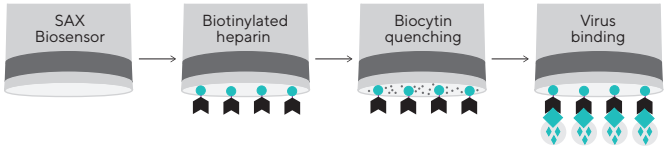

Figure 1: Octet® AAV2 assay workflow. The assay method can be extended to any AAV serotype using an appropriate biotinylated capture molecule and following the steps described herein.

Method Description

Sartorius' Octet® AAV2 virus assay is designed for monitoring AAV2 virus concentrations in crude lysate and cell culture supernatant (Figure 1). High Precision Streptavidin Biosensors (SAX) supplied by Sartorius are first coated with biotinylated heparin and then blocked with biocytin. Heparin-coated biosensors can be batch-prepared in advance using Sartorius' batch biosensor preparation protocols and stored for future use.

Materials Required

- Octet® instrument with Octet® BLI Discovery and Analysis Studio Software (v11.1 or higher).
- High Precision Streptavidin (SAX) Biosensors, Sartorius part no. 18-5117
- Sample plates: 96-well, black, flat bottom, polypropylene microplate, Greiner Bio-One part no. 655209 for Octet® R8 systems and 384-well tilted (Sartorius part no. 18-5080) or flat-bottom black polypropylene microplate
- 1× PBS, Dulbecco's phosphate buffered saline, Sigma part no. D8662
- Biotin-Heparin (Creative PEGWorks part no. HP-207)
- EZ-Link Biocytin, Thermo Scientific part no. 28022
- Stock of the purified AAV2 virus
- Sartorius sample diluent, part no. 18-1104 (recommended) or buffer of choice. A dilution of 1:100 of the sample in diluent is recommended for best performance.

AAV2 Assay Development and Optimization

The first step in developing a working assay is the identification of a capture molecule herein referred to in this procedure as a ligand. The ligand should bind specifically to the antigen of interest and should be screened for optimal capture concentrations. In selecting a ligand for the Octet® AAV2 assay, biotinylated versions of various receptors and anti-AAV antibodies were screened against an AAV2 positive control sample (data not shown). From the screening experiment, biotin-heparin was selected as the ligand to be loaded on the biosensor, due to good performance on AAV2 detection sensitivity and quantitation dynamic range.

We also evaluated and determined the following conditions as optimal for assay performance:

- Fully equilibrate all reagents and samples to room temperature prior to sample preparation. Thaw frozen samples completely and mix thoroughly prior to use.
- Hydrate the biosensors with 1× PBS for a minimum of 10 minutes prior to use.
- Sartorius recommends running assays at 30°C, and using other temperatures may require modifying the assay times discussed in this protocol. To set the sample plate temperature in Octet® System BLI Discovery Software, select **File > Experiment > Set Plate Temperature** and enter the desired temperature.
- Shaking speed and assay time should be optimized depending on the AAV serotype. For AAV2, a shaking speed of 400 rpm was selected and used for the assay.
- Setting up **Basic Kinetics** experiment in the Experiment Wizard.

The loading level of biotinylated heparin on the SAX Biosensor should be screened in a scouting experiment with varying concentrations of biotinylated heparin (Figure 2). For AAV2, 25 µg/mL of biotinylated heparin was used to achieve ligand loading signal saturation in a 5-minute incubation step. A 30-second quenching step using 25 µg/mL of biocytin prepared in PBS follows before the biosensors can be used to detect AAV2. The quenching step is critical to the prevention of non-specific binding (NSB).

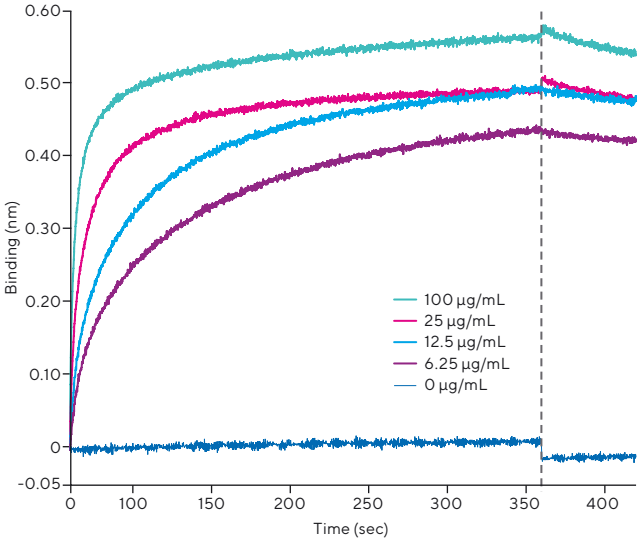

Figure 2: Biotin-heparin loading concentration scouting assay.

AAV2 Standard Curve Preparation

AAV2 standard curve is prepared using purified AAV2 of known concentration. A series of standard dilutions of purified AAV2 is used for the dynamic range determination study.

Prepare 1 mL of each standard sample by diluting the stock solution with an appropriate amount of sample diluent. Standard sample concentrations can be adjusted to reflect the working range of the standard curve. A typical plate map is shown in Figure 3 while Figures 4 and 5 show suggested calibrator sample dilutions to determine the dynamic range and assay workflow design.

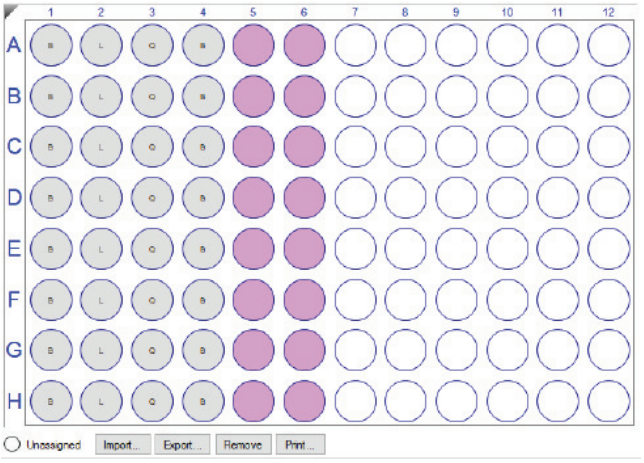

Figure 3: Plate map with standard samples. Sample wells are shown in pink (B: buffer; L: loading; Q: quenching).

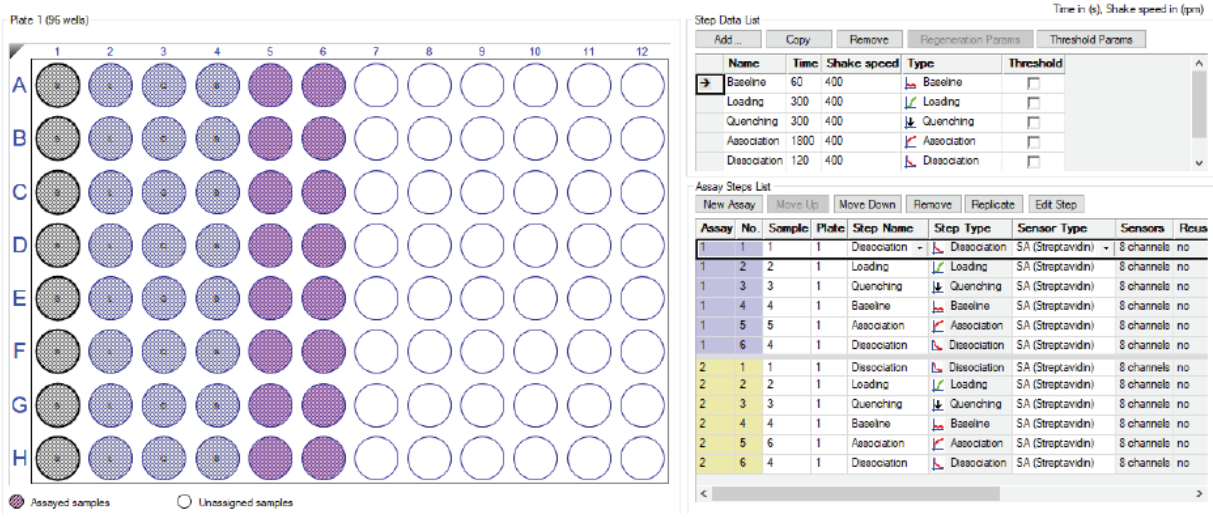

Figure 4: Setting up the AAV2 virus quantitation experiment. Figure highlights plate map and concentrations of the known samples.

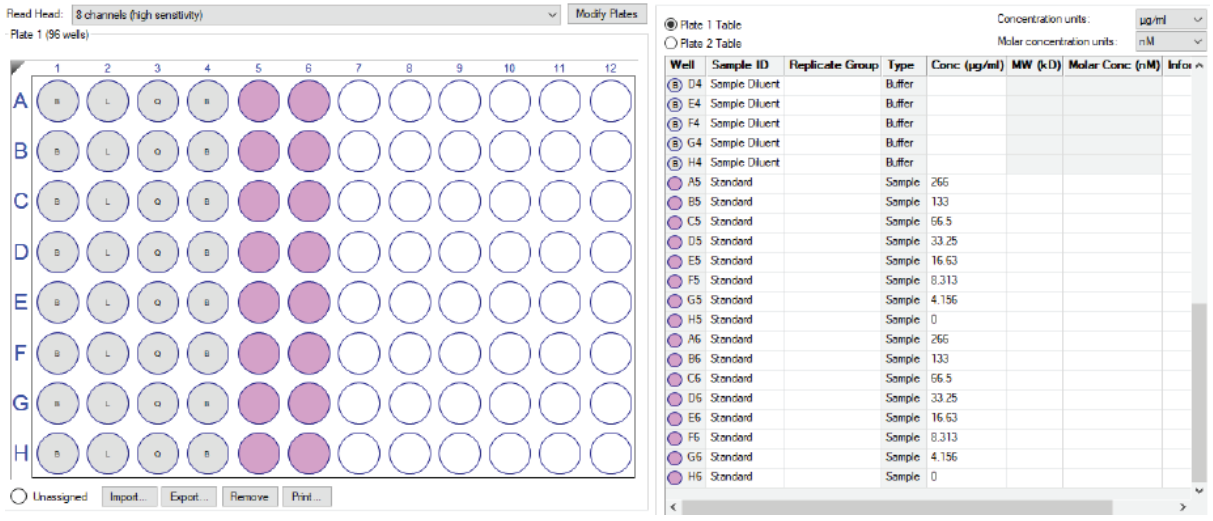

Figure 5: Octet® Software set up for AAV2 virus quantitation experiment. Figure shows plate design and assay step sequence and parameters.

AAV2 Virus Binding and Standard Curve Generation

Using the described assay setup and the standard biosensor hydration protocols (10 minutes hydration in assay buffer), the AAV2 virus binding assay is run at an rpm of 400. Assay times for each step is shown in Figure 5. A zero gc/mL (sample diluent only) sample well is used as a negative control. An example of binding raw data for calibrator or test samples is shown in Figure 6.

The virus capture step in Figure 6 is analyzed using the Octet® BLI Discovery Software to generate a calibration curve as shown in Figure 7. The analysis software uses the established calibration curve to generate test sample concentrations. Table 1 shows an example of an AAV2 Virus quantitation assay with good precision up to 2.66×10^{10} gc/mL obtained. The standard curve should cover the range of unknown samples. To obtain the most accurate results one standard sample needs to be above the range of quantitation detected for the assay. In the AAV2 example, the recommended range for best precision and accuracy is 4.15×10^{8}–2.66×10^{10} gc/mL. Some AAV virus serotypes may have a more limited dynamic range while a broader dynamic range could be appropriate for others. Dynamic range may also be influenced by the nature of the AAV2 virus sample. In general, the actual dynamic range will be dependent on the virus of interest.

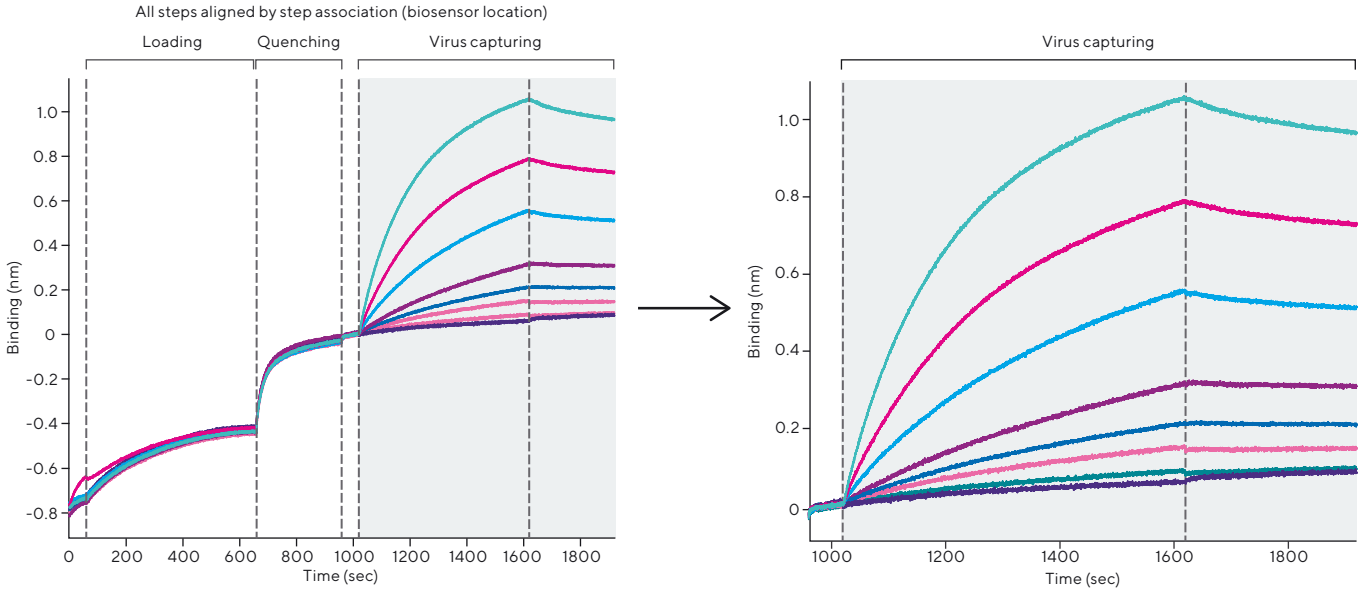

Figure 6: Full binding sensorgram of AAV2 detection on SAX Biosensors: The SAX Biosensors are first immobilized with biotinylated-heparin, quenched with Biocytin and associated with different concentrations of AAV2 samples. The Octet® assay is complete within 30 minutes.

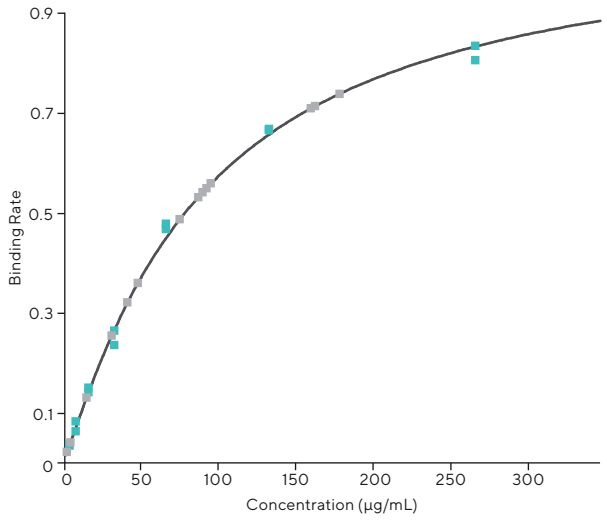

Figure 7: AAV2 standard curve.

Sample ID	Known conc. (*10^8 gc/mL)	Conc. avg (*10^8 gc/mL)	Conc. CV (n=2)
Standard 1	266	206.5	0.8
Standard 2	133	133.1	1.7
Standard 3	66.5	71.4	1.1
Standard 4	33.2	30.5	8.7
Standard 5	16.6	17.6	3.3
Standard 6	8.3	8.4	9.5
Standard 7	4.3	4.3	5.4
Neg Control	0	0	0

Table 1: Displays calculated average concentrations and % CV determined for a quadruplicate calibrator series ranging from 4.15×10^{8}–2.66×10^{10} gc/mL.

It is also important to ensure that there is sufficient separation between each standard level and that they do not overlap.

Note: If the unknown virus samples you want to quantify are bioreactor AAV2 virus with a changing media matrix throughout the bioreactor run (for example, media supplementing), dilute the sample 1:100 to eliminate the matrix interferences.

Data Analysis

1. Open the Octet® Analysis Studio Software. Select the column(s) from **Data Selection/Sensor-Assay** and click on **Processing**. Click on the association step and choose **Quantitate Selected Step**. Then click on **Yes** to the pop up window and the quantitation window will display. Click on **Results** and choose **4PL (weighted Y2)** for Standard Curve Equation use **Requilibrium** for Binding Rate Equation and click on the **Calculate Binding Rate!** Save report.
2. Copy the data table into Microsoft® Excel® and determine the % CV of the calculated calibrator calculation. % CV = (standard deviation/average)*100. CV can also be automatically calculated by the data analysis software when the **Replicate Group** information is filled during assay setup.
3. The dynamic range of the AAV2 virus assay is the continuous range for which the % CV of quantitation is ≤ 10%.
4. Examine the calibration curve and verify that replicate data points on the graph are not overlapping. Overlapping data points would indicate that the calibrators are not clearly distinguishable.
5. Select the dynamic range according to the criteria listed prior or based on your assay criteria. In order to quantify correctly in the highest desired range, add the next higher level to your calibration curve.
6. Unknown AAV2 virus samples should be subsequently diluted into the established assay range (see Table 2).

Spike Recovery Test

To test the effect of different matrices, the AAV2 sample was subjected to dilution in different buffers. A control sample was first diluted 1:5 in different buffers, then diluted again 1:20 in Sample Diluent as the starting material. A spike and recovery test was performed using Sample Diluent. Good recovery (> 90%) with low CV (< 15%) above 6.25×10^8 gc/mL (Table 2) across different buffer systems indicate that the assay works well in different bioprocess buffer systems (Figure 8).

Table 2: AAV2 spike recovery test in different media.

Assay buffers	Spiked conc. ($*10^8$ gc/mL)	Calculated conc. ($*10^8$ gc/mL)	Recovery (%)
Sample diluent	Assay buffers	99.1	99.1
TE 0.1% pluronic	100	104.6	104.6
Medium	100	103.6	103.6
Lysis buffer	100	92.7	92.7
Sample diluent	50	49.9	99.8
TE 0.1% pluronic	50	51.7	103.4
Medium	50	51.5	103.0
Lysis buffer	50	47	94.0
Sample diluent	25	23.2	92.8
TE 0.1% pluronic	25	29	116.0
Medium	25	22.3	89.2
Lysis buffer	25	25.4	101.6
Sample diluent	12.5	11.9	95.2
TE 0.1% pluronic	12.5	14.1	112.8
Medium	12.5	10.6	84.8
Lysis buffer	12.5	13.8	110.4
Sample diluent	6.25	6.41	102.6
TE 0.1% pluronic	6.25	6.12	97.9
Medium	6.25	4.83	77.3
Lysis Buffer	6.25	8.28	132.5
Sample diluent	3.13	2.75	87.9
TE 0.1% pluronic	3.13	3.09	98.7
Medium	3.13	3.1	99.0
Lysis buffer	3.13	4.72	150.8

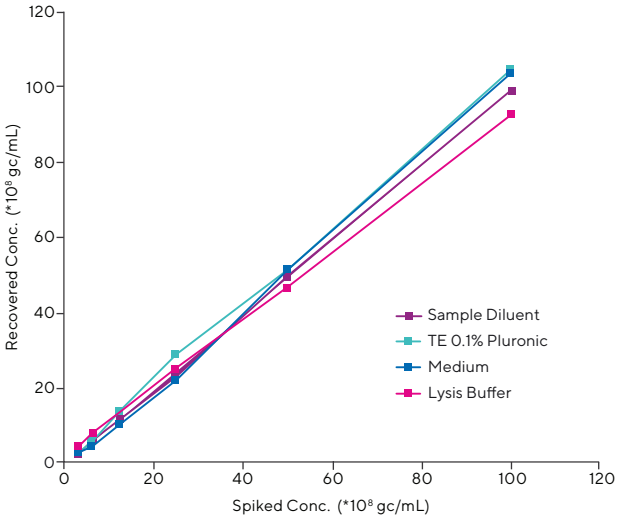

Figure 8: AAV2 spike recovery comparison.

AAV2 Virus Quantitation Reproducibility Assessment

For reproducibility assessment, two samples, before (sample A) and after (sample B) the filtration enrichment were analyzed in duplicate. Results in Table 3 show good consistency with R^2 values at 0.99 and CVs of less than 10%.

A comparison between the Octet® assay, ddPCR and ELISA is highlighted in Figure 9. The data suggests very comparable results between the three techniques; Smilar trends were obtained using ddPCR, ELISA and Octet® methods for both sample A (before filtration enrichment) and sample B (after filtration enrichment). Results further clearly show the AAV vector titer improves with enrichment.

A key application in bioprocess optimization is the monitoring of recovery during virus purification. To test whether the Octet® assay could be used to assess the purification profile of AAV2, 7 different samples from various purification steps were diluted 100x in Sample Diluent and analyzed in the same run. Figure 10 shows that different bioprocess samples can be detected simultaneously in less than 30 minutes. This assay time can be further optimized for at-line monitoring of bioprocesses.

Sample	Binding rate	Calc conc. (*10^8 gc/mL)	Dilution factor	Conc. (*10^{12} gc/mL)	Avg. conc. (*10^{12} gc/mL)	%CV
S2-1	0.5554	41.0	200	0.82	0.88	9.64
S2-2	0.5928	47.0	200	0.94	0.88	9.64
S5-1	0.7609	82.7	200	1.65	1.75	7.69
S5-2	0.7942	92.3	200	1.84	1.75	7.69

Table 3: Two samples from different virus purification processes were analyzed in duplicate.

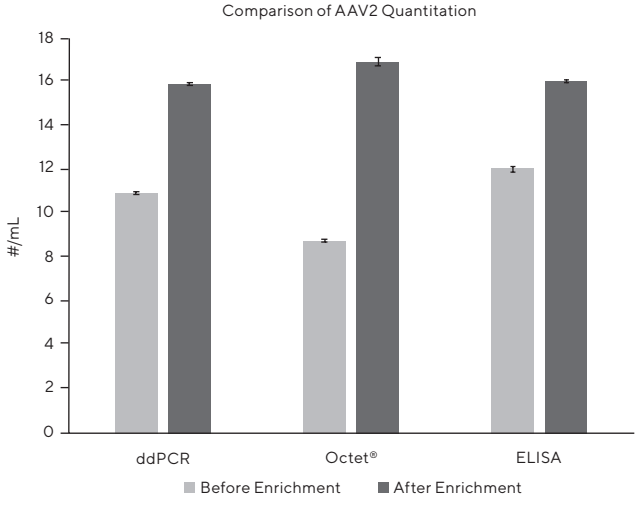

Figure 9: Comparison of ddPCR, ELISA, and Octet® platforms [#/mL: gc/mL(ddPCR and Octet); capsids/mL(ELISA)].

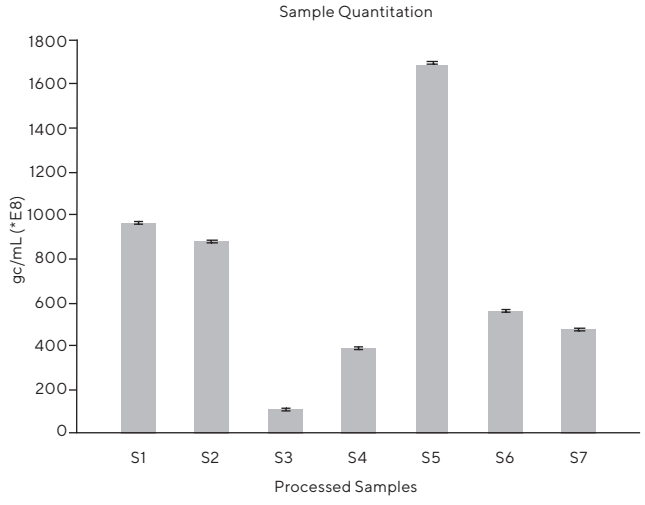

Figure 10: Octet® AAV2 titer determination for bioprocessing samples. Samples were obtained from different purification steps and assayed simultaneously.

Discussion

The operating procedure for Octet® instruments is simple and straightforward. Since it is a Dip and Read method with samples placed in microtiter plates, sample purification is not necessary, resulting in significant sample preparation time savings. The technology is highly versatile and can be used broadly to design multiple AAV serotype-specific titer assays. Here we have developed a fast, high-throughput and robust AAV2 capsid quantification method using the Octet® platform. The titer values measured by the Octet® assay correlate well with results obtained from ELISA and ddPCR. The Octet® assay afforded an approximately 2-log dynamic range. The assay is capable of quantifying process-related samples. Samples can be quantified in less than one hour compared to approximately 5 hours for ELISA and about 8 hours for ddPCR for a similar number of samples. The Octet® quantitation assay is a robust analytical method for titer measurement of any virus particle by coating the appropriate capture ligand on a biosensor. We postulate that rapid and easy viral titer measured at-line enables near real-time feedback on the bioprocess, thereby saving significant resources and time for virus manufacturing.

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