gator

Application Note

High-Throughput Titer Assay for AAV Capsids

Purpose

This document represents a comprehensive analysis of the high-throughput adeno-associated virus (AAV) capsid titer, utilizing the Gator® HS AAVX and HS AAV9 kit in conjunction with the Gator® Pro, a high-throughput instrument designed for antibody characterization and viral vector analytics.

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Introduction

Adeno-associated virus (AAV) is a small, non-pathogenic virus belonging to the Parvoviridae family, characterized by its small size and single stranded DNA genome with a maximum cargo size of approximately 4.7 kilobases (kb). Due to its ability to infect a broad spectrum of cell types, low immunogenicity, and site-specific integration into the host genome which reduces the risk of insertional mutagenesis, AAV has gained significant attention as a promising vector for gene therapy.

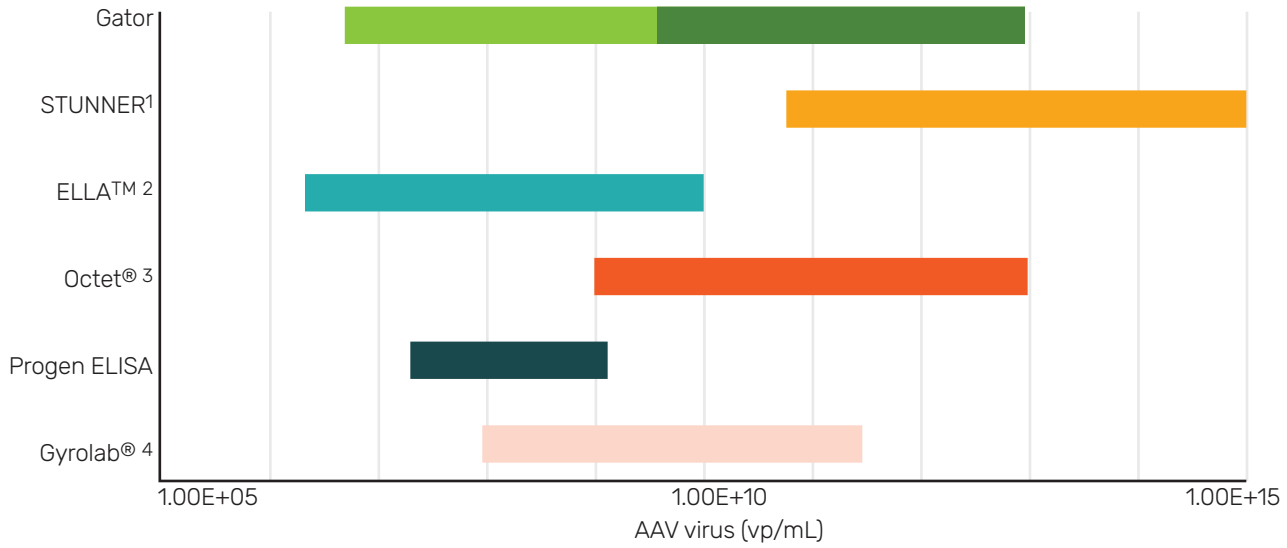

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Figure 1: Dynamic range of AAV capsid titer for different companies

Gator Bio currently offers two distinct methods for quantifying AAV capsid titers. The first is the direct binding assay, which utilizes Gator AAVX/AAV9 probes to achieve high dynamic range quantitation of AAV serotypes across a broad range of concentrations (1.00E+09 - 1.00E+13 vp/mL). Alternatively, the HS AAV/HS AAV 9 kits are employed for AAV capsid titers in crude matrixes or for lower capsid titers (<1.00E+10 vp/mL). The combination of these two methods enables Gator Bio to offer a wide range of AAV capsid titer quantification options in comparison to our competitors (Figure 1). Both methods entail immobilization of AAV-specific nanobodies onto a biosensor surface, followed by exposure to a sample containing AAV particles. As AAV particles bind to the immobilized antibodies, changes in the interference pattern of light are detected by the biosensor, enabling the real-time measurement of AAV concentrations in the sample. Rapid and accurate quantification of AAV particles is critical for gene therapy applications, as it allows for precise dosing of the therapeutic vector.

With 32 high frequency parallel measurement, the Gator® Pro provides high-throughput, label-free detection for AAV capsid titer. The instrument is designed with four plates on the deck, where one plate is used for the biosensors and the other three plates are solely dedicated to samples. The system can read from 1 - 32 wells in parallel format, offering advantages for assay design and allowing for the optimization of analytical throughput or sensitivity. The 32-biosensor mode enables rapid whole-plate detection, providing high sensitivity AAV quantitation data for 96-well or 384-well plates in just 100 minutes, significantly reducing screening time. Gator® Pro's 32 spectrometers ensure accurate low sensitivity sample detection, making it an ideal tool for high-throughput screening.

AAV quantitation assay	Through-put	Time	Serotype specific
GatorBio AAVX/AAV9-Direct format	96 assay	15 minutes	All serotypes (1-10)
GatorBio AAVX/AAV9-High sensitivity format	96 assay	100 minutes	All serotypes (1-10)
ELLA	96 assay	90 minutes	Serotype specific
GyroLabs	96 assay	60 minutes	All serotypes
Progen ELISA	96 assay	5/6 hours	Serotype specific

Table 1: Assay time comparison of AAV capsid titer for Gator® Pro and different companies

Materials Required

- High Sensitivity AAV Kit (Part Number: 350003, Gator Bio)
- High Sensitivity AAV9 Kit (Part Number: 350005, Gator Bio)
- Quantitation (Q) Buffer (Part Number: 120010, Gator Bio)
- Microplate, 384 Wells, PP, F-Bottom (Part Number: 781209, Greiner Bio-One)
- Max Plate (Part Number: 130062, Gator Bio)
- BLI 96-Flat Plate, Polypropylene (Part Number: 130150 (Pack), 130260 (Case), Gator Bio)

Method

1.1. Basic parameter

The first step in the assay setup is to select the plate type, the number of probe columns to monitor, temperature, and other basic parameters. An example of the basic parameter window is shown below.

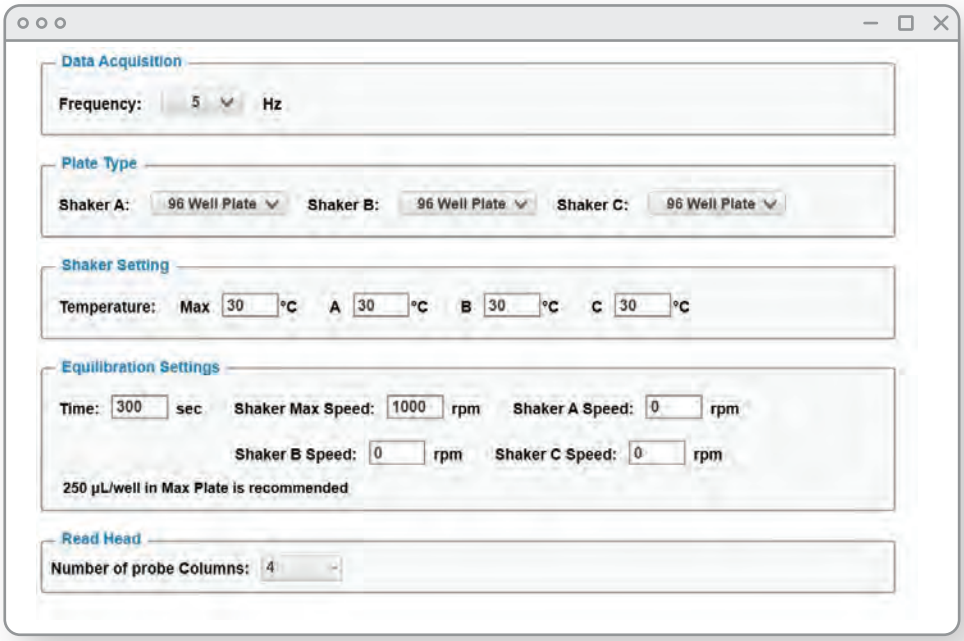


Figure 2: Basic parameter setup in the Gator® Pro for HS AAV assay format

Setup

1.2. Sample plate and Max plate setup for HS AAV/ HS AAV9 assay format: In-direct binding 32 sample assay format

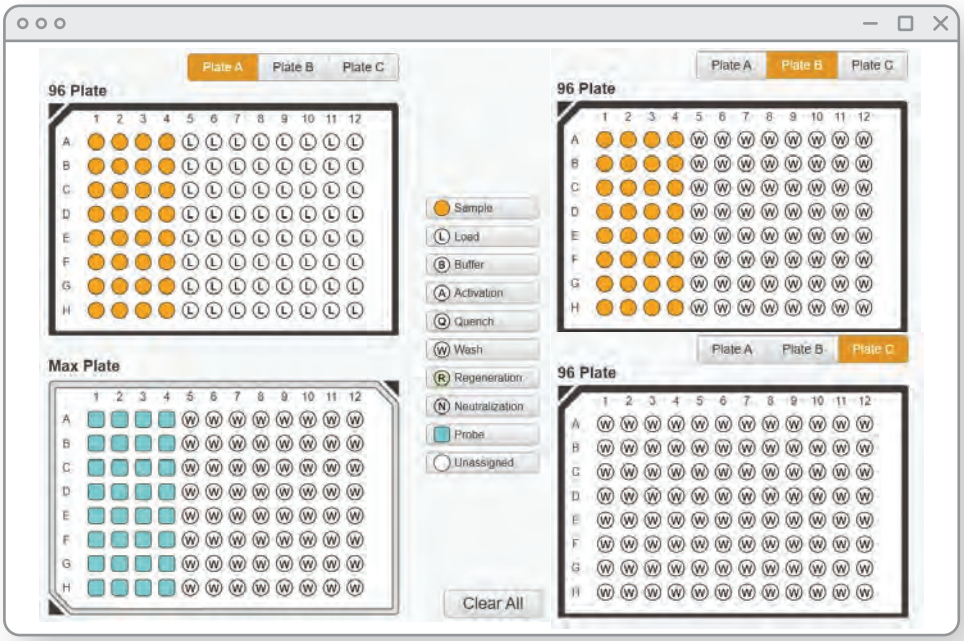


Figure 3: Basic parameter setup in the Gator® Pro for HS AAV (96-well plate, 32 sample assay format)

Max Plate Setup:

1. Columns 1-4 of the Max Plate contain Q Buffer (254 µL/well)
2. Transfer new probes from the HS Assay Kit tray into Max Plate Columns 1-4
3. Columns 5-12 of the Max Plate contain Q Buffer (200 µL/well), which will be used as wash solution

Assay Plate Setup:

- Three 96-well assay plates are required for 32 samples.

Plate A

- Column 1 contains the AAV standard with one blank well
- Column 2-4 contain unknown AAV samples
- Columns 5-8 contain Detection Solution (200 µL/well)
- Columns 9-12 contain Amplification Solution (200 µL/well)

Plate B

- Columns 1-4 contain Substrate Mixture (200 µL/well)
- Columns 5-12 contain Q Buffer (200 µL/well)

Plate C

- Columns 1-12 contain PBS Buffer (200 µL/well)

1.3. Sample plate and Max plate setup for HS AAV/ HS AAV9 assay format: In-direct binding 96 sample assay format

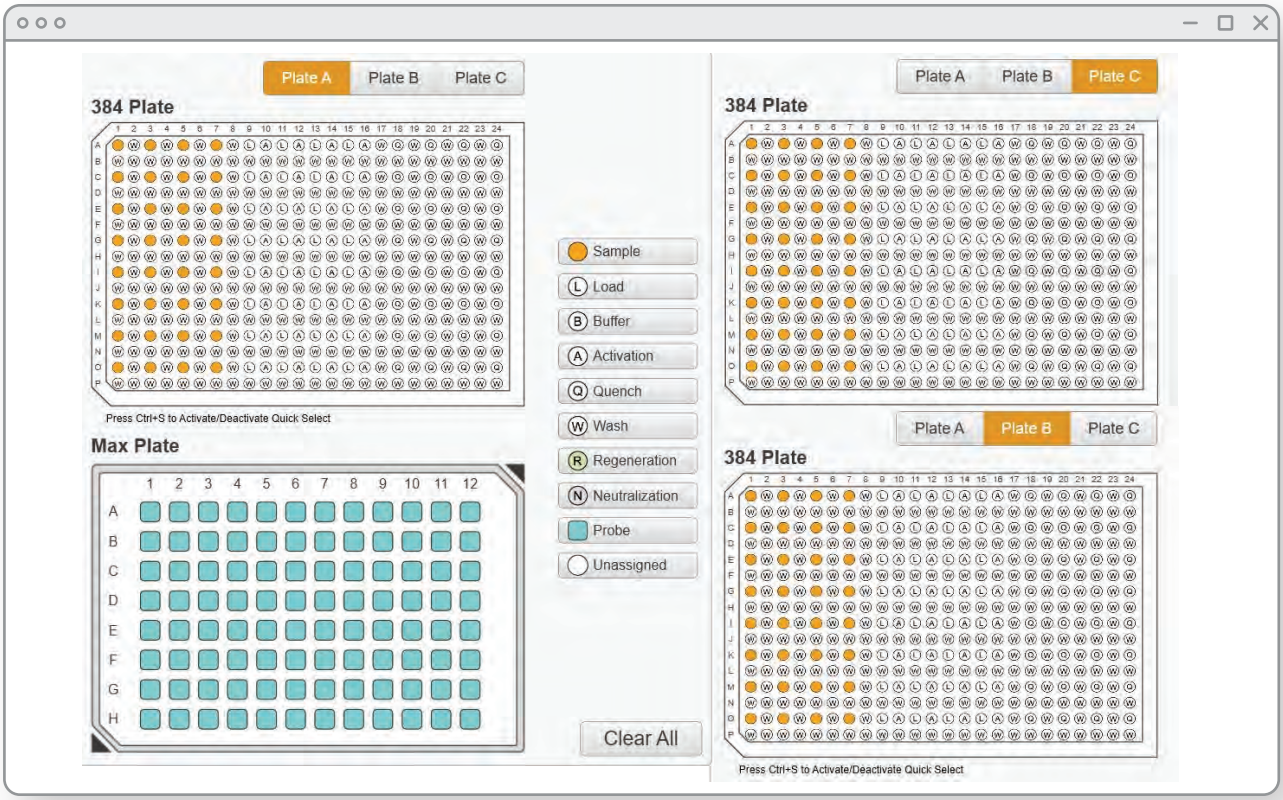


Figure 4: Basic Parameter setup in the Gator® Pro for HS AAV (384-well plate, 96 sample assay format)

Max Plate Setup:

1. Columns 1-12 of the Max Plate contain Q Buffer (254 µL/well)
2. Transfer new probes from the HS Assay Kit tray into Max Plate Columns 1-12

Assay Plate Setup:

- Three 384-well assay plates are required for 96 samples.

Plates A, B, C

- Odd rows of Columns 1, 3, 5, 7 contain AAV samples (80 µL/well)
  - *Odd rows of Column 1 in Plate A contain the AAV standard with one blank well*
  - *The remaining columns contain unknown AAV samples*
- Odd rows of Columns 9, 11, 13, 15 contain Detection Solution (80 µL/well)
- Odd rows of Columns 10, 12, 14, 16 contain Amplification Solution (80 µL/well)
- Odd rows of Columns 18, 20, 22, 24 contain Substrate Mixture (80 µL/well)
- Columns 2, 4, 6, 8 and Even rows of Columns 1, 3, 5, 7, 9-16 contain Q Buffer (80 µL/well)
- Columns 17, 19, 21, 23 and Even rows of Columns 18, 20, 22, 24 contain PBS Buffer (80 µL/well)

Note: For a 32 sample assay format in 384-well plate, add 4 columns of probes in Max plate and prepare only Plate A as mentioned above.

Data Analysis

Standard Curve Analysis

1. **Under the Results & Analysis tab, open the assay corresponding to a completed standard curve.**

2. **Click "New K Analysis 1", select "Set Reference", and then click on graph in the bottom right corner.**

3. **Select the "Quantitate Selected Step".**

Note: The following message may appear: "Multiple assays are detected." If "Yes" is selected, the other assays from the same run will be included in this standard curve. Select "No" if this is not applicable.

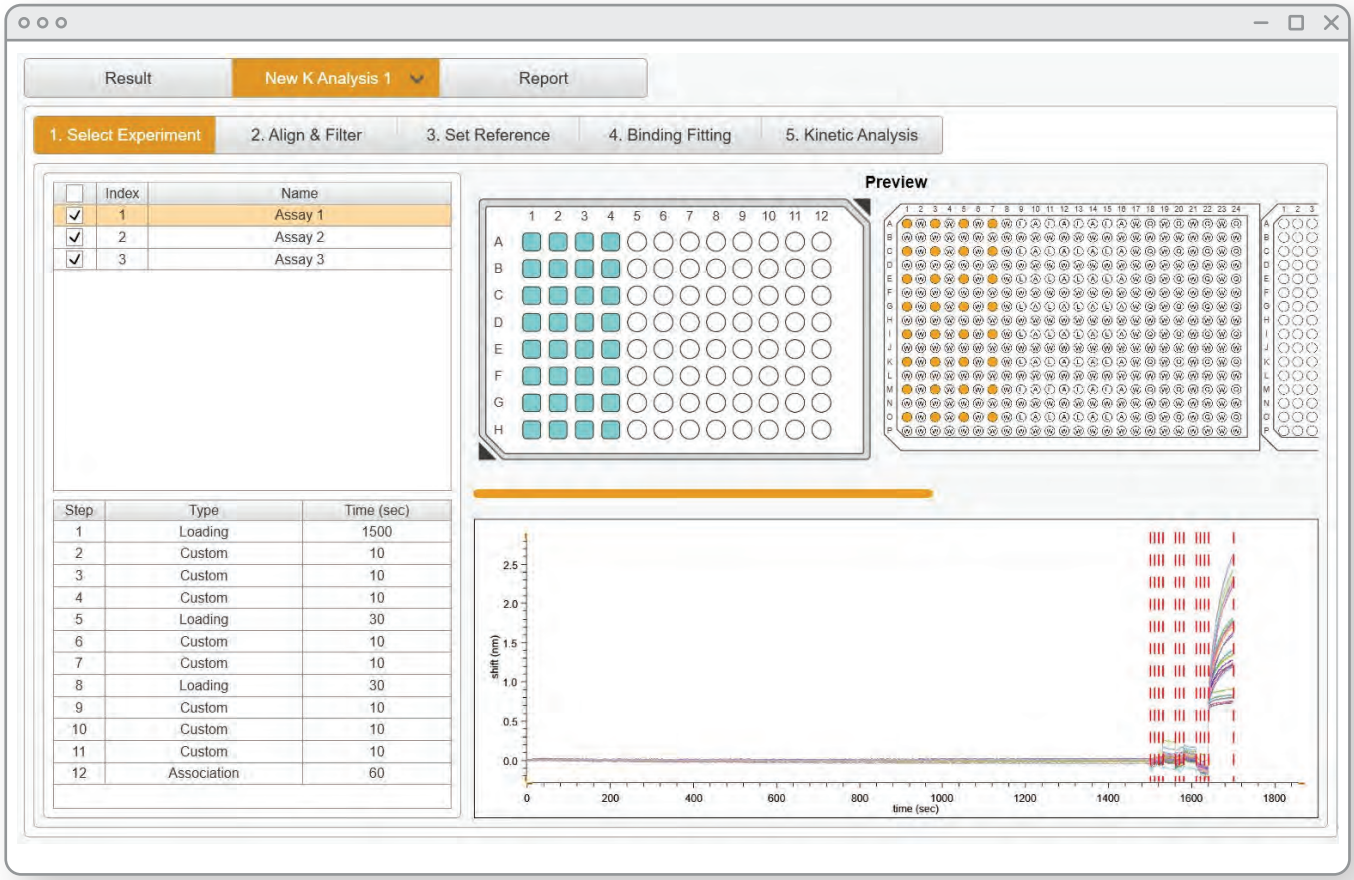


Figure 5: Analysis steps of HS AAV in GatorOne software

4. Under the Sample ID tab, adjust the following parameters to match those listed below (refer to the image for additional clarification):

- Lower Limit: 5 sec.
- Upper Limit: 35 sec.
- Highlight the buffer blank samples and label as “Reference”
- Reference subtraction: column
- Highlight the remaining wells and label as “Standard”
- Enter the known concentrations for the standards

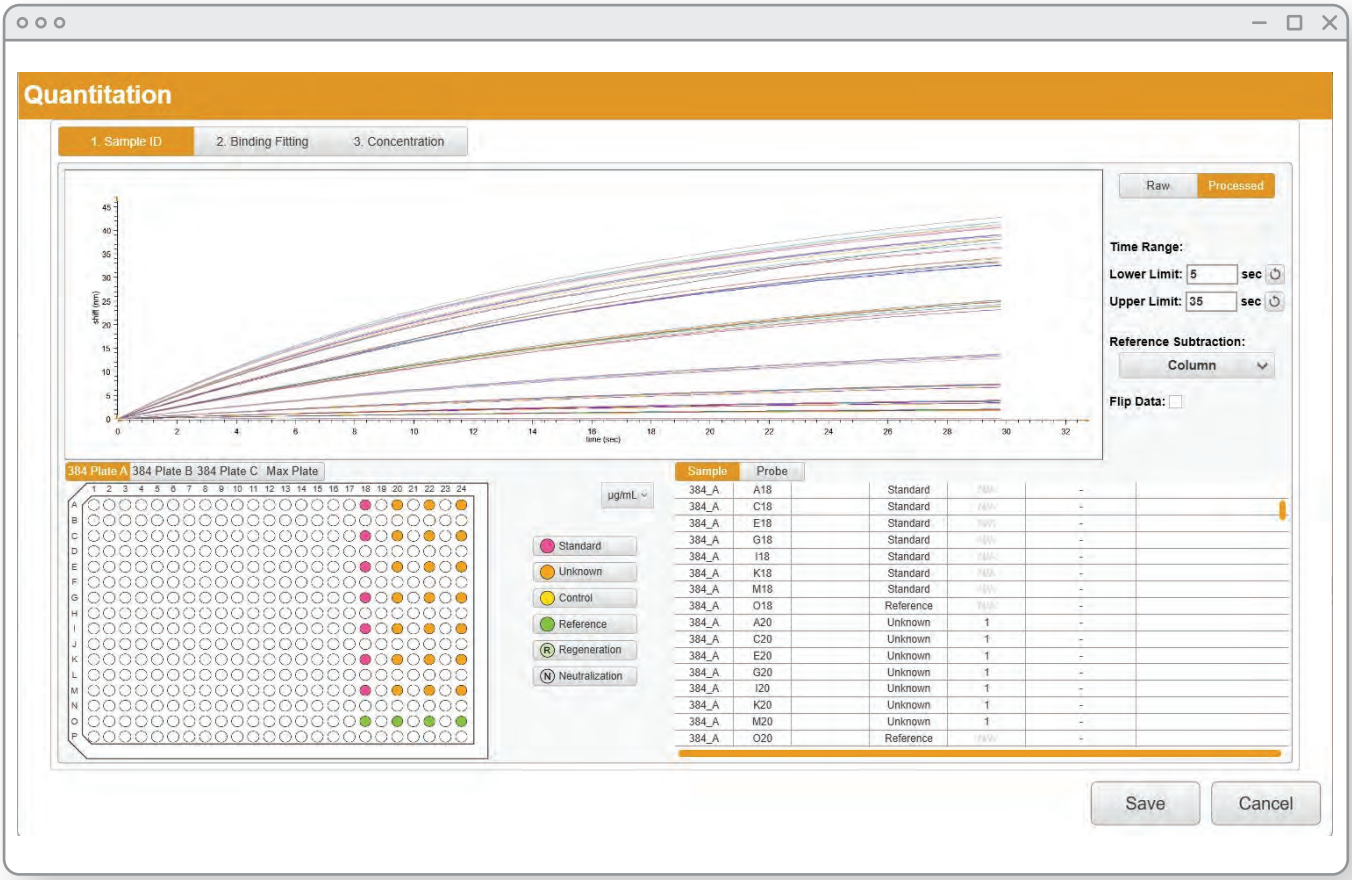


Figure 6: Quantitation steps of HS AAV in GatorOne software

5. Under the Binding Fitting tab, click “Parameters”. The following are the recommended parameters:

- Equation: REquilibriumOptimal
- Optimal: Leave as default value
- Low Conc: Leave as default value
- Click “Confirm” to proceed
- Click “Binding Curve Fit” and allow a moment for the graph to recalibrate

6. Under the Concentration tab, click “Calculate Conc” and wait for the calculated concentration values to auto-populate in the table below

7. Click “Save Std Curve” for future assays

Experimental Sample Analysis

1. Repeat steps 1-3. under Standard Curve Analysis.

2. Under the Sample ID tab, input the following parameters:

- Lower Limit: 5 sec.
- Upper Limit: 35 sec.
- Reference Subtraction: None/column (in case any blanks are included in the same run)
- All highlighted wells labeled as unknown

3. Under the Binding Fitting tab, select parameters. Recommended parameters are:

- Equation: REqulibriumOptimal
- Optimal: Leave as default value
- Low Conc: Leave as default value
- Click confirm to proceed
- Select "Binding Curve Fit" and allow a moment for the graph to recalibrate

4. Under the Concentration tab, click "Parameters"

5. Click "Load" to upload the AAV standard curve from the desktop. In case of standard curve run in the same assay, one doesn't need to click "Load" and continue to the next step

6. Identify the preferred fitting model and select "Confirm"

7. Select "Calculate Conc" and wait for the calculated concentration values to auto-populate in the table below

8. Select the "Save" icon to save analysis report

Notes:

Plate Setup

- Ensure that that appropriate wells have been filled with the respective reagents and correspond correctly to the wells in the GatorOne software.
- Confirm that the pin placement in the Max Plate matches that in the GatorOne software.

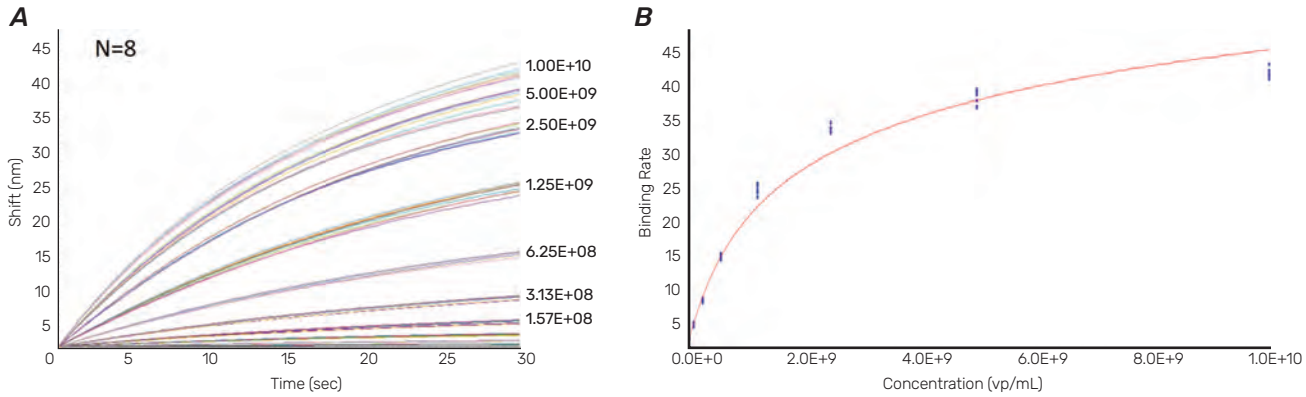
Software

- If running multiple consecutive assays, change the probe column for every run under "Assay Set Up". The regeneration error code will appear if the user fails to specify this information.
- Ensure that step 12 from every assay is labeled as "Association" under "Assay Set Up" before running assay. If not, user can change step 12 from every assay when doing data analysis under "1. Select Experiment." as Fig.5 shown above. The data analysis will be unavailable if this step is labeled incorrectly.

Results

Titration of purified AAV5 viral capsids

The titration of Virovek AAV5 viral capsids was performed using the HS AAV kit on the Gator® Pro instrument. Taking advantage of its capacity to run 32 samples simultaneously, a total of 96 probes were tested in three consecutive assays, with a total assay time of 100 minutes. AAV5 capsids were serially diluted across a concentration range of 1.00E+10 to 9.81E+06 vp/mL, and a blank reference was included as a control. For each concentration, 8 probes were run, and the binding rates were calculated using REquilibriumOptimal fitting. Subsequently, the binding rate data was fitted with the AAV5 titer values to generate a standard curve using the five-parameter logistic (5PL) model, as illustrated in Figure 7.



Conc. of AAV5 (vp/mL)	Binding rate (nm)	% CV (n=8)
1.00E+10	41.96	2.27
5.00E+09	38.50	3.49
2.50E+09	33.73	2.17
1.25E+09	24.46	3.26
6.25E+08	14.36	3.28
3.13E+08	7.62	3.30
1.57E+08	3.91	5.54
7.83E+07	1.89	5.96
3.92E+07	0.95	6.60
1.96E+07	0.51	4.15
9.81E+06	0.28	5.96

Figure 7: Titration of AAV5 viral capsids using HS AAV kit in Gator® Pro instrument in 384-well format. *A. Binding profile of different AAV5 viral capsids is demonstrated. B. After fitting the binding rates of all the concentrations, standard curve is generated which is fitted with 5PL fitting equation with R^2 of 0.99.*

The binding rates of AAV5 capsid titer, along with the corresponding percent coefficient of variation (% CV), are summarized in Table 2. The precision of each titer measurement is well below 10%, indicating high reproducibility of the HS AAV kit. The accuracy of the HS AAV kit, when used with the high-throughput Gator® Pro instrument, makes the process of AAV titration easy and reliable, ensuring robust and consistent results.

Table 2: Binding rates and % CV (n=8) of 11 concentrations of AAV5 viral capsids.

Titration of Crude AAV2 capsids

Measuring AAV titer in crude matrices can be challenging due to the presence of impurities and contaminants that can interfere with the accuracy and precision of the assay. To overcome these issues, the HS AAV kit was utilized, which provides more accurate readings without matrix interference.

AAV2 titer is particularly challenging among other AAV serotypes due to aggregation issues. In this study, we successfully quantified AAV2 titer in crude samples provided by our collaborators using the HS AAV kit on the Gator® Pro instrument in a 384-well format. The crude samples were obtained from different stages of upstream AAV2 production and were diluted 1 to 1000 times with Q buffer. Standards provided by the collaborator were used to back-calculate the unknown AAV2 titer in the crude samples. Binding graphs of the standard and the AAV2 samples can be seen in Figure 8.

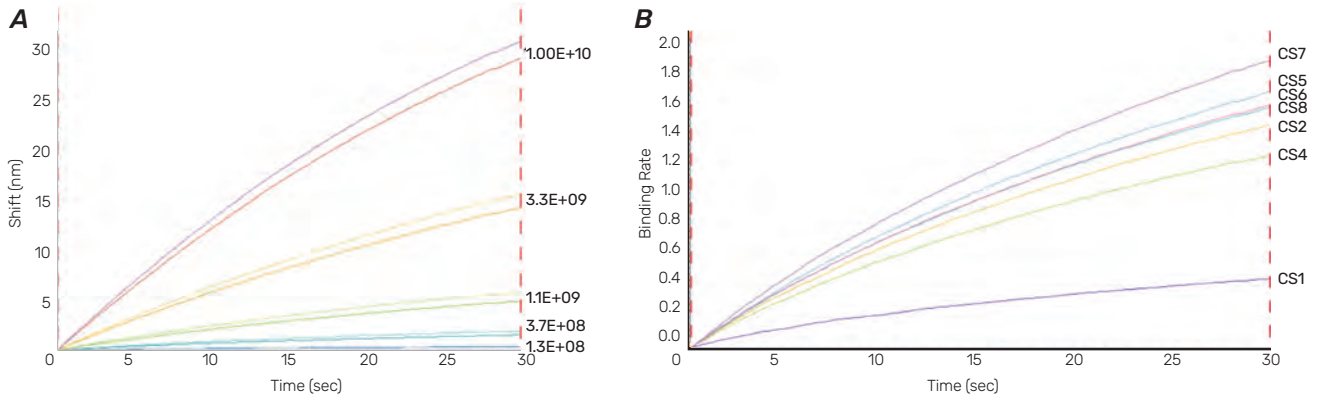


Figure 8: AAV2 capsids titration and unknown crude sample titer calculation. *A. Standard Curve of the samples provided by the collaborator. Two different standards were provided and a standard curve was run in duplicates . B. Binding rate graph of all the unknown crude samples from different phases of AAV2 productions (collaborator's samples).*

The back-calculated titer of the crude samples is presented in Table 3. A total of 2 experiments were run for each sample. Additionally, the titer of some of the crude samples was also calculated using the PROGEN AAV2 ELISA kit. The samples were diluted 1 to 1000 times in 1x ASSB buffer (provided in the ELISA kit), and PROGEN AAV2 standard and collaborator-provided standards were used to back-calculate the titer (Table 3). Two of the crude samples were saturated during the reading and their titers could not be calculated. The titers of the other crude samples were comparable to the titers obtained from the HS AAV kit. Thus, the higher dynamic range of the HS AAV kit proves beneficial in unknown samples as the real-time analysis platform provides readings that can be adjusted later according to the linear fitting range of the assay.

Sample Label	Gator: HS AAV Calculated Titer (vp/mL) n=2	PROGEN: AAV2 ELISA Calculated Titer (vp/mL) n=1
CS1	1.11E+12	OFR
CS2	1.46E+11	OFR
CS3	3.63E+11	1.23E+11
CS4	3.80E+11	Not performed
CS5	3.80E+11	4.48E+11
CS6	4.12E+11	3.96E+11
CS7	4.60E+11	Not performed
CS8	3.88E+11	5.35E+11

Table 3: Comparison between AAV2 titer in HS AAV kit and PROGEN AAV2 ELISA kit.

When using the Gator AAVX/AAV9 probes, HS AAV/AAV9 kit, and Gator® Pro instrument in combination, the titration of AAV serotypes becomes a straightforward and precise process, even in various stages of AAV production (refer to Figure 9). Furthermore, Gator® Pro offers built-in templates for both direct and indirect HS AAV assay formats, further enhancing the convenience and versatility of the quantitation process.

Note: The same assay setup and analysis can be done with the Gator HS AAV9 kit.

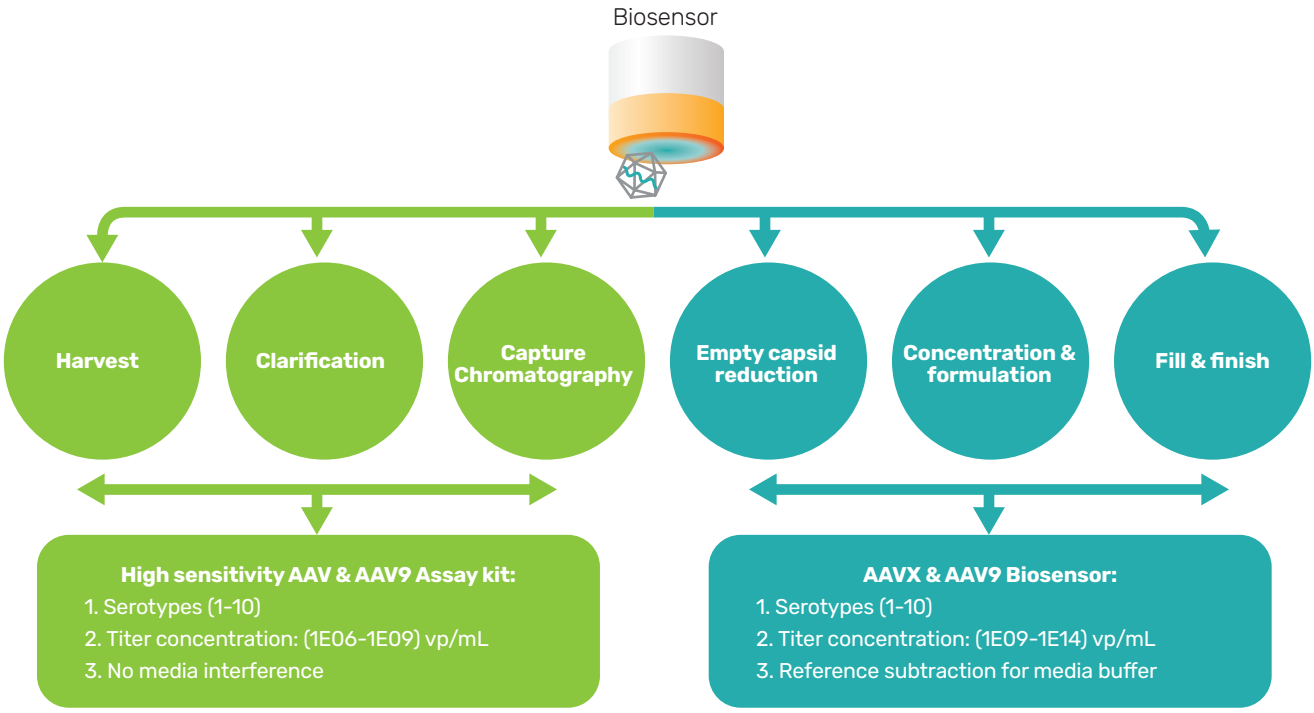


Figure 9: AAV Biosensor / AAV Assay Kit Selection Guide for AAV Titer. Gator Bio offers AAV titer solutions to support the entire AAV production pipeline. *With both rapid and high sensitivity assays available, the automated AAV titer solutions enable titer determination with wide dynamic range for serotypes 1-10 in both crude and purified samples.*

Key Takeaways

- Gator® Pro has three sample plates on the deck, allowing for simultaneous analysis of up to 96 upstream samples, saving time and improving efficiency.
- The instrument supports both 96-well and 384-well assay formats, providing flexibility in assay design to suit specific experimental needs.
- With 32 high-frequency parallel measurements, Gator® Pro enables high-throughput sample analysis, making it particularly useful for large-scale studies.
- Gator® Pro allows for 32 sample quantitation in as little as 2 minutes for direct binding assays, enabling quick and accurate measurements.
- For high sensitivity assay formats, Gator® Pro can perform 32 upstream sample quantification in as little as 35 minutes, which is considerably faster than other systems.
- The assay format of Gator® Pro is designed to conserve precious samples for other assay development, which is especially important for rare or limited samples.

