Rapid Titer Measurement of AAV Vectors by AAVX Probes

Purpose

This document applies to the Gator® AAVX probes (Part Number: 160017 Gator Bio) for quantifying Adeno-Associated Virus (AAV) serotypes. Included in this application note is a detailed protocol for (i) the rapid quantitation assay of AAV particles, (ii) built-in data analysis software, and (iii) tips for troubleshooting.

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Introduction

AAV are non-enveloped viruses with a small, single-stranded DNA genome. AAV is widely used in viral gene therapy because the vectors are non-integrating and non-immunogenic, thereby reducing the risk for insertional mutagenesis in the host genome or an immune response. Gator AAVX probes quantitate AAV particles, ranging from serotypes AAV1 to AAV10, via label-free Bio-Layer Interferometry (BLI) technology. BLI quantitation provides a variety of advantages over an enzyme-linked immunoassay (ELISA), such as a simple "one-step assay" format, reduced assay time, and minimal hands-on time to decrease user-dependent variability. Moreover, Gator AAVX probes are pre-made to exacting specifications, ensuring that each assay performed will yield highly reproducible results.

The Gator AAVX probes are highly specific for AAV particles with a Limit of Quantitation (LOQ) of 1E+09 vp/mL and a Limit of Detection (LOD) of 5E+08 vp/mL. In addition to serotypes AAV1 to AAV10, quantitation is also accurate for any AAVX-compatible synthetic serotype. As shown in the figure below, differences in binding rate with respect to time (A) are proportional to the concentration (titer) of AAV particles. By plotting these binding rates as a standard curve (binding rate versus concentration- B), accurate quantitation can be obtained from viral titers ranging from 1E+09 to 1E+13 vp/mL. Each Gator AAVX probe can be regenerated up to 10 times using Gator Regeneration (R) Buffer with No Salt (Part Number: 120063, Gator Bio) while retaining its consistent binding rate, which yields accurate, repeatable, and budget-friendly quantitation of viral particles.
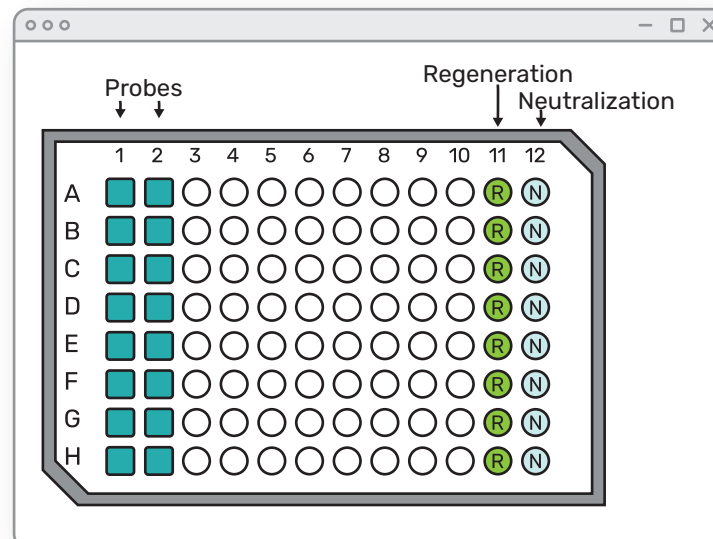
Materials Used

- AAVX Probes (Part Number: 160017, Gator Bio)

- Quantitation (Q) Buffer (Part Number: 120010, Gator Bio)

- Regeneration (R) Buffer with No Salt (Part Number: 120063, Gator Bio)

- Max Plate (Part Number: 130062, Gator Bio)

- BLI 96-Flat Plate, Polypropylene (Part Number: 130150 (Pack), 130260 (Case), Gator Bio)
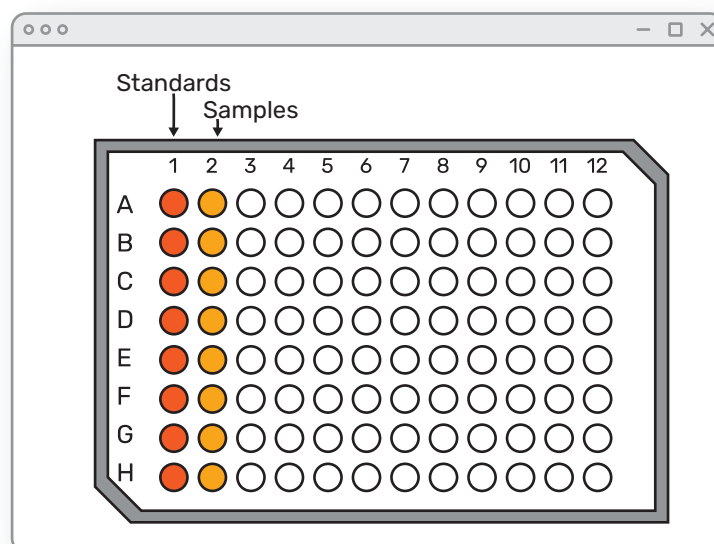
Storage

- AAVX probes should be stored at room temperature in their accompanying foil packaging with desiccants to avoid moisture. In high-humidity environments, storage in a dry cabinet is recommended.

Using Gator Bio AAVX probes, the binding rates with respect to time (A) are proportional to the concentration (titer) of AAV particles. Plotting these binding rates versus concentration (B), enables accurate quantitation to be obtained from viral titers ranging from 1E+09 to 1E+13 vp/mL.

AAVX Titer Protocol

Max Plate Setup

1. In two columns of a Gator Max plate (columns 1 and 2 in the example below), add 250 μL/well of Quantitation (Q) Buffer or sample diluent (i.e., the media that matches the media containing the viral particles).

2. Using tweezers, remove fresh probes from the tray container and place probes into these wells. Leave two columns (e.g., 11 and 12 in the example below) for Regeneration (R) and Neutralization (N) reagents.

3. Add 250 μL/well of Regeneration Buffer to the (R) wells of the Max plate.

4. Add 250 μL/well of Neutralization Buffer (or sample diluent) to the (N) wells of the Max plate.

5. An example of the Max plate map is shown below.

Sample Plate Setup

1. Prepare standards in the same sample diluent. Add 200 µL/well of AAV standards to column 1 of the 96-Flat Plate.

• It is highly recommended to also include a zero or blank sample among your standard curve.

• NOTE: AAVX probes can be used to quantitate AAV particles in a variety of buffer conditions or matrices. To enable direct comparison of samples to the standards, standards should be run using the same matrix as the samples are in.

   • *Example: when using 50 µL of AAV in media/serum, the sample should be diluted in 150 µL of Q buffer. The standards should then be comprised of 1 volume of blank media/serum and 3 volumes of Q buffer containing the standard AAV serotype. This will minimize background noise and inter-sample variability.*

2. Add 200 µL/well of AAV unknown samples into the remaining columns. For accurate results, test several dilutions of the samples to ensure that the concentrations fall within the dynamic range of the assay.

3. An example of the sample plate map is shown below.

Assay Setup

1. On the Quick Start menu in the Gator software, select Q to create a new quantitation assay setup file.

2. Rename the assay and click on Browse to select the folder where the data file will be saved.

3. Open the lid of Gator instrument and confirm that Shaker A is in the tilt position.

  • GatorPrime: Lift the lever next to Shaker A until it clicks – it is now in the tilt configuration.

  • GatorPlus: Tilt/Flat is controlled by an internal actuator – no action is required.

4. Place the sample plate in Shaker A (left side) and Max plate in Shaker B (right side).

5. Click on Description and input the required information: Assay Name, Description, and User.

6. Click on Basic Parameters and input the following settings:
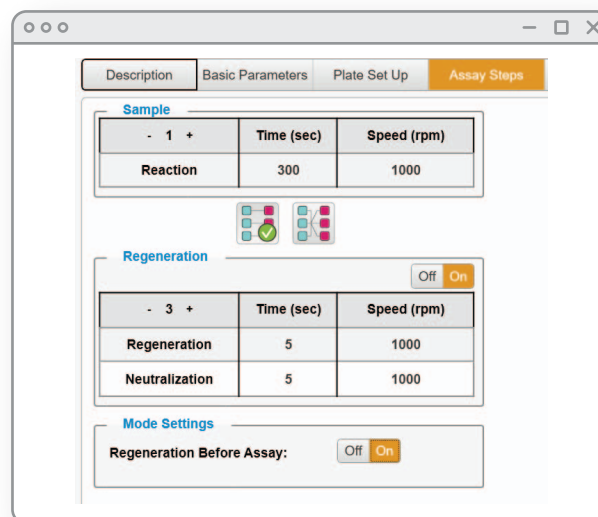
  • Data Acquisition Frequency: 5 Hz

  • Shaker Setting Status: Tilt

  • Shaker Setting Temperature: 30°C for Shaker A and Shaker B

  • Equilibrium (Pre-Wet and Pre-Mix) Setting Time: 600 sec

  • Equilibrium Setting Speed: 1000 rpm for Shaker A and Shaker B

7. Click on Plate Set Up. Set up the assay plate maps to specify the locations of the standards and samples in the Sample plate, as well as the Regeneration and Neutralization reagents and Probes in the Max plate. (See Max Plate Setup Section). Remember to set blank sample as reference.

  • Enter the concentrations for the standards in the Known Conc. column of the spreadsheet in the bottom-right.

  • Enter dilution factor of unknown samples.

8. Click on Assay Steps and input the following parameters:

  • Sample Reaction Time: 300 sec

  • Sample Reaction Speed: 1000 rpm

  • Regeneration Cycle: 3

  • Regeneration Time: 5 sec

  • Regeneration Speed: 1000 rpm

  • Neutralization Time: 5 sec

  • Neutralization Speed: 1000 rpm

  • Regeneration Before Assay: On

  • The parameters in the Assay Steps are shown on the right:

9. Open the Preview tab.

  • Use the arrows at the top of the screen to observe the movement of the probe throughout the assay.

    • *Probes in column 1 of the Max plate should appear to enter column 1 of the Sample plate, regenerate, and be returned to their place.*

    • *Probes in column 2 of the Max plate should appear to enter column 2 of the Sample plate, regenerate, and be returned to their place.*

  • If all steps are correct, click Start in the top-right of the Preview tab to begin the assay.

Data Analysis

After the completion of the assay, perform the data analysis as follows:

1. Click on Q Results, find your file, open the Analysis folder, then double-click on "New Q Analysis 1".

2. Under the Sample ID tab, perform the following:

• If not done previously, enter the concentrations for the standards in the Known Conc. column of the spreadsheet in the bottom-right.

• In the 96-well plate map, select the wells that contained zero viral particles. While the well is selected, click Reference. This function will highlight those cells in green.

• Under the Reference Subtraction area to the right of the graph, select "Column", "Row", or "All" depending on whether the samples in the same column, same row, or all the samples in the plate are to be reference subtracted.

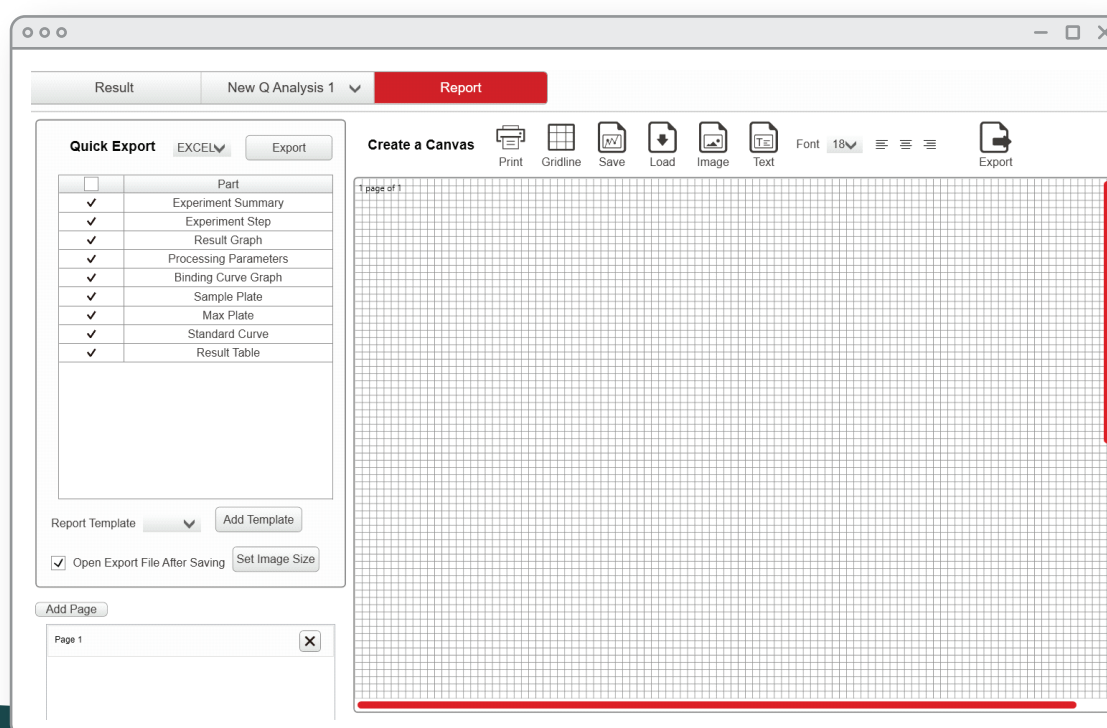**3. Click Processed to display the reference-subtracted data.**

4. Under the Binding Fitting tab, click on Parameters and select Initial Slope Optimal. Click Binding Curve Fit to generate binding rate graphs. This function will generate the binding rate data for all the standards and unknown samples.

5. On the Concentration tab, click Parameters and select "FivePLRgressionWeightedY2". Click Confirm to be returned to the quantitation screen. Click on Calculate Conc.

• The standard curve can be saved for future analysis by clicking on Save Standard Curve. This function will save the standard curve as a .csv file, which can be loaded and used when performing future analysis.

• To load a previously saved standard curve, click Parameters, click Load, and select the files previously saved as standard curve and then select Confirm.

6. Under the Report tab, you can export the data as an Excel file.

• For large data sets, exporting data may take a while if all boxes are checked in the Quick Export section. Only export the portions that are necessary for later reference.

• An example of a data report exported as an Excel file is shown below.

Result Table

Plate	b96plate	Position	Sample N	Type	Dilution F	Known C (	Calculated	Orig C (µg	Residual	Binding Ra	R2	Relative X	X2	Optimal M	Probe	Information
1	True	A1	'AAV9	Standard	NA	1.00E+012	9.66E+011	NA	-3.4428	19.481	0.9992	53.201	76.3684	Model1To	Probe	'
1	True	B1	'AAV9	Standard	NA	3.33E+011	3.55E+011	NA	6.5466	11.054	1	4.9247	0.6544	Model1To	Probe	'
1	True	C1	'AAV9	Standard	NA	1.11E+011	1.00E+011	NA	-9.7449	4.4757	0.9999	4.0559	0.4439	Model1To	Probe	'
1	True	D1	'AAV9	Standard	NA	3.70E+010	3.24E+010	NA	-12.5657	1.7397	0.9963	9.6911	2.5349	Linear	Probe	'
1	True	E1	'AAV9	Standard	NA	1.23E+010	1.24E+010	NA	0.6892	0.8011	0.9804	9.5016	2.4368	Linear	Probe	'
1	True	F1	'AAV9	Standard	NA	4.12E+009	4.73E+009	NA	14.9633	0.4581	0.952	8.0501	1.7491	Linear	Probe	'
1	True	G1	'AAV9	Standard	NA	1.37E+009	2.01E+009	NA	46.6222	0.3603	0.8974	8.4524	1.9283	Linear	Probe	'
1	True	H1	'AAV9	Standard	NA	NA	4.10E+005	NA	∞	0.2647	0.8506	7.6817	1.5927	Linear	Probe	'
1	True	A1	'AAV9	Standard	NA	1.00E+012	9.46E+011	NA	-5.4282	19.2781	0.994	142.3617	546.8403	Model1To	Probe	'
1	True	B1	'AAV9	Standard	NA	3.33E+011	3.59E+011	NA	7.615	11.1239	0.9982	48.4958	63.4575	Model1To	Probe	'
1	True	C1	'AAV9	Standard	NA	1.11E+011	1.25E+011	NA	12.4669	5.314	1	2.1333	0.1228	Model1To	Probe	'
1	True	D1	'AAV9	Standard	NA	3.70E+010	3.80E+010	NA	2.6836	1.9964	0.9998	2.646	0.1889	Model1To	Probe	'
1	True	E1	'AAV9	Standard	NA	1.23E+010	1.28E+010	NA	3.5574	0.8177	0.9931	6.0406	0.9849	Linear	Probe	'
1	True	F1	'AAV9	Standard	NA	4.12E+009	4.12E+009	NA	0.0371	0.4339	0.976	5.713	0.8809	Linear	Probe	'
1	True	G1	'AAV9	Standard	NA	1.37E+009	5.22E+008	NA	-61.9187	0.3244	0.9341	6.3453	1.0868	Linear	Probe	'
1	True	H1	'AAV9	Standard	NA	NA	1.45E+005	NA	∞	0.2588	0.8903	6.4127	1.1099	Linear	Probe	'

Common Issues and Troubleshooting Tips

Issue	Potential Cause	Troubleshooting
No binding signal in samples	AAV concentration is too low	• Increase assay time • Include positive control sample into the assay
Binding signal in samples is lower than expected	Interference or matrix effects from sample diluent (serum/media)	Check that the standards and samples are assayed in the same diluent with proper dilution
Binding rates are too fast and clustered together	AAV concentration in samples is too high	Dilute sample and test different dilutions (e.g., 100x dilution)
Standard curve nm shift is too low	Standard concentration is too low	Recheck standard concentration and verify that it is in the range of 1E+09 - 1E+13 vp/mL
Standard curve nm shift is too low	Standards are degraded	• Prepare both fresh AAV stock and standards by serial dilution • Repeated freeze/thaw of purified samples may degrade sample • Prolonged shaking pre-assay may cause increase due to aggregation
Binding curves shows Non-standard binding patterns (Linear vs. Pseudo first order, etc.)	Non-specific binding from the matrix	Place probes in matching condition in Pre-wash step and increase the pre-wet time from 10 to 30 minutes
Reference/Blank showing binding signals	Non-specific binding from matrix	In Data Analysis, use reference subtraction to eliminate the background binding
No AAV for standard	Unable to calculate the unknown	Using the software, please recall an earlier standard if it is in the same matrix
Binding shows low nanometer shift with each regeneration	Regeneration conditional not optimal	pH scouting for optimal regeneration buffer (recommended pH range: 1.5 – 3.0)
Binding curves has a sudden jump at the beginning of the assay	Non-specific binding	During data processing, exclude the initial non-linear signal to normalize the effect of non-specific binding
Binding signal has significant background binding	Dilute the sample in Q buffer	The Q buffer helps reduce non-specific binding to the probe

Note: The sample protocol and troubleshooting tips can be adopted for Gator AAV9 probes (Part No: 160021, Gator Bio) which is specific to AAV9 serotype only.

The samples with concentrations lower than the limit of quantification of AAVX probes should be quantified using the HS AAV kit (Part No: 350003) for serotypes 1-8 and 10, and HS AAV9 (Part No: 350005) kit for serotype 9.