PROTOCOL

Gator® High Sensitivity (HS) AAV Kit

Scope

This document provides a detailed protocol for running a titer assay for adeno-associated virus (AAV) particles using Gator® High Sensitivity AAV Kit and analyzing AAV titer data using the GatorOne software. It also includes common issues and troubleshooting tips.

Introduction

Adeno-associated viruses (AAV) are non-enveloped viruses with a small single-stranded DNA genome. AAV is widely used in viral gene therapy since it is non-integrating and non-immunogenic, reducing the risk for insertional mutagenesis in the host genome or an immune response. Gator® High Sensitivity AAV Kit along with Gator® Biolayer Interferometry (BLI) technology is highly useful for the quantification of low titer samples of AAV viral particles (vp) from different AAV serotypes. Quantification through BLI offers many advantages over an ELISA such as a simpler assay format, reduced assay run time, and decreased hands-on labor, thus minimizing user-dependent variability. The kit has a limit of quantitation (LoQ) of 1E+07 viral particles (vp)/mL and a limit of detection (LoD) of 5E+06 vp/mL. The dynamic range is over four orders of magnitude (1E+07 – 5E+10). The kit enables the quantification of 8 samples per run of the instrument in 35 minutes.

Materials Provided

- Gator® High Sensitivity AAV Kit, Part No: 350003
    - HS AAV probes (96 probes/tray)
    - HS AAV Detection Solution
    - AAV Amplification Solution
    - AAV Substrate Solution
    - AAV Substrate Diluent
    - Q Buffer

User Supplied Materials

- 1x PBS buffer
- Gator® Max Plate, Part No: 130062
- Gator® BLI 96-Flat Plate, Polypropylene, Part No: 130260 (Case)
- Precision pipettes and tips
- Tweezer, Fisher Scientific, Cat No: 14955032

Storage

- HS AAV probes - Shelf life of one year at room temperature when kept in a sealed pouch.
- Store the HS AAV Reagent Set - The unopened reagent set has a shelf life of 6 months at 4°C.

HS AAV Kit Quantification Protocol

Reagent Preparation

1. Bring the HS AAV Detection solution, Amplification solution, Substrate solution, Substrate diluent, Q Buffer and 1x PBS Buffer to RT.
2. Prepare the working solution of 1x Detection solution (from 10x) and 1x Amplification solution (from 10x) in Q Buffer just before the start of the experiment. Mix thoroughly. The volume should be adjusted according to the number of samples in the experiment. If the working solutions of the Detection and Amplification Solutions do not get used up, they may be stored at 4°C for up to 1 month.
3. Prepare the Substrate mixture (1:1 of Substrate Solution: Substrate Diluent) just before the start of the experiment. The volume should be adjusted according to the number of samples in the experiment.

Max Plate Setup

Note: Each fresh Max Plate can accommodate 24 probes.

1. Pipette 250 uL/well of Q Buffer into columns 1-6 of a new Max Plate.
2. Pipette 250 uL/well of 1X PBS into columns 7-9 of the same Max Plate as above
3. Pipette 250 µL/well of Q buffer into columns 10 before adding the probes.
4. Using a probe picker, pick out fresh probes from the tray and place them into column 10. Refer to the image below for Max Plate layout.
   Note: For running different assay, same washes can be re-used and one needs to pipette fresh Q buffer in column 11 or 12 and add the fresh probes

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="6" rowspan="8">Q Buffer</td><td colspan="3" rowspan="8">1X PBS</td><td rowspan="8">Q Buffer + Probes</td><td rowspan="8"></td><td rowspan="8"></td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

AAV Standard Preparation

Prepare a standard curve for a particular serotype when using the kit for the first time. Standard curves for each serotype can be saved for the future.

Refer to table below to determine the optimal AAV standard range. The recommended range for each serotype is:

AAV1	1E+08 vp/mL – 5E+10 vp/mL
AAV2	5E+07 vp/mL – 5E+10 vp/mL
AAV3	1E+07 vp/mL – 5E+10 vp/mL
AAV4	8E+07 vp/mL – 5E+10 vp/mL
AAV5	1E+07 vp/mL – 5E+10 vp/mL
AAV6	1E+07 vp/mL – 5E+10 vp/mL
AAV8	5E+07 vp/mL – 5E+10 vp/mL

Quantitation Plate Setup

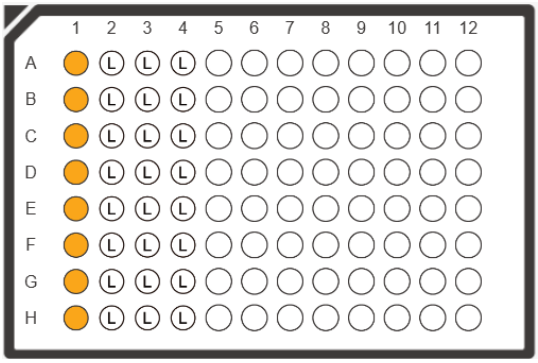
Mix all the reagents gently but thoroughly prior to pipetting them EXCEPT for Substrate Mixture. Prepare the Black Plate according to the following plate design.

1. Add 180 uL/well of standards/samples into column 1 of a new Black Plate.
2. Add 180 uL/well of 1x Detection Solution into column 2 of the Black Plate.
3. Add 180 uL/well of 1x Amplification Solution into column 3 of the Black Plate.
4. Add 180 uL/well of Substrate Mixture (1:1 Substrate Solution: Substrate Diluent) into column 4 of the Black Plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standards / Samples 180 µL	1X HS AAV Detection Solution 180 µL	1X HS AAV Amplification Solution 180 µL	HS AAV Substrate Solution + Substrate diluent 180 µL								
B												
C												
D												
E												
F												
G												
H												

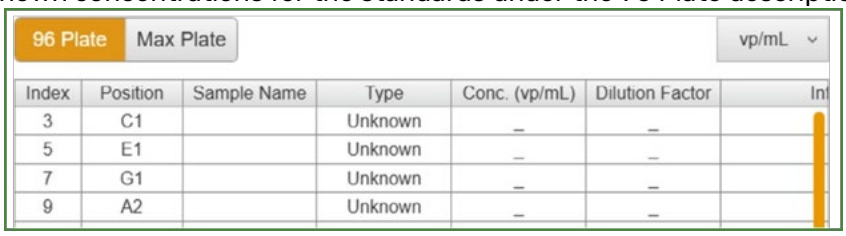
Assay Setup on Gator® Prime or Gator® Plus Instrument

1. Open the lid of the instrument.
2. Place the Black Plate into Shaker A and the Max plate into Shaker B. Column numbers should be oriented to face the right side.
3. Close the lid.
4. On the Quick Start menu in the GatorOne software, select “K assay” to set up a new experiment and name your assay.
5. Under the Description tab, input assay details and description.
6. Under the Basic Parameters tab, specify the following parameters:
    - Data Acquisition: 5 Hz
    - Shaker Setting: Flat – A & B at 30°C
    - Pre-wet and Pre-Mix Setting: 300 sec
    - Shaker A at 1000 RPM and Shaker B at 1000 RPM
7. Under Plate Set Up, set up the 96-well plate map. Refer to image below.
    - Highlight column 1 on the 96-well plate and select the Sample icon.
    - Highlight columns 2, 3, and 4 on the 96-well plate and select the Load icon.



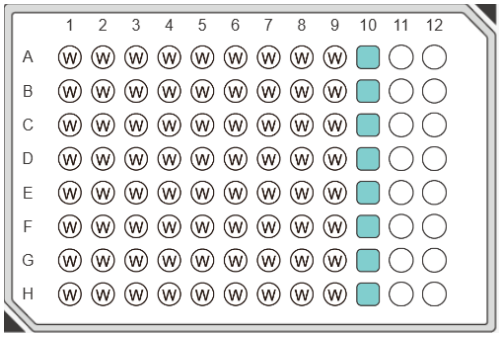
.

- Assign each well its position with the name of the corresponding sample/solution. Refer to the image below.
- Assign the known concentrations for the standards under the 96 Plate description box.

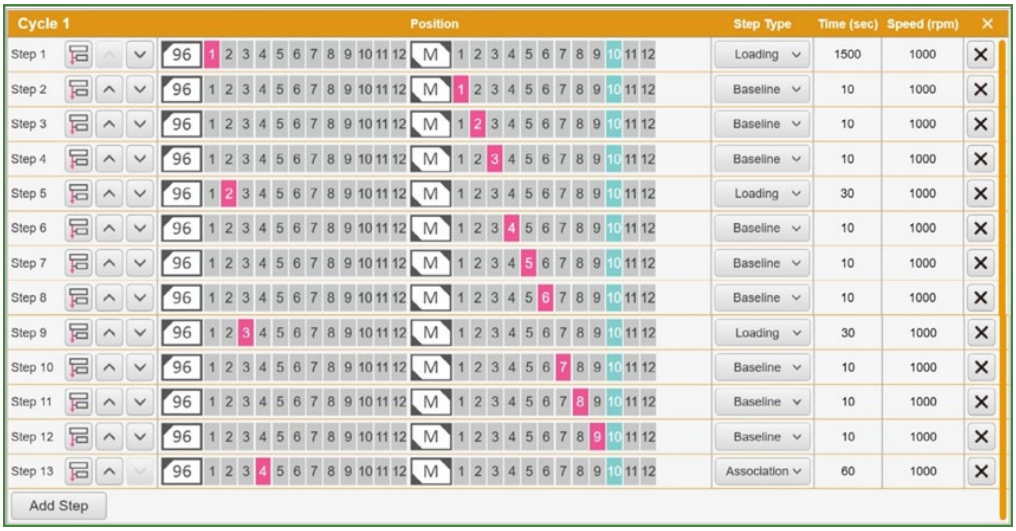


Index	Position	Sample Name	Type	Conc. (vp/mL)	Dilution Factor	Int
3	C1		Unknown	_	_	
5	E1		Unknown	_	_	
7	G1		Unknown	_	_	
9	A2		Unknown	_	_	

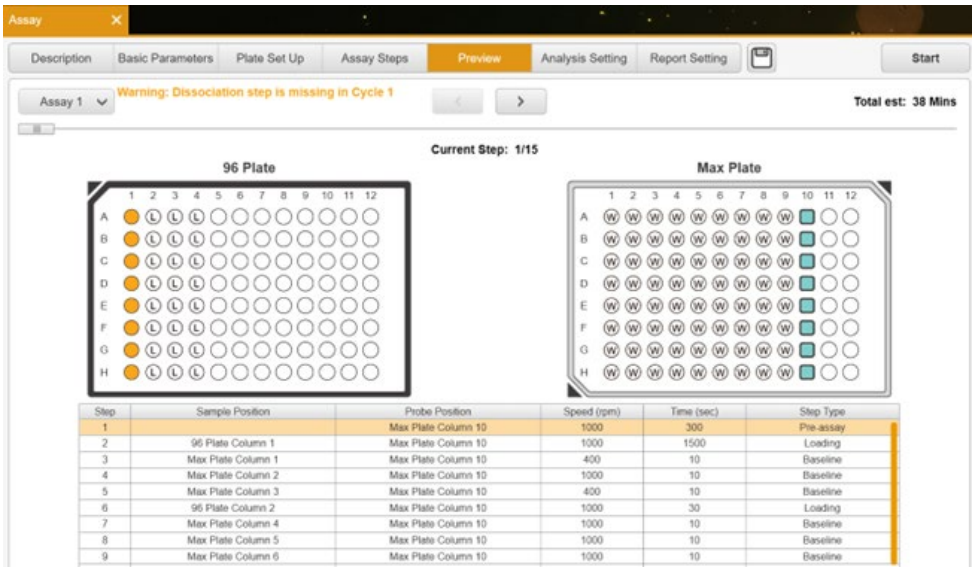
8. Under Plate Set Up, set up the Max Plate map.
   - Highlight columns 1-9 on the Max Plate and select “Wash”. Refer to image below.
     Highlight columns 10 and select “Probe”.



9. Under the Assay Steps tab, set up the experimental cycle as follows:
   - 1500 sec for sample
   - 10 sec for each wash step
   - 30 sec for HS AAV Detection Reagent
   - 30 sec for AAV Amplification Reagent
   - 60 sec for Substrate Mixture
     Note: Refer to the image below to set up the step positions



10. Under the Preview tab, confirm all the step positions, times, and RPM. Refer to the image below.

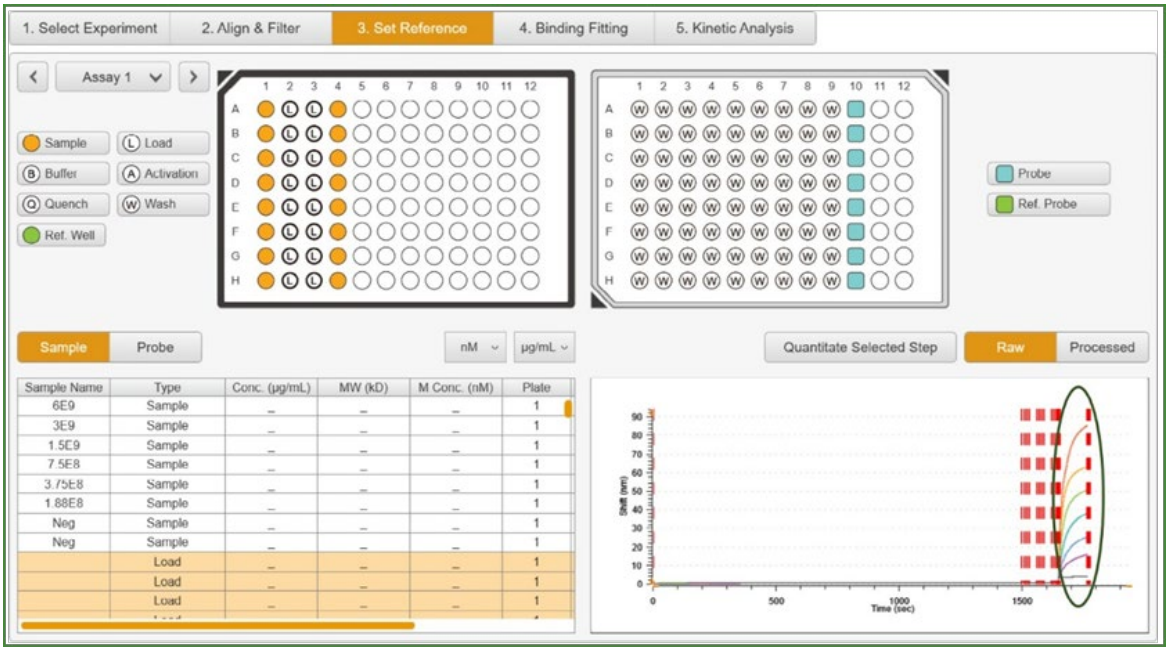


11. The assay will be completed in 35 minutes. After the assay is completed, the required data analysis can be performed.

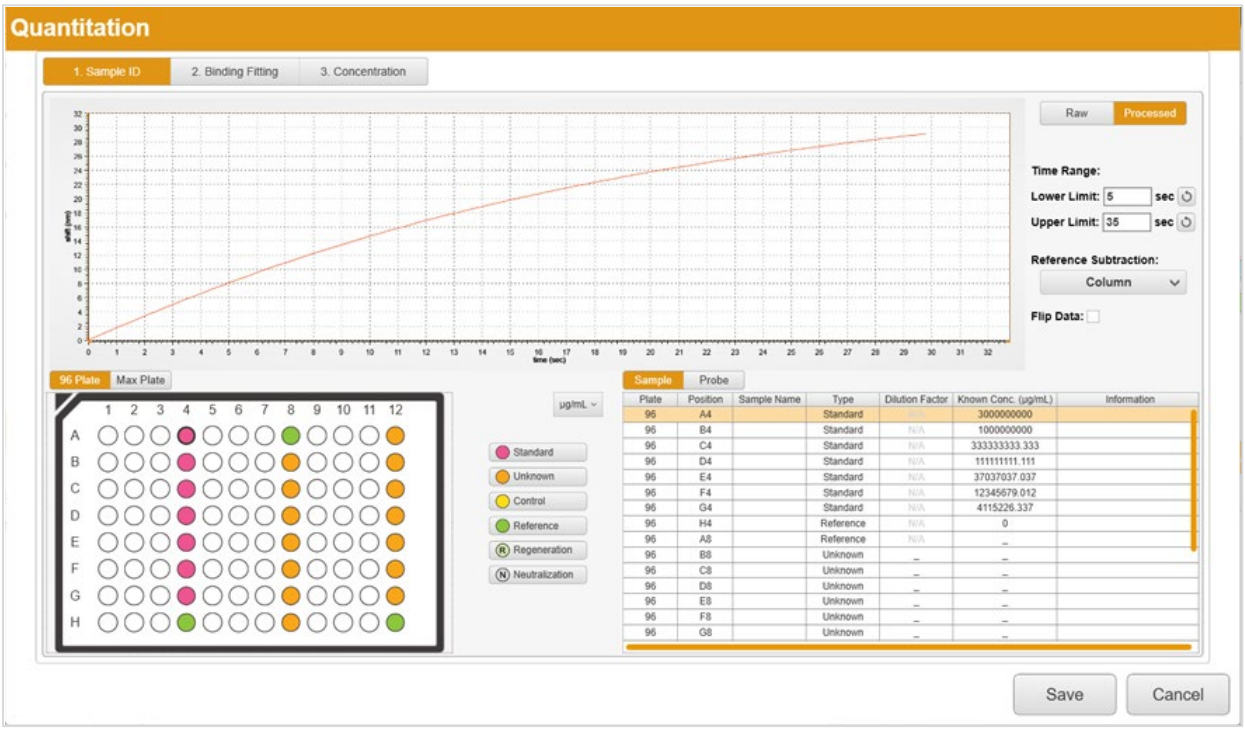
Data Analysis

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.
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



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 when working with the same AAV serotype if the same lot of the HS AAV kit is being used and the reagents have been stored as recommended.

Experimental Sample Analysis

1. Repeat steps 1-3. under Standard Curve Analysis.
2. Under the Sample ID tab, input the following parameters:
    - Low Limit: 5 sec
    - Upper Limit: 35 sec
    - Reference Subtraction: None/column (in case if any buffer blank is included in the same run)
    - All highlighted wells labeled as unknown
3. Under the Binding Fitting tab, select parameters. Recommended parameters are:
    - Equation: "REquilibriumOptimal"
    - 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 desired serotype specific standard curve from the desktop. In case of standard curve run in the same assay (for example a five-point standard curve and three samples), one doesn't need to click "Load" and continue to the next step.
6. Identify the preferred fitting model and select "Confirm"Select "Calculate Conc" and wait for the calculated concentration values to auto-populate in the table below.
7. Select the "Save" icon to save analysis report.

Notes

Plate Set Up

- Ensure that the 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.
- Only 1 column of standard/samples can be quantified per run of the instrument. Additional samples need to be run separately and pipetted into 96 well plates immediately before the run. Running more than one column of samples per run leads to sub optimal performance of the assay.

Software

- Ensure that step 13 for every assay is labeled as "Association" under "Assay Set Up." The data analysis will be unavailable if this step is labeled incorrectly.

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

Common Issues and Troubleshooting Tips

Issue	Potential Cause	Troubleshooting
Inconsistent signal shifts	Nonhomogeneous reagents	• Mix gently but thoroughly AAV stock solutions well (minimum 30 seconds recommended) prior to preparing the AAV samples • Mix HS AAV Detection Solution and Amplification Solution well before pipetting them into the Black Plate wells
Insufficient signal separation from the negative control	Matrix interference contributing to an elevated background	• If the sample is suspended in a matrix, consider diluting up to 1000-fold (the working concentration should remain within the serotype's dynamic range). The extent of the dilution will depend on the concentration and the matrix
Minimal/no signal shift	Dead probe(s)	• Rerun the assay with fresh probes