Gator® GeneSwift Kit

Introduction

Adeno-associated virus (AAV) is a common vehicle in gene therapy to treat diverse human diseases. Titer determination is a critical parameter governing therapeutic efficacy. Although ddPCR is often used to determine titer, this method is laborious, time-consuming, costly and requires extensive dilution series. In addition, its accuracy is significantly influenced by PCR inhibitors and various sample matrices. Gator Bio has developed a novel GeneSwift titer assay that leverages DNA hybridization, immunochemistry and biolayer interferometry technology (BLI). This simple and fast assay is a reliable method to determine the AAV viral genome titer (dynamic range 4E+9 to 1E+12 vg/mL) for all AAV serotypes.

Materials

Gator® GeneSwift AAV Kit GoI, Part No: 350006

Part 1 at 2-8° C

- Biotin PolyA, Part No: 130166
- Biotin Detection Reagent 200X, Part No: 120077
- GeneSwift Substrate, Part No: 120079
- GeneSwift Diluent, Part No: 120080
- Hybridization Buffer, Part No: 120076
- Q Buffer, Part No: 120010

Part 2 at Room Temperature

- Anti-Fluorescein Biosensors, Part No: 160045
- Lysis Tubes (x12), Part No: 130134
- Probe Picker, Part No. 130298

User Supplied Materials

- AAV capsid samples
- Benzonase, EDTA and TE Buffer
- Nuclease Free Water
- Single channel and 8-channel pipettes and tips
- 5 mL Eppendorf tubes and 25 mL reservoirs
- Heat block
- Ice bath
- Vortex Mixer
- Centrifuge for spinning tubes and plates
- 96-Well MAX and black plates

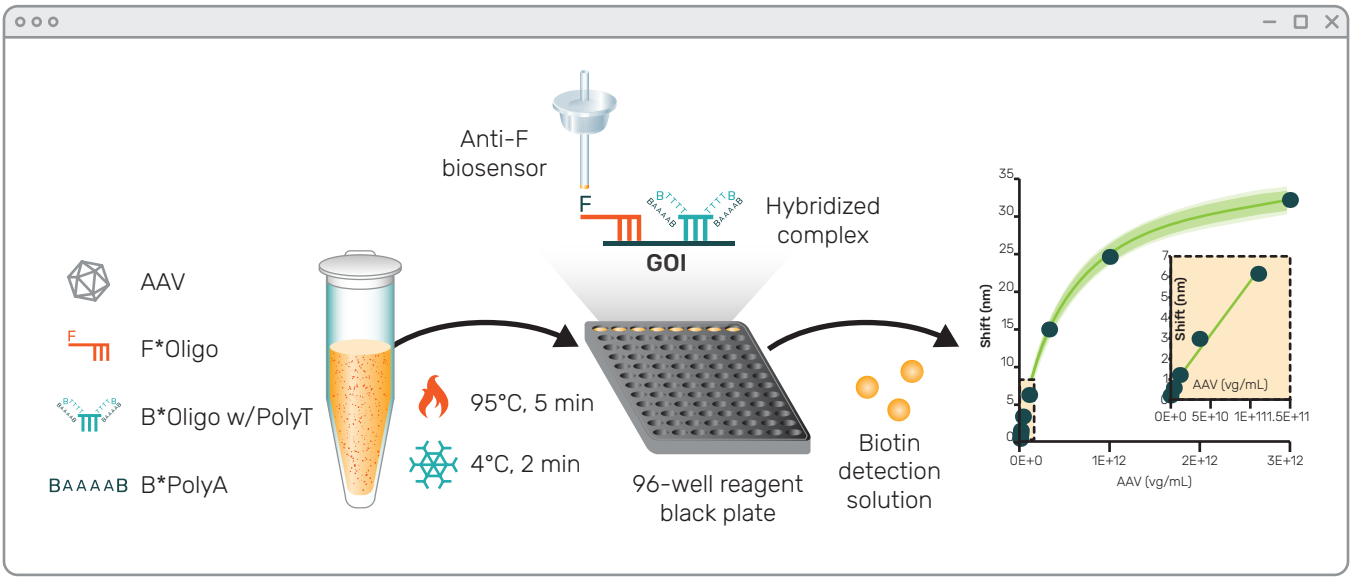
Storage

- GeneSwift Kit Part 1 components are stable at 2-8° C for 6 months in the unopened reagent package. For long-term storage, store reconstituted oligos at -20° C. **Keep Biotin Detection Reagent 200X at 2-8° C when use and after use.**

- GeneSwift Kit Part 2 components are stable at room temperature for 1 year. **Keep Anti-Fluorescein Biosensors in the sealed pouch after use.**

Workflow

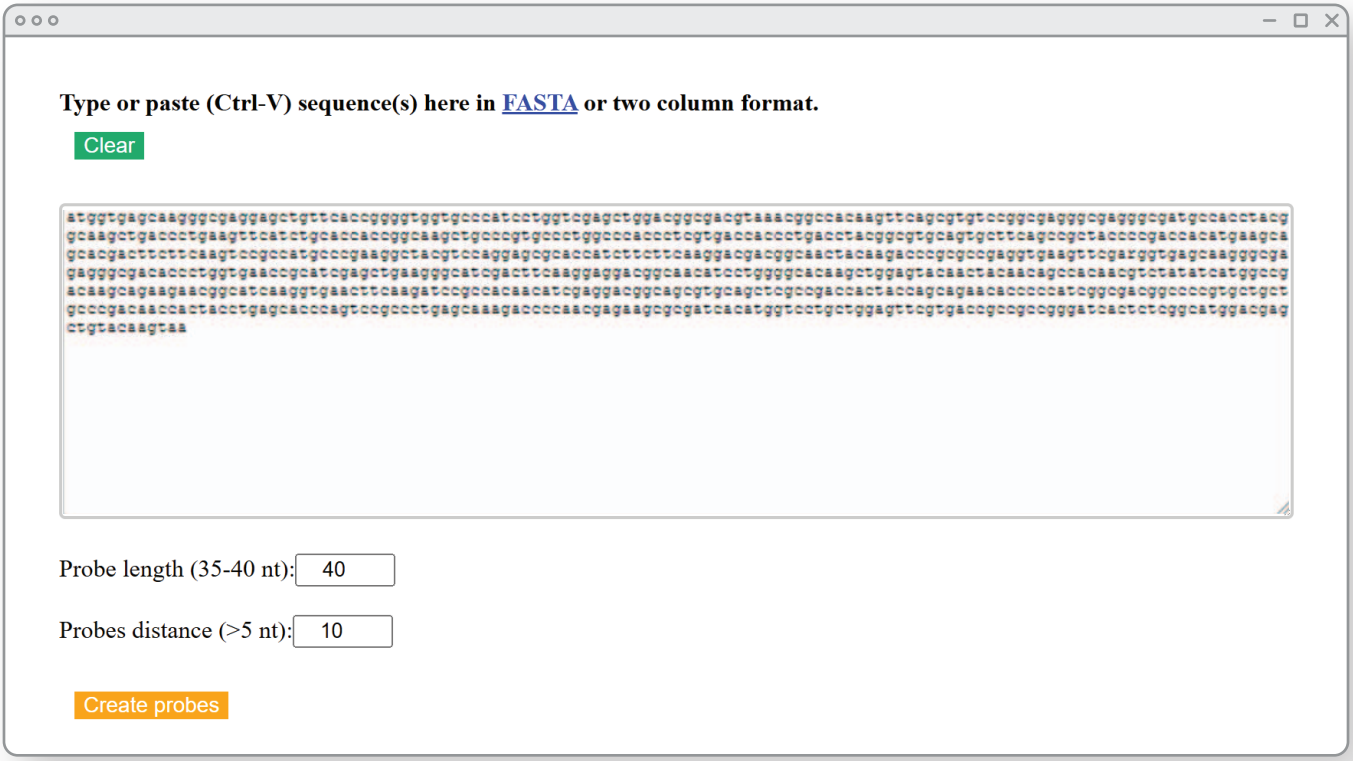
AAV samples are first mixed with two gene specific oligos (one fluorescein F*Oligo and one biotin B*Oligo) and lysed by heating in Hybridization Buffer. After lysis, the samples are cooled down to allow oligo hybridization to the gene of interest (GoI). The hybridized complexes are then fished out by binding to Anti-Fluorescein (Anti-F) Biosensors. After washing, the biotin molecules of the hybridized complexes are detected by Biotin Detection Solution and the signals are measured by a Gator BLI instrument. As the assay sensitivity increases with the number of detected biotins, we recommend the designed B*Oligo (B*Oligo w/PolyT) to add a polyT region at both ends so that it can also hybridize to the Biotin PolyA to form a complex with more biotins to improve the detection sensitivity .



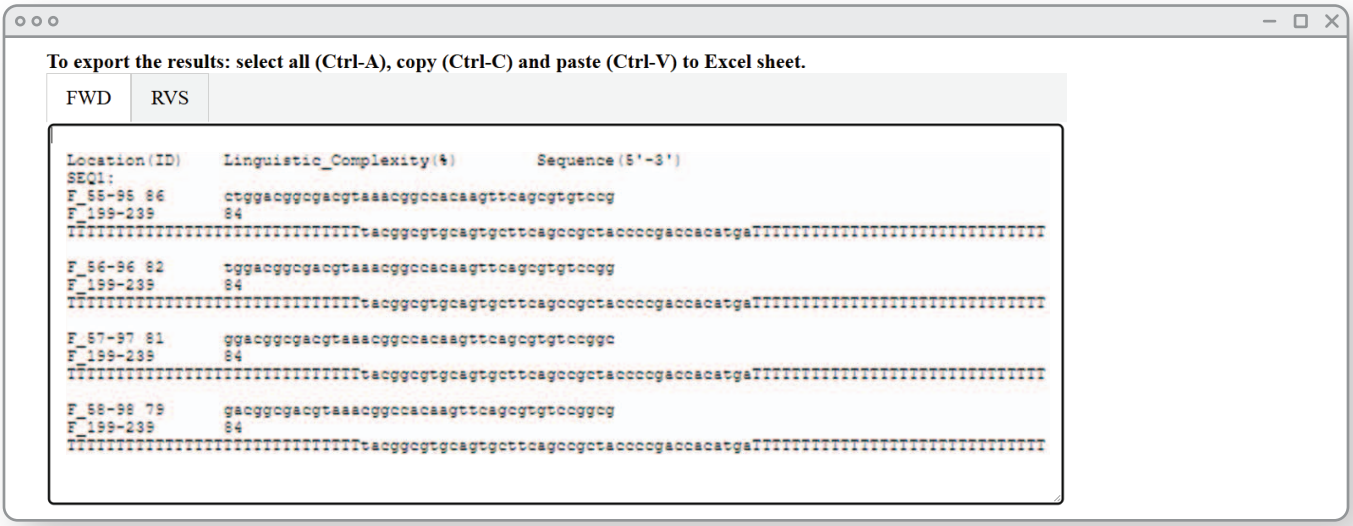
Oligo Design

Users need to design and prepare the F*Oligo and B*Oligo w/PolyT specific to their target genome. Please use the PrimerDigital tool (https://primerdigital.com/tools/gator2.html) and follow the instructions to design oligos.

1. In **Sequences Box**, copy and paste your DNA sequence.
2. In **Probe length**, enter the desired oligo length (eg. 40 nt). Minimum is 35 nt.
3. In **Probes distance**, enter the minimum gap distance between two oligos (eg. 10 nt).



4. Click **Create probes**. Different suggested oligo pairs will be displayed as shown below.



5. Choose one pair of either forward (FWD) or reverse (RVS) oligos for synthesis. Typically 5-10 nmol of oligo amount is sufficient.

6. Remember to add 5'-end labeled 5' 6-FAM modification for the F*Oligo.

7. Add both 5'- and 3'-end labeled biotin for the B*Oligo w/PolyT.

*Purification by HPLC or SDS-PAGE is recommended. If necessary, test one more pair of oligos for comparison so that you can choose to use the pair showing better signals.

Preparation

Crude AAV Samples

Always pretreat crude AAV samples with benzonase (or DNase) to remove interference from production plasmids. Incubate AAV samples with 50 U/mL of benzonase at 37°C for ≥30 min and then add 2 mM EDTA to inactivate benzonase.

AAV Standards

Purified AAV sample with a known titer (eg. 1E+12 vg/ml or above) determined by dPCR, ddPCR or qPCR can be used as a standard stock. For the best accuracy, AAV standards and unknowns should be prepared and/or diluted using the same and suitable matrix (eg. PBS w/0.01% F-68). Perform serial dilutions (eg. 3-fold) to prepare various standard concentrations (eg. 1E+12, 3.33E+11, 1.11E+11, 3.7E+10, 1.2E+10, and 0 vg/ml). For unknown samples, duplicates are recommended.

Oligo

i. Calculate and add TE Buffer to reconstitute your specific F*Oligo and B*Oligo w/PolyT to 40 µM stocks.

ii. Add 50 µL of TE Buffer into the vial of Biotin PolyA (kit provided) and mix to prepare 40 µM stock.

iii. Mix 1 µL of each oligo stock with 39 µL of TE Buffer to prepare 1 µM as the working concentration.

Assay Protocol (16 Assays)

Bring the required reagents to room temperature and set up a heat block at 95° C. The following scheme is for 16 assays using one 96-Well Black Plate. For higher throughputs, design your assays using 384-Well Plates (not provided) and/or using Gator Pro BLI instrument.

MAX Plate Preparation

1. In the MAX Plate, add 250 µL of Q Buffer into columns 1 and 2.
2. Place 16 anti-F biosensors into columns 1 and 2 and pre-wet them for 10 min.

Tested Sample Preparation

1. Add the following components in a 1.5 mL eppendorf tube to prepare the specific Oligo Pre-Mix.

   Note: 1 µM is a suggested concentration, and users can further optimize this concentration if necessary.

   - 1 µM F*Oligo, 20 µL
   - 1 µM B*Oligo w/PolyT, 20 µL
   - 1 µM Biotin PolyA, 40 µL

2. Add 320 µL of Hybridization Buffer into the tube. Make sure the Hybridization Buffer is clear at room temperature.
3. Vortex and centrifuge the tube briefly.
4. Use a single channel pipette to add 20 µL/well of the specific oligo mixture into two strips of Lysis Tubes.

5. Add 20µL of AAV standards or unknowns into the Lysis Tubes.

6. Heat the samples for 5 min in a heat block at 95° C. The samples may become cloudy.

7. After 5 min, briefly spin the samples and cool them down in an ice-bath for ≥2 min.

96-Well Black Plate Preparation

1. Pour some Q Buffer into a 25 mL reservoir. Use a 8-channel pipette to add 180 µL/well into the empty wells in columns 2, 4, 8 and 10 (Plate Map below). Pour back excess Q Buffer into the original bottle.

2. Mix 16 µL of Biotin Detection Reagent 200X with 3.2 mL of Q Buffer in a 5 mL tube to prepare the Biotin Detection Working Solution.

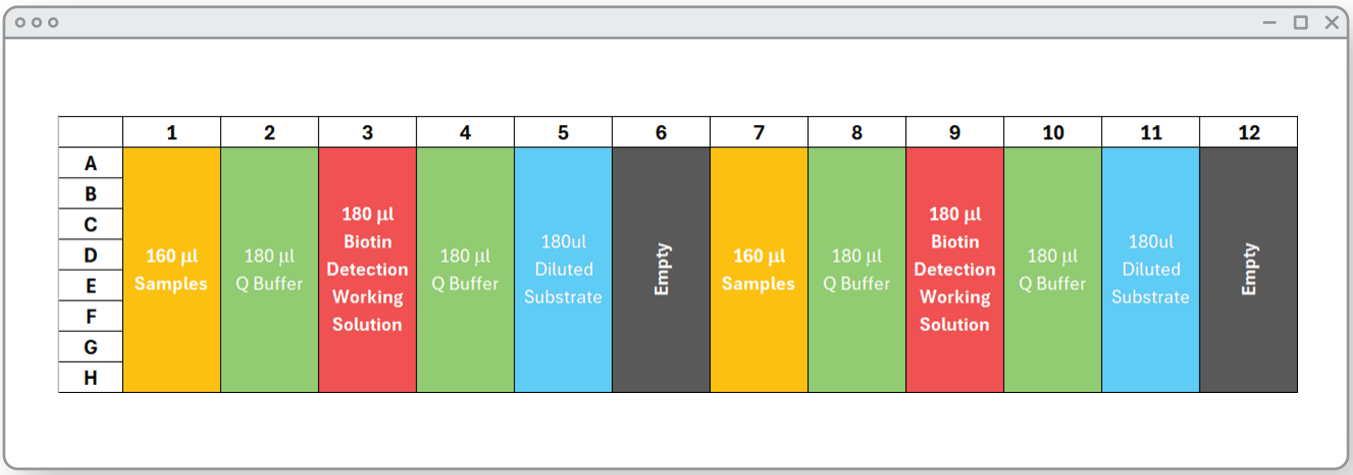
3. Pour the Biotin Detection Working Solution into a 25 mL reservoir. Use a 8-channel pipette to add 180 µL/well into the empty wells in columns 3 and 9. Do not reuse the solution after 1 day.

4. Mix 1.6 mL of GeneSwift Substrate and 1.6 mL of GeneSwift Diluent in a 5 mL tube to prepare the Diluted Substrate.

5. Pour the Diluted Substrate into a new 25 mL reservoir. Use a 8-channel pipette to add 180 µL/well into the wells in columns 5 and 11. Do not reuse the solution after 1 day.

6. Pour some Nuclease Free Water (or DI water) into a new 25 mL reservoir. Use a 8-channel pipette to add 160 µ L/tube into the first strip of Lysis Tubes. Pipette up and down carefully a few times to mix well and then directly transfer 160 µL / well of the samples into the empty wells in column 1.

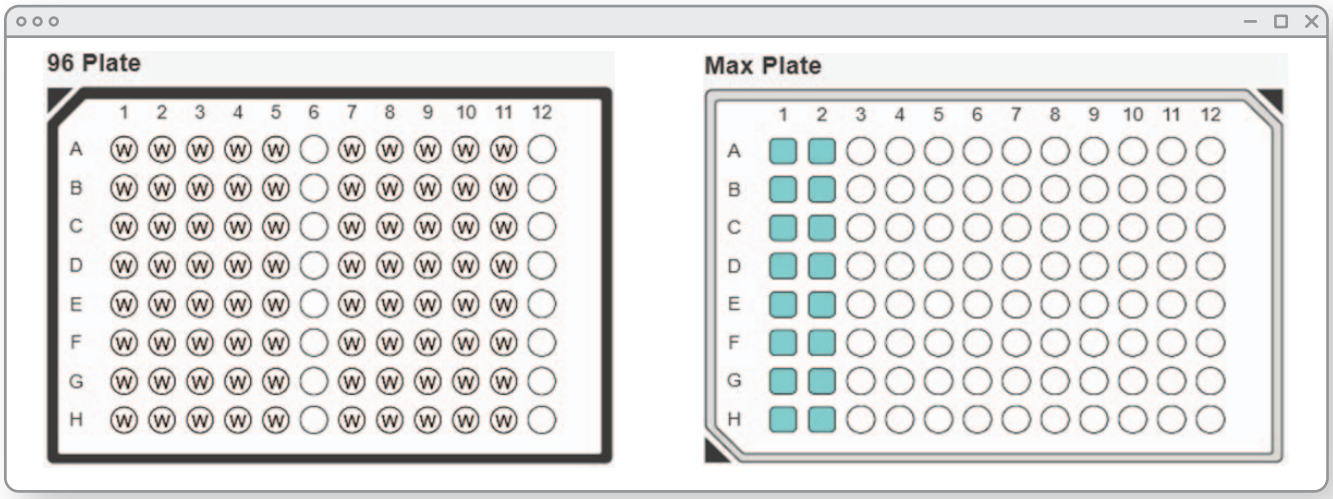
7. Repeat the same procedure (step 6) using new tips for the second strip of Lysis Tubes. Transfer 160 µL/well of the samples into the empty wells in column 7. The final plate components are shown below.



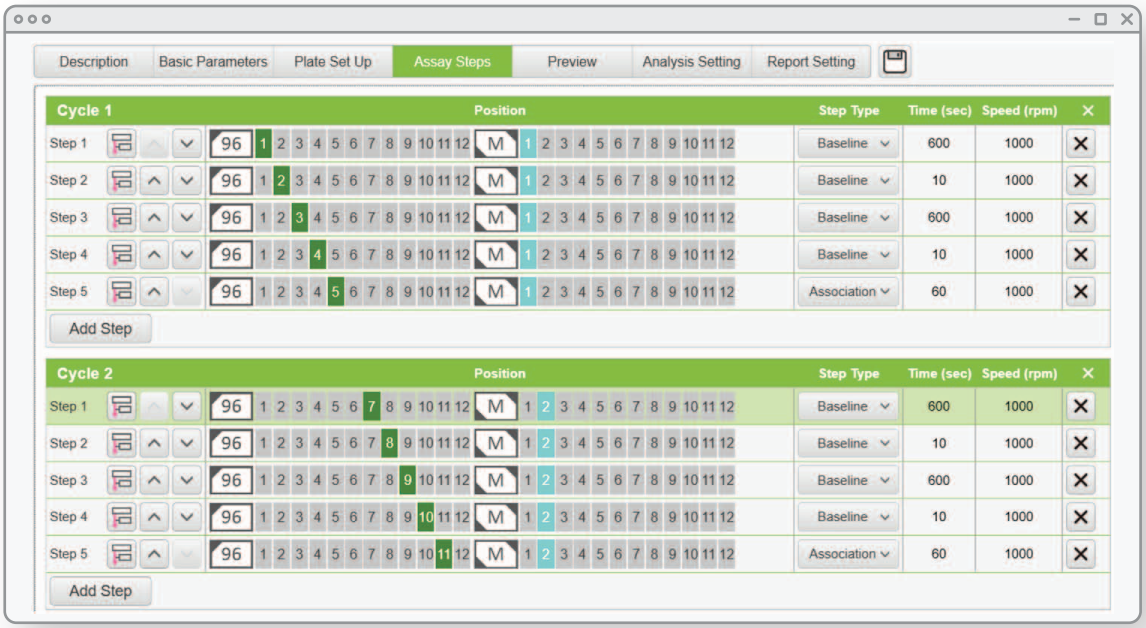
	1	2	3	4	5	6	7	8	9	10	11	12
A	160 µl Samples	180 µl Q Buffer	180 µl Biotin Detection Working Solution	180 µl Q Buffer	180ul Diluted Substrate	Empty	160 µl Samples	180 µl Q Buffer	180 µl Biotin Detection Working Solution	180 µl Q Buffer	180ul Diluted Substrate	Empty
B												
C												
D												
E												
F												
G												
H												

Instrument Setup on Gator® Prime or Plus (16 Assays)

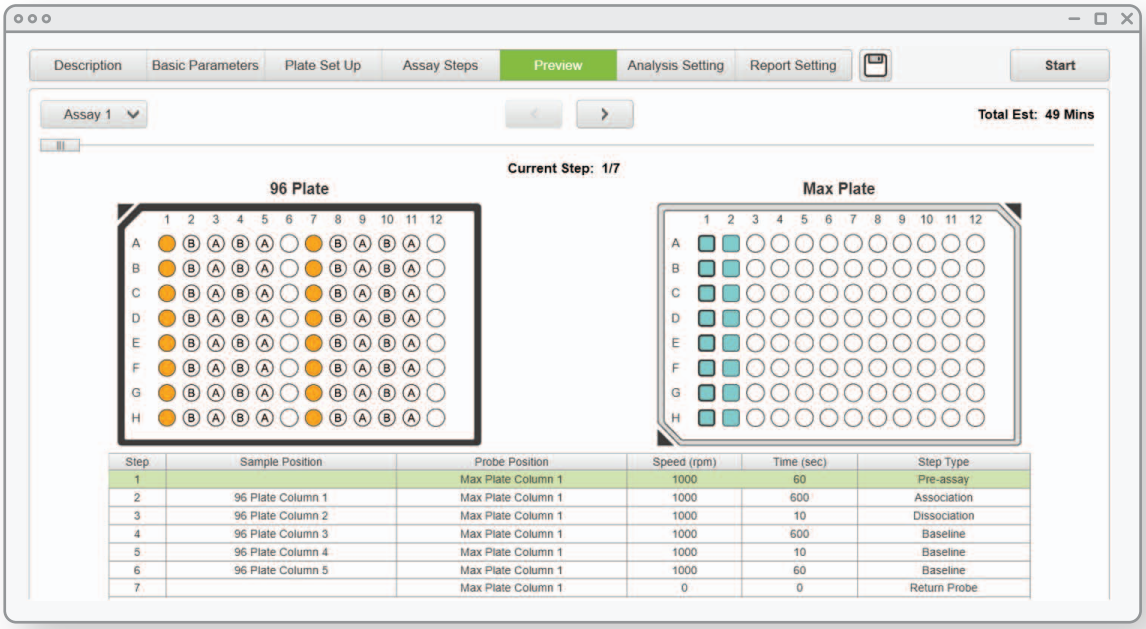
- Place the GeneSwift Ready Plate into Shaker A (Left) and MAX Plate into Shaker B (Right).
- Make sure the plate orientations are correct (row letters on left) and close the lid.
- Open the GatorOne software, click on Kinetics “K”.
- Go to **Description**, input the assay details and user’s name.
- Go to **Basic Parameters**, input following parameters:
  - Data Acquisition: 5 Hz
  - Plate Type: 96 Well Plate
  - Shaker Setting: Status Flat
  - Temperature: A & B at 30°C
  - Equilibration Settings: Time 60 sec, Shaker A and Shaker B Speed: 1000 RPM
- Go to **Plate Set Up**.
  - In the 96-Well Plate, highlight columns 1-5 and 7-11 and select the Wash icon.
  - In the MAX plate, highlight columns 1-2 and select Probe icon.



- Go to **Assay Steps.**
- Click **Add Step** to add up to 5 new steps in Cycle 1.
  - Highlight Positions 1-5 correspondingly for the Steps 1-5 in 96-Well Plate. Highlight Position 1 in MAX Plate.
  - Change the Step Type for Step 5 to Association.
  - From Step 1 to 5, set the time to 600, 10, 600, 10 and 60 sec respectively. Keep the Speed at 1000 rpm.
  - Right click Cycle 1 and select Copy cycle.
  - Then right click in the blank space after Cycle 1 and select Paste a cycle.
- In Cycle 2, highlight Positions 7-11 correspondingly for the Steps 1-5 in 96-Well plate and highlight Position 2 in MAX Plate. Other parameters are the same in Cycle 1 as shown below.



- Go to **Preview**, check the steps, positions, time and speed shown below. **Start**.



Data Analysis by GatorOne

1. Click **Results & Analysis**. Double click the desired **New K Analysis** to open the data file.

2. Go to **Set Reference**, select the last step on the sensorgram and then click **Quantitate Selected Step**. (Insert an image)

3. A Quantitation window will open up and click **Yes** in "Multiple assays detected. Do you want to include other assays?"

4. In **Sample ID,**

    i. Select the appropriate unit if applicable.

    ii. Select the standard wells as Standard. Right click to add sample information and input the known titer values (Insert an image).

    Note: For faster value input, highlight all standard wells, right click them and open **Sample Info editor**. Check the box in **Dilution Series**, enter the first Starting Value (eg. 1E+12). If the dilution factor is 3, enter 3 in **Operand**. Click **Confirm**.

    iii. In **Time Range**, keep the Lower Limit at 0 sec and enter 30 sec as the Upper Limit.

5. Go to **Binding Fitting** and click **Parameters**.

    i. In **Equation**, select **REquilibriumOptimal** and click **Confirm**.

    ii. Click **Binding Curve Fit**.

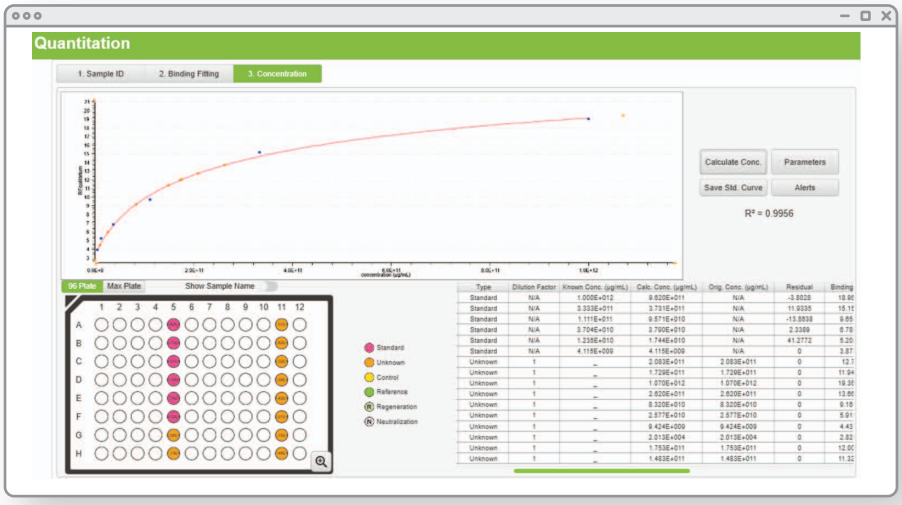
6. Go to **Concentration** and click **Parameters**.

    i. In **Fitting Model**, select an appropriate model such as FivePLRegression.

    ii. Click **Confirm** and then click **Calculate Conc**.

    iii. The Standard Curve will be generated, and the titers of the unknowns will be calculated and shown in the table shown below.

7. To export the data, **click** Save and save it in an appropriate location.



Common Issues and Troubleshooting Tips

Issue	Potential Cause	Troubleshooting
High background signal even without AAV capsids used	The designed oligos may have homodimer or heterodimer issue	• Select another pair of oligos identified by the software to test • Enter a new sequence region to design oligos
The calculated titer of the unknown sample differs from the expected value	AAV standards and unknowns are not prepared or diluted using the same matrix	• Use the same medium to prepare or dilute both AAV standards and samples • Try higher dilution factors (eg. 5-10 folds) of AAV samples to minimize the matrix effect
Inconsistent signal shifts	Non-homogeneous AAV samples or reagents	• AAV capsids sometimes can aggregate easily. Use 0.05% of Pluronic F-68 for dilution and vortex the sample • Mix solutions well before pipetting into the Black Plate wells
Minimal/no signal shift	Dead probe(s)	• Rerun the assay with fresh probes • Check to make sure the reagents used are correctly prepared and placed in the 96-well plates
The throughput of the assay is not high enough	The maximum number of assays using Gator® Prime or Plus is 8 samples/cycle at a time	• Prepare all standards and unknowns using the same reagents and conditions together and then test 8 samples/run for several runs. Standards do not need to be repeated in every run and can be applied to the determine the titer for unknowns in different runs • Use Gator Pivot to test up to 16 samples/run or Gator Pro to test up to 32 samples/run

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