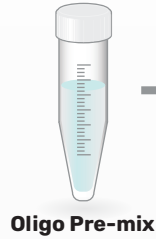# Gator® GeneSwift Kit

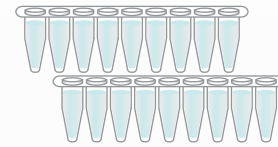## 16 Assay Format

Follow the protocol below for **GeneSwift Kits:** **GFP** 350007    **CMV Pr-En** 350008    **SV40** 350010

## Oligo Pre-mix

**Oligo Pre-mix**

20µl

**8-Strip Lysis Tubes X 2**

**Heat to 95°C**
5 minutes

**Cool to 4°C**
2 minutes

## AAV Standards

**1/3 dilution**
3E12 vg/ml

20µl

**AAV Sample**
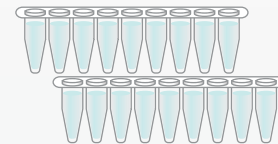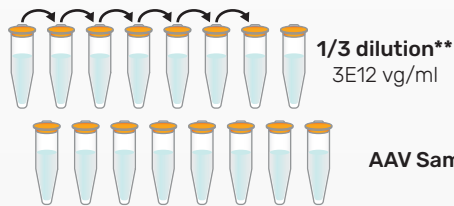
**Refer to detailed protocol document*

**Add water**
160µl / tube
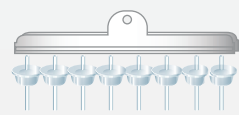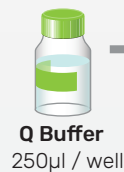
## Detection Reagent Working Solution

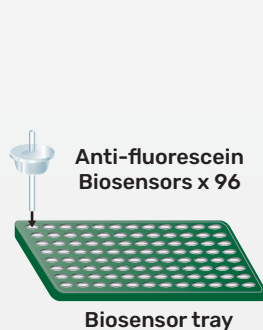**Q Buffer**
3.2 mL

+

**Biotin detection stock**
16 µl

**Biotin detection working solution**

## Max Plate Preparation

**Anti-fluorescein Biosensors x 96**

**Q Buffer**
250µl / well

**Transfer 16 biosensors to the Max Plate after Q buffer has been added to its wells**

**Max Plate®**

**Biosensor tray**
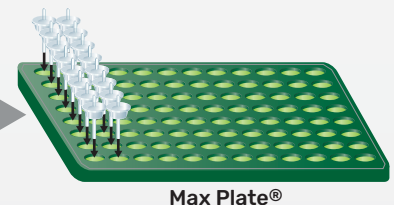
# Gator Supplied 96-well Ready Plate



**96 well ready plate**

Q Buffer • Substrate • Diluent
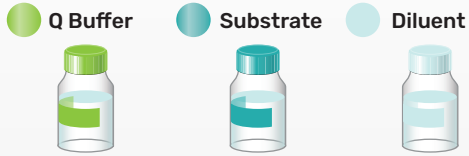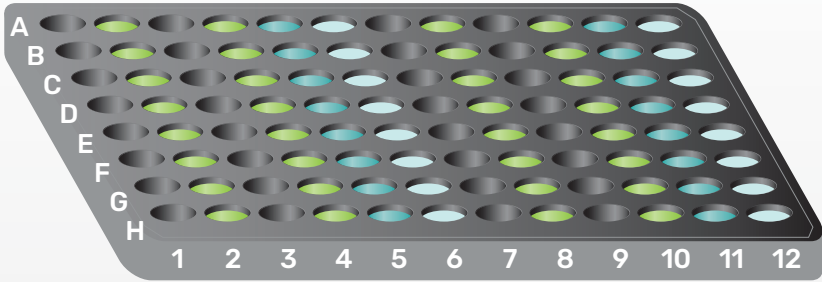
# Final Plate Map

Add standards / samples and biotin detection solution as shown in the illustration below



Load 180 μL of standard / sample

Load 180 μL Biotin detection solution

Q Buffer • Q Buffer

Transfer 100 μL • Substrate • Diluent

Load 180 μL of standard / sample

Load 180 μL Biotin detection solution

Q Buffer • Q Buffer

Transfer 100 μL • Substrate • Diluent

**96 well ready plate**

Q Buffer • Substrate • Diluent

Standard / Sample • Biotin detection solution
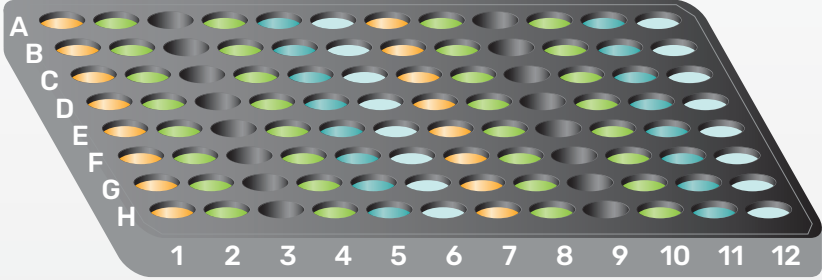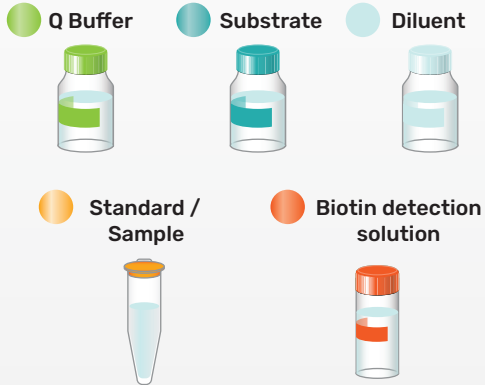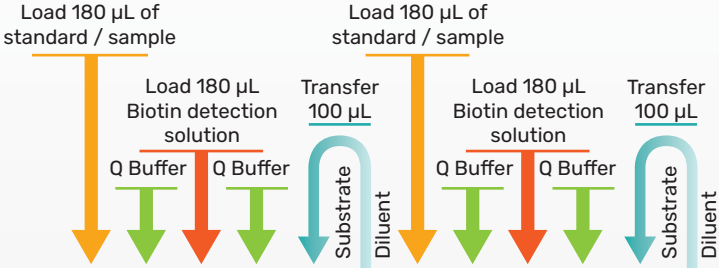
# Gator® GeneSwift Kit Protocol

## 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 dd PCR is often used to determine titer, this method is laborious, time-consuming, costly and requires extensive dilution series. Titer accuracy is significantly influenced by PCR inhibitors and various sample matrices. Gator Bio has developed a novel AAV genome titer assay that leverages DNA hybridization, immunochemistry and biolayer interferometry (BLI). This simple and fast assay is reliable method to determine the AAV titer with a dynamic range of 4E +9 – 3E+12 vg/mL for all AAV serotypes.

## Materials Provided with GeneSwift Kits, Part No: 350006, 350007, 350008, 350010

- o Anti-Fluorescein Biosensors, Gator Bio, Part No: 160045
- o B*PolyA Oligo, Gator Bio, Part No: 120078
- o Biotin Detection Reagent, Part No: 120077
- o Hybridization Buffer, Gator Bio, Part No: 120076
- o Q Buffer, Gator Bio, Part No: 120010
- o 96-Well Ready plate (x6), Gator Bio, Part No: 120094
- o 96-Well Reagent Black Plate Map
- o 96-Well MAX Plate (x1), Gator Bio, Part No: 130297
- o 8-Strip Tubes (x12), Gator Bio, Part No: 130134
- o Picker, Gator Bio, Part No. 130298

## Kit Specific Materials

- **Gator® GeneSwift Kit - GFP, Part No: 350007**,
  - o GFP Oligo Pre-mix, Gator Bio, Part No: 120089
  - o GFP Calibrator, Gator Bio, Part No: 130301

- **Gator® GeneSwift Kit - CMV-Pr-En, Part No: 350008**
  - o CMV Pr-En Oligo Pre-mix, Gator Bio, Part No: 120090
  - o CMV Pr-En Calibrator, Gator Bio, Part No: 130302

- **Gator® GeneSwift Kit - SV 40, Part No: 350010**
  - o SV 40 Oligo Pre-mix, Gator Bio, Part No: 120096
  - o SV 40 Calibrator, Gator Bio, Part No: 130305

## User Supplied Materials

- AAV capsids
- PBS and TE Buffer
- 8-channel and single channel pipettes and tips
- Vortex Mixer
- Centrifuge for spinning tubes and plates

- Heating block
- Ice with ice bucket or refrigerator
- DNase and EDTA for AAV sample pretreatment

## Storage

- **Anti-**Fluorescein **Biosensors**, Hybridization Buffer, B*PolyA (lyo) and Calibrator (lyo) are stable at room temperature (RT) for 1 year. After reconstitution, B*PolyA (lyo) and Calibrator (lyo) should be stored at -20°C and stable for 6 months.  Anti-Fluorescein Biosensors should be stored in a sealed pouch**.**
- **Genome Titer Kit Reagent Set** – 96-Well Ready plates (sealed) are stable at 4° C for 6 months. Q Buffer is stable at 4° C for 1 year.
- GFP/CMV Oligo Pre-mix and Biotin Detection Stock should be aliquoted and kept at -20°C after shipment arrival and are stable for 6 months.

## Dynamic Range

4E +9 – 3E+12 vg/mL for all AAV serotypes.

## Workflow

AAV capsids are first mixed with gene specific oligos and lysed by heating in Hybridization Buffer. After lysis, the mixture is cooled down to allow the fluorescein labelled and biotinylated oligos to hybridize with gene of interest (GoI). This hybridized complex is then fished out by binding to the anti-fluorescein (anti-F) biosensor. After washing, the biotin molecule of the hybridized complex is detected by using a Biotin Detection Solution and the signal is then measured by a Gator BLI instrument. Since the sensitivity of the assay can be boosted by increasing the number of biotins in the hybridized complex, we recommend a poly T region at both ends of the biotinylated oligo (B*Oligo w/PolyT) during oligo design so that this oligo can also hybridize to the kit's biotinlylated Poly A Oligo (B*PolyA) to form a complex with more biotins.
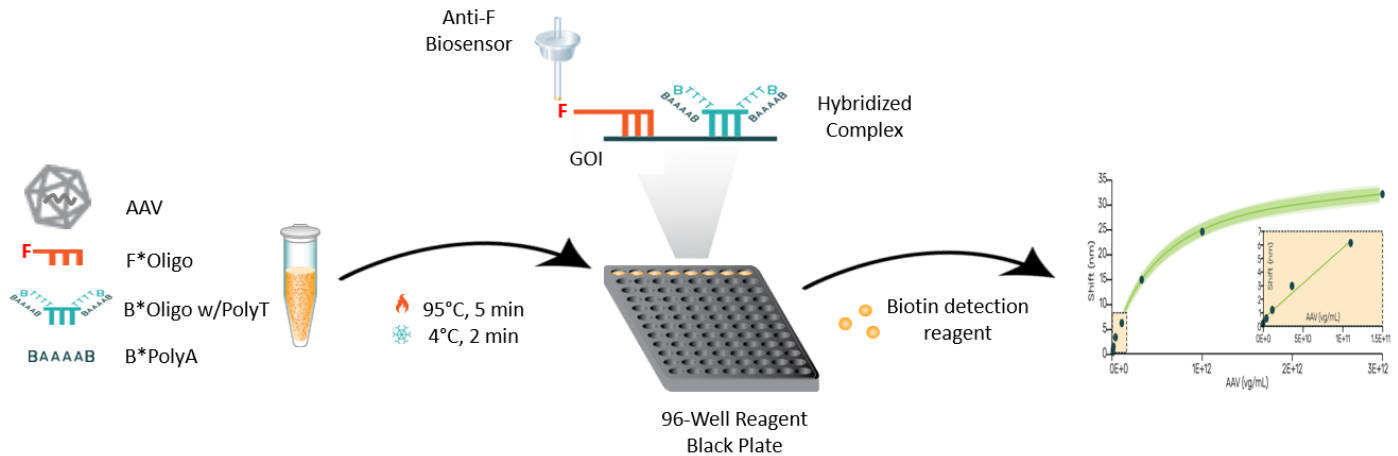


## Oligo Design

Customers are responsible for designing and preparing the F* and B* oligos specific to their target genome. Please use the PrimerDigital tool on Gator Bio website (Gatortool - Gator Bio) and follow the steps below to design the oligos.

1. In the Sequences Box, copy and paste your desired DNA sequence.
2. In the Probe length, enter the oligo length (eg. 35-40 nt).
3. In the Probes distance, enter the gap distance (eg. 10 nt) between F* and B* oligos you want.
4. Press "Create probes".
5. Different oligo pairs will be shown. You can choose either forward (FWD) and reverse (RVS) oligo pairs.

6. When placing an order with any DNA synthesis provider, remember to add 5'-end labeled 5' 6-FAM modification for the F*Oligo. Add both 5'- and 3'-end labeled biotinylation for the B*Oligo. Oligo purification by HPLC or SDS-PAGE is recommended.
7. We recommend ordering at least two different pairs and comparing them to determine the oligo pair that gives better signal. Signals above 5 nm for a titer of 1E+12 vg/mL will give the best results.

**Type or paste (Ctrl-V) sequence(s) here in FASTA or two column format.**

Clear

>EGFP

Cttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgaccccgcccattgac
gtcaataatgacgtatgttcccatagtaacgCcaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgc

Probe length (35-40 nt): 35

Probes distance (>5 nt): 10

Create probes

**To export the results: select all (Ctrl-A), copy (Ctrl-C) and paste (Ctrl-V) to Excel sheet.**

| FWD | RVS |
| --- | --- |

```
Location(ID)     Sequence(5'-3')
EGFP:
F_71-106    tacggtaaatggcccgcctggctgaccgcccaacg
F_128-163   TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTaatgacgtatgttcccatagtaacgccaatagggaTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

F_72-107    acggtaaatggcccgcctggctgaccgcccaacga
F_128-163   TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTaatgacgtatgttcccatagtaacgccaatagggaTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

F_73-108    cggtaaatggcccgcctggctgaccgcccaacgac
F_128-163   TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTaatgacgtatgttcccatagtaacgccaatagggaTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

F_74-109    ggtaaatggcccgcctggctgaccgcccaacgacc
F_128-163   TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTaatgacgtatgttcccatagtaacgccaatagggaTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
```

## Crude AAV Pretreatment

When analyzing crude AAV samples, it is important to pretreat the crude samples with benzonase/DNase to remove the interference from production plasmids. Incubate the samples with 50 U/mL of benzonase/DNase at 37°C for 30 min and then add 2 mM EDTA to inactivate benzonase/DNase. To determine the titer accurately, AAV standards and unknowns should be prepared and diluted using the same matrix or buffer.

## Oligo, AAV and Standard Preparation

1. For specific GoI, use TE Buffer to reconstitute the F*oligo and B*oligo w/PolyT to 40 µM stocks.

   Add 15 µL of TE to reconstitute the kit's B*polyA oligo to 40 µM stock. Then dilute them in TE buffer to 0.8 µM as the recommended working concentration. When running the GFP/ CMV Pr-En/ SV 40 assay, you can skip this step and directly use GFP/CMV Pr-En/ SV 40 Oligo Pre-mix instead.

2. Use your AAV standard (eg. 1E+12 vg/ml) to prepare the standards at different concentrations. Initial dilution can be done by performing 3-fold serial dilutions for 4 times to generate 1E+12, 3.33E+11, 1.11E+11, 3.7E+10, 1.2E+10, and 0 vg/ml. For unknown samples, duplicates are recommended.
3. If no AAV is available, you may use the CMV/GFP Calibrator (a short dsDNA CMV/GFP gene fragment) from the kit as a standard if your sample's genome has the CMV/GFP gene. Reconstitute the Calibrator by adding 400 μL of TE (or nuclease free water) into the vial to prepare a Calibrator Stock (approximately 1E13 vg/ml). Then dilute the Calibrator Stock by performing 3-fold serial dilutions for 4 times to generate 1E13, 3.33E12, 1.11E12, 3.7E+11, 1.2E11, and 0 vg/ml. Note that the Calibrator is a synthesized DNA and its signals may be different from the signals generated by ssDNA from AAV.

## Assay Protocol (16 Assays)

Bring the required reagents to room temperature and set up the heat block to 95°C. Before doing the assay, estimate the quantity of materials needed for the number of assays to be performed. The following scheme is for 16 assays using one 96-Well Ready plate.

1. Mix the following components in a 1.5 mL Eppendorf tube. Note that 0.8 μM is a suggested concentration, and users can further optimize the oligo concentration if necessary.
   - 0.8 μM F*Oligo, 20 μL
   - 0.8 μM B*Oligo, 20 μL
   - 0.8 μM B*PolyA Oligo , 40 μL
2. Add 320 μL of Hybridization Buffer into the tube to prepare the Oligo Mix.
   Note - If you are using PN 350007, 350008, or 350010, the skip the above steps and directly use 20 μL of the Oligo Pre-mix instead.
3. Vortex and centrifuge the tube briefly.
4. Use a single channel pipette to add 20 μL of the Oligo Mix into 8-Strip Tubes.
5. Add 20 μL of various diluted standards and unknowns into the 8-Strip Tubes.
6. Heat the samples at 95°C for 5 min in a heat block.
7. After 5 min, cool down the tubes at 4°C (eg. ice-bath) for 2 min, or at RT for 5 min.
8. After cooling, use a single-channel or 8-channel pipette to add 160 μL of water into each tube.
9. In the MAX plate, add 250 μL of Q Buffer into columns 1-2 for hydration of biosensors.
10. Place 16 anti-F biosensors into columns 1 and 2 (see plate map below) and pre-wet them briefly for 2-10 min.

| MAX | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-----|---|---|---|---|---|---|---|---|---|----|----|----|
| A | Anti-F biosensors in 250 mL/well of Q Buffer | Anti-F biosensors in 250 mL/well of Q Buffer | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

11. Centrifuge a 96-Well Ready plate at 2000g for 15 sec to make sure all solutions inside are in the bottom of the plate.
12. Peel off the aluminum foil carefully. The top of the plate wells may look flattened due to sealing but it will not affect the assay performance.

13. Place the plate on the top of a 96-Well Ready Plate Map to align them. The plate well components are shown below.

| 96 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | Q Buffer | | Q Buffer | Substrate | Diluent | | Q Buffer | | Q Buffer | Substrate | Diluent |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

14. Prepare biotin detection reagent working solution by mixing 16 μL of Biotin Detection Stock with 3.2 mL of Q Buffer.
15. Add 180 μL of the Biotin Detection Solution into the wells in columns 3 and 9.
16. Next use an 8-channel pipette to mix Substrate and Diluent together by transferring 100 μL of Diluent (wells in columns 6 and 12) into the Substrate wells (columns 5 and 11) respectively.
17. Finally, use an 8-channel pipette to add 180 μL of sample from 8-Strip Tubes into the wells in columns 1 and 7.
18. The final plate components are shown below.

| 96 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | 180 μL/ well of Biotin Detection Solution) | | | | | | 180 μL/ well of Biotin Detection Solution) | | | |
| D | 180 μL/ well of Sample) | Q Buffer | | Q Buffer | Diluted Substrate | | 180 μL/ well of Sample) | Q Buffer | | Q Buffer | Diluted Substrate | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

## Gator Instrument Setup

Assay Setup on Gator® Prime or 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."
5. Under the Description tab, input the description details and user's name.
6. Under the Basic Parameters tab, input the following parameters:
   - Data Acquisition: 5 Hz
   - Plate Type: 96 Well Plate
   - Shaker Setting: Status Flat, Temperature: A & B at 25°C
   - Equilibration Settings: Time 60 sec, Shaker A and Shaker B: 1000 RPM
7. Under Plate Set Up tab,
   - 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.
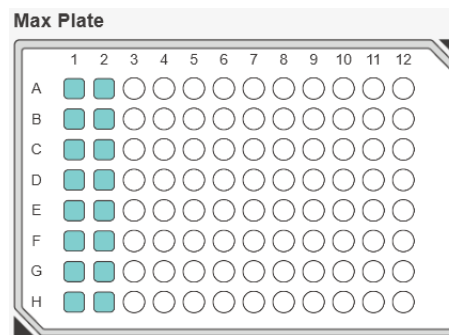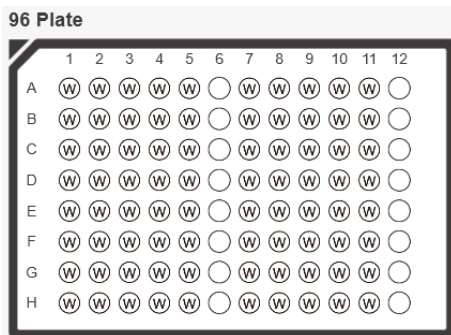
96 Plate / Max Plate

8. Under the Assay Steps tab,
   - Use the Add Step tab to add 5 new steps for Cycle 1.
   - Click "Add Cycle" and add 5 new steps again.
   - In Cycle 1, highlight Positions 1-5 for the Steps 1-5 in 96-Well plate and highlight Position 1 in the Max plate.
   - Change the Step Type for Step 5 to Association.
   - In these 5 steps, set the corresponding time to 600, 10, 600, 10 and 120 sec respectively. Keep the Speed at 1000 rpm.
   - In Cycle 2, highlight Positions 7-11 for the Steps 1-5 in 96-Well plate and highlight Position 2 in the Max plate. Other parameters are the same in Cycle 1. The correct setting is shown below.



Under the Preview tab, check to confirm the steps, positions, time and RPM. Note that the warning "Dissociation step is missing in Cycle 1" can be ignored.

| Step | Sample Position | Probe Position | Speed (rpm) | Time (sec) | Step Type |
|---|---|---|---|---|---|
| 1 | | Max Plate Column 1 | 0 | 0 | Pick up Probe |
| 2 | 96 Plate Column 1 | Max Plate Column 1 | 1000 | 600 | Baseline |
| 3 | 96 Plate Column 2 | Max Plate Column 1 | 1000 | 10 | Baseline |
| 4 | 96 Plate Column 3 | Max Plate Column 1 | 1000 | 600 | Baseline |
| 5 | 96 Plate Column 4 | Max Plate Column 1 | 1000 | 10 | Baseline |
| 6 | 96 Plate Column 5 | Max Plate Column 1 | 1000 | 120 | Association |
| 7 | | Max Plate Column 1 | 0 | 0 | Return Probe |

## Data Analysis by GatorOne

1. Open the correct data file from "Results & Analysis". Under "New K Analysis", go to Set Reference.
2. Click to select the last step on the sensogram and click on "Quantitate Selected Step".
3. A Quantitation window will open up
   a. Under "Sample ID",
      i. Select the appropriate unit if applicable.
      ii. Identify the sample wells as Standard or Unknown.
      iii. For the Standards, enter the titer values.
      iv. Select the time range, select 0 sec as the Lower Limit and 120 sec as the Upper Limit.
   b. Go to Binding Fitting and click on "Parameters"
      i. Under "Equations" select "ResponseAverage0" and click "Confirm".
      ii. Click "Binding Curve Fit".
   c. Go to "Concentration" and click "Parameters". A "Parameter" window will pop up.
      i. Go to "Fitting Model" window and select the appropriate model such as FourPLRegression. If FourPLRegression does not show the best fitting, try to use FivePLRegression..
      ii. Click "Confirm" and then press "Calculate Conc."
      iii. The Standard Curve will be generated, and the titer of the unknown will be calculated and shown in the generated table as shown below.



4. To export the data, click the button "Save" and save it using the appropriate file name and 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 PrimerDigital Olio tool.<br>• Enter a new sequence region to design oligos, |
| The calculated titer of the unknown sample differs from the expected value. | AAV standards and unknown samples are not prepared or diluted using the same matrix, | • Use the same medium to prepare both AAV standards and samples<br>• 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,<br>• Mix solutions well before pipetting into the Black Plate wells, |
| Minimal/no signal shift. | Dead probe(s), | • Rerun the assay with fresh biosensors.<br>• Check to make sure the reagents used are correctly prepared and placed in the 96-well plates, |