

Gator[®] AAV Ratio Kit

Scope

This document provides a detailed protocol for performing empty vs full determination of adeno-associated virus (AAV) capsids using the Gator® AAV Ratio Kit and GatorOne software. It also includes common issues and troubleshooting tips.

Introduction

Gene therapy has the potential to treat a host of diseases from cancer to neurodegenerative disorders. Disease caused by mutated or defective genes can be treated by therapeutic genes that are packaged and delivered to target cells via viral vectors such as the adeno-associated virus (AAV).

As vectors, AAVs are non-integrating and non-immunogenic, which reduces the risk for insertional mutagenesis in the host genome or an immune response. Thus, AAV vectors are one of the most investigated vehicles for gene delivery.

The production of AAV vectors has challenges. The viral particles/capsids are a mix of fully loaded and less ideally, partially loaded and empty capsids. AAV production requires accurate, precise, and high throughput determination of capsid titer and empty versus full (E/F) content ratio. These features ensure enhanced efficacy, the safety of AAV gene therapy products, and good quality control to promote the desired therapeutic effect.

Gator[®] AAV Ratio Kit along with biolayer interferometry (BLI) technology determines empty (E) versus full (F) content without relying on additional techniques to obtain data on different AAV critical quality attributes (CQA).

Materials Provided

- Gator[®] AAV Ratio Kit, Part No: 350004
 - o Gator® AAVX Probes, Gator Bio, Part No: 160017
 - Gator[®] SSB Probes, Gator Bio, Part No: 160026
 - o Gator[®] Lysis Buffer, Gator Bio, Part No: 120064
 - Gator[®] DNA Detection Solution, Gator Bio, Part No: 120065
 - o Gator® AAV Ratio Substrate, Gator Bio, Part No: 120061
 - Gator[®] Q Buffer, Gator Bio, Part No: 120062
 - Gator[®] SS DNA Standard, Part No: 120066
 - Lysis tubes, Part No: 130134

User Supplied Materials

- AAV-empty
- AAV-full with payload size same as that of the sample
- Gator[®] MAX Plate, Gator Bio, Part No: 130062
- Gator[®] BLI 96-Flat Plate, Gator Bio, Part No: 130260 (Case)
- Precision pipettes and tips
- Tweezer, Fisher Scientific, Cat No: 14955032
- 1x PBS
- Heating block, Fisher Scientific, Cat No: 88870001 or similar device.



Storage

- **AAVX probes** room temperature (RT), stable for 1 year in the sealed pouch.
- **AAV Ratio Kit Reagent Set** (all components except DNA Detection Solution) stable at 4° C for 6 months in the unopened reagent package. After dilution, keep on ice and use within the same day.
- **DNA Detection Solution** at 4°C stable for 3 months and at -20°C for longer-term. After dilution keep on ice and use within the same day.

AAV Ratio Kit Protocol

E/F AAV Workflow for Processing 1-8 Samples in One Batch

The complete workflow of E/F AAV ratio determination consists of 3 steps: I) AAV Capture, II) AAV Lysis, and III) ssDNA Detection. AAV capsids are first captured and measured by using Gator[®] AAVX biosensors. The captured AAV capsids are then lysed to release the ssDNA and quantitated using the ssDNA using DNA probe.

Crude sample pre-treatment: When analyzing crude lysates, it is important that the lysate solution is treated with Benzonase/DNAse. This is to avoid nonspecific binding of the free DNA in the lysate to the capture probe. Add 50 U/mL Benzonase/DNase to the lysate containing AAV capsid solution and incubate for at least 30 minutes in a 37°C water bath.

I. AAV Capture

Bring the reagent set to RT before performing the assay.

Capture Preparation

1. Pipette 250 μL/well of Q Buffer into column 1 of a new Max Plate. Refer to the image below for Max Plate Layout for AAV Capture.

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D E F G H	Q Buffer 250µL + AAVX Probes											

- 2. Using a probe picker/tweezer, pick out fresh AAVX probes from the tray and place them into column 1 of the Max Plate. Pre-wet the AAVX probes for 15 minutes.
- 3. Pipette 200 $\mu L/well$ of Q Buffer into column 1 of a new Black Plate.

Calibrators Preparation

- 1. Calculate the volume and mix different amounts of empty and full AAV capsids in PBS to prepare the AAV standards with various fixed E/F ratios (e.g., 0%, 20%, 40%, 60%, and 80%).
- 2. It is recommended that samples are prepared at the same concentration as calibrators.
- 3. Pipette 200 µL/well of the AAV standards/test sample into column 2 of the Black Plate. Refer to the image below for Black Plate Layout for AAV Capture.



	1	2	3	4	5	6	7	8	9	10	11	12
A B C D E F G H	Q Buffer 200µL	Standards/ sample 200µL	Additional assay									

Assay Setup on Gator[®] Prime or Plus Instrument for AAV Capture

- 1. Open the lid of the instrument.
- 2. Place the Black Plate into Shaker A and the Max Plate into Shaker B.
- 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: 60 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 Buffer icon.
 - Highlight column 2 on the 96-well plate and select the Sample icon.
 - Assign each well to its position with the name of the corresponding sample/solution. Refer to the image below.





96 Pl	ate Max	Plate		Simulat	e µg/mL ~	nM ~
Index	Position	Sample Name	Туре	Conc. (µg/mL)	MW (kD)	M Conc. (nl
1	A1		Buffer	_	_	_
2	B1		Buffer	_	_	_
3	C1		Buffer	_	_	_
4	D1		Buffer	_	_	_
5	E1		Buffer	_	_	_
6	F1		Buffer	_	_	_
7	G1		Buffer	_	_	_
8	H1		Buffer	_	_	_
9	A2		Sample	_	_	_
10	B2		Sample	_	_	_
11	C2		Sample	_	_	_
12	D2		Sample	_	_	_
13	E2		Sample	_	_	_
14	F2		Sample	_	_	_
15	G2		Sample	_	_	_
16	H2		Sample	_	_	_

- 8. Under Plate Set Up, set up the Max Plate map.
 - Highlight column 1 on the Max Plate and select "Probe". Refer to image below.



- 9. Under the Assay Steps tab, set up the experimental cycle(s) as follows:
 - 60 sec for wash step
 - 300-3600 sec for sample step. Capture should be stopped when the binding signal reaches 5-6 nm signal shift.

Note: Refer to the image below to set up the step positions for Cycle 1.

Cycle	1	Position	Step Type	Time (sec)	Speed (rpm)	×
Step 1		96 1 2 3 4 5 6 7 8 9 10 11 12 M 1 2 3 4 5 6 7 8 9 10 11 12	Baseline 🗸	60	1000	×
Step 2		96 1 2 3 4 5 6 7 8 9 10 11 12 M 1 2 3 4 5 6 7 8 9 10 11 12	Association ~	2400	1000	×
Add	Step					

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



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II. AAV Lysis

- 1. Add 200 µL of Lysis Buffer into 8 Lysis tubes each.
- 2. After the AAV capture is completed, use the tweezer to manually transfer and dip the AAVX probes into the Lysis tubes containing 200 µL of Lysis Buffer.
- 3. Transfer the Lysis tubes into a heating block pre-set at 95°C and heat the samples for 5 minutes to lyse the capsids and release the ssDNA.
- 4. After 5 minutes, remove the Lysis tubes and let them cool to RT for 5 minutes.

III. ssDNA Detection

- 1. Allow the DNA Detection Solution and Q Buffer to reach RT before performing the assay.
- 2. Prepare the working solution of DNA Detection Solution by diluting 12.5 µL of the stock solution into 1.24 mL of Q buffer. Vortex for 5 seconds. This amount is enough for 8 samples

Reagent Preparation for the Max Plate

- 1. Pipette 250 µL/well of Q Buffer into columns 1-4 of a new Max Plate.
- 2. Pipette 250 µL/well of 1X PBS into columns 5-6 of the Max Plate.
- Pipette 250 µL/well of Q Buffer into column 7. Pick out fresh DNA probes from the tray and place them into column 7 and pre-wet the probes for 2 minutes. Refer to the image below for Max Plate Layout for ssDNA Detection.

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D E F G H	Q Buffer 250µL	Q Buffer 250µL	Q Buffer 250µL	Q Buffer 250µL	PBS Buffer 250µL	PBS Buffer 250µL	Q Buffer 250µL + DNA Probes					



Reagent Preparation for the Black Plate

- 1. Pipette all the lysed samples from the Lysis tubes into column 1 of a new Black Plate.
- 2. Pipette 150 μL/well of the DNA Detection Solution prepared in Q buffer into column 2. Pipette 200 μL/well of the Substrate into column 3. Refer to the image below for Black Plate Layout for ssDNA Detection.

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D E F G H	Standard /samples 200µL	DNA detection solution 150µL	Substrate 200µL		Additional assay			Additional assay			Additional assay	

- 3. In the ssDNA Detection process, program the GatorOne software to transfer and dip the SSB probes into the wells containing the standards and sample. Incubate the probes in the lysed sample for 15 minutes, followed by 3 washes. Next, incubate the probes in DNA Detection Solution for 5 minutes, followed by 3 washes. Lastly, incubate the probes in substrate for 5 minutes.
- 4. Measure the wavelength shift signal of the standards and sample (refer to the section: Assay Setup on Gator® Prime or Plus Instrument).

Assay Setup on Gator[®] Prime or Plus Instrument for ssDNA Detection

- 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 and 3 on the 96-well plate and select the Load icon.
 - Assign each well to 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-well plate description box.

96 Pl	ate Max	Plate		Simulat	e µg/mL ∽	nM v
Index	Position	Sample Name	Туре	Conc. (µg/mL)	MW (kD)	M Conc. (nl
1	A1		Sample	_	_	_
2	B1		Sample	_	_	_
3	C1		Sample	_	_	_
4	D1		Sample	_	_	_
5	E1		Sample	_	_	_
6	F1		Sample	_	_	_
7	G1		Sample	_	_	_
8	H1		Sample	_	_	_
9	A2		Load	_	_	_
10	B2		Load	-	_	_
11	C2		Load	-	-	_
12	D2		Load	_	_	_
13	E2		Load	_	_	_
14	F2		Load	_	_	_
15	G2		Load	-	_	_
16	H2		Load	_	_	_
17	A3		Load	_	_	_
18	B3		Load	_	_	_
19	C3		Load	_	_	_
20	D3		Load	_	_	_
21	E3		Load	_	_	_
22	F3		Load	_	_	_
23	G3		Load	_	_	-
24	H3		Load	_	_	_

- 8. Under Plate Set Up, set up the Max Plate map.
 - Highlight columns 1-4 on the Max Plate and select "Buffer".
 - Highlight columns 5-6 and select "Wash".
 - Highlight column 7 and select "Probe". Refer to image below.





- 9. Under the Assay Steps tab, set up the experimental cycle(s) as follows:
 - 900 sec for the sample step
 - 10 sec for each wash step
 - 300 sec for the DNA Detection Solution step
 - 300 sec for the Substrate step

Note: Refer to the image below to set up the step positions for Cycle 1.

EvF AAV	' Capture	×	EvF	DNA D	etec	tic	×				2	•						•	•	•			•••									
Des	cription	Ba	isic P	aramete	ers	1	Plat	e Se	t Up)		Assa	iy Ste	eps			Pre	viev	v		An	alys	sis Set	tting	Repo	ort Se	etting		0			
																															~	
Сус	le 1													Pos	ition											s	tep Ту	ре	Time (sec)	Speed (rpm)	X	
Step 1	F	^	~	96	1	2	3 4	5	6	78	9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		L	oading	, v	900	1000	X	
Step 2		^	~	96	1	2	3 4	5	6	78	3 9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		в	aseline	e ~	10	1000	X	
Step 3		^	~	96	1	2	3 4	5	6	78	3 9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		В	aseline	e ~	10	1000	×]
Step 4		^	~	96	1	2	3 4	5	6	78	9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		в	aseline	e ~	10	1000	×	J
Step 5		^	~	96	1	2	3 4	5	6	78	9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		L	oading	, v	300	1000	×	J
Step 6		^	~	96	1	2	3 4	5	6	78	9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		В	aseline	e 🗸	10	1000	X	J
Step 7		^	~	96	1	2	3 4	5	6	78	3 9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		в	aseline	e 🗸	10	1000	×	
Step 8		^	~	96	1	2	3 4	5	6	78	9	10 1	1 12	N	1	1 2	3	4	5	6	8	9	10 11	12		в	aseline	e ~	10	1000	×	
Step 9		^		96	1	2	3 4	5	6	78	3 9	10 1	1 12	N	1	1 2	3	4	5	6 7	8	9	10 11	12		As	sociati	on 🗸	300	1000	×	ļ
Ad	ld Step																															





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

vF DNA Detecti	c X					
Description	Basic Parameters Plate Set U	p Assay Steps	Preview	Analysis Setting	Report Setting	Start
Assay 1	 ✓ Warning: Dissociation step is n 	issing in Cycle 1	<			Total est: 35 M
			Current Step: 1/1	1		
_	96 Plate				Max Pla	ate
	A E E C C C B E E C C C C E E C C C D E E C C C F E E C C C G E E C C C H E E C C C			A 6 6 B 6 6 C 6 6 D 6 6 F 6 6 F 6 6 H 6 6		
9,	Step Sample Position	Prot	e Position	Speed (rpm)	Time (sec)	Step Type
	1	Max Pl	ate Column 7	1000	300	Pre-assay
	2 96 Plate Column 1	Max Pi	ate Column 7	1000	900	Loading
	4 Max Plate Column 1	Max Pl May Di	ate Column 7 ate Column 7	1000	10	Baseline
	5 Max Plate Column 3	Max Pi	ate Column 7	1000	10	Baseline
	6 96 Plate Column 2	Max Pi	ate Column 7	1000	300	Loading
	7 Max Plate Column 4	Max Pl	ate Column 7	1000	10	Baseline
	8 Max Plate Column 5	Max Pl	ate Column 7	1000	10	Baseline
	9 Max Plate Column 6	Max Pl	ate Column 7	1000	10	Baseline
	10 96 Plate Column 3	Max Pl	ate Column 7	1000	300	Association
	11	Max Pl	ate Column 7	0	0	Return Probe

11. The assay will be completed in 31 minutes. After the assay is completed, the required data analysis can be performed.

Data Analysis Using MS Excel

The amount of ssDNA released should be proportional to the % full ratio of the standards or test sample. A standard curve is required to determine the E/F ratio of the test sample. To set up a standard curve, the AAV Capture signal of the standards and test sample is first compared to generate normalization ratios. Then the ssDNA shift signal of the standards and sample is multiplied by the normalization ratios to yield the normalized signals. The normalized signals on the y-axis are then plotted versus the known E/F ratio of the standards on the x-axis. The E/F ratio of the standard curve (refer to the Excel Calculation Template for E/F AAV Ratio Determination). The template will be provided during installation of the assay by the Field Application Scientist. Refer to the image below for an example.

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4	Standard		AAVX	Capture			ssDNA Detect	ion	_		16						
5	Sample	Full (%)	Detected Shift	Net Shift	Normalization	Detected Shift	Normalized Shift	Calculated Full (%)			14			Ŷ	R ² = 0.9974	1506	
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11	6	0.00	0.3481	0.35	0.00	15463	0.00	0		lized				and and the second			
12	7	0.00	0.00	0.00	0.00	0.00	0.00	0		ST I	6						
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14												S					
15											2						
16	Unknown		AAVX	Capture			ssDNA Detect	ion			0						
17	Sample	Sample ID	Detected Shift	Net Shift	Normalization	Detected Shift	Normalized Shift	Calculated Full (%)			0	20	40	6	0	80	100
18	1	1	7.4/19	141	1.00	15.528/	15.55	98.54561834						Full (%)			
20		2	8.5050	8.57	0.87	A 342A	6.90	20.1900/055									
21	4	4	9 5046	9.50	0.70	24444	210	14 38370132	-								
22	5	5	9.5153	9.52	0.79	2.1234	167	1122622323	-								
23	6	6	0.3481	0.35	21.46	15463	33.19	243.2939548	-	-							
24	7	7	0.00	0.00	#DIV/0I	0.00	#DIV/0I	#DIV/0I									
25	8	8	0.00	0.00	#DIV/0	0.00	#DIV/0I	#DIV/0	1								
26																	
27																	
28										_							
4	▶ Sta	ndard AAV Capt	ture Report	Standard DN/	A Capture Report	Unknown AAV C	apture Report	Unknown DNA Capture Re	port		Summar	+				-	

Data Analysis Using GatorOne Software

Capture File

- 1. Open the Capture File form K result.
- 2. Under "New K Analysis", go to Set Reference



- 3. Click to select the loading step on the sensogram and double click on "Quantitate Selected Step".
- 4. A Quantitation window will open up
 - a. On the Quantitation window click "Sample ID".
 - i. Change the unit from mass/volume to "%".
 - ii. Enter the sample name if not already entered. In case of standards, enter the E/F ratio (%)



- b. Go to Binding Fitting and click on "Parameters". A Parameters window will open up.
 - i. Under "Equations" select "ResponseAverage0" and click "Confirm"
 - ii. Click "Binding Curve Fit".



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- c. Go to "Concentration" and click "Normalization E/F". A Normalization window will open up.
 - i. Either "multi"-select or click certain well to assign Normalization denominator.
 - ii. Check the box "Selected well to normalize samples".
 - iii. Click "Normalize".



5. Click Save as csv file to be imported to calculate standard curve.

Re	sult	New K Analysis 1 🔍	Report				
ntitation	🗢 Save As				×		
1. Sample ID	$\leftarrow \rightarrow ~~ \wedge$	Ben Data 102722b > 221027 E&F Captu	ire 10-27-2022 13-15-48 > Report >	ン C Search Report	م :		
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Ratio File

- 1. Open Ratio file from K result.
- 2. Under "New K Analysis", go to Set Reference
- 3. Click to select the loading step on the sensogram and double click on "Quantitate Selected Step".
- 4. A Quantitation window will open up
 - a. Under "Sample ID", change the unit from mass/volume to "%"
 - i. Enter the sample information including % E/F ratio. This information should match that on the Capture file.
 - 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 "E/F Ratio". An "E/F Ratio" window will open up.
 - i. Go to "Normalization Factor" window
 - ii. Click "Load" and find the pre-saved normalization csv file
 - iii. Click "Open"
 - iv. Click "Confirm" to normalize the corresponding data set

Note: GatorOne will determine if the sample information in the Capture and Ratio files match one another, if not, the GatorOne will not allow user to load the normalization file.

221027 E&F Capture 1 Result	0-27-202: X 221027 E&F DNA (exp New K Analysis 1 V	et 2) 10-27 X Report	100 C. C. P	
Quantitation	/E Pation			
	ter Conc 0.0001 BR FILING MOdel FILING MODEL	Image: constraint of the second sec	To have a set of the s	Prote

5. Click on "Cal. E/F Ratio". Ratio has been determined and file can be saved

Result New K Ana	ysis 1 🗸	Report		
antitation				
1. Sample ID 2. Binding Fitting 3. Concentration				
				12
			Cal. E.F. Ratio Parameters	8
			Save Std. Curve Alerts	ŏ
			R ^a = 0.9929	O Probe
			Normalization E/F E/F Ratio	Ref. Probe
				8
M Pare Max Pare Show Sample Name	•	Plate Position Sample Name Type Dilution Factor 96 87 75% full Standard NA	ElF ratio (%) Galo, ElF ratio (%) Orig, 15 El Alda	ŏ
A 000000000000000000000000000000000000		96 C7 50% full Standard NA 96 D7 25% full Standard NA 99 E7 5% full Standard NA	50 55.85 25 20.467	
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Notes

Plate Set Up

- Ensure 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.

Common Issues and Troubleshooting Tips

Issue	Potential Cause	Troubleshooting
Inconsistent signal shifts	Nonhomogeneous reagents	 Mix the AAV stock solutions gently but thoroughly for at least 30 seconds Mix solutions well before pipetting 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 1:1000-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
Negative signal shift	Using only Q Buffer for washes	• Make sure the last two wash steps are in PBS
Standard curve showing a plateau at the high end instead of being linear	% full ratio close to 100% or large payload size	• Cut down the AAV capture time by half

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