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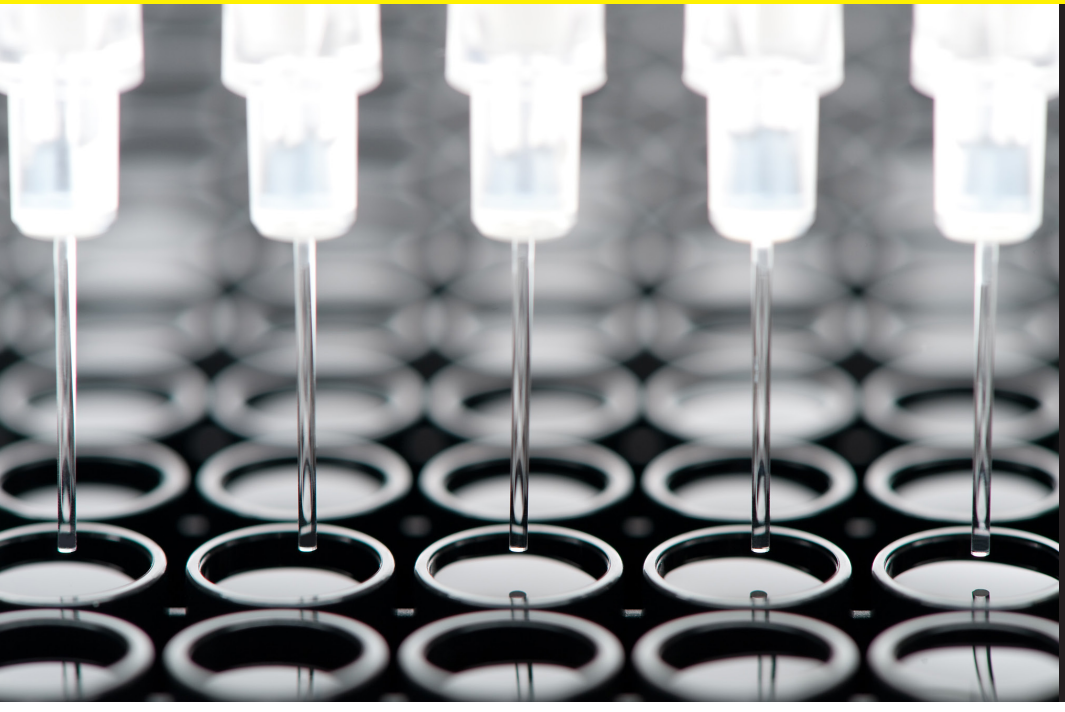
AAV Empty/Full Ratio Assessment Using the Octet® AAVX Biosensors



Technical Note

Scope
A rapid, high-throughput assay for analyzing the ratio of empty to full Adeno-Associated Virus (AAV) capsids using Octet® AAVX biosensors during bioprocess development for gene therapy.

Introduction

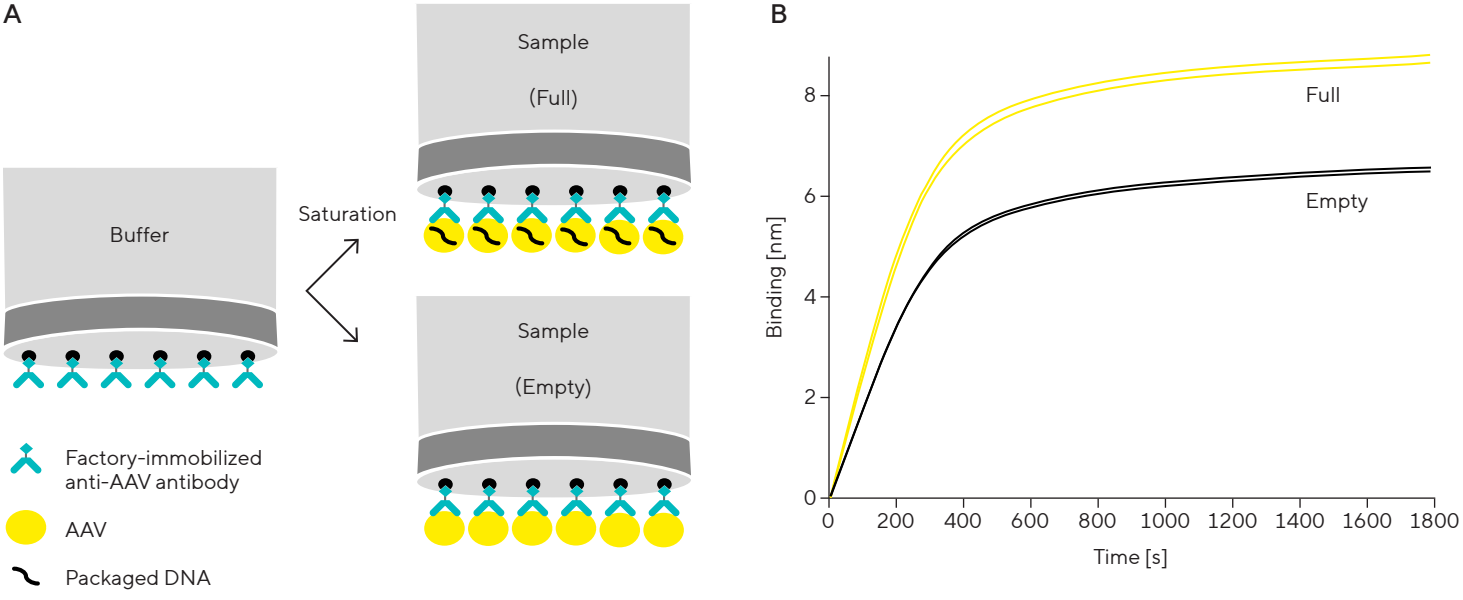
Adeno-associated virus (AAV) is a leading modality for in vivo gene delivery. Full capsids containing the gene cargo deliver therapeutics to target cells. However, the presence of empty capsids in the AAV drug product can compromise therapeutic efficacy; hence, these need to be detected at different stages of the AAV production cycle. Conventional methods for determining Empty/Full (E/F) ratio, such as analytical ultracentrifugation (AUC), electron microscopy (EM) and chromatography (IEX, SEC) and emerging technologies such as mass photometry or UV-Vis/light scattering have limitations that include lack of throughput, time constraints or work only with purified samples. Methods that measure released viral DNA and total capsids such as dPCR/ELISA require extensive optimization. Therefore, a method that reliably measures crude AAV samples with high throughput is urgently needed.

This Technical Note introduces a method for a rapid, high-throughput E/F ratio determination on the Octet® BLI platform using the Octet® AAVX Biosensors. The method is particularly suited for screening both crude and purified AAV samples in the upstream and downstream bioprocessing stages of AAV development and manufacturing. It analyzes intact viral capsids thereby avoiding the challenges associated with releasing and measuring viral DNA. This method relies on the high precision and consistency of the Octet® AAVX biosensors and it involves the saturation of the biosensor surface with equal amount of AAV particles, effectively normalizing capsid titer between samples. As a result, only one measurement is used to determine E/F ratio, eliminating compounded error — a limitation intrinsic to other methods that rely on accurate determination of both capsid titer and DNA content (two-measurement approaches). While this method can provide absolute quantitation with high precision, under certain circumstances where the signal window may be too small, it is better suited for screening purposes (see the section "Factors that Determine Signal Window and Assay Precision" below).

Principles of AAV E/F Ratio Detection

Octet® Bio-Layer Interferometry (BLI) deciphers light interference between reflections from the molecular layer and an internal reference layer of the biosensor. Analyte binding to biosensor surface changes the thickness and density of the molecular layer, which in turn alters light reflected off it and the interference pattern that ensues. Capitalizing on the fact that empty and full capsids have nearly identical size and protein composition, the Octet® AAV E/F assay saturates the biosensor surface with sufficiently high concentrations of AAV (≥ 2E11 vp/mL) to normalize the thickness factor between samples. As a result, BLI signal difference is solely a result of density difference between bound AAV particles, with samples with a higher percentage of full capsids generating higher signal (Figure 1). A standard curve can be generated from mixtures of Full and Empty reference materials and used to determine the E/F ratio of unknown samples in a typical Octet® BLI dip-and-read assay.

Figure 1: *Schematic of the Octet® AAV E/F Assay Workflow*



Note. (A) the AAVX biosensor is dipped into an AAV sample (Empty or Full). The unique aspect of the Octet® AAV EF assay is that AAV binding is allowed to reach saturation, at which point the amount of bound AAV is equal between samples, and the signal difference between a test sample and the matching Empty reference material can be attributed solely to density (in turn, E/F ratio) differences. (B) Example of binding response signal of Full (yellow) and Empty capsids (black) normalized to identical capsid titer (N = 2).

Materials Required

- Octet® BLI instrument with Octet® BLI Discovery and Analysis Studio Software, version 13.0.1 or newer

- Octet® AAVX Biosensors (Sartorius Part No. 18-5160)
  - Note: for best results, use the same lot of sensors within the same experiment

- For all Octet® instruments:
  - 96-well, black, flat bottom microplate, Greiner Bio-One Part No. 655209 (Minimum volume: 200 µL)

- Optional for Octet® RH16 and RH96 instruments:
  - 384-well, black, flat bottom, polypropylene microplate, Greiner Bio-One Part No. 781209 (Minimum volume: 80 µL)
  - Octet® 384 Well Tilted bottom Plate, Sartorius Part No. 18-5166 (pack); 18-5167 (case) (Minimum volume: 40 µL)

- Assay Buffer
  - Octet® Sample Diluent (Sartorius Part No. 18-1104) (recommended to dilute purified samples such as AAV reference materials)
  - Custom Assay Buffer (matches the sample matrix as closely as possible, recommended to dilute crude or in-process samples)

- Regeneration solution (10 mM Glycine, pH 1.7, Sartorius Part No. 18-1184)

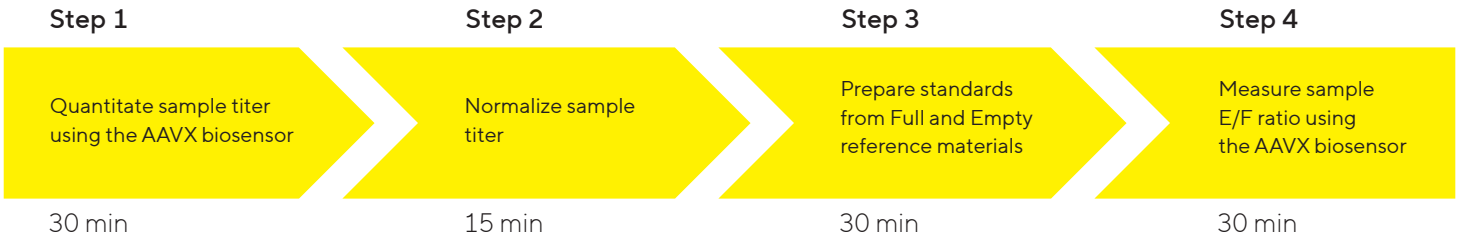
- Neutralization solution (same as the Assay Buffer of choice)

- AAV Reference Materials to make calibration standards:
  - Capsid titer of the two paired reference materials (Full vs. Empty) needs to be accurately determined by validated methods using Octet® AAVX Biosensors or ELISA.
  - The E/F ratio of the reference materials should be pre-determined by an orthogonal method. There is no minimal requirement of the %Full level of reference materials, so long as they provide a test window to accommodate the expected %Full range of unknown samples.
  - The reference materials should be of the same serotype, contain the same or similarly sized genome (± 0.5 kb), and be produced from the same host cell line as the unknown samples.
  - Empty and Full AAV reference materials of various serotypes are readily available from third-party vendors, commonly with GFP as insert. It is recommended to purchase products with pre-determined capsid titer and E/F ratio. For other insert sizes, users are encouraged to use internally produced AAV as reference.

* Data showcased in this Tech Note were generated using reference materials from Progen (AAV2, 5, 8 produced in HEK293 cells) and Virovek (AAV2, 5, 8 produced in Sf9 cells).

- AAV Samples:
  - Capsid titer requirement to approach binding saturation under 30 minutes: ≥ 2E11 vp/mL for AAV5 or AAV8, ≥ 5E11 vp/mL for AAV2. Lower titer can be used but longer assay time should be expected.
  - Due to high binding variability, this assay is not recommended for AAV9.
  - For serotypes not listed here such as AAV1, AAV3, AAV4, AAV6, AAV7 and AAVrh10, the user is encouraged to determine optimal assay conditions with reference materials.

Assay Steps and Workflow



Step 1: Quantitate Sample Titer Using the Octet® AAVX Biosensor

1. Choose one of the two AAV reference materials (Full or Empty) as the titer standard. The reference material of choice should be close to the expected % Full levels of the samples. For example, if the samples are expected to be 10% Full, the Empty reference should be used as titer standard; if the samples are expected to be 70% Full, the Full reference should be used.
2. Use the AAVX biosensors to determine titer of the unknown samples. Refer to the Technical Note: Octet® AAVX Biosensors for Quantitation of AAV Capsids (1).

Step 2: Normalize Sample Titer

1. Normalize unknown samples to the lowest capsid titer of the set (higher than the minimum titer requirement as outlined in the "Materials Required" section: ≥ 2E11 vp/mL for AAV5 or AAV8, and ≥ 5E11 vp/mL for AAV2). Unknown samples are best diluted in the same buffer that they are already in (referred to as "Custom assay buffer"). An example is provided in the Appendix.
2. Dilute Empty reference material to the same capsid titer as the normalized unknown samples using the same assay buffer. This material is to be analyzed simultaneously with the rest of the samples and to be used as reference for subtraction (referred hereinafter as Matching Reference, see Designing the Assay for more details).

Step 3: Prepare Standards from Full and Empty Reference Materials

The Octet® AAV EF Calculator App (requires Windows Operating System, version 10 or above) can be used to generate mixing scheme of standards with different %Full ratio using the steps below. Note that E/F ratio standards are created by taking two main steps: 1) normalizing capsid titer of the Full and Empty reference materials by diluting each stock in the same diluent, 2) mixing titer-normalized Full and Empty reference sample dilutions at volumes calculated by the EF Calculator App to make user-specified standards.

1. In Parameters tab, enter sample ID, pre-determined percent full value (%) and sample titer (vp/mL) for both Full and Empty reference materials (Figure 2A). Click Next (>>>) to proceed to the Standards tab.
2. In Standards tab, first define the desired normalized titer of standards (in vp/mL), the desired number of standards to construct standard curve, and the desired volume per standard (Figure 2B). Note that the normalized titer should be equal to or above the minimum titer requirement for the particular serotype (see Materials Required).
3. Next, enter desired %Full values at each standard level in Column 1 of the table below, which needs to be a number in-between the %Full value of Full and Empty references as defined in the previous tab (Figure 2B). The mixing volume of titer-normalized Full and Empty reference samples to form each standard (Column 2 and 3, respectively) and the total volume needed for each titer-normalized reference material (second table below) will automatically populate based on user inputs.

4. To prepare reference sample dilutions, enter "Volume to Prepare" based on the calculated "Total Volume Needed" from the step above, taking void volume into consideration according to specific user needs. Based on this, the required volume of reference material stock and diluent will be calculated.
5. To display and save all parameters and mixing scheme for the standards, click Next (>>>) to move to the Results tab. Right click inside the table to copy the %Full values of standards and paste into Octet® BLI Discovery Software under Plate Definition setup (inside the Concentration column of wells corresponding to the standards, see Assay Settings). To save or print the entire window, click Copy to take a screenshot.

Note: the capsid titer of standards does not need to match unknown samples, but each set (standards or unknowns) should maintain constant titer within themselves, each including a Matching Reference for subtraction purpose. This provides the user with flexibility to use a single saved standard curve to calibrate multiple batches of samples whose titer, buffer and experimental date are different, given that 1) the same Empty reference is used to make Matching References for both standards and unknown samples, and 2) assay settings are kept constant between experiments.

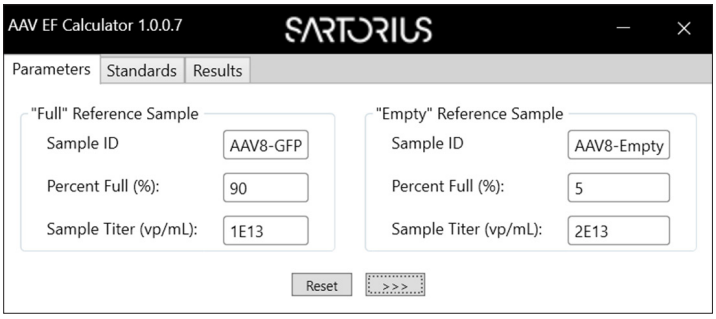
Step 4: Measure Sample E/F Ratio Using the AAVX Biosensor

Designing the Assay

One critical aspect of the assay is reference subtraction, in which the binding signal of a Matching Reference is subtracted from the rest of the samples. The subtraction step not only amplifies the relative signal change upon changes in %Full, but more importantly, it serves to normalize such variables as sample titer, buffer matrix, incubation time and experiment day. To properly implement reference subtraction, ensure that enough Empty reference wells are reserved to pair with each sample condition AND each sample read step. It is helpful to adopt the concept of "paired real-time reference subtraction" in assay design, requiring the Empty reference well used for subtraction (subtrahend) matches the samples for which it is to be subtracted from (minuend) as closely as possible in both composition (titer, matrix, volume, etc.) and detection conditions (plate, sensor lot, instrument time). Two examples are given below.

Figure 2: *AAV EF Calculator Interface*

A



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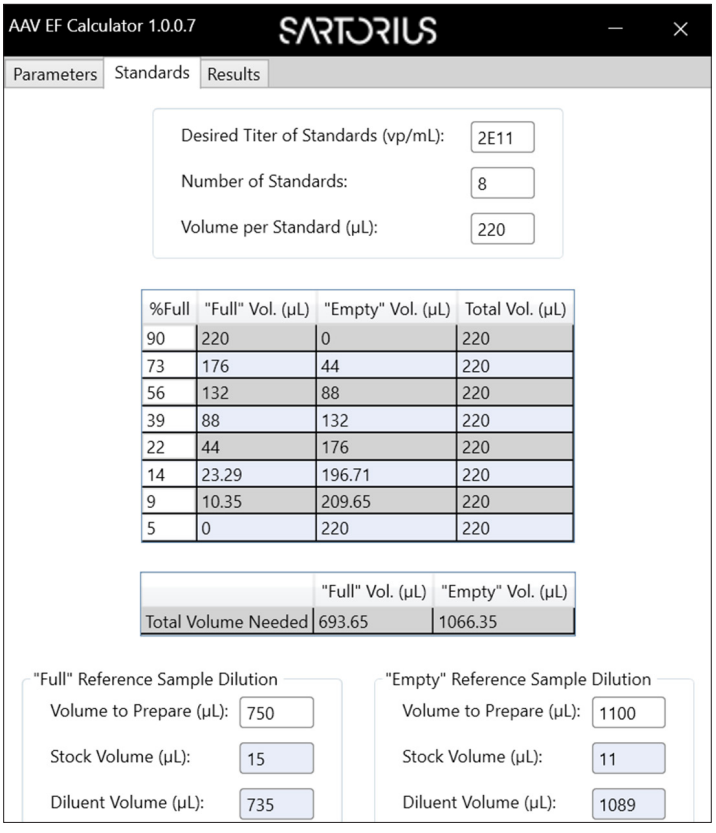


Example 1:
If standards are diluted in Sample Diluent to titer A and unknown samples are diluted in cell lysis buffer to titer B, Empty reference diluted in Sample Diluent to titer A should serve as Matching Reference to subtract the standards, while the same Empty reference diluted in cell lysis buffer to titer B should serve as Matching Reference to subtract the unknowns. This is the example applied in Appendix: EXAMPLE WORKFLOW TO DETERMINE E/F RATIO OF AAV8-GFP SAMPLES.

Example 2:
To process 56 samples on the Octet® R8 system, ensure that one of the 8 channels is always reading the Matching Reference during each sample read step. Therefore, instead of designing the assay with 7 read steps (each processing 8 samples), run 8 read steps (each processing 7 samples and one Matching Reference) (Figure 3). This practice eliminates volume (and in turn, concentration) differences between sample read steps due to evaporation, ensuring a fair comparison.

Assay Settings

- In the Octet® BLI Discovery software version 13.0.1 or higher, select appropriate method template by clicking Experiment → New Experiment Wizard → Advanced Quantitation → AAV Quantitation.
- Under the "Plate Definition" tab, enter Sample ID and Replicate Group for standards and unknown samples. Enter %Full values of the standards (from Octet® AAV EF Calculator App) into the respective Concentration fields. Any concentration unit can be used but the actual unit is %Full.
- Ensure that each sample read step contains at least one Matching Reference and change its Type to Reference. Two examples of the Plate Map and Assay Settings are shown in Figure 3 to perform the assay on the Octet® R8 system (A - without regeneration; B – with regeneration). Two additional examples are shown in Figure 8 to perform the assay in high-throughput Octet systems (A - RH16; B- RH96).
- For the baseline step in Assay Buffer, assay time should be set in the range of 60 – 180 s to allow sufficient equilibration.
  - Note: If standards and samples have different matrix, the corresponding assay buffers in baseline wells should also be different to eliminate buffer mismatch between baseline and sample read steps. If this is the case, click on "Modify" under the "Plate Definition" tab to open the "Assay Parameters" window, then navigate to the "Assay Parameters" tab. Change Step Options of Buffer from "Reuse position" to "Use once" so that each baseline well is used only for the corresponding sample reading step.
- For the sample read step, time to reach saturation depends on AAV serotype and concentration. The default assay time of 1800 s is generally sufficient for saturation at the minimum titer requirement (see Materials Required). However, given the complexity of AAV material and titer determination methods, longer or shorter assay time may be optimal and should be evaluated on a case-by-case basis. For example, if shorter assay time such as 15 minutes is desired to accommodate large number of AAV8 samples, the titer needs to be increased to 5E11 vp/mL.
- (Optional) To regenerate biosensors, click on "Modify" under the "Plate Definition" tab to open the "Assay Parameters" window, then navigate to the "Assay Parameters" tab. Change the default Regeneration time from 5 s to 20 s, and the number of regeneration cycles at the "Pre-conditioning sensors" step from 10 cycles to 3 cycles. See Figure 6.

Performing the Assay

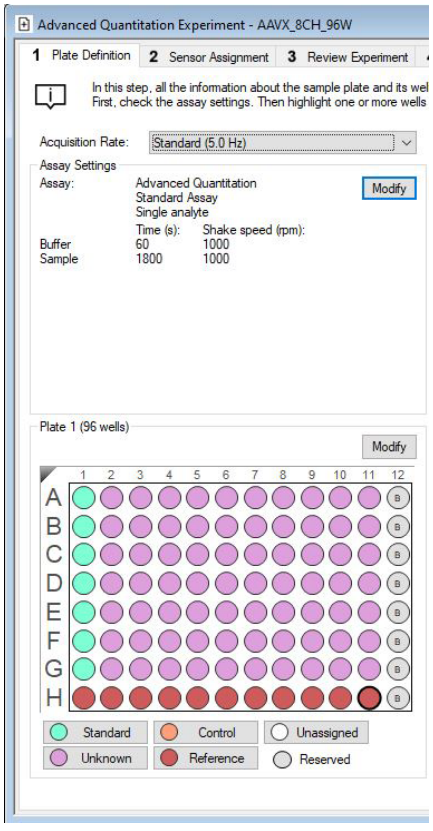
- Following the plate layout in the BLI Discovery Software, aliquot assay buffer, titer-normalized samples and | or standards from Assay Step 2 and 3 into corresponding plates.
- Aliquot regeneration and neutralization buffers to corresponding wells (if applicable).
- Confirm that the assay temperature is set to 30 °C under Experiment → Set Plate Temperature.
- Prepare a hydration tray in a 96-well plate by dispensing 200 µL of the matching assay buffer(s) in wells that match the locations of the biosensors and corresponding standard/sample wells in this assay.
- Place the hydration plate in the instrument with or without the plate holder (depending on instrument model). Place the green biosensor tip tray on top of hydration plate.
- Place the prepared sample plate(s) in the instrument.
- Set the delay to 10 minutes to allow the biosensors to hydrate and the samples to warm up to the assay temperature (30 °C).
- In the software select the location where the data should be stored. Click GO button to start the experiment.

Data Analysis

- Open the Octet® Analysis Studio Software version 13.0.1 or higher. Browse and load the data to be analyzed.
- In Preprocessed Data tab, select all wells corresponding to Matching Reference, and confirm or change its type to "Reference".
- Select all and only wells belonging to the same sample read step, of the same titer and share the same matrix, right click and select "Subtract Reference for Selected Wells → By Average".
  a. Note: the "By Average" command works even when only one reference well is present.
- Repeat the same for all other sample read steps/titers/matrices combinations (if applicable).
- In the Quantitation Analysis tab, select the appropriate Standard Curve Equation to use. Typically, "Linear" or "Dose Response – 4PL unweighted" is suitable.
- Select "End Point" for Binding Rate Equation and verify that saturation is reached at the default or user-specified data analysis read time.
- Once the parameters are set, the standard curve and unknown values will be tabulated automatically.
- (Optional) If using previously saved standards, click "Load Standard" and select the saved Standard Curve File (.fsc) and click Open.
- Click Export button to generate and save a Microsoft Excel report of the data.

Figure 3: *Two examples of Plate Map and Assay Setting on the Octet® R8 Instrument*

A



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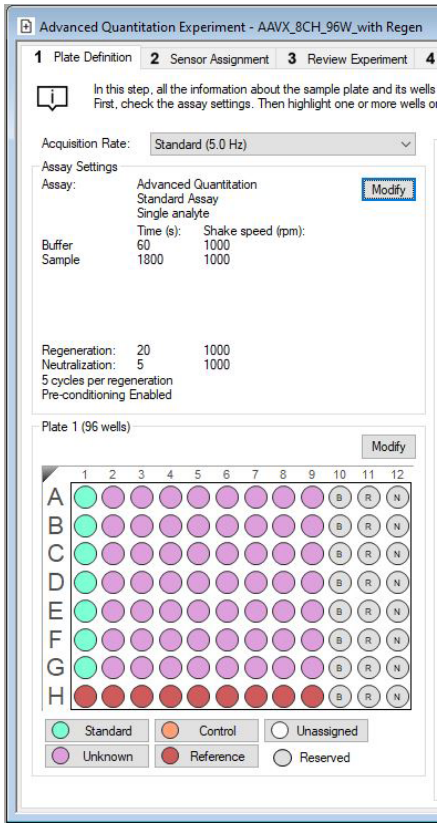


Note. (A) Plate layout in R8 instrument without regeneration of biosensors. (B) With regeneration. Cyan: standards; Pink: samples; Red: matching reference wells. Assuming each sample read step consists of 8 wells (one column), each reference well is matched to the standards or samples within the same column because they share the same sample read step.

Representative Data

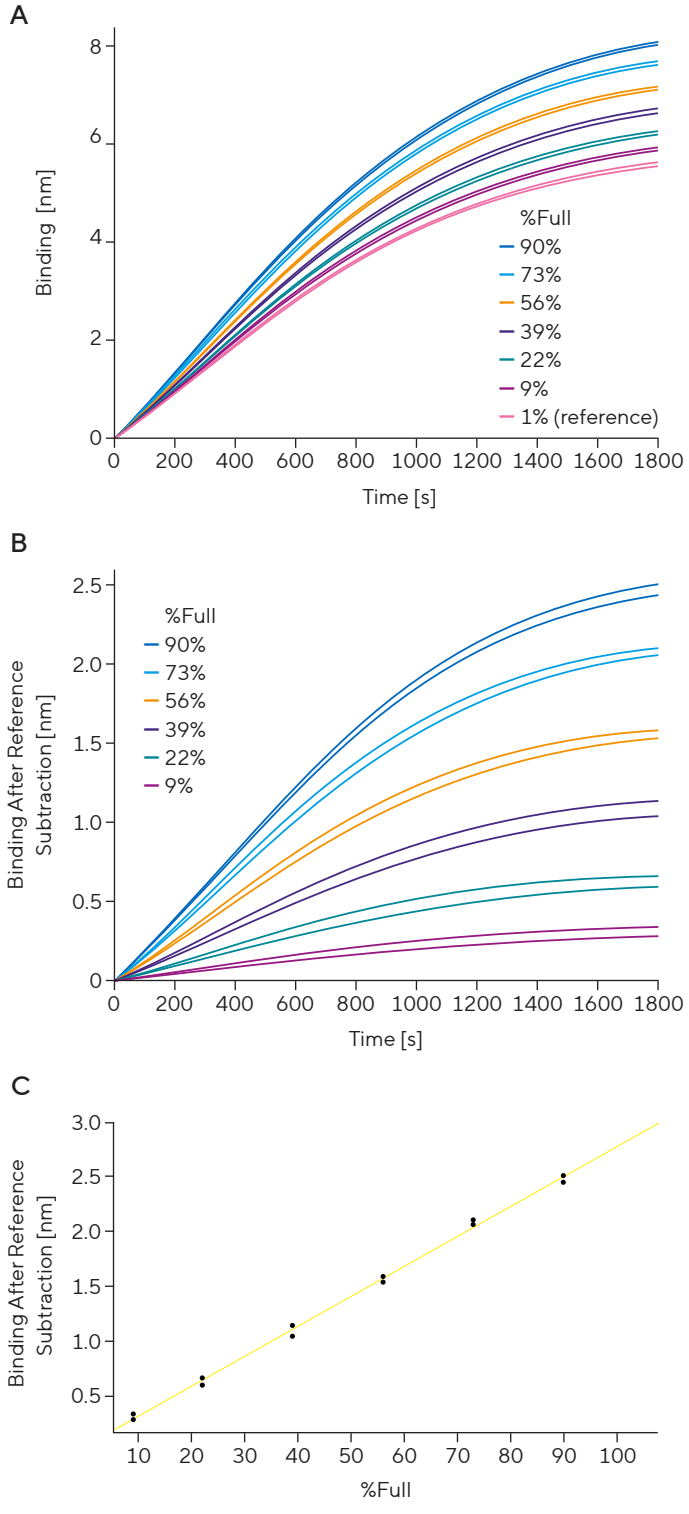
A representative Octet® AAV E/F Assay performed on AAV8 samples with %Full ranging from 9 – 90% is presented. Different %Full levels were obtained by mixing Full and Empty reference materials at different ratio, as designed by the Octet® AAV EF Calculator App. The samples were normalized to 2.5E11 vp/mL in Sample Diluent and captured onto the AAVX biosensor for 30 minutes. Figure 4 shows the binding curves before and after reference subtraction and the standard curve derived from curves post-subtraction. Table 1 shows accuracy (Recovery 100 ± 10%) and precision (%CV) of the 6 standards.

Table 1: *Results of the Octet® E/F Assay Analyzing AAV8 at Different %Full Levels (N = 2)*

Known %Full	90%	73%	56%	39%	22%	9%
Average Calculated %Full	89%	74%	55%	38%	21%	10%
%CV of Calculated %Full	1.8%	1.4%	2.5%	6.7%	8.3%	14.5%
%Recovery*	99%	102%	99%	98%	97%	108%

* Recovery refers to the percentage ratio of determined %Full (mean of two replicates) and theoretical %Full values.

Figure 4: *Representative Octet® AAV E/F Assay Analyzing AAV8*



Note. (A) Raw binding traces of AAV8 standards at 90%, 73%, 56%, 39%, 22% and 9% full (N = 2 by the same color). The Empty reference traces are shown in pink. (B) Same dataset as A after reference subtraction. (C) Standard curve generated from end point signal in B using linear fit equation.

Assay Performance

Matrix Compatibility

Table 2: *Octet® AAV E/F Assay is Directly Compatible with Common Matrices in AAV Manufacturing*

Matrix Category	Matrix Type	AAV Serotype tested	Recommended Dilution Factor
Buffer	Octet® Sample Diluent	AAV2/5/8	Neat
Culture media	Chemically defined, protein-free medium (Viral Production Media*, FreeStyle 293 Expression Media*)	AAV5/8	Neat
	DMEM+10% Fetal Bovine Serum	AAV5/8	5-fold in Octet® Sample Diluent
Cell lysis solutions	1x Lysis Buffer with 1% Tween-20 in protein-free culture media	AAV5	Neat
	0.5 M NaCl in protein-free culture media	AAV5/8	Neat
Cell lysate	2 mg/mL HEK293 cell lysate with 1x Lysis Buffer and 0.5 M NaCl	AAV5/8	Neat

* Reagent from Thermo Fisher Scientific

The Octet® AAV E/F Assay analyzes the end point signal and is therefore more tolerant to matrix interference than the Octet® AAV Titer Assay. In addition, matrix effect can be normalized by subtracting a Matching Reference diluted in the same matrix. As a result, most matrices do not require dilution to maintain recovery comparable to the Sample Diluent (Table 2). Taking advantage of this, a single standard curve generated in Sample Diluent can be used to measure samples in different matrices. An example is shown in Figure 5 and Table 3.

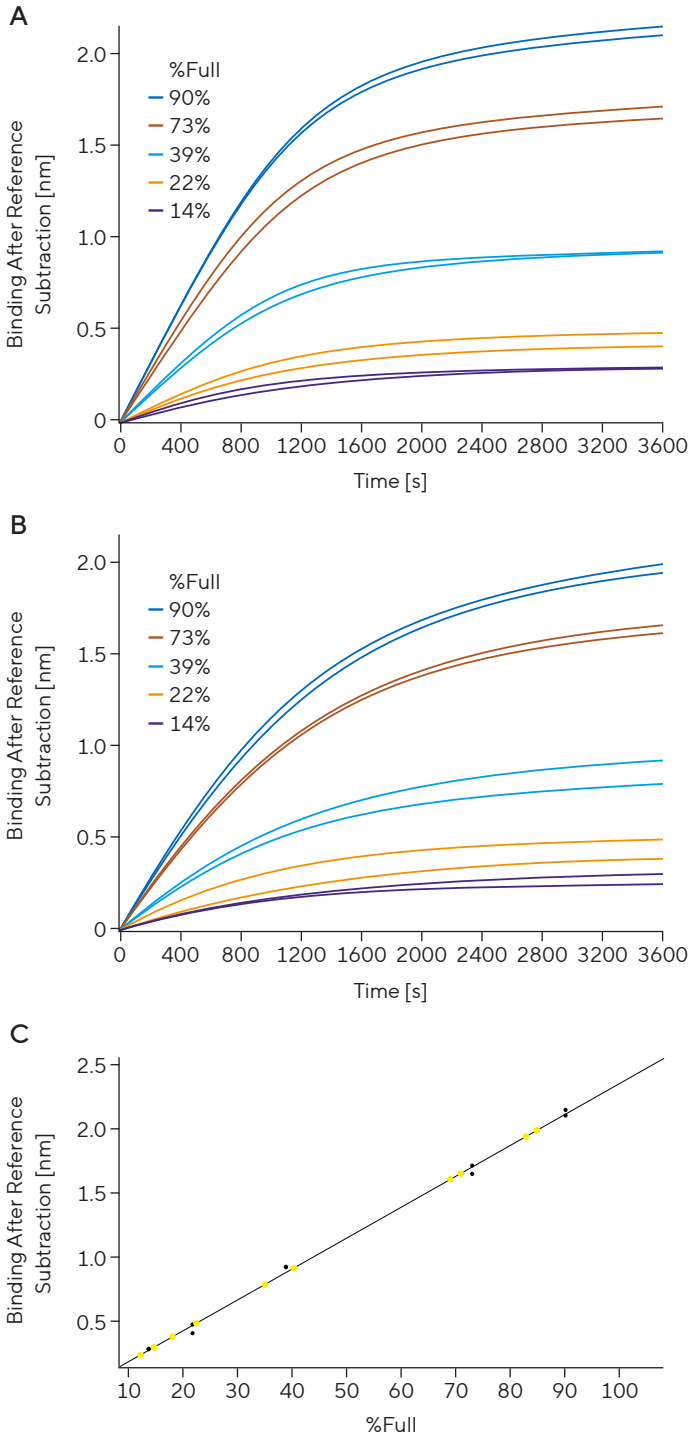
Table 3: *Results of the Octet® AAV E/F Assay Analyzing AAV8 in Standards and Spiked Samples with Different %Full Levels (N = 2)*

	Known %Full	90%	73%	39%	22%	14%
Standards (in Octet® Sample Diluent)	Average Calculated %Full (N=2)	90%	72%	41%	21%	14%
	%CV of Calculated %Full (N=2)	1.4%	2.6%	0.3%	9.6%	0.7%
	%Recovery*	100%	99%	104%	94%	102%
Spiked samples (in cell lysate)	Average Calculated %Full (N=2)	84%	70%	38%	20%	14%
	%CV of Calculated %Full (N=2)	1.6%	1.9%	9.9%	15.0%	12.7%
	%Recovery*	93%	96%	97%	93%	98%

* Recovery refers to the percentage ratio of determined %Full (mean of two replicates) and theoretical %Full values.

Note. All standards and samples have a titer of 2.5E11 vp/mL.

Figure 5: *Octet® AAV E/F Assay Analyzing AAV8 in Cell Lysate Matrix Using Standard Curve Generated in the Octet® Sample Diluent*



Note. (A) Reference-subtracted binding traces of AAV8 standards at 90%, 73%, 39%, 22% and 14% full (N = 2 by the same color). All standards were diluted in Sample Diluent. (B) Reference-subtracted binding traces of the same AAV8 standards spiked-in to neat HEK293 cell lysate (culture media supplemented with 1% Tween-20 and 0.5 M NaCl) at 1:160 ratio. (C) Spiked samples in B (yellow dots) and standards in A (black dots) generated comparable signal. Calculated %Full of the spiked samples (presented as the average recovery relative to the standards) and %CV is shown in Table 3.

Regeneration of the Octet® AAVX Biosensors for E/F Ratio Measurement

The Octet® AAVX Biosensors can be regenerated and re-used for E/F ratio measurement, providing an efficient and cost-effective solution for high-throughput applications (Table 4). The end point measurement requires more stringent removal of analyte captured in the previous cycle, therefore a modified regeneration scheme from the one outlined in Octet® AAV Titer Assay is recommended. The new scheme consists of 3 regeneration cycles before the experiment starts (pre-conditioning) and 5 regeneration cycles between assay steps. Each regeneration cycle consists of a regeneration step (10 mM glycine, pH 1.7 for 20 seconds) and a neutralization step (assay buffer for 5 seconds), as shown in Figure 6. Note that a Matching Reference should be dedicated to each regeneration round and used to subtract samples from the same sample read step (see Designing the Assay). The number of possible regeneration rounds should be determined by the user as it depends on the sample, buffer and assay conditions used, especially for serotypes that are prone to aggregation. Figure 7 and Table 5 show an example of AAV5 EF assay in which the biosensor is regenerated for 4 rounds.

Figure 6: *Recommended Regeneration Scheme for E/F Ratio Measurement*

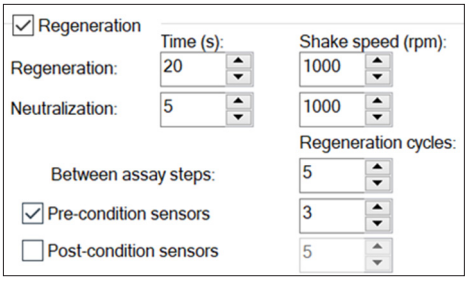
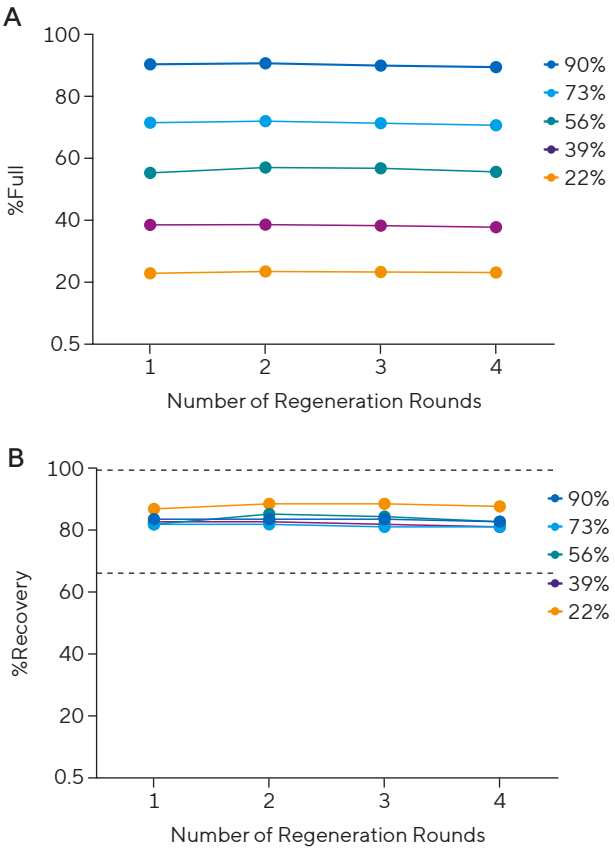


Table 4: *Regeneration Capacity Towards Different AAV Serotypes.*

AAV serotype	AAV2	AAV5	AAV8
Number of Rounds AAVX Biosensor can be Regenerated	2	4	*2*

Note. Assay buffer: Octet® Sample Diluent; Regeneration buffer: Octet® 10 mM glycine, pH 1.7.

Figure 7: *Octet® AAV E/F Assay with Biosensor Regeneration*



Note. (A) Four replicate wells of each of the five standards (at 90%, 73%, 56%, 39% and 22% full, respectively) were measured by the same biosensor over 4 rounds of regeneration. Round 1 denotes the first sample read step after the biosensors went through pre-conditioning and is used to generate the standard curve using linear fit equation. Rounds 2 – 4 were treated as unknowns. Calculated %Full values were plotted against the number of rounds of regeneration. (B) The same data plotted as %Recovery relative to the expected %Full.

Table 5: *Results of the Octet® AAV E/F Assay Analyzing AAV5 Samples with Different %Full Levels Over Four Biosensor Regeneration Rounds*

Known %Full	90%	73%	56%	39%	22%
Average Calculated %Full	91%	72%	57%	39%	23%
%CV of Calculated %Full	0.6%	0.7%	1.5%	1.0%	1.2%
%Recovery*	101%	98%	101%	99%	106%

* Recovery refers to the percentage ratio of determined %Full (mean of four replicates) and theoretical %Full values.

Note. All standards and samples have a titer of 2.5E11 vp/mL.

High-Throughput Compatibility

The Octet® AAV EF Assay can be performed on various Octet® BLI Systems, including R8, RH16 and RH96 that offer a wide range of sample throughput capabilities and allow to analyze 96 samples in as little as 30 min. Table 6 shows the comparison of total assay time required to analyze one fully filled 96-well plate depending on the Octet® instrument capacity. As outlined in the Assay Settings section, the assay time should be adjusted based on the AAV titer range. When using the Octet® R2, R4 or R8 system to analyze a large number of samples, it is important to consider the total experiment time. For example, if one fully filled 96-well plate is to be analyzed on the Octet® R8 instrument, the total assay time is 6 hours (30 min per assay x 11 assays). Evaporation resulting from the extended experiment time can be minimized either by using an Octet® Evaporation Cover, or by splitting the samples between two 96-well plates and analyze these plates consecutively. Data from the two plates can then be overlayed in the Octet® Analysis Studio Software for analysis.

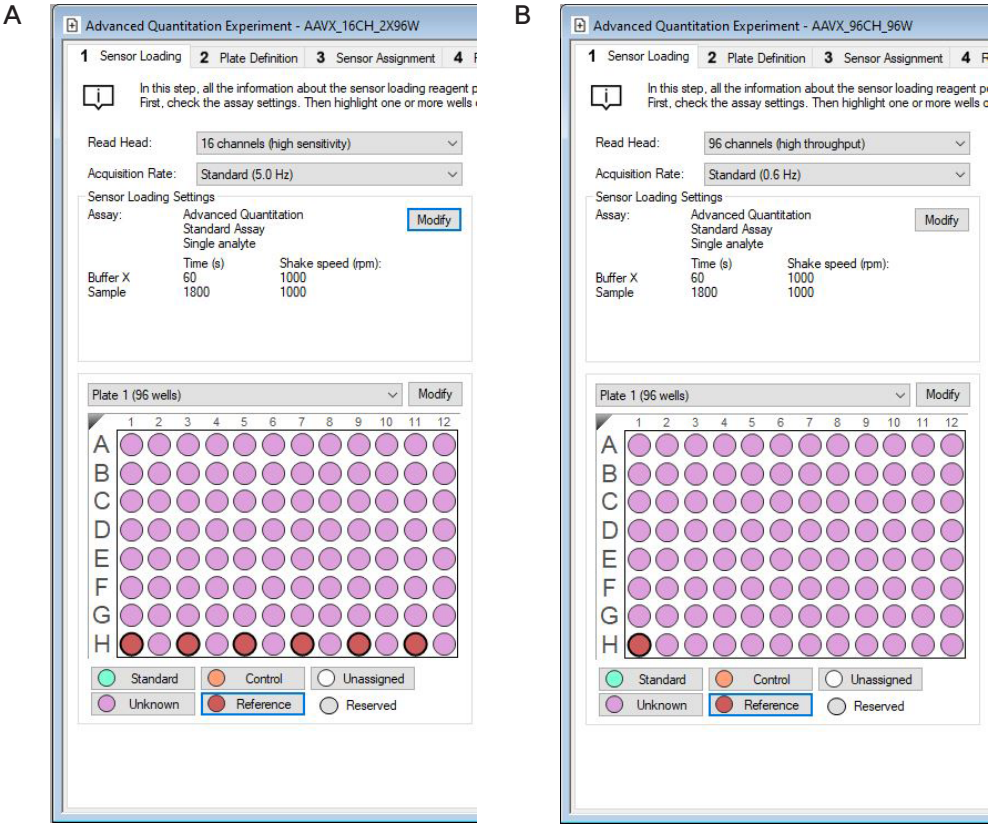
Table 6: *Total Time Required to Analyze One Fully Filled 96-Well Plate on the Octet® BLI Platform*

Octet® BLI System (Acquisition Mode)	Octet® R8 (8-channel)	Octet® RH16 (16-channel)	Octet® RH96 (96-channel)
Number of Analyzed Samples in one 96-well plate	77 Samples + 11 Matching Reference + 8 Buffer[#]	90 Samples + 6 Matching Reference [^]	95 Samples + 1 Matching Reference [&]
Assay time × Number of Assays Required	30 min × 11	30 min × 6	30 min × 1
Total Assay Time	6 hours*	3 hours	30 min

[#] Figure 3A shows an example layout.
[^] Figure 8A shows an example layout. Buffer on separate plate.
[&] Figure 8B shows an example layout. Buffer on separate plate.
* With Octet® Evaporation Cover on; see text for details.

Note. As noted in Designing the Assay, it is highly recommended that at least one well containing Matching Reference is included in each sample read step, therefore the upper limit on the number of samples a 96-plate can accommodate is dependent on the BLI System in use. For example, in Octet® BLI R8, 11 assays are needed to process 77 samples, so at least 11 wells should be reserved to hold Matching Reference, each dedicated to one sample read step.

Figure 8: *Examples of Plate Map and Assay Settings on the High-Throughput Octet® Instruments*



Note. (A) Plate layout in the Octet® RH16, assuming each sample read step fully utilizes all 16 wells (2 adjacent columns). (B) Plate layout in RH96, assuming each sample read step fully utilizes all 96 wells (whole plate). Pink: samples; Red: matching reference wells. Each reference well is matched to the standards or samples within the same sample read step.

Factors that Determine Signal Window and Assay Precision

The signal window refers to the end point BLI signal difference (in nm) between full and empty reference materials (e.g., Figure 4C, Figure 5C). A bigger signal window provides higher precision and better sensitivity towards the low %Full range. A side-by-side comparison of three AAV serotypes and two DNA insert sizes is shown in Table 7. AAV genome size is the primary factor in determining the signal window, as can be expected by the change in capsid density. Serotype plays a minor role, with AAV2 and AAV5 generating bigger signal window than AAV8.

Furthermore, producer cell does not significantly affect the signal window. Typically, samples greater than 20% full generate > 0.5 nm signal window, where CV < 10% can be expected. For samples that generate < 0.5 nm signal window, such as low % full samples or AAV with a small genome, larger variation such as 20 – 30% CV should be expected. In these scenarios, the assay should not be intended for absolute quantitation. Rather, it is better suited for screening applications where the ranking of candidates is desired.

Table 7: *Signal Window and Precision Comparison*

	2.4 kb AAV Genome (GFP)			4.4 kb AAV Genome		
Serotype	**Signal window (Full – Empty, nm)**	**%Full range**	**%CV range of calculated %Full**	**Signal window (Full – Empty, nm)**	**%Full range**	**%CV range of calculated %Full**
AAV2/AAV5	1.9 – 2.1	> 20% full	2 – 13%	2.5 – 2.7	> 20% full	2 – 9%
		< 20% full	11 – 25%		< 20% full	12 – 26%
AAV8	1.3 – 1.5	> 20% full	9 – 30%	2.3 – 2.5	> 20% full	3 – 12%
		< 20% full	10 – 20%		< 20% full	15 – 20%

Note. Each %Full standard was measured by 3 – 4 biosensors in separate aliquots, from which the standard curve, %Full recovery and CV is calculated. All measurements were made in 384-well microtiter plates in an Octet® BLI RH96 system using the 32-channel high throughput acquisition mode. AAVs with GFP insert produced from either HEK293 or Sf9 cells yield similar results.

Patent pending on methods disclosed in this Technical Note.

References

1. Technical Note: Octet® AAVX Biosensors for Quantitation of AAV Capsids.

Appendix

Example Workflow to Determine E/F Ratio of AAV8-GFP Samples

Goal: to rank E/F ratio of five crude cell lysate samples expressing AAV8-GFP (named Unknown 1 – 5), the %Full of which is expected to be < 20%.

1. Locate the Octet® instrument and AAVX biosensors.

Decision point (instrument choice): Throughput of the Octet® system impacts total assay length and design factors such as plate format, number of replicates and assay volume. See Materials Required for details. In this example, only Octet® R8 is available.

2. Locate Full & Empty AAV8 reference materials.
   a. Full: AAV8-GFP, 90% full (determined by third party), capsid titer of 1E13 vp/mL (determined by Octet® AAVX or ELISA)
   b. Empty: AAV8-Empty, 5% full (determined by third party), capsid titer of 2E13 vp/mL (determined by Octet® AAVX or ELISA)
3. Choose one of the two reference materials as titer standard for Octet® AAV Titer Assay.

Decision point (titering reference choice): Since unknown samples have expected %Full of < 20%, empty reference is better suited as the standard than the Full reference.

4. Create serial dilutions of the Empty reference and of each unknown samples in Octet® Sample Diluent (SD) buffer, according to instructions of Technical Note: Octet® AAVX Biosensors for Quantitation of AAV Capsids (1).
5. Run AAV Titer Assay and analyze data following instructions (1).
6. Record titer of each unknown sample:
   i. Unknown 1: 4E11 vp/mL
   ii. Unknown 2: 6E11 vp/mL
   iii. Unknown 3: 8E11 vp/mL
   iv. Unknown 4: 1E12 vp/mL
   v. Unknown 5: 1E11 vp/mL
7. Normalize capsid titer among unknown samples and a Matching Reference.

Decision point (sample titer choice): For AAV8, a minimum titer of 2E11 vp/mL is recommended to reach binding saturation in 30 min (see Materials Required). While assay time can be extended to accommodate lower titer, in this example the decision is to keep sample read time at 30 min to allow running multiple assays from the same sample plate and keep evaporation to minimum (see High Throughput Compatibility). Therefore, Unknown sample 5 is excluded from the rest of the assay due to insufficient titer. Among Unknown samples 1 – 4, the lowest titer of 4E11 vp/mL is chosen as the normalized titer, both for the unknown samples and for the matching Empty reference.

Decision point (sample diluent and volume choice): Since the samples are crude cell lysate themselves, dilutions (if any) are made with mock cell lysate (lysate made with identical lysis buffer on cells not transfected with AAV) to keep matrix constant between samples. In addition, there is no need to dilute the matrix per se because crude cell lysate can be used as neat (see Matrix Compatibility). Samples are titer-normalized to final volume of 420 µL because 200 µL is needed for each replicate well in 96-well plate (the only plate format compatible with Octet® R8, see Materials Required) and two replicates are chosen to provide more confidence in the ranking result (given expected CV of 10 – 20% for this serotype and insert size, see Factors that Determine Signal Window and Assay Precision).

Table 8: *Dilution Scheme to Normalize Sample Titer with the Matching Reference*

Sample	Starting titer (vp/mL)	Normalized titer (vp/mL)	Dilution factor	Vol. of original sample (µL)	Vol. of diluent (µL)	Final vol. (µL)
Unknown 1	4E11	4E11	1x	420	0	420
Unknown 2	6E11	4E11	1.5x	280	140	420
Unknown 3	8E11	4E11	2x	210	210	420
Unknown 4	1E12	4E11	2.5x	168	252	420
Empty reference	2E13	4E11	50x	8.4	411.6	420

8. Prepare EF standards.
    a. Follow Step 3: Prepare Standards from Full & Empty Reference Materials under Assay Steps in this Tech Note to set up mixing scheme using the Octet® EF Calculator App. The exact example is shown in Figure 2.

Decision point (number of standards, titer, diluent, and volume): In this example, 7 standards at %Full ranging from 9–90% were chosen, in addition to the matching Empty reference. If sensor or reagent availability is a concern, the number of standards can be reduced. Since the titers of standards and unknowns do not need to match, standards were mixed at 2E11 vp/mL, the minimum titer recommended for this serotype (see Materials Required Section) for cost saving purpose. For the same reason, dilutions are prepared for only one replicate well at each standard level (220 µL, Figure 2B). In addition, in order to reuse the standard curve for future experiments, standards and their Matching Reference are diluted in the Octet® Sample Diluent.

9. Set up method file to measure E/F ratio. Follow instructions described in Assay Settings under Step 4: Measure Sample E/F Ratio Using AAVX Biosensor to set up experimental method file. An example created for the Octet® R8 instrument is shown in Figure 9.
    a. Ensure that each sample read step has a designated Matching Reference well containing the Empty reference material. See Table 9 for details.
    b. Ensure that the buffer well used in the first "Buffer" read step contains the same matrix as the corresponding sample well in the second "Sample" read step.

Decision point (Buffer step option choice): Due to different matrices being included in this experiment, the buffer wells are not reused but rather set to "Use Once" under Step Options (instead of the default "Reuse position"). See Assay Settings.

Decision point (Biosensor reuse choice): When analyzing AAV8, only 2 rounds of regeneration is recommended (see Regeneration of Biosensor for E/F Ratio Measurement), less than the 3 back-to-back assays required to perform this experiment (Figure 9B). Assuming supply is sufficient, a new biosensor is used here for each sample read instead of going through regeneration.

10. Perform the assay. Follow instructions described in Performing the Assay under Step 4: Measure Sample E/F Ratio Using AAVX Biosensor.
11. Analyze the data. Follow instructions described in Data Analysis under Step 4: Measure Sample E/F Ratio Using the AAVX Biosensor.
    a. Select only the samples and matching reference belonging to the same sample read step to apply reference subtraction. In this example, three such groups exist (Figure 9A).
    b. %Full of Unknown samples 1–4 are calculated automatically based on the standard curve.

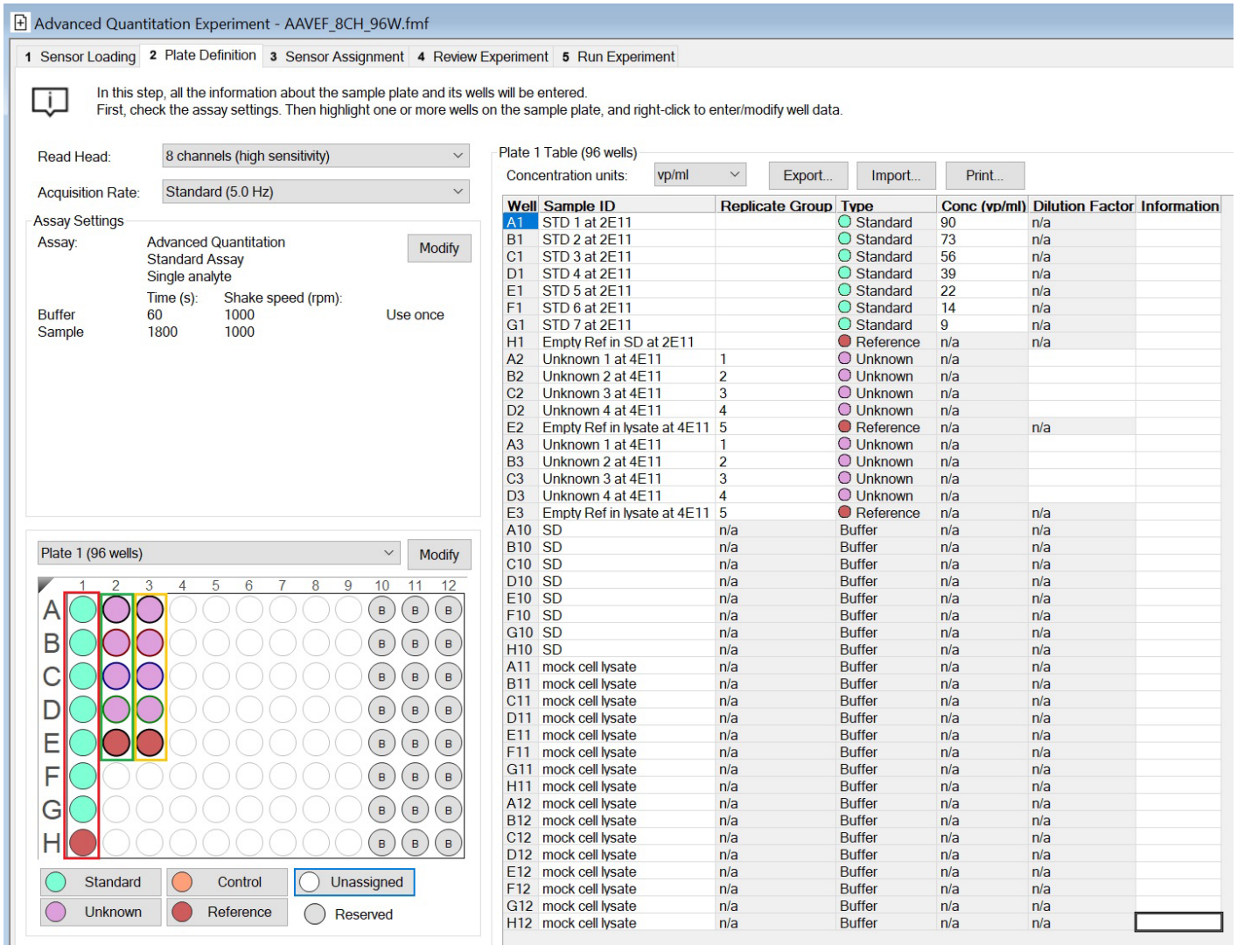
Table 9: *Description of the Plate Map and Well Content in Figure 9*

	Samples			Corresponding Buffer well		Sample read step
	Well no.	Titer	Matrix/Buffer	Well no.	Content	Assay no.
Standards 1–7	A1–G1	2E11	SD	A10–G10	SD	1
Empty Ref for Standards	H1	2E11	SD	H10	SD	1
Unknowns 1–4 Rep 1	A2–D2	4E11	Crude cell lysate	A11–D11	Mock cell lysate	2
Empty Ref for Unknowns Rep 1	E2	4E11	Mock cell lysate	E11	Mock cell lysate	2
Unknowns 1–4 Rep 2	A3–D3	4E11	Crude cell lysate	A12–D12	Mock cell lysate	3
Empty Ref for Unknowns Rep 2	E3	4E11	Mock cell lysate	E12	Mock cell lysate	3

Note. It is recommended to prepare samples in quantities sufficient for analysis of two replicates in 96 well plates.

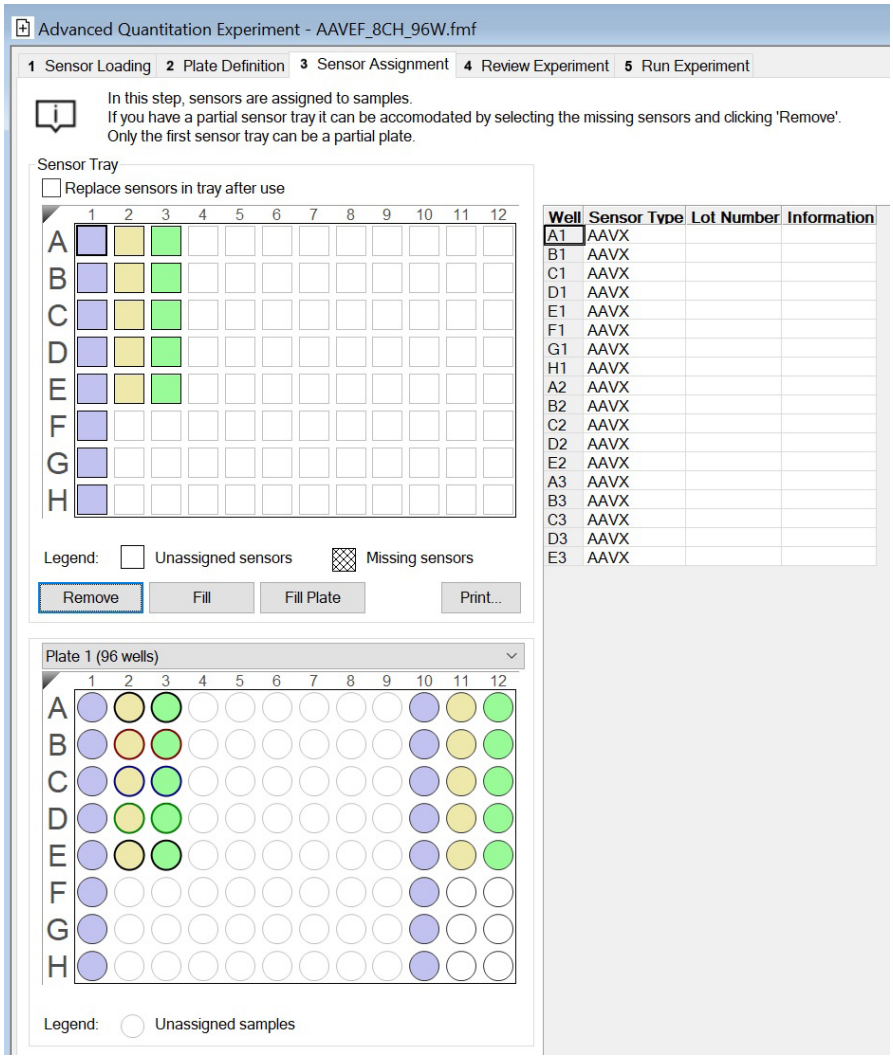
Figure 9: *Method File to Carry Out the Example Experiment Using the Octet® R8 Instrument*

A



Well	Sample ID	Replicate Group	Type	Conc (vp/ml)	Dilution Factor	Information
A1	STD 1 at 2E11		Standard	90	n/a	
B1	STD 2 at 2E11		Standard	73	n/a	
C1	STD 3 at 2E11		Standard	56	n/a	
D1	STD 4 at 2E11		Standard	39	n/a	
E1	STD 5 at 2E11		Standard	22	n/a	
F1	STD 6 at 2E11		Standard	14	n/a	
G1	STD 7 at 2E11		Standard	9	n/a	
H1	Empty Ref in SD at 2E11		Reference	n/a	n/a	
A2	Unknown 1 at 4E11	1	Unknown	n/a		
B2	Unknown 2 at 4E11	2	Unknown	n/a		
C2	Unknown 3 at 4E11	3	Unknown	n/a		
D2	Unknown 4 at 4E11	4	Unknown	n/a		
E2	Empty Ref in lysate at 4E11	5	Reference	n/a	n/a	
A3	Unknown 1 at 4E11	1	Unknown	n/a		
B3	Unknown 2 at 4E11	2	Unknown	n/a		
C3	Unknown 3 at 4E11	3	Unknown	n/a		
D3	Unknown 4 at 4E11	4	Unknown	n/a		
E3	Empty Ref in lysate at 4E11	5	Reference	n/a	n/a	
A10	SD	n/a	Buffer	n/a	n/a	
B10	SD	n/a	Buffer	n/a	n/a	
C10	SD	n/a	Buffer	n/a	n/a	
D10	SD	n/a	Buffer	n/a	n/a	
E10	SD	n/a	Buffer	n/a	n/a	
F10	SD	n/a	Buffer	n/a	n/a	
G10	SD	n/a	Buffer	n/a	n/a	
H10	SD	n/a	Buffer	n/a	n/a	
A11	mock cell lysate	n/a	Buffer	n/a	n/a	
B11	mock cell lysate	n/a	Buffer	n/a	n/a	
C11	mock cell lysate	n/a	Buffer	n/a	n/a	
D11	mock cell lysate	n/a	Buffer	n/a	n/a	
E11	mock cell lysate	n/a	Buffer	n/a	n/a	
F11	mock cell lysate	n/a	Buffer	n/a	n/a	
G11	mock cell lysate	n/a	Buffer	n/a	n/a	
H11	mock cell lysate	n/a	Buffer	n/a	n/a	
A12	mock cell lysate	n/a	Buffer	n/a	n/a	
B12	mock cell lysate	n/a	Buffer	n/a	n/a	
C12	mock cell lysate	n/a	Buffer	n/a	n/a	
D12	mock cell lysate	n/a	Buffer	n/a	n/a	
E12	mock cell lysate	n/a	Buffer	n/a	n/a	
F12	mock cell lysate	n/a	Buffer	n/a	n/a	
G12	mock cell lysate	n/a	Buffer	n/a	n/a	
H12	mock cell lysate	n/a	Buffer	n/a	n/a	

B



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