AAV Reference Standards

AAV1, AAV2, AAV5, AAV6, AAV8, AAV9
Empty & Full Capsids
User Manual

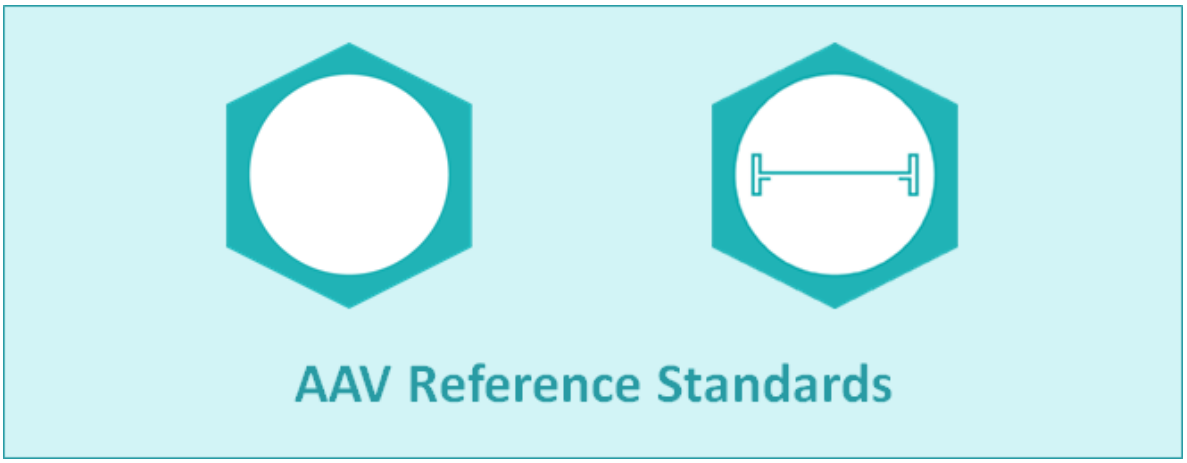


Table of Contents

Package Contents and Storage Conditions

AAV Standard Materials	Catalog #	Volume (UL)	Titer	Shipping	Storage
AAV1 reference standards (full capsids)	RS-AAV1-FL	100	5.33X10^11 GC/mL	Dry ice	-80°C
AAV2 reference standards (full capsids)	RS-AAV2-FL	100	1.82X10^11 GC/mL	Dry ice	-80°C
AAV5 reference standards (full capsids)	RS -AAV5-FL	100	2.60X10^11 GC/mL	Dry ice	-80°C
AAV6 reference standards (full capsids)	RS -AAV6-FL	100	4.10X10^11 GC/mL	Dry ice	-80°C
AAV8 reference standards (full capsids)	RS -AAV8-FL	100	7.97X10^11 GC/mL	Dry ice	-80°C
AAV9 reference standards (full capsids)	RS -AAV9-FL	100	3.86X10^11 GC/mL	Dry ice	-80°C
AAV1 reference standards (empty capsids)	RS -AAV1-ET	100	2.26X10^12 VP/mL	Dry ice	-80°C
AAV2 reference standards (empty capsids	RS -AAV2-ET	100	1.27X10^12 VP/mL	Dry ice	-80°C
AAV5 reference standards (empty capsids)	RS -AAV5-ET	100	1.46X10^12 VP/mL	Dry ice	-80°C
AAV6 reference standards (empty capsids)	RS -AAV6-ET	100	1.79X10^12 VP/mL	Dry ice	-80°C
AAV9 reference standards (empty capsids)	RS -AAV9-ET	100	1.76X10^12 VP/mL	Dry ice	-80°C

Related Products

Custom AAV Reference Standards – Any serotype, any size, same quality
AAV Custom Packaging Service – High quality, quick turnaround time
cGMP AAV Production – High-yield producer cells, experienced GMP team

Product Description

Recombinant Adeno-associated virus (rAAV) is a widely used gene delivery tool for research and clinical applications. To be able to compare the pharmacokinetics and efficacy of rAAV, well characterized reference standard materials are needed for assays and calibration of internal reference standards. Reference standard (full capsids) with accurate vector genome concentration can be used in qPCR-based vector genome titrations. Since AAV is known to produce empty vial particles without a gene payload, the full-to-empty ratio of standard materials is also very critical. High quality and high purity empty capsid reference standards can be used as reference standards in assays, such as HPLC, ELISA, etc. Vigene Biosciences is a leader in viral vector manufacturing and analytic assays. Vigene provides extensively analyzed AAV reference standard materials with viral particle concentration and full/empty ratio.

Six AAV serotypes of reference standards are offered, AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9. For most of the serotypes, both full capsids and empty capsids reference materials are available. Each standard material is well characterized, including endotoxin, bioburden, mycoplasma testing, silver staining, ELISA, qPCR, and transmission electron microscopy. Our AAV reference standards are quantified based on the reference standard material from ATCC.

Using AAV Reference Standards for Quantification, Viral Vector Genome and Total Viral Particle Titration

Quantitative PCR (qPCR) is commonly used to quantify viral genome copies. Linearized plasmid DNA may not be the best qPCR standard for AAV quantification. Viral vector standards with known vector genome copies, such as our full capsids reference standards, serve as better qPCR template standards. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the total viral particle numbers. Both our full and empty reference standards with accurate titers can be used as standards in ELISA assays.

Using Reference Standards for Accurate Full/Empty Ratio Quantification

One common challenge in AAV production is that AAV packages a significant number of empty viral particles without gene payloads. There is still a great percentage of empty capsids in the final product even after a series steps of purification/enrichment procedures. Therefore, a well-characterized AAV standard with an accurate full-to-empty ratio is needed to characterize the final viral vector production. The full/empty ratio is calculated with transmission electron microscopy (TEM).

Percentage of Empty Capsids of Reference Standards (Empty Capsids)

Our AAV empty particles are enriched to an extremely high percentage of empty capsids, approaching 85%-99.5%, depending on the serotype. For most serotypes, we can achieve equal or greater than 92% empty capsid purity, including AAV1, AAV2, and AAV9.

Percentage of Full Capsids of AAV Reference Standard (Full Capsids)

CMV-GFP is used to produce AAV full capsids standard materials. Viral particles are purified to a very high percentage of full capsids, ranging from 55% to 82%, depending on the serotype.

Experimental Protocols

The protocols below may be used as a point of reference, in addition to, or in lieu of your own protocols.

SDS PAGE and Silver Staining

AAV Sample Preparation for SDS PAGE Gel

1. Preheat thermocycler to 95°C

2. Dilute AAV samples to match the concentration of the AAV reference standard (~1X10^12 viral particles/mL, exact titer can be found on the COA and tube label)

3. Aliquot 10 uL of AAV reference standard and diluted purified viral sample(s) into new PCR tubes. Add 10 uL of 2x Laemmli Sample Buffer into each vial. Vortex briefly.

4. Place the AAV standard and AAV sample(s) in thermocycler for 15 minutes.

5. Remove sample(s) from thermocycler and briefly spin down for 5 seconds to collect the sample.

Loading and running samples on SDS-PAGE Gel

NuPAGE™ 4-12% Bis-Tris precast gels are used (ThermoFisher NP0321PK2) to separate the proteins. Load the gel and run in 1x MOPS running buffer at 120V for 60 minutes.

Silver Staining Protocol

Pierce Silver Stain Kit (ThermoFisher 24612) is used for silver staining. Here is the manufacture's protocol.

1. Wash gel in ultrapure water for 5 minutes. Replace water and wash for another 5 minutes.

2. Fix gel in 30% ethanol:10% acetic acid solution (i.e., 6:3:1 water: ethanol: acetic acid ratio by volume) for 15 minutes. Replace the solution and fix for another 15 minutes.

   Note: Gel may be kept in fixing solution overnight without affecting stain performance.

3. Wash gel in 10% ethanol solution for 5 minutes. Replace solution and wash for another 5 minutes.

4. Wash gel in ultrapure water for 5 minutes. Replace water and wash for another 5 minutes.

5. Prepare Sensitizer Working Solution by mixing Silver Stain Sensitizer and ultrapure water 1:500 by volume (e.g., mix 5 µL Sensitizer with 10 mL water).

6. Incubate gel in Sensitizer Working Solution for exactly 1 minute, then wash with two changes of ultrapure water for 1 minute each.

7. Prepare Stain Working Solution by mixing Silver Stain Enhancer with Silver Stain, 1:50 (e.g., 0.5mL of Enhancer with 25mL Stain).

8. Incubate gel in Stain Working Solution for 30 minutes.

   Note: Gel may be incubated in Stain Working Solution for as short as 5 minutes or as long as overnight without affecting stain performance.

9. Prepare Developer Working Solution by mixing Silver Stain Enhancer with Silver Stain Developer, in 1:50 ratio by volume, (e.g., mix 0.5mL of Enhancer with 25mL Developer).

10. Prepare 5% acetic acid solution as a Stop Solution.

11. Immediately add Developer Working Solution and incubate until protein bands appear (2-3 minutes).

    Note: Protein bands will begin to appear within 30 seconds and then continue to develop. Between 2 and 3 minutes, protein detection vs. background is optimal. After 3 minutes, lane background signal may increase to undesirable levels.

12. When the desired band intensity is reached, replace Developer Working Solution with prepared Stop Solution (5% acetic acid). Wash gel briefly, then replace Stop Solution and incubate for 10 minutes.

ELISA Procedures

AAV serotype specific ELISA kits should be used for the corresponding AAV serotype. The following ELISA procedure is modified from the Progen ELISA kits (Wayne, PA) ELISA kit protocol. We recommend following your manufacturer's protocol. This protocol uses Vigene's AAV reference standards as reference material, while the KC control from the original kit is diluted for as standards. If you desire to use Vigene's AAV reference standards to replace the original kit standards, please skip step 2 and dilute the standard materials from Vigene following the kit control dilutions in step 1, section "KC control, AAV standard material and sample dilutions".

Preparing Reagents

Allow all kit reagents to reach room temperature (20-26^{o}C) before use.

Note: Buffer concentrates may contain salt crystals, which dissolve quickly at 37°C. Allow buffers to reach room temperature and ensure that reagents are mixed well before use.

1. **1X working Assay Buffer solution**

Prepare 1X working Assay Buffer solution by diluting the 20X Assay Buffer stock provided in the kit with distilled water. The volume prepared should be adjusted so it is appropriate for the number of test samples, about 30 mL of 1x assay buffer per strip is needed.

2. **Reconstitute and dilute the Kit Control**
   Reconstitute the Kit Control in 500 µL 1X Assay Buffer. Follow the dilution scheme, 2-fold dilution from 1:1 to 1:64. Refer to the kit control label and the lot-specific Quality Control Certificate to determine the amount of particles/mL present in the reconstituted kit control. Mix carefully and do not vortex the AAV reference standard dilution.

3. **Reconstitute Anti-AAV Biotin Conjugate and dilute 1:20**
   Reconstitute Anti-AAV Biotin Conjugate with 750 µL 1X Assay Buffer.
   Immediately before use (not more than 10 minutes before use), dilute 1:20 with 1X Assay Buffer.

   Note: The Biotin Conjugate dilution could change based on the specific serotype instructions.

4. **Strep -HRP 20x** (Streptavidin Peroxidase Conjugate 20x)
   Immediately before use, dilute 1:20 with 1x Assay Buffer, the diluted component is named Strep-HRP 1x. Store in the dark until use.

Kit Control, Vigene's AAV reference standard material and sample dilutions

1. **Kit control dilution.**
   The kit control is diluted for use as kit standards. We recommend 2-fold serial dilution with 1X Assay Buffer, undiluted, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64.

2. **Vigene's AAV reference standard material dilution**
   The viral particle titer of the AAV reference standard material is approximately 1X10^12 VP/mL. The AAV standard material can be diluted 1:1000-1:10,000 with 1X Assay Buffer. We recommend 1:1,000, 1:5,000 and 1:10,000 dilutions.

3. **Experiment sample dilutions**
   Samples can be prepared and tested at several concentrations to ensure they fall within the calibration curve range. It may be necessary to perform a pre-experiment to determine the approximate titer of an unknown specimen before analysis with more fine-tuned dilutions.

Testing Procedure

1. Pipette 100 µL of 1X Assay Buffer (Blank), each serial dilution of the Kit Control, AAV reference standard materials and test samples into each of the corresponding wells of the microtiter strips. Seal the strips with adhesive foil and incubate for 1 hour at 37°C.

2. Prepare 1x Biotin conjugate by dilute 20x biotin stock 1:20 with 1X Assay Buffer.

3. Empty the contents of the microtiter strips by inverting the plate over a waste reservoir. Blot the inverted plate on absorbent paper. Fill each well with 200 µL of 1X Assay Buffer,

incubate approximately 5 seconds, empty and tap the inverted plate onto absorbent paper. Perform this washing step a total of 3 times.

4. Pipette 100 µL of 1x Biotin Conjugate into each well. Seal strips with adhesive foil and incubate for 1 hour at 37°C.

5. Prepare 1x Strep-HRP solution. Immediately before use (not more than 10 minutes before use), dilute the Streptavidin Peroxidase Conjugate stock solution 1:20 with 1X Assay Buffer.

6. Repeat the washing step as described in step 3.

7. Pipette 100 µL of 1:20 diluted Streptavidin Conjugate into each well. Seal strips with adhesive foil and incubate for 1 hour at 37°C.

8. Repeat the washing step as described in step 3.

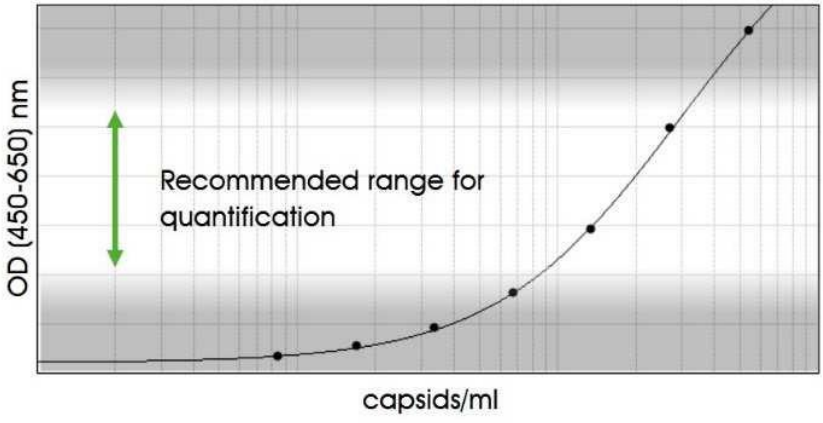
9. Pipette 100 µl of the ready-to-use TMB substrate into each experimental well. Incubate at room temperature for 15 minutes.

10. Pipette 100 µl of ready-to-use Stop Solution into each well.

11. Within 30 minutes of adding the Stop Solution, measure intensity of color reaction with a microplate reader at a wavelength of 450 nm (optional: reference wavelength 650 nm).

Calculating Results

Subtract the blank (buffer) reading from the calibrator and sample readings. Create a standard curve by plotting the mean absorbance value of each Kit Control dilution (y-axis) against the corresponding concentration (x-axis) using the SoftMax Pro 7.0.3 software. Use a best fit curve (4-parameter or 5-parameter logistic fit) to calculate the results. Calculate the viral particle titer of your specimens by multiplying the results by the dilution factor.



Quantitative PCR (qPCR)

The qPCR protocol below uses Vigene's AAV reference standards (full capsids) as qPCR standards to create the standard curve. A similar dilution method may be used If linearized DNA is used as PCR standards. If you use Vigene's AAV standard material as a positive control/calibration tool, then they may be treated the same as testing AAV samples. Note that, linearized DNA is used as the standard, the final sample titer needs to be multiplied by 2, as the linearized DNA is double-stranded.

DNase I Digestion of AAV Samples

Treat the purified AAV reference standard materials and AAV samples with DNase I to eliminate any contaminating plasmid DNA carried over from the production process (DNase does not penetrate the virion).

Components	Volume
AAV sample /standard	5 µL
10x DNase buffer	5 µL
DNase	1 uL
dH2O	39 µL
Total	50 uL

Gently mix sample (do not vortex) and incubate 30 minutes at 37°C. Inactivate DNase by heating samples at 95°C 10 minutes, then transfer to ice.

Proteinase K Digestion of AAV Samples

Treat DNase-digested AAV samples with proteinase K to release viral DNA from the viral particles.

Components	Volume
DNase-treated AAV sample /standard	5 µL
10x DNase buffer	5 µL
Proteinase K (5 mg/mL)	4 uL
dH2O	36 µL
Total	50 uL

Gently mix sample (do not vortex) and incubate at 55°C for 30 minutes. Inactivate the Proteinase K by heating the sample at 95℃ for 15 minutes, followed by cooling at 4℃ for at least 5 minutes. Keep the treated samples on ice until ready to proceed.

AAV Reference Standard and Sample Dilutions

1. Dilute DNase/Proteinase K-treated AAV standard materials as qPCR standards

The concentration of AAV reference standards from Vigene is approximately 1X10^12 GC/mL. The exact titer can be found on COA and the vial label. 1x10^12 GC/mL equals 1x10^9 GC/uL. There is a 100-fold dilution of the reference standards and virus samples (10-fold dilution in

DNase digestion and 10-fold dilution in Proteinase K digestion step). Therefore, the starting virus concentration of the reference standards to make PCR standards is 1x10^7 GC/uL. Carry a series of 10-fold dilutions to make dilutions according to the table below.

Note: Accuracy and precision of the sample dilution series is critical. Make sure to pipette each dilution up and down at least 10 times.

Table 1: Example of Standard Curve Dilutions

Initial Concentration (GC/µL)	Dilution	Volume of Stock Dilutions (µL)	dH_2O (µL)	Final Concentration (GC/µL)	Volume Used in qPCR (µL)
1x10^7 (DNase/Proteinase K treated stock)	No dilution			1x10^7	5
1x10^7	1:10	10	90	1x10^6	5
1x10^6	1:10	10	90	1x10^5	5
1x10^5	1:10	10	90	1x10^4	5
1x10^4	1:10	10	90	1x10^3	5

2. Dilute DNase/Proteinase K-treated AAV Test Samples

At the end of the DNase/Proteinase K digestion, the samples are diluted 1:100. Complete a 2-step 1:10 serial dilutions for each sample according to the table below. Final dilutions are 1:1000, and 1:10,000.

Table 2. Example of Sample Dilutions

Dilution Series	Dilution	Volume of Stock (µL)	dH_2O (µL)	Total Dilutions	Volume Used in qPCR (µL)
Dilution 1 (DNase/Proteinase K treated, 100x)	1:10	10	90	**1,000x**	5
Dilution 2	1:10	10	90	**10,000x**	5

Preparing PCR SYBR Green Master Mix and Running qPCR

Prepare enough PCR mix for 3 reactions of each sample, including standards, test samples, and no template (blank) control. Follow the table below to prepare your PCR master mix. Aliquot 15 uL of the master mix into each well of qPCR plate, then add 5 uL of standards, test samples and no template (blank) controls (nuclease free water) into each well. Use nuclease-free water

for the blank control. Mix the sample with the reagents in the well by gentle pipetting. Seal the plate, spin briefly before loading into the qPCR machine.

Run the following protocol in your qPCR instrument using SYBR detection:

95°C 10 min
40 cycles of 95°C 15 sec / 60°C 60 sec
Melt curve: 95°C 15 sec / 60°C 60 sec / 95°C 1 sec

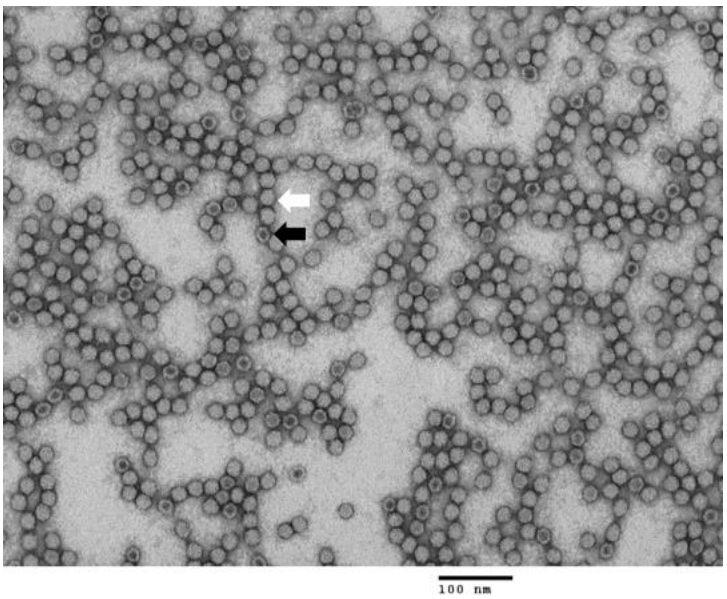
Perform data analysis using the instrument's software. Determine the physical titer of samples (genome copies (GC)/mL) based on the standard curve and the sample dilutions.

Table 3. Example of PCR Master Mix Preparation

Reagent	**Final Concentration**	**Vol. per 20 uL reaction**
2x SYBR Green master mix	1x	10 uL
Forward Primer (10 uM)	0.5 uM	1 uL
Reverse Primer (10 uM)	0.5 uM	1 uL
Nuclease Free Water		3 uL
Total PCR Master Mix		15 uL

Transmission Electron Microscopy (TEM)

TEM can be used to measure the full/empty ratio of AAV reference standards. AAV viral particles can be visualized using electron microscopy. Viral particles are negatively stained with uranyl acetate. Empty particles exhibit an electron-dense central region of the capsid – a black dot in the center of the particle. Full particles exclude the negative stain and display as white spheres. Images are taken after negative staining of the AAV particles. The percentage of full or empty capsids can be readily calculated via the ratio of full or empty capsids to total viral particles, respectively.



TEM image of AAV9 reference standards (full capsids).
AAV particles were negatively stained with uranyl acetate. Full capsids are shown as white spheres (white arrow). Empty capsids shown with a black dot in the center (dark arrow).

Analytical Ultracentrifugation (AUC) for Full/Empty Ratio Quantification

AUC is an accurate and quantitative analytical method for defining the full-to-empty capsid ratio of AAV viral vectors. AUC uses sedimentation velocity to separate capsids. The AAV samples are measured at 260 nm at a speed of 20,000 rpm. The empty and full capsids will exhibit as distinct peaks based on mass. The percentage of empty and full capsids can be accurately measured using AUC, thus AUC is the gold standard in measuring the empty/full ratio of AAV viral vectors. The disadvantage of this method is that it usually requires a larger amount of viral particles at higher concentrations, approximately 0.5 mL of 1X10^12 GC/mL viral particles.

Terms and Conditions

Products are for research use only, Vigene Biosciences' products are to be used for research purposes only. They may not be used for any other purposes, including, but not limited to, in vitro diagnostic purposes, therapeutics, or in humans.

Cancellation and Changes. Once orders are placed, cancellation or changes cannot be made without our written consent.

Use restrictions. You must not resell, transfer, distribute, or aliquot the products directly or indirectly to any third party for any purpose or use, unless otherwise authorized by Vigene in writing.

Warranties. Vigene warrants that the products will meet specifications listed. At Vigene's discretion, free replacement/ or credit for any nonconforming product will be issued if Vigene Biosciences is notified within 30 days of product delivery, which shall constitute the sole remedy.

IN NO EVENT SHALL VIGENE BIOSCIENCES BE LIABLE FOR LOST PROFITS, OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, PUNITIVE, INDIRECT, OR DIRECT DAMAGES, ARISING FROM ANY USE OF THE PRODUCTS.