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AAV titration with qPCR

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Transcriptional crosstalk

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Abstract

Titration of AAV genomes in a purified sample is critical for ensuring accurate dosing. Titering AAV samples is typically accomplished by first treating samples with a DNase to degrade DNA not contained within the AAV capsid (i.e. unencapsidated DNA). Subsequently, the DNase is inactivated and encapsidated genomes are solubilized by denaturing and digesting the capsid. The number of DNase-resistant genomes in the treated sample(s) can then be absolutely determined using qPCR, with comparison to a standard of known concentrations. Alternatively, a droplet PCR method can be used, which bypasses the need for a standard. Here, we describe how to titer AAV samples using qPCR.

Guidelines

This protocol is based on the titering protocol from Challis et al., Nat Protoc 14: 379, published in 2019. In the original protocol, 1.5 mL microcentrifuge tubes were used for all sample incubation and dilution steps, and for generating the standard DNA. This protocol presents a scaled-down version using 96-well plates, which saves on reagent and consumable costs and on plastic waste. We have not seen any systematic differences in titers obtained with this protocol vs. the previous protocol.

Materials

Reagents

- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Calcium chloride anhydrous ≥96.0% (by argentometric titration) BAKER ANALYZED® ACS J.T.Baker® **JT Baker Catalog #**1311-01
- Magnesium chloride hexahydrate 99.0-102.0%, crystals, AR® ACS, Macron Fine Chemicals™ **VWR International Catalog #**MK5958-04
- 1M Tris-HCl pH=7.5 **Invitrogen - Thermo Fisher Catalog #**15567-027
- Sodium chloride **Merck Millipore (EMD Millipore) Catalog #**SX0420-1
- N-Lauroylsarcosine sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L9150
- Restriction enzyme appropriate for linearizing the standard DNA plasmid.

Note

We often use ScaI-HF to cut within the ampicillin resistance gene, but any single cutting restriction enzyme that cuts outside of the amplified sequence should work.

ScaI-HF - 1,000 units **New England Biolabs Catalog #**R3122S

- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854
- DPBS, no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14190144
- Pluronic F-68 **Gibco - Thermo Fischer Catalog #**24040-032
- DNase I recombinant, RNase-free **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**04716728001
- UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #**15575020
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #**P8107S
- qPCR master mix

Note

We use a SybrGreen-based master mix:

FastStart Universal SYBR Green Master (Rox) **Roche Catalog #**4913850001

However, any qPCR master mix should work. Probe-based qPCR assays can also be used.

DNA

- Plasmid DNA containing the target sequence to be amplified

Note

This can be the pAAV that was used for packaging, or another plasmid that contains the target sequence. Note though that the presence of the ITR and it's proximity to the target sequence can affect amplification efficiency. Thus, a pAAV that has a similar sequence and structure to the packaged genome is ideal as a standard.

- Primers to amplify the target sequence

Note

We frequently use the following to amplify the WPRE sequence (will also work for the commonly used W3 truncation of the WPRE):

- Forward: 5'-TGGTATTCTTAACTATGTTGCTCCT-3'
- Reverse: 5'-AAGCCATACGGGAAGCAATAG-3'

The following can be used to amplify the EGFP sequence:

- Forward: 5'-TCTTCAAGTCCGCCATGC-3'
- Reverse: 5'-CGCCCTCGAACTTCACC-3'

Before using these primers, ensure that they will work for your standard and sample sequences. Note that titers obtained with different primer sets are not directly comparable to one another.

Consumables

- Low binding barrier pipette tips
- DNA clean up kit

Note

Use a kit with large binding capacity, such as:

DNA Clean & Concentrator-25 (Capped) **Zymo Research Catalog #**D4033

to purify large quantities of linearized standard in a single prep.

- 1.5 mL Microcentrifuge tubes
- PCR tube strips (for storing single-use aliquots of DNA standard)
- Qubit assay tubes **Thermo Fisher Scientific Catalog #**Q32856
- 250 mL sterile vacuum bottle top filters
- 96-well plates
- Adhesive foil seals for 96-well plates
- 96-well qPCR plate
- Optical seals for qPCR plates

Equipment

- Heat blocks capable of heating 96-well plates, with heated lid to prevent evaporation

Note

Alternatively, a thermocycler can be used, which can provide better protection against evaporation

- PCR plate spinner
- Real-time PCR system

Equipment

Qubit™ 3 Fluorometer	NAME
Spectrophotometer	TYPE
Invitrogen	BRAND
Q33216	SKU

Safety warnings

- AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. Experiments involving AAVs were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

- rAAVs, although replication-incompetent, are potent gene-delivery vehicles and must be handled according to governmental and institutional regulations. The safety of packaged transgenes (e.g., oncogenic genes) should be carefully considered. Perform all procedures in a certified biosafety cabinet and clean AAV-contaminated equipment, surfaces, and labware with fresh 10% (vol/vol) bleach.

General

1 This protocol is comprised of 3 sections:

1. Reagent preparation (can be done months ahead of time)
2. Sample preparation (should be done the day of, or 1-2 days before running qPCR)
3. Sample and standard dilutions, and qPCR

The reagent preparation can be done on bench top. Sample preparation should be done in a Biosafety cabinet and in compliance with biosafety guidelines. We prepare sample and standard dilutions and qPCR reactions in a biosafety cabinet as well.

Reagent preparation

2 Prepare DNase I buffer, by combining:

- $CaCl_2$: 55.5 mg
- 1 M Tris-HCl: 2.5 mL
- $MgCl_2 \cdot 6H_2O$: 508 mg

With 250 mL of UltraPure water in a sterile bottle. Shake to mix, filter sterilize, and store at Room temperature for several months.

3 Prepare Proteinase K buffer, by combining:

- NaCl: 14.61 g

With 250 mL of UltraPure water in a sterile bottle. Shake to mix until NaCl is dissolved. Add:

- N-lauroylsarcosine sodium salt: 2.5 g

And gently swirl to mix. Filter sterilize and store at Room temperature for several months.

4 Prepare DNA standard stock. Linearize a plasmid DNA containing the target sequence, using a single cutter restriction enzyme that does not cut within the PCR amplicon (e.g. something that cuts within the ampicillin resistance gene). Digest 20 µg of plasmid DNA with

60-80 U of enzyme, preferably in an overnight reaction to ensure complete digestion. Purify the reaction using two DNA clean-up columns, and measure the DNA concentration with a spectrophotometer. Dilute the DNA to 10 ng/µL, and measure the diluted DNA concentration in triplicate using a high sensitivity Qubit assay. Record the mean value and use for subsequent calculation. Pipette into 20 µL aliquots in PCR strip tubes and store at -20 °C.

Sample preparation

1h

5 Determine how much DNase I buffer (Step 1) and Proteinase K buffer (Step 2) you will need. Each AAV sample and a negative control (virus storage buffer: DPBS + 0.001 % Pluronic F-68) should be titered in technical triplicates. You will need 50 µL of DNase I buffer and 60 µL of Proteinase K buffer per well. For example, if you have 8 viruses to titer you will need to prepare at least:

(8 samples + 1 negative control) x 3 = 27 rxn

DNase I buffer: 27 rxn x 50 µL/rxn = 1350 µL
Proteinase K buffer: 27 rxn x 60 µL/rxn = 1620 µL

Prepare extra to account for pipetting error and loading method. If using a repeater pipette or single channel pipette, 3 extra wells worth should be adequate. If using a multichannel pipette, 10 extra wells worth should be adequate.

Pipette required volumes of DNase I buffer and Proteinase K buffer into clean tubes.

6 Prepare DNase I solution. For every mL of DNase I digestion buffer needed, add 50 U of DNase I. Mix by inverting or by pipetting. Briefly spin down.

7 With a P10 pipette, place 2 µL of AAV sample into wells of a 96-well plate in triplicate. Load 2 µL of DPBS + 0.001% Pluronic F-68 into 3 more wells. Do not load samples into wells at the edge of the plate. Add 50 µL of DNase I solution (Step 5) to wells with AAV or DPBS + 0.001% Pluronic F-68. Surround the sample wells with 200 µL of UltraPure water, then seal plate and centrifuge briefly. Incubate at 37 °C for 01:00:00.

1h

Note

Surrounding the sample wells with UltraPure water helps to mitigate volume loss due to evaporation. Because the sample is relatively high concentration at this stage (it will be diluted more later), small changes to the sample volume will have large effects on the resulting titer. Ensuring that the plate is well sealed will also help to reduce evaporation.

8 After incubation, briefly centrifuge plate and carefully remove and discard the seal. Add 2.5 µL of 0.5M EDTA, pH 8 to each sample well, and mix by pipetting. Reseal plate with a clean seal, centrifuge briefly, and incubate at 70 °C for 00:10:00 . 10m

Note

We do not re-use seals, as even small amounts of liquid transfered between wells can have a large effect on the measured titer.

CRITICAL
Incubation at 70 °C inactivates DNase I, but also denatures AAV capsids, leading to AAV genome release. Adding EDTA chelates Ca^{2+} and Mg^{2+} ions that are necessary for DNase I function. Failure to add EDTA to samples can lead to digestion of AAV genome by DNase I molecules that are still active, leading to a reduction in the measured titer.

9 Prepare proteinase K solution. For every mL of Proteinase K buffer needed, add 100 µg of Proteinase K. Mix by inverting or pipetting, and briefly spin down.

10 Once 70 °C incubation is completed, allow plate to cool to room temperature, then centrifuge. Carefully remove and discard the seal. Add 60 µL of Proteinase K solution to each sample well. Reseal plate with a clean seal, centrifuge briefly, and incubate at 50 °C for 02:00:00 to Overnight . 2h

Note

Ensure that the plate is well sealed, as evaporation at this stage will confound accurate measurement of sample titer. In addition, using a heating block or thermocycler with heated lid can help to reduce evaporation. If a heat source with a heated lid is not available, limit incubation time to 02:00:00 to mitigate sample evaporation.

11 Inactivate the Proteinase K by incubating plate at 95 °C for 00:10:00. Following incubation, plate can be stored at 4 °C for a few days. 10m

Note

If using a thermocycler, this step can be programmed in and followed by a 10 °C hold.

At this stage, the AAV genomes have been released from the capsid and are in solution. The samples are stable at 4 °C, but some DNA loss can occur due to adsorption of the DNA to the plastic of the plate. Thus, it is not recommended to leave processed samples at 4 °C for longer than a few days.

Sample and standard dilution, and qPCR

12 Before starting, create a "plate map" and determine the number of qPCR reactions you will run. In total three 96-well plates will be needed:

1. The sample plate: this is the plate that you have already prepared, containing the DNase I- and Proteinase K-treated AAV samples, as well as the negative control.
2. The dilution plate: this plate will be used to create the standard dilution series and to further dilute the samples. As with the samples, each standard concentration is run in triplicate.
3. The qPCR plate: this plate will be used to set up and run the qPCR reactions.

The dilution plate and the qPCR plate will have the same layout. 24 wells will be used for the standard, which will occupy wells A1-B12. The processed samples will be added starting at well C1. Finally, a water control should also be used, loaded after the samples.

13 Retrieve sample plate and centrifuge to collect liquid at bottom. Carefully remove and discard seal. Add 120 µL of UltraPure water to sample wells, and mix by pipetting. Set plate aside.

Note

This dilution (i.e. adding 120 µL of UltraPure water) and the subsequent 1:100 dilution are to ensure that the sample loaded into the qPCR reaction falls within the linear range of the standard. Failure to dilute the samples can yield CT values that are outside the CT values for the standard.

14 Prepare standard dilutions. In a new 96-well plate, add 5 µL of linearized standard to wells B10-B12. Then add 45 µL of UltraPure water to each well of rows A and B (24 wells total). When adding 45 µL of UltraPure water to wells B10-B12, mix well by pipetting. This will create a 1:10 dilution of the standard stock in wells B10-B12.

Seal plate and centrifuge, then carefully remove and discard seal. Using 3 tips on a P10 multichannel pipette, prepare serial dilution of the standard. Working backwards, pipette 5 µL from B10-B12 into B7-B9 (i.e. the standard from B10 will be diluted in B7), mix by pipetting up and down 20 times to mix, then transfer 5 µL from wells B7-B9 into wells B4-B6, pipetting up and down 20 times to mix. Continue this process until wells A1-A3.

Note

CRITICAL

Use the same three P10 tips for the entire dilution series. When pipetting, only expel to the first stop; expelling to the second stop can add air bubbles to the wells that can prevent accurate transfer of 5 µL to the next 3 wells. When transferring liquid visually check the liquid level in each pipette and ensure no bubbles are present.

After transferring liquid from the previous 3 wells, be sure to thoroughly mix the contents by pipetting at least 20 times. Avoid bubbles. Thorough mixing is critical to making a standard curve that is linear over a wide dynamic range and with little variation between replicates.

Once completed, you should have a standard dilution series encompassing 8 orders of magnitude, with the highest concentration around 1 ng/µL (wells B10-12). All the wells should have 45 µL of liquid, except wells A1-A3, which will have 50 µL .

15 Prepare sample dilutions. In the same 96-well plate as the standard, add 2 µL of DNase I-, Proteinase K-digested samples (Step 13) to the wells below the standard, starting at well C1. Add 198 µL of UltraPure water to the 2 µL and mix by pipetting. Seal and spin down the dilution plate.

The sample plate can be sealed and stored at 4 °C in case it needs to be used again.

Note

When transferring the 2 µL visually inspect each tip to ensure there are no bubbles and that the liquid level is the same for each tip. Small variations in the amount of transferred liquid can lead to large errors in the measured titer.

16 Set up the qPCR mastermix, according to manufacturer directions. Transfer 2 µL of diluted standards and samples (from dilution plate, Step 15) to qPCR plate, add master mix, and seal with an appropriate seal. Run the qPCR reaction according to manufacturer directions.

Note

When transferring the 2 µL visually inspect each tip to ensure there are no bubbles and that the liquid level is the same for each tip. Small variations in the amount of transferred liquid can lead to large errors in the measured titer.

17 Analyze results using Supplementary Table 4 from Challis et al., *Nature Protocols* 14: 379.

Note

When analyzing titration results, ensure that the relationship between CT values and standard DNA concentration is linear over the region in which the sample CTs fall. If it is not, repeat the protocol starting from Step 14, re-using the sample plate. Generally, we achieve a strong linear relationship between standard concentration and resulting CT values (R^2 > .99).

The technical replicates, for both standard and sample dilutions, should not vary by more than 0.5 CTs. If you observe variation >0.5 between CT values of technical replicates, consider excluding certain values, or repeating the entire protocol.

Ensure that all information in Column B is correct. The molecular weight of the DNA standard and Qubit concentration are necessary to determine how many molecules are in each dilution of the standard. Likewise, the dilution factors are necessary to determine the concentration of the original AAV stock. If the volumes in this protocol are used, change the values for:

- Cell B16 to 234.5 µL (2 µL of AAV sample + 50 µL of DNase I solution + 2.5 µL of EDTA + 60 µL of Proteinase K solution + 120 µL of UltraPure water). Also change the value
- Cell B17 to 100 (i.e. a 1:100 dilution, Step 15).

If following this protocol, the final dilution factor should be 11725.

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