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Absolute quantification of AAV genomes from total DNA with digital droplet PCR (ddPCR)

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We use this protocol and it's working

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Abstract

Accurate and absolute determination of AAV transduction from total DNA is important for understanding gene transfer efficiency across multiple experimental manipulations. As compared to relative measurements (e.g. through comparison of qPCR CT values between experimental conditions), absolute quantification can provide biological insights (e.g. actual average MOIs) that can guide further development of these vectors. This protocol enables absolute measurement of AAV genome number from total DNA samples extracted from animal tissues. Because total DNA is extracted, it is possible to estimate the number of AAV genomes per cell, using DNA content per cell and an estimate of transduction efficiency.

Materials

UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625

Phosphate buffered saline powder, pH 7.4, for preparing 1 L solutions **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3813

Heparin sodium salt from porcine intestinal mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3149

Total DNA extraction

QIAgen DNeasy Blood and Tissue Kit, 50 rxn **Qiagen Catalog #**69504

Probe and primer sets to amplify target sequences.

Note

We purchase primer and probes as pre-mixed assays from IDT (PrimeTime qPCR Assays), with 2.5 nmole of a double-quenched FAM- or HEX-labeled probe and 9 nmole of each primer.

Re-suspending this in 200 µL of TE buffer yields a 50x stock for the suggested ddPCR mix, with primer concentrations of 45 µM and a probe concentration of 12.5 µM .

Digital Droplet PCR reagents

ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863024

Droplet Generation Oil for Probes **Bio-Rad Laboratories Catalog #**1863005

ddPCR™ Droplet Reader Oil **Bio-Rad Laboratories Catalog #**1863004

ddPCR 96-well plates **Bio-Rad Laboratories Catalog #**12001925

PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #**1814040

DG8™ Cartridges and Gaskets **Bio-Rad Laboratories Catalog #** #1864007

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.

Ethics statement

Animal husbandry and all procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) and by the Office of Laboratory Animal Resources at the California Institute of Technology.

Reagent set-up

1 Before beginning, ensure that you are able to specifically detect your AAV genome and design PCR reactions. We recommend using probe-based assays and designing 2 separate assays against distinct sequences in your target genome, with 2 separate fluorophores to detect each target sequence separately. During analysis, only count spots positive for **both** target sequences as true positives.

> **Note**
>
> This protocol is for quantifying absolute AAV genome count from total DNA, and thus can be used to estimate average MOI by incorporating per cell DNA content and transduction rate.
>
> Because total DNA is used, amplification of off-target sequences in host genome can increase the false positive rate. This protocol mitigates this by amplifying 2 separate target sequences, readout by spectrally distinct probes. Only droplets positive for both target sequences are considered as true positives.

Sample preparation and total DNA extraction

2 General note on sample preparation

> **Note**
>
> This protocol should work with either fixed or fresh tissues. However, because DNA recovered from fixed tissue will be more fragmented, for best results we recommend processing fresh tissue. Thus, if fixed samples are also required for parallel analyses, it may be best to post-fix only.
>
> E.g. for analysis of transduction in brain by both ddPCR and by fluorescence microscopy, post-fix one hemisphere overnight in 4% PFA, 1x PBS, and microdissect the other hemisphere fresh for ddPCR.

3 Transduce animals with AAVs, carrying sequence that will be amplified in droplet PCR reaction.

4 On day of harvest, prepare work area. You will need:

- ice-cold 1x PBS

- ice-cold 1x heparinized PBS
- clean 10 cm petri dishes
- ice
- tools for perfusion and microdissection
- 1.5 mL tubes for tissue collection
- dry ice (optional; if choosing to snap freeze tissue, rather than processing immediately)
- dry ice-ethanol slurry (optional; if choosing to snap freeze tissue, rather than processing immediately)

Pre-label collection tubes. If planning to freeze tissue, use an ethanol-resistant marker

If analysis of fixed tissue is also desired (e.g. for detection of an AAV-encoded fluorescent protein), prepare tubes with appropriate volumes of 4% PFA in 1x PBS and keep on ice. In addition, tissue may be collected and homogenized, dissociated, or snap-frozen for other downstream analyses.

Note

Collection time is dependent upon experimental question. This protocol detects the AAV's DNA genome, so does not rely on reporter gene expression.

5 Begin tissue collection. Euthanize animals according to your lab's approved protocol, then transcardially perfuse animals with 30 mL of heparinized PBS. Working quickly, dissect out and place tissue of interest into a petri dish with ice-cold PBS, then isolate regions of interest. Dissect tissues of interest into 2-3 mm cubes (maximum 25 mg of wet tissue weight), and transfer to 1.5 mL tubes.

If processing immediately, keep tissue on ice until ready to process (Step 6).

If not processing immediately, snap-freeze tissue by immersing tubes in the dry ice-ethanol slurry, and store on dry ice until ready to transfer to -70 °C . Once ready to begin processing, remove from -70 °C and proceed to step 6.

If also collecting tissue to fix, gently drop tissue into 4% PFA in 1x PBS, and post-fix at 4 °C Overnight , then proceed with standard procedures. Fresh tissue may also be harvested and homogenized, dissociated or snap-frozen for other downstream analyses.

6 Process samples with Qiagen DNeasy Blood and Tissue total DNA purification kit. Follow manufacturer instructions for total DNA extraction.

Measure [DNA] using a spectrophotometer.

If processing immediately, total DNA samples can be kept on ice or at 4 °C until ready to proceed. Alternatively, store at -20 °C or -70 °C until ready to proceed.

Restriction enzyme digest

7 Note about restriction enzyme digest

> **Note**
>
> ddPCR detects molecules that contain 1 or more copies of each target sequence. Because, AAV genomes concatemerize in host cells (one AAV molecule containing multiple genome copies), it is necessary to digest AAV concatemers into constituent genomes so that they can be partitioned into separate droplets and accurately quantified.

8 Choose restriction enzymes that cut AAV genome in or near ITR regions, and that do not cut within target amplicons. Ideally, choose enzymes that:

1. can be used in a single reaction
2. can be safely used in Overnight digests
3. can be heat inactivated

> **Note**
>
> We have used (a) single digests with SmaI, which digests within the AAV2 ITR, and (b) double digests with KpnI-HF and SpeI-HF, which cut beside the AAV2 ITR in our vector genomes. When used on the same samples, both of these digest paradigms produced comparable results.

9 Set up digest reactions.

Combine:

- Total DNA: 1000 ng
- Restriction enzyme 1: 20 U
- Restriction enzyme 2 (if necessary): 20 U
- Appropriate buffer (e.g. CutSmart): 2 µL

- UltraPure water: Bring to 20 µL

Incubate in a thermocycler with a heated lid. Use manufacturer-suggested incubation temperature (e.g. 37 °C), and heat-inactivate, if possible.

To ensure complete digestion, we recommend digesting samples Overnight (if allowable by chosen restriction enzymes).

Note

Given the multitude of parameters that can be optimized (restriction enzyme choice(s), enzyme concentrations, incubation times, etc.), we recommend initially testing two separate restriction enzyme choices in overnight reactions, and comparing results.

10 Following digest and heat-inactivation, samples can be stored at -20 °C or -70 °C , or at 4 °C for immediate processing.

DNA dilution and digital droplet PCR

11 Note about optimal dilution

Note

CRITICAL It is important to dilute samples so that the target molecules are within the dynamic range of ddPCR. The optimal dilution will be dependent on multiple factors (viral dose, tissue, etc.) and may need to be determined empirically. We recommend either (a) running a 10-fold serial dilution on *all* samples, starting from a 1:10 dilution or (b) optimizing dilution on a single sample and then repeating with all samples at optimal dilution.

For measuring AAV transduction of cortex and liver by AAV-PHP.eB, injected retro-orbitally into C57BL/6J male mice at 3e11 vg, we found that a 1/10 dilution of a 50 ng/µL digested total DNA prep was optimal.

12 Make a plan for digital droplet PCR. We recommend running at least 2 technical replicates per sample, and multiplexing 2 separate target sequences in the same wells, using different fluorophores. Prepare extra to account for pipetting error.

For each reaction, you will need:

- Diluted DNA sample(s): 5 µL
- 50x probe/primer mix 1: 0.5 µL
- 50x probe/primer mix 2: 0.5 µL
- UltraPure water: 6.5 µL
- ddPCR Supermix for Probes: 12.5 µL

Combine all components, except DNA samples. Aliquot 5 µL of DNA to PCR strip tubes, then add 20 µL of above mastermix. Mix by tapping and spin down.

Note

We purchase primer and probes as pre-mixed assays from IDT (PrimeTime qPCR Assays), with 2.5 nmole of a double-quenched FAM- or HEX-labeled probe and 9 undetermined of each primer.

Re-suspending this in 200 µL of TE buffer yields a 50x stock for the suggested ddPCR mix, with primer concentrations of 45 µM and a probe concentration of 12.5 µM .

13 Prepare droplets with 23 µL of PCR reaction and 70 µL of droplet generation oil. Follow manufacturer directions for droplet generation.

Once droplets are generated, transfer 46 µL of droplets to PCR plate. Once all samples are prepared, seal PCR plate with a pierceable heat seal and run PCR according to manufacturer instructions.

14 Once PCR is complete, analyze droplets with droplet reader. Follow manufacturer directions for droplet reader.

Data can be analyzed using Bio-Rad QuantaSoft Software. Count droplets that are positive for both target sequences as true positives.

The following example may help in converting from copies/µL in the ddPCR reaction to a more meaningful metric (copies/ng of original total DNA sample):

- You digested 1000 ng of total DNA in a 20 µL, then diluted that 1:10 and loaded 5 µL into a 25 µL ddPCR reaction. 23 µL of the ddPCR reaction was used to generate droplets, and 46 µL of emulsification was read by the droplet reader.
- After analysis, you measured 500 copies/µL. Note that this number corresponds to the concentration in the ddPCR mix.
- If there were 500 copies/µL in the 25 µL ddPCR reaction, then there were 12500 copies total in the PCR reaction (500 x 25 = 12,500)
- Those 12500 copies were loaded in 5 µL of diluted DNA sample; thus the diluted DNA sample had 2500 copies/µL (12,500 / 5 = 2500).
- That diluted sample was a 1:10 dilution of the original, so the original sample had 25000 copies/µL
- 1000 ng of total DNA was digested in a 20 µL reaction, so the undiluted sample had a DNA concentration of 50 ng/µL.
- Thus, the original sample had an AAV genome concentration of 25000 copies/µL / 50 ng/µL = 500 copies/ng