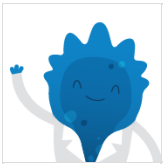

Jul 02, 2020

ddPCR titration of AAV vectors

Addgene The Nonprofit Plasmid Repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.bef8jbrw

Addgene The Nonprofit Plasmid Repository
Addgene

ABSTRACT

This protocol describes ddPCR titration of AAV vectors. To see the full abstract and additional resources, visit https://www.addgene.org/protocols/aav-ddpcr-titration/.

Sample Data:

- When analyzing data there should be a clear distinction between negative droplets (black) and positive droplets (blue).
- The no template control (NTC) should be close to zero (B08). At Addgene, runs with an NTC >5 are invalid.
- To reduce NTC values, we recommend wiping down all pipettes and equipment with 10% bleach prior to use and keeping all reagents and samples on ice or pre-chilled 96-well freezer blocks during use.
- In this protocol, a dilution series is prepared for each AAV sample and the 3 final dilutions are assayed. The samples that are assayed are diluted 2-fold serially therefore, the concentration obtained by ddPCR should decrease by a factor of 2 across the dilutions.
- In the example below, 2-fold serial dilutions of a sample were loaded in wells A04, A05 and A06. As shown in the image and table below, the concentration of positive droplets decreases by a factor of ~2.
- To increase the accuracy of the titer, calculate an average of several dilutions.

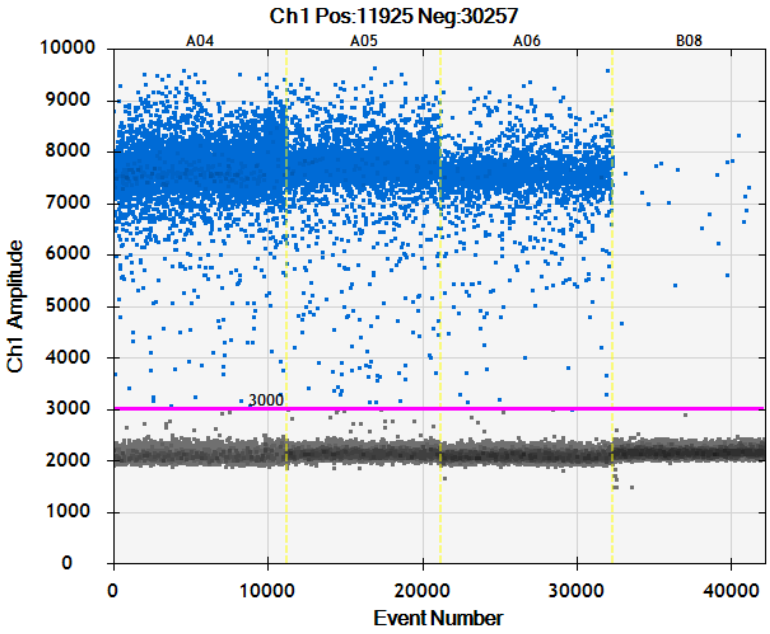

Figure 1: ddPCR sample data

Citation: Addgene The Nonprofit Plasmid Repository (07/02/2020). ddPCR titration of AAV vectors. https://dx.doi.org/10.17504/protocols.io.bef8jbrw

Sample	Dilution	Concentration	Titer (GC/mL)	Average GC/ml
AAV Sample 1	6400000	965	3.09E+13	3.2E+13
	12800000	474	3.03E+13	
	25600000	271	3.47E+13	
NTC	N/A	2.5	N/A	N/A

Table: Example dilutions and titration table

For additional tips on AAV titering using ddPCR, read our blog post.

EXTERNAL LINK

https://www.addgene.org/protocols/aav-ddpcr-titration/

DOI

dx.doi.org/10.17504/protocols.io.bef8jbrw

PROTOCOL CITATION

Addgene The Nonprofit Plasmid Repository 2020. ddPCR titration of AAV vectors. **protocols.io** dx.doi.org/10.17504/protocols.io.bef8jbrw

EXTERNAL LINK

https://www.addgene.org/protocols/aav-ddpcr-titration/

KEYWORDS

ddPCR, titer, AAV, ITR

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GUIDELINES

- This protocol was modified from the publication Lock M, Alvira MR, Chen SJ, Wilson, 2014.
- The dilution series outlined in this protocol is based on an AAV titer range of 5E+12 - 5E+13 GC/mL. Users may need to run lower or higher dilutions depending on their particular sample.

MATERIALS TEXT

Equipment:

- Class II, Type A2 Biological Safety Cabinet, Labconco 302411100
- Droplet Digital PCR System, Bio-Rad, DX200
- Thermal Cycler, Bio-Rad, T100
- PCR Plate Sealer, Bio-Rad, PX1
- 1-10µL single channel pipette
- 20-200µL single channel pipette
- 200-1000µL single channel pipette
- 1-10µL multichannel pipette
- 2-50µL multichannel pipette

- 20-200µL multichannel pipette
- Vortex, VWR, 10153-688
- Mini Centrifuge, Thermo Scientific, 10199-452
- Ice bucket
- 96-well freezer blocks (x3)

Reagents and Consumables:

- Molecular Biology Grade Water, Hyclone, SH30538.02
- GeneAmp 10X PCR Buffer, Applied Biosystems, N8080129
- ddPCR Supermix for Probes no dUTP, Bio-Rad,1863023
- 10% Pluronic F-68, Thermo Fisher, 24040032
- Droplet generation oil, Bio-Rad, 1863005
- DG8 cartridge, Bio-Rad, 1864008
- DG8 gasket, Bio-Rad, 1863009
- DG8 cartridge holder, Bio-Rad, 1863051
- 8-strip PCR tubes, Axygen, PCR-02-FCP-C
- ddPCR 96-well PCR plates, Bio-Rad, 12001925
- 48-well dilution plate, Bio-Rad, MLL4801
- Pierceable Foil Heat Seal, Bio-Rad, 1814040
- Microseal adhesive seal, Bio-Rad, MSB1001
- Polystyrene Reservoirs, VWR, 89094-662
- Microcentrifuge tubes, VWR, 87003-294
- Primers/probe targeting ITR:
  - ITR Forward Primer: 5'-CGGCCTCAGTGAGCGA
  - ITR Reverse Primer: 5'-GGAACCCCTAGTGATGGAGTT
  - ITR Probe: -FAM-CACTCCCTCTCTGCGCGCTCG-BBQ-

Reagent Preparation:

- 1X PCR Buffer containing 0.05% Pluronic F-68:
  - Prepare immediately before use and vortex before using.
  - **500 µl** of GeneAmp 10X Buffer
  - **25 µl** of 10% Pluronic F-68
  - **4475 µl** Molecular Biology Grade Water

SAFETY WARNINGS

AAV is generally considered biosafety level 1 but may require BSL-2 handling depending on the insert. Please ensure that you are in compliance with your institution's biosafety regulations.

DISCLAIMER:

BEFORE STARTING

- To reduce the risk of contaminating reagents we recommend making small aliquots of master mixes, primers and probes prior to use.
- Thaw the master mix, primers, and probe on ice before use.
- Wipe down all pipettes and surfaces with 10% bleach.

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Preparation

1 Before handling any viruses get materials ready.

2 Ensure that primers, probe, 10X PCR buffer, and master mix are thawed.

3 Vortex primers, probe and master mix for **00:00:15** then spin **00:00:10** in a mini centrifuge and place **On ice** .

4 Wipe down a DG8 cartridge holder with bleach and place in the Biological Safety Cabinet (BSC).

5 Make sure that the BSC to be used for dilution and the BSC to be used for droplet generation are supplied with sufficient pipette tips and reagent reservoirs.

> ***Pro-Tip***
> Prepare the viral dilution in a separate biological safety cabinet (dilution BSC) than that used for master mix preparation and droplet generation (droplet generation BSC).

6 Pre-warm the 96-well plate sealer by gently touching the screen.

Prepare the Serial Dilution

7 Place a 48-well dilution plate in a chilled 96-well freezer block and place in the dilution BSC.

8 Prepare 1X dilution buffer (see recipe in reagent section).

9 Pour the 1X dilution buffer into a polystyrene reagent reservoir.

10 Using a 20-200µl multichannel pipette, carefully add the dilution buffer to the dilution plate according to the dilution scheme listed in step 12.

11 Use a single channel 1-10µl pipette to add **5 µl** of each viral sample to Dilution 1 in the 48-well dilution plate and pipette 5-10 times to mix.

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12 Dilute the virus as follows: Mix dilutions thoroughly, but pipette slowly to avoid generating aerosols.

- **Dilution 1 (20X):** **5 µl** in **95 µl** H_2O (1:20)
- **Dilution 2 (20X):** **5 µl** in **95 µl** H_2O (1:400)
- **Dilution 3 (20X):** **5 µl** in **95 µl** H_2O (1:8,000)
- **Dilution 4 (20X):** **5 µl** in **95 µl** H_2O (1:160,000)
- **Dilution 5 (20X):** **5 µl** in **95 µl** H_2O (1:3,200,000)
- **Dilution 6 (20X):** **50 µl** in **50 µl** H_2O (1:6,400,000)
- **Dilution 7 (2X):** **50 µl** in **50 µl** H_2O (1:12,800,000)
- **Dilution 8 (2x):** **50 µl** in **50 µl** H_2O (1:25,600,000)

13 Use multichannel pipettes for the dilution series.

- For dilutions 1-5, use the 1-10µL multichannel pipette set to **5 µl** .
- For dilutions 6-8, use the 20-200µL multichannel pipette set to **50 µl** .
- For mixing between dilutions, use the 20-200µL multichannel pipette set to **90 µl** and mix by pipetting the liquid up and down 10-20 times.

14 Gently cover the entire dilution plate with Microseal adhesive seal - do not press the film just gently cover so nothing falls into the plate.

15 Leave dilutions on the chilled 96-well freezer block in the BSC until ready to use.

Prepare the Master Mix

16 Place a ddPCR plate onto a chilled 96-well freezer block and set aside in the droplet generation BSC to cool.

17 Prepare the ITR master mix in a microcentrifuge tube as shown below. For 8 samples prepare enough master mix for 9 samples.

ITR Master Mix	Volume	9X Volume	Final Concentration
2X ddPCR Supermix for Probes, no dUTP	10µL	90µL	1X
10uM ITR probe (FAM)	0.5µL	4.5µL	250nM
Forward ITR Primer (10uM)	1.8µL	16.2µL	900nM
Reverse ITR Primer (10uM)	1.8µL	16.2µL	900nM
Nuclease-free water	7.95µL	71.55µL	
Total Volume	20µL		

Table: ITR Master mix for 9 samples

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18 Vortex the master mix for **00:00:15** and spin in a mini centrifuge for **00:00:10** before use.

19 Place an 8-well PCR tube strip into a chilled 96-well freezer block.

20 Add **20 µl** of the master mix to each PCR tube. Be careful to dispense to the bottom of the tube without collecting drops along the side of the tube.

21 Cap gently - do not push the cap in all the way, just ensure the samples are covered.

22 Bring the PCR tubes to the BSC used for dilutions.

23 Without disturbing the liquid in the tube, gently uncap the PCR tubes.

24 Add **5 µl** of dilutions 6-8 to the appropriate PCR tubes. Pipette back and forth 5 times.

25 Lightly cap the PCR tubes.

Generate the Droplets

26 Bring the PCR tubes to the droplet generation BSC.

27 Without disturbing the liquid in the tube, gently uncap the tubes.

28 Add **5 µl** nuclease-free water to the NTC tube.

29 Place a DG8 cartridge into the cartridge holder.

30 Using a 2-50µL multichannel pipet, load **20 µl** of the reaction mixtures into the middle wells of the cartridge.

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31 Add **800 µl** of droplet generation oil to a polystyrene reagent reservoir.

32 Using the 20-200µL multichannel pipet, load **70 µl** of droplet generation oil into the bottom row of wells.

33 Cover the cartridge with the DG8 gasket, making sure that it is secure.

34 Transfer the cartridge holder to the droplet generator. Close the lid and wait for the droplets to be generated.

35 Once the droplets have been generated, use a 20-200µL multichannel pipet to aspirate **40 µl** of droplets.

Pro-Tip
To ensure that the droplets are not disrupted insert the pipette tips directly in the center of the well and tilt to a 45^{o} angle. Count to 20 while slowly and gently aspirating the droplets.

36 Transfer the droplets to a prechilled PCR plate.

Pro-Tip
To ensure that the droplets are not disrupted insert the pipette tips and gently touch the bottom of the well. Lift the tips ~1mm. Touch the side of the well and tilt the pipette tips at a 45^{o} angle. Count to 20 while slowly and gently dispensing the droplets down the side of the tube.

37 Place a Pierceable Foil Heat Seal on the PCR plate with the red line facing up. If the plate sealer is not at temperature, touch the screen on the plate sealer to allow it to get to temperature. Once the temperature is reached, place the PCR plate with the foil onto the metal support block. Place the block in the plate sealer and press the 'Seal' button.

38 After the plate has been sealed, proceed to thermocycling.

Thermal Cycling

39 Run the following PCR parameters.

Cycling Step	Temperature (deg C)	Time (min)	Ramp Rate (deg C/sec)	# Cycles
Denaturation	95	10	2	1
Denaturation	95	0.5	2	50

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Annealing/ Extension	60	1	2	50
Signal Stabilization	98	10	2	1
Hold	4	∞	2	1

Table: ddPCR parameters

40 After PCR is complete, transfer the plate to the Droplet Reader.

41 Open the QuantaSoft software to set up a new plate layout. Designate the sample name, experiment type, supermix type (ddPCR Supermix for Probes), the target names and target types.

42 When the plate layout is complete, select 'Run' to begin the droplet reading.

43 When the droplet reading is complete, export the data from all wells as a CSV file which will be used to calculate the titer.

T = {[(R*C)(1000/V)]*D}

T = GC/mL R = reaction volume (20µL)
C = Copies/µL
V = volume of virus in reaction mix (4µL)
D = Dilution factor of virus

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