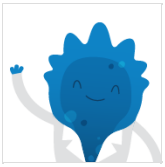

Jul 02, 2020

ddPCR Titration of Lentivirus Vectors

Addgene The Nonprofit Plasmid Repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.be7ijhke

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ABSTRACT

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

This protocol was modified from the publication Wang Y, Bergelson S, Feschenko M, 2018.

Sample Data:

- When analyzing data there should be a clear distinction between negative droplets (black) and positive droplets (blue/green).
- The concentration of RRE positive droplets in the untransduced control should be close to zero (A01).
- In this protocol, the lentiviral particles are serially diluted and used to transduce HEK293T cells. Genomic DNA is extracted from the target cells and assayed for integrated copies of RRE. Since the samples that are assayed are diluted 2-fold serially, the concentration of RRE positive droplets should decrease by a factor of 2 across the dilutions. RPP30 copies should be relatively constant across samples.
- In the RRE example below, 2-fold serial dilutions of a sample were loaded in wells B01-H01.
- As shown in the image and table below, the concentration of RRE positive droplets increases by a factor of ~2 as you progress from the higher dilutions to the lower dilutions (blue).
- The concentration of RPP30 positive droplets stays relatively even across samples (green).
- To increase the accuracy of the titer, calculate and average of several dilutions.

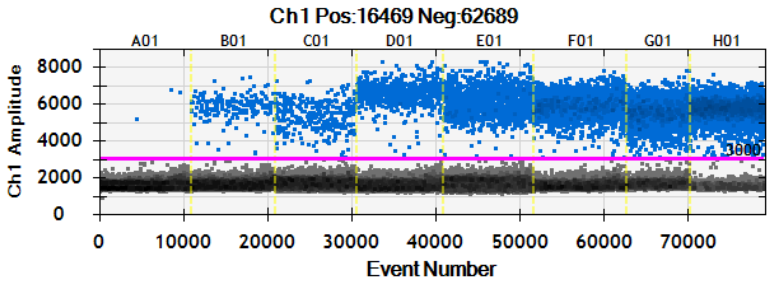

Figure 1: ddPCR Lentivirus sample data, RRE positive (blue) and negative (black) droplets

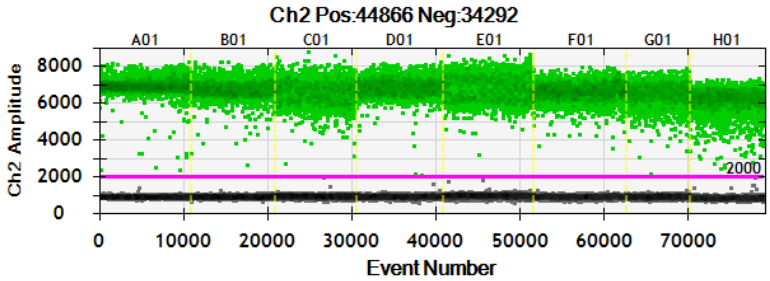

Figure 2: ddPCR Lentivirus sample data, RPP30 positive (green) and negative (black) droplets

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Sample	Dilution	RRE Copies/µL)	RPP30 (Copies/µL)	Viruses/Genome	Titer (TU/mL)	Average TU/mL
Lentivirus Sample 1	800	428	22020	0.0466	7.46E+07	7.4E+07
	400	768	18360	0.0970	7.76E+07	
	200	1620	15840	0.1577	6.31E+07	
	100	3180	20540	0.3724	7.45E+07	
	50	8960	17080	0.7387	7.39E+07	
	25	14440	24260	1.6098	8.05E+07	
Untransduced	N/A	6.4	17940	0.0006	N/A	N/A

Table: Example dilutions and titrations

EXTERNAL LINK

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

DOI

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PROTOCOL CITATION

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EXTERNAL LINK

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KEYWORDS

ddPCR, titer, Lentivirus, RRE

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GUIDELINES

Workflow Timeline

- **Day 1:** Seed and transduce cells
- **Day 4:** Treat cells with benzonase and harvest cells
- **Day 4+:** ddPCR and analysis

MATERIALS TEXT

Equipment:

- class II, Type A2 Biological Safety Cabinet, Labconco 302411100
- aspirating Unit
- microcentrifuge, Eppendorf, 5425R

- 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
- 2-50µL multichannel pipette
- 20-200µL multichannel pipette
- ice bucket
- 96-well freezer blocks
- vortex, VWR, 10153-688
- mini Centrifuge, Thermo Scientific, 10199-452

Reagents and Consumables:

- GeneJet Genomic DNA Purification Kit, Thermo Fisher, K0721
- 6-well tissue culture treated dish, VWR, 29442-042
- DMEM, high glucose, pyruvate, Corning, 10-013-CV
- heat inactivated premium grade, fetal bovine serum, Seradigm, 1500-050H
- Glutagro, 200 mM, Corning, 25-015-CI
- 1X Phosphate Buffered Saline, Corning, 21-040-CV
- TrypLE, Thermo Fisher, 12605010
- ethanol, VWR, EX0276-1
- benzonase 250U/µl, Millipore #71205-3
- polybrene 10mg/ml, Millipore, TR-1003-G
- molecular Biology Grade Water, Hyclone, SH30538.02
- ddPCR Supermix for Probes no dUTP, Bio-Rad,1863023
- 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
- pierceable Foil Heat Seal, Bio-Rad, 1814040
- polystyrene Reservoirs, VWR, 89094-662
- microcentrifuge tubes, VWR, 87003-294
- primers/probe targeting RRE:
  - forward primer: tgtgccttggaatgctagt
  - probe (FAM): tttggaatcacacgacct
  - reverse primer: aatttctctgtcccactccatc
- PrimePCR ddPCR Copy Number Assay: RPP30, Human, Bio-Rad, 10031244

Reagent Preparation:

- DMEM Complete
  - **500 mL** DMEM, high glucose, pyruvate
  - **55 mL** Heat inactivated premium grade, fetal bovine serum
  - **5 mL** Glutagro
- 50U/mL benzonase
  - **15 mL** DMEM Complete
  - **3 µl** of 250U/µL Benzonase

SAFETY WARNINGS

Lentivirus is generally considered biosafety level 2+. Please ensure that you are in compliance with your institution's biosafety regulations.

BEFORE STARTING

- Thaw the master mix, primers/probe mixes and samples on ice before use.
- Wipe down all pipettes and surfaces with 10% bleach.

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Transducing Cells

1 Thaw the virus **On ice** .

2 Prepare the following dilution series of virus in DMEM complete:

- When adding the virus to the diluent, pipette 5 times, to remove virus from pipette tip.
- To mix each dilution, set the P200 pipette to **200 µl** and pipette 10-20 times.
- Use a new pipette tip to transfer the virus to the next dilution.
- Repeat for all dilutions.

Dilution Factor	Virus	Media	Total Volume
2.5	160µL viral stock	240µL	400µL
5	200µL of 2.5-fold dilution	200µL	400µL
10	200µL of 5-fold dilution	200µL	400µL
20	200µL of 10-fold dilution	200µL	400µL
40	200µL of 40-fold dilution	200µL	400µL
80	200µL of 80-fold dilution	200µL	400µL
160	200µL of 160-fold dilution	200µL	400µL

Table: Dilution series of virus in DMEM complete

3 Add **150 µl** of each viral dilution to a well of a 6-well plate (1 dilution per well). Leave one well untransduced.

4 Seed 300,000 cells/well in **1350 µl** media and 11.1µg/mL Polybrene into the wells containing the virus and the untransduced control.

Pro-Tip
For even seeding, prepare a batch for 10 wells with 3,000,000 cells in 13.5 mL of media and 11.1µg/mL Polybrene. Mix the cell suspension well before seeding.

When seeding, **150 µl** of virus is being diluted an additional 10-fold in media therefore the final dilutions range from 25-1600-fold.

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5 Mix each well with a 1mL pipette 5-10 times.

The final volume in the well is 1.5mL and the final Polybrene concentration is 10µg/mL.

6 Mix well and incubate **72:00:00** .

Benzonase Digestion and Cell Harvest

7 Gently aspirate media from the 6-well plates.

8 Add **1.5 mL** of 50U/mL Benzonase in DMEM complete to each well.

9 Incubate at **37 °C** for **00:30:00** .

10 Gently aspirate media from wells.

11 Wash wells with **1 mL** of 1X PBS and aspirate.

12 Detach cells by incubating with **200 µl** TrypLE for **00:01:00** - **00:02:00** .

13 Resuspend cells in **500 µl** DMEM complete and transfer to a microcentrifuge tube.

14 Centrifuge for **00:05:00** at **100 x g** .

15 Gently aspirate supernatant.

16 Wash cell pellets in **500 µl** PBS.

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17 Centrifuge for **00:05:00** at **100 x g** .

18 Gently aspirate supernatant.

19 Extract genomic DNA according to the GeneJet Genomic DNA Purification Kit instructions.

20 Determine the concentration of each sample on a spectrophotometer.

21 Prepare 25ng/µL stocks of each sample. Samples can be used for ddPCR immediately or stored at **-20 °C** until ready to use.

Preparation for ddPCR

22 Thaw samples, primers/probe mixes, and master mix on ice.

23 Before handling any viruses get materials ready.

24 Vortex primers/probe mixes and master mix for **00:00:15** then spin **00:00:10** in a mini centrifuge and place on ice.

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

26 Make sure that the BSC is supplied with sufficient pipette tips.

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

Preparation of the Master Mix

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

29 Prepare the RRE/RPP30 master mix in a microcentrifuge tube as shown below. For 8 samples prepare enough master

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mix for 9 samples.

Component	Volume	9X Volume	Final Concentration
2X ddPCR Supermix for Probes, no dUTP	10µL	90µL	1X
20X RRE target primers/probe (FAM)	1µL	9µL	900nM/250nM
20X RPP30 primers/probe (HEX/VIC)	1µL	9µL	900nM/250nM
Nuclease-free water	4µL	36µL	
Total Volume	16µL	144µL	

Table: RRE/RPP30 Master Mix

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

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

32 Add **16 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.

33 Add **4 µl** of the 25ng/µL samples the appropriate PCR tubes. Pipette back and forth 5 times.

Generating the Droplets

34 Place a DG8 cartridge into the cartridge holder.

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

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

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

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

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

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40 Once the droplets have been generated, use a 20-200µL multichannel pipette 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-degree angle. Count to 20 while slowly and gently aspirating the droplets.

41 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-degree angle. Count to 20 while slowly and gently dispensing the droplets down the side of the tube.

42 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.

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

Thermal Cycling

44 Run the following PCR parameters:

Cycling Step	Temperature (oC)	Time (min)	Ramp Rate (oC/sec)	# Cycles
Enzyme Activation	95	10	2	1
Denaturation	94	0.5	2	40
Annealing/Extension	60	1	2	40
Enzyme Deactivation	98	10	2	1
Hold	4	∞	2	1

Table: PCR parameters

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

46 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.

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

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

Calculations

49 To calculate the titers, first calculate the number of viruses per genome:

Viruses/genome = 2*(Copies/20µL RRE)/(Copies/20µL RPP30)

50 Use the viruses/genome to calculate the infectious titer:

Infectious titer = (viruses/genome)*(#cells/well)*(dilution factor)/(virus volume)

51 Take the average infectious titer obtained from the appropriate dilutions to calculate the final infectious titer.

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