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2nd generation Lentiviral production

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We use this protocol and it's working

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Abstract

This is a protocol to amplify and purify plasmids (with gRNA of interest) and package into lentivirus for future transduction.

Materials

Equipment

Centrifuges/Ultracentrifuge (RT and 4 degree)
Tissue Culture Hood appropriate for viral work
Bunsen burner
37 degree incubators one static and one shaking

Materials

Single-gRNA Expression Lentiviral Vector
pxPAX2 (Addgene, #12260)
pMD2.G (Addgene, #12259)
Plastic loops
Agar plates with the appropriate antibiotic
Ampicillin/Or other appropriate antibiotic
Liquid Broth
Large plastic centrifuge tubes
PureLink Maxi-Prep kits
Isopropanol
Ethanol
TE bufffer
HEK 293T cells
FuGene HD (Promega, cat. no. E2311)
DMEM (Gibco, cat. no. 31966021)
FBS
Pen-Step (cat. no. 15070063)
Glutamax (cat. no. 35050038)
10cm cell culture dishes
Falcon and Eppendorf tubes
0.45um filters
PBS

Vector Design

1 Design Mammalian Single-gRNA Expression Lentiviral Vector. Typically we use Vector Builder which deliver E.Coli stocks.

Bacteria growth on agar plates

1d

2 For this you need plastic loops, bunsen burner and agar plates with the **appropriate** antibiotic based on vector design.

3 Sterilise plastic loop over bunsen flame right before dipping into bacteria stock

4 Dip into stock and streak onto agar plate. Make sure the agar plate is labelled!

5 Incubate agar plate overnight at 37 degrees for bacteria to grow in colonies.

Liquid Broth

1d

6 Add 500uL of ampicillin (**or appropriate antibiotic**) to bottle of LB broth, place LB broth into large glass flask (+1 extra for control)

7 Remove agar plates from 37 degrees and check for successful growth of colonies

8 Take a pipette tip and sterilise over bunsen burner flame, pick colony and place into flask - cover flask with foil (loosely)

9 As a control take a tip, sterilise and place into LB flask without picking a colony

10 Incubate flasks shaking at 37 degrees overnight

Maxi-Prep

2h

11 Large plastic tubes that fit into the centrifuge are needed for this step. Remove the flasks from the incubator - there should be visible growth of bacteria in all but control flask. Transfer contents of flask into plastic centrifuge tube (**without the tip**)

12 Weigh each tube - add PBS to make up difference (this is to ensure the centriguge is balanced. Centrifuge at 4000g 4 degrees for 10 minutes.

13 We use the PureLink Maxi-Prep kits from Thermo Fisher Scientific. Place the red plastic top onto a glass beaker and then the column on top. Add 30mL of EQ1 buffer to each column.

14 Remove platic tubbes from centrifuge - discard supernatant. Resuspend sediment in 10mL of R3 buffer and pipette up and down until cloudy.

15 Add 10mL of L7 lysis buffer to the tube - invert to mix 4-5 times. Incubate for 5 minutes

16 Add 10mL of N3 buffer and shake. Transfer contents to a labelled falcon tube and centrifuge 12000g for 10 minutes (or 4000g for 30 minutes)

17 Remove falcons from centrifuge - there should be clear layers. Pipette liquid into column, avoid top layer and bottom layer by tilting tube. Allow to filter through column

18 Add 60mL wash buffer to each column. Flow through can be discarded.

19 Take washed column and place on top of a labelled falcon tube. Add 15mL elution buffer to each column. Once eluted - throw column.

20 Add 10.5mL isopropanol to each falcon - shake and centrifuge for 30 minutes at 12000g at 4 degrees.

21 DNA should have pelleted at base of falcon. Discard supernatant. Add 5mL 70% ethanol to each DNA pellet. Further centrifuge for 10 minutes to remove any remaining bacteria.

22 Remove all liquid and allow pellet to air dry for 10 minutes.

23 Add 200-500uL TE buffer to resuspend DNA pellet, transfer to eppendorf. Nanodrop DNA to get the concentration. Store in freezer.

Lentiviral Production

1w

24 Prior to this make sure you have HEK 293T cells in culture. Media: 500mL DMEM, 10% FBS, 1% Pen-Step, 1% Glutamax. When confluent, plate into 10cm cell culture dish. Good to do this on a Friday so they are 80% confluent on a Monday ready for transfection. Do multiple dishes per plasmid - i.e. 3 dishes of HEK. Remember to scale the rest of the protocol depending o the number of dishes you are doing.

25 3-4 hours before transfection change media on HEKs (10mL) - aim for transfection to be in the afternoon (4pm)

26 Add 18uL FuGene HD to 300uL DMEM and incubate for 5 minutes.

27 Add 3ug transfer plasmid with 2ug psPAX2 and 1ug pMD2.G in 300uL of DMEM

28 Mix both reactions gently and incubate for 30 minutes at room temp

29 Add mixture (600uL) to HEK cells

30 18 hours after the transfection replace the media

31 Turn on Ultracentrifuge - set to 4 degrees.
Harvest the media after 48 hours into 50mL falcons (curve bottom) and centrifuge 1500rpm for 3 minutes at room temperature

32 Filter supernatant through 0.45um filter

33 Transfer to ultracentrifuge tubes. Weigh to make sure this is balanced.

34 Centrifuge for 3 hours at 48000g

35 Pellet will be hard to see - circle when you remove from centrifuge. Remove supernatant in the hood and resuspened pellet in ice cold sterile PBS. 3 dishes - 850uL of PBS. Leave these on ice in fridge/cold room overnight.

36 Next day aliquot virus - snap freeze and transfer to -80.