The RNAi Consortium (TRC)
Broad Institute

TRC Laboratory Protocols
Protocol Title: **Creation of large-scale shRNA pools**
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Brief Description:

This protocol describes production and amplification of pooled TRC pLKO.1-shRNA pDNAs for use in pooled shRNA screens. It also describes the packaging of lentivirus from pDNA pools.

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Overview:

Starting with normalized pDNA obtained from minipreps done in 96w format, collections of pDNAs are successively aggregated into larger pools stepwise with amplification in bacteria at each step. The steps described for formation of a 50,000 or 100,000 shRNA pool are as follows:

1. Pool normalized pDNA from one 96w plate to make a 'plate-pool'.
2. Pool 40 plate-pools to form a '4k' pDNA pool composed of ~3800 shRNA pDNA, expand in bacteria on agar, harvest pDNA.
3.Pool a dozen(s) 4k pools to form genome scale shRNA pools of 50-100,000 shRNAs, expand in bacteria on agar,, harvest pDNA.
4. Produce lentivirus in high volumes from complex pools of shRNAs.

Materials:

ElectroMax DH5a-E (Invitrogen Cat# 11 319-019)
Gene Pulzer Cuvette (0.1 cm electrode gap, BioRad 165-2089)
GenePulser Electroporation Unit (Bio-Rad)
Hispeed Plasmid DNA Maxiprep kit (Qiagen Cat# 12663)
24 cm bioassay plates (LB-agar + carb selection)
VSVG packaging plasmid (Addgene 12259)
pspax2 packaging plasmid (Addgene 12260)

Part 1: Create pDNA 'plate pools' from normalized miniprep DNAs (plate pool protocol)

After miniprep pDNAs are normalized (we normalize to15 ng/ul), a plate pool can be generated from each 96 well plate of miniprep pDNAs of library constructs.

1. Remove tips corresponding to the control wells on the plate (control pDNAs are not pooled)

2. Using a multichannel pipette, pipette 5ul pDNA from each well/racked tube on the plate except the control wells into a reservoir.(Robotics can be used in place of multichannel pipette)

3. Transfer the plate pool into a screw cap tube. Make separate aliquots if desired.

Part 2: Create 4k library by pooling about 40 plate pools and amplify on agar (4k library electroporation protocol)

1. Create 4k library by pooling 10ul pDNA from each plate pool of about 40 plate pools is pooled to create the starting 4k library. Adjust volume to be pooled from incomplete plates according to relative # of shRNAs on initial plate

2. 2ul of pre-transformed 4k library is mixed with 40ul ElectroMax DH5a-E (Invitrogen) in a chilled tube.

3. The mixture is aliquotted into 2 chilled Gene Pulzer Cuvette (0.1 cm electrode gap, BioRad 165-2089) so that each cuvette contains about 20ul mixture.

4. Wipe cuvette dry with Kim wipe and tap to gently tap the cuvette so the cells to settle to the bottom without any visible bubbles. Keep cuvettes on ice until just before electroporation.

5. Prepare 500ul SOC in pipette so that it can be added immediately after electroporation.

6. Electroporate the first cuvette using GenePulser at Ecl (1.8kV).

7. Add the 500ul SOC immediately into the cuvette and mix up and down. Transfer the content to a Falcon 2059 tube and add 2ml of SOC.

8. Read time constant. The time constant should be higher than 5ms.

9. Repeat for the second cuvette.

10. Rotate the transformation for 1 hour at 37C and 225 rpm.

11. Pool the two tubes to have about 5ml of transformation.

12. Each ml of transformation is plated onto a large bioassay plate (24cm x 24cm) so that each transformation is plated onto 5 plates.

13. For assessing transformation, 10ul, 0.1ul and 0.001ul of transformation mix is plated onto 10 cm Petri dishes. The colonies on the plates are counted after overnight incubation at 37C.
Note: A successful transformation should yield about 5E7 colonies in total.

15. The plates are incubated overnight at 37C. The bioassay plates don't need to be inverted if the plates are wet with liquid.

Part 3: Harvest large-scale transformation for pDNA maxiprep from 4k libraries (4k harvest protocol)

1. One day after the transformation, count the titering plates to determine the number of independent colonies on the confluent plates. Note: A successful transformation should yield about 5E7 colonies in total.

2. Add 10ml LB media per bioassay plate.

3. Scrape cells off using scraper.

4. Pipette and collect liquid and aliquot half harvest from each plate into one of two 50ml conical tubes to ensure equal representation in the two tubes.

5. Add another 10ml LB media per Bioassay plate to wash off any residue cells and aliquot the harvest as above.

6. Spin at 3750 rpm and 4C for 15 minutes on Allegra X-12R centrifuge.

7. Perform one DNA Maxiprep for the content in each 50ml tube so that there are two DNA Maxipreps for each 4k library amplification. The DNA from the two DNA Maxipreps of each 4k library is pooled and the concentration is measured.

Part 4: Creation of ~50k shRNA screening libraries from 4k library Maxiprep DNAs (50k DNA protocol)

Note: To produce a 100k shRNA library follow amounts noted in blue text

1. About 20ng DNA from each 4k library Maxiprep DNA is pooled to create pre-transformation master pool for the screening library so that each construct has similar molar representation in the pool. The final concentration can be from 0.2ug /ul to 2ug/ul.

2. Pre-chill 10 (20) electroporation cuvettes.

3. Add 2ul (2*2ul) (0.4ug to 4ug) master pool DNA to 200ul (2*200ul) ElectroMax DH5a-E cells on ice. Mix gently.

4. Add 20ul of the mixture to each cuvette and tap cells down and keep on ice.

5. For each cuvette, perform electroporation as in 4k library electroporation protocol.

6. Transformation from each cuvette results in one 15ml tube containing 2.5ml liquid.

7. Rotate at 37C and 2250rpm for 1 hour.

8. Pool the 10 (20) tubes to have 25ml (50) in total.

9. Add another 75ml (150) SOC so that the total volume is 100ml (200)

10. Set up titering plates as in 4k library electroporation protocol.
The total number of colonies on the 50 plates can be 2E9

11. Plate 2ml per bioassay plate (50 plates in total). (100 plates)

12. The plates are incubated overnight at 37C. The Bioassay plates don't need to be inverted if the plates are wet with liquid.

Part 5: Harvest large-scale transformation for pDNA maxiprep from screening libraries (genome scale harvest protocol)

1. Harvest the cells from all plates as in 4k harvest protocol and pool liquid culture together.

2. Aliquot into 20 (40) 50ml tubes. Spin to pellet.

3. Perform 20 (40) pDNA Maxipreps from the harvest of 50 plates. 25mg (50mgs) pDNA can be obtained.

3b. Perform 20 pDNA Maxipreps from 20 of the 50 ml aliquots (1 maxi / 50 ml tube). Store remaining 20 50 ml tubes at -80)

Part 6: Large-scale virus production from 50k pooled library DNA (50k pooled library virus protocol)

Note: To produce a 100k shRNA library follow amounts noted in blue text

20+ (40+) T175 flasks of 293T cells can be transfected for pooled library virus production. Transfection is started in the morning and transfection mix stay on 293T cells for 5 to 8 hours (longer time with transfection mix can be toxic to cells)

1. 293T cells are maintained in DMEM+10%FCS (No Pen/Strep) and passaged carefully to avoid confluent at any point prior to viral production. 293T cells are plated with about 14e6 cells / T175 in 24ml DMEM+10%FCS (No Pen/Strep) 16-24 hours before transfection so that the cells are 50 to 70% confluent on the day of transfection.

2. For each T175 flask, make DNA mix in a screw cap tube that contains:
   - 100ul OPTI-MEM
   - 50ug ~50k screening library Maxiprep DNA
   - 50ug pspax2
   - 10ug VSVG

3. For each T175 flask, make transfection reagent mix in a 50ml conical tube that contains:
   - 6ml OPTI-MEM
   - 330ul LTI (Mirus) or Fugene transfection reagent
   - Mix

4. Add DNA mix to the transfection reagent mix, shake gently by hand. Incubate at room temperature for 30 to 60 minutes

5. Gently add transfection mix containing DNA to cells so that the total volume is about 30ml.

6. Incubate at 37C for 5 to 8 hours.

7. Gently remove transfection mix.

8. Gently add 40 to 50 ml DMEM+**30%FCS**+Pen/Strep

9. Forty-eight hours after the start of the transfection, viral supernatant (48 hour harvest) is gently harvested and replaced with another 40 to 50 ml DMEM+**30%FCS**+Pen/Strep. The 48-hour harvest is frozen in 250ml conical tubes at -80C (leave 70ml space for the expansion during freezing)

10. Seventy two hours after the start of the transfection, viral supernatant (72 hour harvest) is gently harvested and mixed with the thawed 48 hour collection, and aliquotted. The large batch of virus should be mixed frequently during this aliquot process to avoid settling. These tubes contain the working pooled library virus stock and should be stored at -80.

Revision notes: