Protocol: propagation of genome-wide lentiviral CRISPR-guide RNA libraries
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MATERIALS

REAGENTS

- Library DNA (50 µl at 20 ng/µl in TE buffer provided via Addgene 50947)
- NEB 10-beta Electrocompetent *E. coli* (NEB, cat. no. C3020K)
- Qiagen plasmid maxi kit (Qiagen, cat. no. 12163)
- Yeast extract (Oxoid, cat. no. LP0021)
- Trypton (Sigma-Aldrich, cat. no. T7293)
- Sodium chloride (Sigma-Aldrich, cat. no. S7653)
- Agar (BD, cat. no. 214040)
- Ampicillin (Sigma-Aldrich, cat. no. A9518-5G)
- Potassium chloride (Sigma-Aldrich, cat. no. P9541)
- Magnesium chloride (Sigma-Aldrich, cat. no. M1028)
- Magnesium sulphate (Sigma-Aldrich, cat. no. M2643)
- Glucose

EQUIPMENT

- BioRad GenePulser Xcell (BioRad, cat. no. 165-2662)
- Electroporation cuvette, 0.1 cm gap (BioRad, cat. no. 165-2083)
- 140-mm bacterial dish (Sterilin, cat. no. 501V)
- 100-mm bacterial dish (Sterilin, cat. no. 101Vr20)
- 15-ml round-bottomed tube (BD, cat. no. 352059)
- 50-ml Falcon tube (BD, cat.no. 352098)
- Bacteria spreader (Fisher Scientific, cat. no. 12908140)

REAGENT SETUP

Ampicillin stock solution (50 mg/ml, 1000x concentrated) Dissolve 500 mg in 5 ml distilled water. Add 5 ml 100% ethanol and store at -20 °C.

LB+amp plate Dissolve 5 g of NaCl, 5g of Yeast extract and 10 g of tryptone in 1 L distilled water and adjust pH to 7.5. Add 20 g of agar and autoclave. When it cools down to 55 °C, add 1 ml of ampicillin (50 mg/ml), pour 20 ml per 100-mm petri dish or 66 ml to 140-mm dish. Let plates sit on the bench for overnight. Store the plates at 4 °C.

2xTY medium Dissolve 5 g of sodium chloride, 10 g of yeast extract and 16 g of tryptone to 1 L of distilled water. Autoclave and store at room temperature.

SOC Dissolve 20 g of tryptone, 5 g of yeast extract, 0.5 g of Sodium chloride , 2.5 ml of 1M potassium chloride, 10 ml of 1 M magnesium chloride and 10 ml of 1 M magnesium sulphate into 1 L of distilled water and autoclave. When the mixture cools down to room temperature, add 20 ml of filter-sterilized 1 M glucose solution. Store at room temperature.

PROCEDURE

Electroporation

1| Pre-warm 30 LB+amp plates (140mm) or 500 ml 2xYT+amp medium in a 1000-ml flask, and SOC recovery medium (supplied with the competent cells) at 37 °C for 1 h.

2| Place four electroporation cuvettes and four 1.5-ml tubes on ice.

3| Dilute the library DNA (obtained from Addgene) by 1:2 by mixing 5 µl of the DNA and 5 µl of sterile water to a final concentration of 10 ng/µl. Add 1 µl to each of three pre-chilled 1.5-ml tubes and keep the tubes on ice.

4| As a positive control for transformation, dilute the control pUC19 DNA (provided with the competent cells) by 1:5 to a final concentration of 10 pg/µl using sterile water. Add 1 µl of the DNA solution to one pre-chilled 1.5-ml tube and keep the tube on ice.
5| Thaw one viral of the frozen electrocompetent cells on ice. This will take for approximately 5 min. Mix the cells by flicking gently. Keep the cells on ice.
6| Add 25 µl of the cells to each of the four pre-chilled DNA and mix gently by pipetting. Place the DNA/cell mixture on ice.
7| Set the electroporation condition: 2.0 kV, 200 Omega and 25 µF.
8| Bring the ice box containing the DNA/cell tubes and the electroporation cuvettes, and the pre-warmed SOC recovery medium next to the electroporator.
9| Transfer 26 ul of the DNA/cell mixture into a pre-chilled cuvette without making bubbles and make sure that the cells deposit across the bottom of the cuvette. Electroporate.
10| Immediately add 974 ul of the pre-warmed SOC recovery medium to the cuvette, gently mix up and down trice, then transfer the cells to a 15-ml round-bottomed tube. Place the tube in bacterial shakers at 37 °C.
11| Perform the second, third, fourth electroporation separately by repeating step 9-10.
12| Shake the tubes for 1 h at 37 °C.
13| Combine two of the gRNA library-transfected bacteria together and mix well by pipetting. Discard the remaining one culture.

Plating transformants for transformation efficiency measurement
14| For pUC19, make serial dilutions (10^{-1} to 10^{-4}) by mixing 50 µl bacteria into 450 µl SOC. Plate 100 µl of each of the diluted bacteria to a 100-mm LB+amp plate. Incubate the plates at 37 °C.
15| For the library DNA, make serial dilutions (10^{-1} to 10^{-6}) by mixing 50 µl bacteria into 450 µl SOC. Plate 100 µl of each of the diluted bacteria (10^{-3} to 10^{-6}) to a 100-mm LB+amp plate. Incubate the plates at 37 °C.
16| On the following morning, count colonies. From pUC19 transformation, calculate the transformation efficiency in colony forming unit (cfu)/µg. This is usually higher than 1.0×10^{10} cfu/µg. From library DNA transformation, calculate the total number of cfu that are produced in 2 ml recovered bacteria (step **13**). If you obtain 25 colonies on the 10^{-5} plate, there are 5.0×10^{7} cfu in the 2 ml recovered bacteria. This should be $>5 \times 10^{7}$ cfu for the faithful library replication.

Culture bacteria transformed with the library DNA (Choose one method)
17a| On-plate culture. Add 2 ml recovered bacterial (step **13**) to 4 ml pre-warmed SOC. Plate 200 µl to each of thirty 140-mm LB+amp plates. Incubate the plates at 37 °C overnight.
17b| Liquid culture. Add 2 ml recovered bacteria (step **13**) to the pre-warmed 500 ml 2xTY+amp (ampicillin, 50 µg/ml) medium and incubate at 37 °C overnight with shaking.

Plasmid purification
A) Bacteria from on-plate culture
18|Harvesting bacteria from plates. Use P1 solution provided with Qiagen plasmid maxi kit for harvesting bacterial colonies. Handle with 5 plates at one time with the following harvesting method: pour 2ml P1 solution to each of the 5 plates and gently but vigorously scrape the surface of the plates with bacteria spreader. Collect the solution (very sticky and viscous) to a 50 ml conical tube. Collect the remaining bacteria on the plates by washing the surface and transferring them to the same 15 ml tube. The conical tube should contain 15 ml bacterial suspension. Repeat this for the remaining plates. There should be 6 conical tubes in the end.
19| Add 15 ml P2 solution to each of the 6 tubes. Mix them by inverting the tubes.
20| Add 15 ml P3 solution to each of the 6 tubes. Mix them by inverting the tubes.
21| Centrifuge them at 10,000 rpm for 30 min at 4 C.
22| Transfer 40 ml supernatant from each tube to a 500-ml clean bottle and mix.

23| Purify plasmid DNA with 4 Maxi columns by following the manufacturer's instruction.

B) Bacteria in liquid culture

24| Use 3 columns from the Qiagen plasmid maxi kit to purify plasmid from 500 ml culture. Follow the manufacturer's instruction.

Normally the yield can be achieved mg order in both methods.

ANTICIPATED RESULTS

We propagated the published genome-wide lentiviral library [1] by following the procedure described above and compared replication fidelity between on-plate and liquid culture. For this comparison, we combined bacteria from all 3 electroporation and obtained 3 ml recovered bacteria; a half of the bacteria (1.5 ml) was plated onto 30 140-mm LB+amp dishes and the other half (1.5 ml) was inoculated into 500 ml 2xTY+amp broth. Plasmid DNA was extracted as described above. The electroporation efficiency was 1.3×10^{10} cfu/μg pUC19. We obtained a total of 5.1×10^7 colonies on 30 dishes. The plasmid yields were 3.4 and 2.1 mg from on-plate and liquid culture, respectively. The original mouse genome-wide lentiviral gRNA library was extracted from 1.5×10^7 bacterial colonies. To faithfully replicate the library, plasmid DNA should be extracted from > 5×10^7 bacterial colonies.

We then deep sequenced these replicated libraries as described in our paper and compared the read counts of each gRNA in the replicated libraries with that in the original library (Fig. 1). Both on-plate and liquid culture of electroporated bacteria faithfully replicated the original library. Overall variation was slightly bigger in the library replicated on plates than in liquied. A small fraction of gRNAs in the library replicated in liquid culture was slightly overrepresented (Fig. 1b). This level of overrepresentation should not cause any problems. Plasmid extraction from liquid culture is much easier than on-plate culture. It is up to individual researcher's choice to choose which culture method is used.

Reference

1. Koike-Yusa, H. et al. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nature Biotechnology 2013 doi:10.1038/nbt.2800

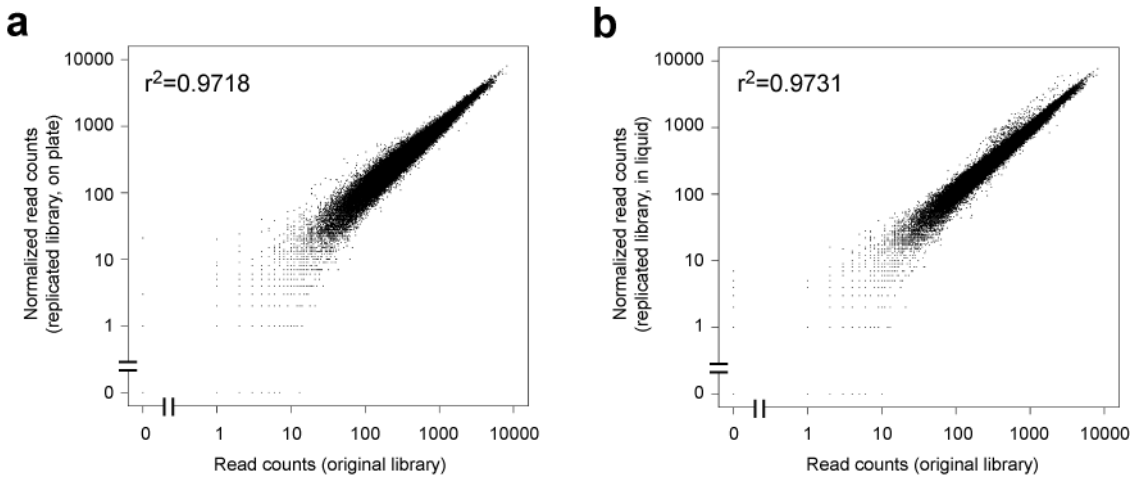

Figure 1 | Comparison between the original library and the replicated libraries. The libraries replicated on plate (**a**) or in liquid (**b**) were deep sequenced and compared with the published original library. Representation of each gRNA is almost evenly replicated in both culture methods.