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Protocol 2: Viral Packaging and Cell Culture for CRISPR-based Screens:

CRISPR-based Screening

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This protocol describes how to perform the tissue culture and high-throughput sequencing library preparation for a CRISPR-based screen. First, pantropic lentivirus is prepared from a sgRNA plasmid pool and applied to the target cells. Following antibiotic selection and a harvest of the initial population, cells are then cultured under the desired screening condition(s) for 14 population doublings. sgRNA barcode sequences integrated in the genomic DNA of each cell population are amplified and subject to high-throughput sequencing. Guidelines for downstream analysis of the sequencing data are also provided.

Materials

Reagents

0.22 μm 150 mL bottle top filter (Corning 430626)

0.45 μm Acrodisc Syringe Filter (VWR 28144-007)

6-well tissue culture-treated plates

15 cm tissue culture-treated plates

Agarose gel, 2.0%

DMEM, high glucose, GlutaMAX Supplement (Gibco 10566-016)

Ethidium bromide

Gel Extraction Kit (Qiagen 28704)

Human embryonic kidney (HEK) 293T cells (ATCC CRL-3216)

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Inactivated Fetal Serum (Sigma Aldrich F4135-500ML)

LB (Luria-Bertani) liquid medium

LB-ampicillin agar plates

Lentiviral sgRNA library (from Protocol 1 or Addgene)

Luer-Lok Tip Syringes (Becton Dickinson, various sizes)

Media and various plastics for screen cell culture

Opti-MEM I Reduced-Serum Medium

pCMV-dR8.2 packaging plasmid (Addgene 8455)

pCMV-VSV-G pantropic viral envelope plasmid (Addgene 8454)

Penicillin-Streptomycin (Sigma-Aldrich P4333-20ML)

Phosphate-buffered saline (PBS)

Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB M0531S)

Plasmid Plus Maxi Kit (Qiagen 12963)

Polybrene (EMD Millipore TR-1003-G)

Puromycin

QIAamp DNA Blood Maxi Kit (Qiagen 51194)

sgRNA barcode PCR primers

Forward: *AATGATACGGCGACCACCGAGATCTACACCGACTCGGTGCCACTTTT*

Reverse:
CAAGCAGAAGACGGCATACGAGATCnnnnnTTTCTTGGGTAGTTTGCAGTTTT
nnnnnn denotes a user-specified sample barcode sequence

Sequencing primers for Illumina HiSeq

Read 1 primer:
CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTC
TAGCTCTAAAAC

Indexing primer:
TTTCAAGTTACGGTAAGCATATGATAGTCCATTTTAAAACATAATTTTAAAACTGC
AAACTACCCAAGAAA

x-tracta gel extractor (USA Scientific 5454-0100)

X-tremeGENE 9 DNA Transfection Reagent (Roche 06365787001)

Equipment

Centrifuge with rotors for 6-well plate spin infection

Erlenmeyer flask, 500 mL

Gel imager

Heat block

NanoDrop spectrophotometer (NanoDrop)

Thermocycler

Tissue culture hood for BL2+ work

Tissue culture incubator

Method

NOTE: You must contact your institution's Bio-Safety office to receive institution-specific instructions. You must follow safety procedures and work in an environment (e.g. BL2+) suitable for handling lentiviruses. A general overview of viral packaging can be found here: https://www.addgene.org/lentiviral/packaging/.

Viral Packaging Vector Preparation

1. Streak out bacterial stab cultures of pCMV-dR8.2 and pCMV-VSV-G obtained from Addgene on LB-amp plates and incubate at 37°C overnight.
2. Pick a single colony and seed into a 500 mL Erlenmeyer flask containing 100 mL LB liquid media with 100 μg/mL ampicillin.
3. Incubate culture at 37°C overnight.
4. Prepare plasmid DNA from the bacterial culture using the Qiagen Plasmid Plus Maxi Kit according to the manufacturer's instructions.

Viral Packaging and Titer Test

Day 1

5. Add the following components to make Virus Production Media (VPM).

   400 mL DMEM, high glucose, GlutaMAX Supplement

   100 mL Inactivated Fetal Serum

   5 mL Penicillin (10,000 U/mL)-Streptomycin (10 mg/mL)

Filter media through 0.22 μm bottle-cap filter in a tissue culture hood.

Seed 750,000 HEK-293T cells in a single well of a 6-well plate in 2 mL of VPM. Incubate cells at 37°C overnight in a tissue culture incubator.

Day 2

Assemble the following transfection mixture.

50 μL Opti-MEM

1 μg Lentiviral sgRNA library

900 ng pCMV-dR8.2

100 ng pCMV-VSV-G

5 μL XtremeGene 9

10 Incubate mixture for 15 minutes at room temperature and add dropwise to cells to transfect.

Incubate cells at 37°C overnight in a tissue culture incubator.

Day 3

11 Change media with 2 mL of VPM. Incubate cells at 37°C overnight in a tissue culture incubator.

Day 4

12 Harvest viral supernatant from cells and filter through 0.45 μm Acrodisc Syringe Filter.

13 Set up the following 5 infections in a 6-well tissue culture-treated plate.

5,000,000 target cells

2 μL polybrene (10 mg/mL)

0, 125, 250, 500, and 1,000 μL filtered virus

Up to 2 mL cell culture media

Note: Some lines may not tolerate spin-infection and overnight incubation this density. Please adjust cell numbers accordingly for cell lines of interest.

14 Spin plate at 1,200 *g* for 45 minutes in a pre-warmed centrifuge. After spinning, incubate cells at 37°C overnight in a tissue culture incubator.

Day 5

15 For adherent cells: aspirate virus-containing media, wash cells with PBS, trypsinize cells, and expand each well into a 15 cm tissue culture-treated plate. Incubate cells at 37°C overnight in a tissue culture incubator. For suspension lines: pellet cells and aspirate virus-containing media. Re-suspend cells into a 15 cm tissue culture-treated plate. Incubate cells at 37°C overnight in a tissue culture incubator.

Day 6

16 Add an appropriate selection dose of puromycin to cells.

Note: The optimal dose should be determined by performing a puromycin kill curve.

Day 9

17 Observe plates. Identify viral dose required for approximately 40% cell survival (multiplicity of infection 0.5) and discard all plates.

Screen Viral Packaging and Infection

Day 1

18 Based on the viral titer test, calculate the volume of virus required to represent the entire library in the cell line of interest 1000-fold (e.g. for a 40,000 sgRNA library = 40,000,000 infected cells = 100,000,000 total cells = 20X test infection volume for 5,000,000 cells)

19 Scale up virus production in 10 cm plates (~10 mL virus produced per plate), seeding 3,750,000 HEK-293T cells per plate in 10 mL VPM. Incubate cells at 37°C overnight in a tissue culture incubator.

20 Day 2

21 For each plate, assemble the following transfection mixture:

250 µL Opti-MEM

5 µg Lentiviral sgRNA library

4.5 µg pCMV-dR8.2

500 ng pCMV-VSV-G

25 µL XtremeGene 9

22 Incubate mixture for 15 minutes at room temperature and add dropwise to cells to transfect.

Incubate cells at 37°C overnight in a tissue culture incubator.

Day 3

23 Change media in plates with 10 mL fresh VPM. Incubate cells at 37°C overnight in a tissue culture incubator.

Day 4

24 Harvest viral supernatant from cells and filter through 0.45 µm Acrodisc Syringe Filter.

Note: Viral supernatants can be stored at −80°C for long term storage but freezing/thawing will cause a reduction in viral titers (typically ~30–50% reduction)

25 Calculate the number of wells in a 6-well tissue culture-treated plate required for infection (e.g. for a 40,000 sgRNA library = 40,000,000 infected cells = 100,000,000 total cells = 20 wells of 5,000,000 cells each).

26 Assemble a large-scale cell-virus infection mixture according to the following amounts per well: 5,000,000 target cells

2 μL polybrene (10 mg/mL)

Viral dose required for approximately 40% cell survival

Up to 2 mL cell culture media

Note: Some lines may not tolerate spin-infection and overnight incubation this density. Please adjust accordingly for your lines of interest.

27 Dispense 2 mL aliquots of the mixture into 6-well plates.

28 Spin plates at 1,200 *g* for 45 minutes in a pre-warmed centrifuge. After spinning, incubate cells at 37°C overnight in a tissue culture incubator.

Day 5

29 For adherent cells: aspirate virus-containing media, wash with PBS, trypsinize cells, and expand each infection into 15 cm tissue culture-treated plates. Incubate cells at 37°C overnight in a tissue culture incubator. For suspension lines: pellet cells and aspirate virus-containing media. Re-suspend cells into 15 cm tissue culture-treated plates. Incubate cells at 37°C overnight in a tissue culture incubator.

30 As a control, seed uninfected cells at an identical confluence into a 15 cm tissue culture-treated plate. Incubate cells at 37°C overnight in a tissue culture incubator.

Day 6

31 Add an appropriate selection dose of puromycin to library-infected and uninfected control cells.

Note: The optimal dose should be determined by performing a puromycin kill curve. Day 9

32 Observe plates after 3 days. If cell survival is 40% (multiplicity of infection 0.5) in the infected population and <5% in the uninfected population, passage the infected cells into fresh media. Be sure to maintain a 1000-fold coverage of the library. With the remaining cells, freeze 2 pellets for DNA extraction. These cells will serve as the initial reference population.

Screen Cell Culture and Library Preparation

Note: After infection and selection of the cell population, all subsequent tissue culture work can be performed in a BL2 environment.

33 Continue to passage cells, maintaining a 1000-fold coverage of the library at each seeding.

NOTE: For positive selection-based screens, the selection agent should be added approximately 1 week after infection to allow sufficient time for knockouts to be generated.

34 After 14 population doublings, collect final cell pellets.

35 Extract genomic DNA from the initial and final cell pellets using the QIAamp DNA Blood Maxi Kit according to the manufacturer's instructions.

36 Calculate the total number of PCR reactions required. A 250-fold coverage of the library should be used as input for sgRNA amplification with 3 μg genomic DNA per 50 μL reaction. (e.g. for a 40,000 sgRNA library = 10,000,000 genome equivalents 66 μg for diploid human cells = 22 reactions with 3 μg genomic DNA each.)

37 Use the following per-sample recipe to assemble the total reaction mixture and dispense into PCR strip tubes in 50-μL aliquots on ice.

3 μg Genomic DNA

2 μL forward sgRNA PCR primer (10μM)

2 μL sample-specific barcoded reverse sgRNA PCR primer (10μM)

25 μL Phusion PCR Master Mix

Up to 50 μL H2O

38 Amplify reactions in a thermocycler using the following program.

1 cycle	98°C	2 minutes
30 cycles	98°C	10 seconds
	60°C	15 seconds
	72°C	45 seconds
1 cycle	72°C	5 minutes
1 cycle	4°C	HOLD

39 Pool reactions and run them on an ethidium bromide-stained 1% agarose gel. Visualize the PCR bands using a standard gel imager.

40 Cut the amplified PCR product using an x-tracta gel extractor tool.

Note: The expected product should be 274 base pairs.

41 Extract DNA using the Qiagen Gel Extraction Kit according to the manufacturer's instructions, eluting in 30 μL H2O.

42 Submit extracted PCR products for high-throughput sequencing on an Illumina HiSeq using the custom sequencing primers list in the Materials section. A single end run with a 6 base pair indexing read should be performed.

Data Analysis

Note: The procedure below describes a simple method for calculating gene scores. A suite of tools (originally designed for analyzing shRNA-based screens) exist for more sophisticated gene score tabulation, hit identification, and pathway analysis (Subramanian et al. 2005; Luo et al. 2008; Shao et al. 2012).

43 For each individual sample:

- **a.** Enumerate sgRNA library barcodes using Bowtie.
- **b.** Add 1 to each sgRNA count.
- **c.** Calculate the $\log_2$ fractional abundance of each sgRNA.

44 For each sgRNA of each final sample, subtract the fractional abundance of the sgRNA in the intial sample to determine the $\log_2$ fold-change in abundance.

45 To calculate gene scores for each final sample, find the average $\log_2$ fold-change of all sgRNAs targeting each gene.

46 To compare between samples, subtract the genes scores between the samples to identify the differentially scoring genes.

Troubleshooting

Problem: Viral titers are too low.

Solution: Low viral production is typically the result of unhealthy HEK-239T packaging cells. Be sure to check the health of the HEK-239T cells before and after transfection. Ethanol precipitation of the packaging and transfer vectors can also help eliminate bacterial endotoxin, which strongly inhibits viral production.

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