Protocol

Viral Packaging and Cell Culture for CRISPR-Based Screens

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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 single guide RNA (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. The 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

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agarose gel (1.0%)
Cell-culture medium appropriate for target cells
Dulbecco's Modified Eagle Medium (DMEM), high-glucose, GlutaMAX Supplement (Gibco 10566-016)
Ethidium bromide
Fetal bovine serum (inactivated) (Sigma-Aldrich F4135)
Gel Extraction Kit (QIAGEN 28704)
Human embryonic kidney (HEK) 293 T cells (ATCC CRL-3216)
LB-ampicillin agar plates <R>

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Cite this protocol as *Cold Spring Harb Protoc;* doi:10.1101/pdb.prot090811

LB (Luria–Bertani) liquid medium <R>
Lentiviral sgRNA library

*Acquire library either by following the associated methodology (see Protocol: **Single Guide RNA Library Design and Construction** [Wang et al. 2016]) or by sourcing library from Addgene (www.addgene.org).*

Opti-MEM I Reduced-Serum Medium (Thermo Fisher Scientific 31985-062)
pCMV-dR8.2 packaging plasmid (Addgene 8455)
pCMV-VSV-G pantropic viral envelope plasmid (Addgene 8454)
Penicillin–streptomycin (Sigma-Aldrich P4333)
Phosphate-buffered saline (PBS) <R>
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)
Sequencing primers for Illumina HiSeq

Read 1 primer:
CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTC-TAGCTCTAAAAC

Indexing primer:
TTTCAAGTTACGGTAAGCATATGATAGTCCATTTTAAAACATAATTTTAAA ACTGCAAACTACCCAAGAAA

Single guide RNA (sgRNA) barcode polymerase chain reaction (PCR) primers

Forward: AATGATACGGCGACCACCGAGATCTACACCGACTCGGTGCCACTTTT
Reverse: CAAGCAGAAGACGGCATACGAGATCnnnnnTTTCTTGGGTAGTTTGCAGTTTT

The sequence "nnnnnn" denotes a user-specified sample barcode sequence.

Target cells and appropriate cell-culture medium
Trypsin (for adherent cells)
Water (H_2O), PCR grade
X-tremeGENE 9 DNA Transfection Reagent (Roche 06365787001)

Equipment

Access to Illumina HiSeq
Acrodisc syringe filter (0.45-µm; VWR 28144-007)
Bottle top vacuum filter (0.22-µm pore, 150-mL) (Corning 430626)
Centrifuge with rotors for six-well-plate spin infection
Erlenmeyer flask (500-mL)
Gel imager
Heat block (for Step 39)
Luer-Lok Tip syringes (Becton Dickinson)
NanoDrop spectrophotometer (NanoDrop)
Thermocycler
Tissue-culture hood for BL2+ work
Tissue-culture incubator set at 37°C
Tissue-culture-treated plates (six-well, 10- and 15-cm)
Vacuum aspirator
x-tracta gel extractor (USA Scientific 5454-0100)

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METHOD

We emphasize that you should follow appropriate safety procedures and work in an environment (e.g., BL2+) suitable for handling lentiviruses. A general overview of viral packaging, and issues relating to safety, can be found at https://www.addgene.org/lentiviral/packaging/.

Preparation of Viral Packaging Vector

1. Streak out bacterial stab cultures of pCMV-dR8.2 and pCMV-VSV-G obtained from Addgene on LB-amp plates and incubate overnight at 37°C.
2. Pick a single colony and seed into a 500-mL Erlenmeyer flask containing 100 mL of LB liquid medium containing 100 µg/mL ampicillin.
3. Incubate culture overnight at 37°C.
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 medium (VPM).

DMEM (high glucose, GlutaMAX Supplement)	400 mL
Fetal bovine serum (inactivated)	100 mL
Penicillin (10,000 U/mL)-streptomycin (10 mg/mL)	5 mL

6. Filter medium through a 0.22-µm bottle-top vacuum filter in a tissue-culture hood.
7. Seed 750,000 HEK-293 T cells in a single well of a six-well plate in 2 mL of VPM. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 2

8. Assemble the following transfection mixture.

Opti-MEM I	50 µL
Lentiviral sgRNA library	1 µg
pCMV-dR8.2	900 ng
pCMV-VSV-G	100 ng
X-tremeGENE 9 DNA Transfection Reagent	5 µL

9. Incubate mixture for 15 min at room temperature and add dropwise to cells to transfect. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 3

10. Replace medium with 2 mL of VPM. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 4

11. Harvest viral supernatant from cells and filter through a 0.45-µm Acrodisc syringe filter.

    See Troubleshooting.

12. Set up the following five infections in a six-well tissue-culture-treated plate.

Target cells	5,000,000
Polybrene (10 mg/mL)	2 µL
Filtered virus	0, 125, 250, 500, or 1000 µL
Cell-culture medium	to 2 mL

    Some cell lines might not tolerate spin infection and overnight incubation at this density. Adjust cell numbers accordingly for the cell line of interest.

13. Centrifuge plate at 1200*g* for 45 min in a prewarmed centrifuge. After centrifugation, incubate cells overnight at 37°C in a tissue-culture incubator.

Day 5

14. Proceed as follows.
    - For adherent cells, aspirate virus-containing medium, wash cells with PBS, trypsinize cells, and expand each well into a 15-cm tissue-culture-treated plate. Incubate cells overnight at 37°C in a tissue-culture incubator.
    - For suspension lines, pellet cells and aspirate virus-containing medium. Resuspend cells into a 15-cm tissue-culture-treated plate. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 6

15. Add an appropriate selection dose of puromycin to cells.

    The optimal dose should be determined by performing a puromycin kill curve. The concentration of puromycin that causes death of 100% of unmodified cells in 48–72 h should be used as a selection dose.

Day 9

16. Observe plates. Identify the viral dose required for ~40% cell survival (multiplicity of infection ≍ 0.5), and discard all plates.

Screen Viral Packaging and Infection

Day 1

17. 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, this requires 40,000,000 infected cells—i.e., 100,000,000 total cells, equivalent to 20 times the test infection volume for 5,000,000 cells).

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

Day 2

19. For each plate, assemble the following transfection mixture:

Opti-MEM I	250 µL
Lentiviral sgRNA library	5 µg
pCMV-dR8.2	4.5 µg
pCMV-VSV-G	500 ng
X-tremeGENE 9 DNA Transfection Reagent	25 µL

20. Incubate mixture for 15 min at room temperature and add dropwise to cells to transfect. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 3

21. Replace medium in plates with 10 mL of fresh VPM. Incubate cells overnight at 37°C in a tissue-culture incubator.

Day 4

22. Harvest viral supernatant from cells and filter through a 0.45-µm Acrodisc syringe filter.

    Viral supernatants can be stored at −80°C for long-term storage, but freeze–thawing will cause a reduction in viral titers (typically ~30%–50% reduction).

23. Calculate the number of wells in a six-well tissue-culture-treated plate required for infection (e.g., for a 40,000 sgRNA library, one needs 40,000,000 infected cells, equivalent to 100,000,000 total cells, thus requiring 20 wells each containing 5,000,000 cells).

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24. Assemble a large-scale cell-virus infection mixture according to the following amounts per well:

Target cells	5,000,000
Polybrene (10 mg/mL)	2 µL
Viral dose	giving ~40% cell survival (from Step 16)
Cell culture medium	to 2 mL

Some lines might not tolerate spin infection and overnight incubation at this density—adjust accordingly for your lines of interest.

25. Dispense 2-mL aliquots of the mixture into six-well plates.
26. Centrifuge the plates at 1200*g* for 45 min in a prewarmed centrifuge. After centrifugation, incubate the cells overnight at 37°C in a tissue-culture incubator.

Day 5

27. Proceed as follows.
    - For adherent cells, aspirate virus-containing medium, wash with PBS, trypsinize the cells, and expand each infection into 15-cm tissue-culture-treated plates. Incubate the cells overnight at 37°C in a tissue-culture incubator.
    - For suspension lines, pellet the cells and aspirate virus-containing medium. Resuspend the cells into 15-cm tissue-culture-treated plates. Incubate the cells overnight at 37°C in a tissue-culture incubator.
28. As a control, seed uninfected cells at an identical confluence into a 15-cm tissue-culture-treated plate. Incubate the cells overnight at 37°C in a tissue-culture incubator.

Day 6

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

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

Day 9

30. Observe the plates after 3 d. 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 medium. Be sure to maintain 1000-fold coverage of the library. With the remaining cells, freeze two pellets for DNA extraction.

    These cells will serve as the initial reference population.

Screen Cell Culture and Library Preparation

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

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

    For positive-selection-based screens, the selection agent should be added at 1 wk after infection to allow sufficient time for knockouts to be generated.

32. After 14 population doublings, collect the final cell pellets.
33. Extract genomic DNA from the initial and final cell pellets using the QIAamp DNA Blood Maxi Kit according to the manufacturer's instructions.
34. Calculate the total number of polymerase chain reactions (PCRs) required. Use a 250-fold coverage of the library as input for sgRNA amplification, with 3 µg genomic DNA per 50 µL reaction (e.g., for a 40,000 sgRNA library, requiring 10,000,000 genome equivalents, one needs 66 µg for diploid human cells—i.e., 22 reactions, each with 3 µg of genomic DNA).

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

Genomic DNA	3 µg
Forward sgRNA PCR primer (10 µM)	2 µL
Sample-specific barcoded reverse sgRNA PCR primer (10 µM)	2 µL
Phusion PCR High-Fidelity Master Mix with HF buffer	25 µL
H_2O	to 50 µL

36. Amplify reactions in a thermocycler using the following program.

1 cycle	98°C	2 min
30 cycles	98°C	10 sec
	60°C	15 sec
	72°C	45 sec
1 cycle	72°C	5 min
1 cycle	4°C	hold

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

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

The expected product size is 274 bp.

39. Extract DNA using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions, eluting in 30 µL of water.

40. Submit extracted PCR products for high-throughput sequencing on an Illumina HiSeq using the custom sequencing primers detailed above (see Reagents).

A single end run with a 6-bp indexing read should be performed.

Data Analysis

The procedure below describes a simple method for calculating gene scores. A suite of tools (originally designed for analyzing short-hairpin-RNA-based screens) exists for more sophisticated tabulation of gene scores, hit identification, and pathway analysis (Subramanian et al. 2005; Luo et al. 2008; Shao et al. 2013).

41. Process each sample as follows.

    i. Enumerate sgRNA library barcodes using the Bowtie alignment program.

    ii. Add 1 to each sgRNA count.

    iii. Calculate the $\log_2$ fractional abundance of each sgRNA.

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

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

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

TROUBLESHOOTING

Problem (Step 11): The viral titers are too low.

Solution: Low viral production is typically the result of unhealthy HEK-239 T packaging cells. Be sure to check the health of the HEK-239 T 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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RECIPES

Ampicillin Stock Solution (100 mg/mL)

Ampicillin (sodium salt [sodium ampicillin], m.w. = 371.40)

Dissolve 1 g of sodium ampicillin in sufficient H_2O to make a final volume of 10 mL. If sterilization is required, prewash a 0.45- or 0.22-µm sterile filter by drawing through 50–100 mL of H_2O. Then pass the ampicillin solution through the washed filter. Store the ampicillin in aliquots at −20°C for 1 yr (or at 4°C for 3 mo).

LB-Ampicillin Agar Plates

Ampicillin, filter-sterilized (10 mg/mL stock)
LB agar

Autoclave 1 L of LB agar. Cool to 55°C. Add 10 mL of ampicillin stock. Pour into Petri dishes (~ 25 mL per 100-mm plate).

LB Agar

Agar (20 g/L)
NaCl (10 g/L; Sigma-Aldrich S9625)
Tryptone (10 g/L; BD 211705)
Yeast extract (5 g/L; BD 212750)

Add H_2O to a final volume of 1 L. Adjust the pH to 7.0 with 5 N NaOH. Autoclave. Pour into Petri dishes (~ 25 mL per 100-mm plate).

LB (Luria-Bertani) Liquid Medium

Reagent	Amount to add
H_2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Combine the reagents and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL). Adjust the final volume of the solution to 1 L with H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.

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