**Genome-scale CRISPR-Cas9 knockout screening**

**Library Description**

The genome-scale library targets more than 18, 000 genes with 185634 guide RNAs. The library is divided into 2 parts (h1 and h2). The h1 and h2 libraries contain in total 10 sgRNAs per gene (5 sgRNAs in each library). The library is in 1 vector (lentiCRISPRv2) format.

**Library Amplification**

Follow these instructions for each library (A and B). Amplify and prepare half-libraries separately as follows:

1. Dilute the library to 50 ng/µL in water or TE (if not already diluted).
2. Test transformation efficiencies of the bacteria by adding 0.5 µL and 1µL of each gRNA library into 25µL highly competent bacteria (XL-10 gold is recommended for chemical transformation; Lucigen Endura cells are recommended for transformation by electroporation). After the recovery period of the transformation, perform dilution plating to determine transformation efficiency using 0.01% and 0.001% of the total reaction.
	1. To ensure no loss of representation, 20 parallel transformations were performed and plate onto 10 plates of 245 mm\* 245mm.
	2. The transformation efficiency must be at least 50-fold greater than the pool size to ensure adequate library representation. If this efficiency is not achieved, discard the bacterial culture and re-transform.
	3. Harvest bacteria prior to reaching stationary phase (after 12-16 hours of growth)
3. Extract plasmid DNA from pelleted bacteria using by GenEluteTM HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma, Catalog #NA0410-1KT). It is critical that transfection-grade DNA is obtained
4. Identify the quality of amplified libraries by sequencing.
First round PCR:
Primer\_R: TCTACTATTCTTTCCCCTGCACTGTACCTGTGGGCGATGTGCGCTCTG
Primer\_F: AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG
	1. Using 50 ng plasmids as template. Perform a 50-µL reaction using Q5® High-Fidelity DNA Polymerase (Biolabs®) and then combined the resulting amplicons. Recommended PCR begin with 8 cycles. Run 2% E-Gel (Invitrogen) or do qPCR to decide appropriate number of PCR cycles, where the yield of amplicons are about to saturate. If amplicons are still in an upswing, add 1-2 more cycles. But the number of cycles should not exceed 16.
	2. Perform a second PCR to attach Illumina adaptors and to barcode samples. Perform the second PCR in a 100 µL reaction volume using 2 µL of the product from the first PCR. The thermocycling Conditions is the same as first round PCR.

	Primers for the second PCR:

Cri\_library\_F: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGAAACACCG

Cri\_library\_index1:

CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCACGTCTACTATTCTTTCCCCTGCACTGTACC

Cri\_library\_index2:

CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGATGTTCTACTATTCTTTCCCCTGCACTGTACC

Followed by Gel purification of the amplicons using Qiagen gel purification kit.

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| **Component** | **100 µL Reaction** |
| 5X Q5 Reaction Buffer | 20 µL |
| 10 mM dNTPs | 2 µL |
| 10 µM Forward Primer | 2 µL |
| 10 µM Reverse Primer | 2 µL |
| Template DNA | variable |
| Q5 High-Fidelity DNA Polymerase | 1 µL |
| Nuclease-Free Water | to 100 µL |

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| **STEP** | **TEMP** | **TIME** |
| Initial Denaturation | 98°C | 30 seconds |
| 8–10 Cycles | 98°C | 10 seconds |
| 68°C | 20 seconds |
| 72°C | 25 seconds |
| Final Extension | 72°C | 2 minutes |
| Hold | 4°C |  |

**Lentivirus Packaging**

***Materials needed:***

* X-tremeGENE transfection reagent
* psPAX2 packaging plasmid
* pMD2.G envelope plasmid
* lentiCRISPR V2 plasmid for each library half
* OPTI-MEM
* DMEM
* 293FT cells
* Tissue-culture grade BSA
* Polypropylene centrifuge tubes

Each 15-cm plate of 293FT cells yields 50mL of virus. If a high titer is achieved, each two-condition screen should require use of 5-10mL of virus.

Package the two halves of the library separately as follows:

1. For each library to be transfected, plate 1-1.25 x 107 293FT cells in 20 mL of media in a 15-cm tissue culture plate.
2. Dilute 200 µL transfection reagent X-tremeGENE in 6 mL serum-free OPTI-MEM in 15 mL polypropylene microfuge tubes. In the tube, add OPTI-MEM first. Pipette X-tremeGENE directly into the OPTI-MEM. Do not allow X-tremeGENE to come in contact with the walls of the tube before it has been diluted. Mix by swirling, gently flicking, or inverting the tube. Incubate for 5 minutes at room temperature.
3. In two 15-mL polypropylene centrifuge tubes (do NOT use polystyrene tubes), make a cocktail for each transfection:
	1. 3 mL serum-free OPTI-MEM
	2. 20 µg lentiCRISPR V2 plasmid
	3. 15 µg psPAX2 packaging plasmid
	4. 6 µg pMD2.G envelope plasmid
4. Add 3 mL of diluted X-tremeGENE to each tube from step 3 for a total volume of 6 ml per transfection. Pipette diluted X-tremeGENE directly into the liquid and not onto the walls of the tube. Mix by swirling or gently flicking the tube.
5. Incubate for 10-20 minutes at room temperature.
6. Without touching the sides of the dish, gently add the plasimd and X-tremeGENE mix dropwise to the plated cells, resulting in a final volume of 26 mL per plate. Swirl to disperse mixture evenly. Do not pipette or swirl too vigorously, as you do not want to dislodge the cells from the plate.
7. Incubate cells at 37°C, 5% CO2 for 12-15 hours in lentiviral room.
8. After 12-15 hr, change the media to remove the transfection reagent. Replace with 25 mL fresh DMEM + 10% FBS + 1%penicillin/streptomycin+ 1%BSA per plate. Pipette the media onto the side of the plate so as not to disturb the transfected cells.
9. Incubate cells at 37°C, 5% CO2 for 31~36 hours.
10. Harvest media from cells and transfer to a polypropylene storage tube. The media contains your lentiviral particles. Store at 4°C.
11. Add 25 ml of fresh media containing antibiotics and 1%BSA to the cells and incubate at 37°C, 5% CO2 for 24 hours.
12. Harvest media from cells and pool with media from Day 4. Spin media at 1000 rpm for 5 minutes to pellet any 293FT cells that were inadvertently collected during harvesting.
	1. In lieu of centrifugation, you may filter the media through a 0.45 µm filter to remove the cells. Do not use a 0.2 µm filter, as this is likely to shear the envelope of your virus.
13. Virus may be stored at 4°C for a few days, but should be frozen at -20°C or -80°C for long-term storage.
	1. Freeze/thaw cycles decrease the efficiency of the virus, so Addgene recommends that you use the virus immediately or aliquot the media into smaller tubes to prevent multiple freeze/thaw cycles.
	2. If not using the virus immediately, 10 mL aliquots are usually optimal to avoid thawing more virus than necessary for a screen. Aliquot at least one 5mL aliquot for testing the MOI in order to avoid unnecessary thawing.

**Lentivirus Infection**

***Materials needed:***

* Polybrene transfection reagent
* Cells from cell lines being screened (at least 3x106-7.2x107)
* Appropriate media for cell line
* Puromycin
* 6-well plate
* 15-cm plates

Prior to lentiviral infection, ensure that you know the minimal dose of puromycin that will kill all cells for your cell line.

Infect cells with 2 libraries separately as follows:

1. Test the MOI every time for each cell line before proceeding with screening.
	1. To find optimal virus volumes for achieving an MOI of 0.3–0.5, each new cell type and new virus lots should be tested by infecting cells with several different volumes of virus. In two 6-well plate, plate between 5x105 and 1.2x106 cells per well in 2 ml media supplemented with 1 µg/ml polybrene (Sigma). Then add virus to of the wells at different volumes. (i.e. have two no-transduction controls, and one well for 2.5 µL, 5µL, 10 µL, 15 µL, 25 µL, 50 µL, 100 µL, 200 µL, 400 µL, and 800 µL.)

Incubate cells at 37°C, 5% CO2 for 48 hours in lentiviral room.

* 1. Select all transduced cells and one well of non-transduced cells (negative control) with puromycin. For most cell types, a final concentration of 1-4 µg/ml puromycin works well, although the minimum dose that kills all cells without any viral transduction should be determined in advance and the minimum concentration should be used for selection. Change the media for all wells, adding puromycin to each well except for one of the non-transduced wells—this will be the positive control for measuring your MOI.
	2. Incubate cells at 37°C, 5% CO2 for 48 hours. Change media, maintaining the same dose of puromycin, and incubate another 24 hours.
	3. Make sure cells in the puromycin-treated, non-transduced control well are all dead. Then calculate MOI and find out under what volume of virus. For the optimal MOI select the well that has 30-50% cell survival after selection as compared to the non-transduced, non-selected well.
	4. If cells in the negative control well are still alive, replace media with puromycin again, and check after 24 hours. Then calculate MOI.
1. Transduce 1x108 to 2x108 cells with each library
	1. It is optimal to have at least 1x108 cells per library in a screen with two conditions (i.e. one control condition and one experimental condition). Scale up the number of cells based on the number of conditions to be screened).
	2. After trypsinizing the cells, collect in two 50 ml polypropylene microfuge tubes (with ~1x108 to 2x108 for each library). Keep ~2x107 cells to plate for for control plate.
	3. Spin cells at 1000 rpm for 5 minutes. Discard supernatant. Add 10-20 ml fresh media to cells. Pipet up and down to re-suspend. Then add appropriate volumes (multiply the tested volume above by 54) of virus and add polybrene into each tube at a concentration of 1 µg/mL. Pipet up and down.
	4. Incubate cell solutions at 37°C, 5% CO2 for 0.5-1 hour. Gently invert every 10 minutes 6–8 times to mix. Alternatively, you can rock the cells or shake at 260 rpm.
	5. Plate cells for each library in six 15-cm tissue culture plates separately. Add media to 25 ml.
	6. Plate 2x107 cells in a 15 cm tissue culture plate as control.
	7. Select cells with puromycin as above.
	8. Incubate cells at 37°C, 5% CO2 for 72 hours.
	9. Check the control cells. Make sure all the control cell are dead.
	10. For screening cells, there should be 30%-50% cells survived.
	11. Incubate cells at 37°C, 5% CO2 for 48 hours.

**CRISPR Screen**

1. Harvest day 0 samples and culture cells in particular conditions according to the interest.
	1. Trypsinize the cells, and collect in one tube pre library. Count the number of cells for each library. The cells should be more than 6x107 for one library.
	2. Re-plate 3x107-5x107 cells per condition per library.
	3. Store pellet of 3x107 or more cells for each library at -20°C as a Day 0 timepoint sample.
	4. Culture the rest of cells for more than 2 weeks. (If the cell doubling time is about 2~3 days, it better to culture the cells for 1 month.)
	5. When the cells reach confluency or at selected timepoints, collect cells, count, and re-plate as above, always maintaining a population of at least 3x107 cells in culture to have adequate coverage of the sgRNA library. It is ideal to track the numbers of doublings for each cell population.
	6. When the desired number of doublings has been reached, collect the final samples and freeze in a 15-mL centrifuge tube.

**DNA Extraction**

***Materials needed:***

* PBS
* Qiagen Blood and Cell Culture Midi kit
* Or
* DMS Lysis Buffer (ddH2O, NaCl2, SDS, EDTA, Tris-HCl)
* RNAase A (Qiagen)

1. Thaw the 0 day sample and any selected timepoint samples. Extract genomic DNA with a Blood & Cell Culture Midi kit (Qiagen) **or** follow these instructions:

* 1. Wash cells with PBS (if not washed during collection) and spin at 1000 rpm for 5 minutes. Discard supernatant. Add DMS lysis buffer to each of the time point samples and pipette up and down. Use 3 mL for 3x107 cells, 5 mL for 7.5x107-1x108 cells.

DMS Lysis Buffer (ddH2O as solvent)

400 mM NaCl2

0.2% SDS

1 mM EDTA

10 mM Tris-HCl (ph 8.0)

* 1. Add RNAase A at a final concentration of 100 µg/mL. Incubate tubes at 65°C for 1 hour.
	2. Add protein kinase or protease K to a final concentration of 100µg/mL. Incubate tubes at 55°C for at least 5 hours or overnight, either rotating constantly or inverting the tubes every hour. The solution should become more transluscent.
	3. Add one volume phenol/chloroform/isoamyl alcohol (PCI) solution (25:24:1)
	4. Invert 6–8 times, Incubate at room temperature for 5 minutes.
	5. Spin tubes at maximum speed for 15 minutes.
	6. Move supernatant to a fresh tube.
	7. Add 1 V isopropanol. Mix by inverting.
	8. Incubate at -20°C for at least 1-2 hours. Spin tubes at maximum speed for 15 minutes. Discard supernatant.
	9. Wash 2 times with 70% ethanol. Open tubes, dry them in the air for 5-20 minutes until all ethanol is gone.
	10. Add 200-500 µL water or elution buffer to dissolve DNA. Wait at least one hour.
	11. Secondary extraction: Transfer re-dissolved DNA to an Eppendorf tube or a 2mL Phase Lock Gel Light tubes for better stratification.
	12. Add 1/10 V NaAc (3mol/L).
	13. Add one volume of PCI and mix thoroughly by repeated inversion.
	14. Spin at maximum speed and transfer to fresh tube.
	15. Repeat steps o.-r. to clean and re-dissolve DNA.
	16. Store at 4°C to dissolve completely.
	17. Measure the concentration. At least 200 µg is needed for adequate coverage during the library-amplification.

**Two-step PCR for DNA Sequencing preparation DRAFT**

1. For the first-round PCR, calculate the amount of input genomic DNA (gDNA) for each sample in order to achieve 300X coverage over the library, which results in 200 µg DNA per sample (assuming 6.6 µg of gDNA for 106 cells). For each sample, perform 25-30 separate 100 µL reactions with 6-8 µg genomic DNA in each reaction using Q5® High-Fidelity DNA Polymerase (Biolabs®) and then combine the resulting amplicons (~2.5-3mL) in a 15-mL centrifuge tube.

Primers sequences to amplify lentiCRISPR sgRNAs for the first PCR are:

lentiCRIPR\_F1: AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG

lentiCRISPR\_RV2: TCTACTATTCTTTCCCCTGCACTGTACCTGTGGGCGATGTGCGCTCTG

Recommended reaction setup and thermocycling Conditions as follows:

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| **STEP** | **TEMP** | **TIME** |
| Initial Denaturation | 98°C | 30 seconds |
| 16 Cycles | 98°C | 15 seconds |
| 68°C | 25 seconds |
| 72°C | 25 seconds |
| Final Extension | 72°C | 2 minutes |
| Hold | 4°C |  |

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| **Component** | **100 µL Reaction** |
| 5X Q5 Reaction Buffer | 20 µL |
| 10 mM dNTPs | 2 µL |
| 10 µM Forward Primer | 2 µL |
| 10 µM Reverse Primer | 2 µL |
| Template DNA | variable |
| Q5 High-Fidelity DNA Polymerase | 1 µL |
| Nuclease-Free Water | to 100 µL |

2. A second PCR is needed to attach Illumina adaptors and to barcode samples. Perform the second PCR in a 100 µL reaction volume using 2 µL of the product from the first PCR after mixing well.

* + 1. Primers for the second PCR include both a variable length sequence to increase library complexity and a 6-bp barcode for multiplexing of different biological samples:
		2. Recommended reaction setup and thermocycling conditions are the same as for the first PCR except to reduce the number of cycles to 8-12.

3. Purify PCR product by 2% agarose gel and Gel purification Kit. For the optimal yields for sequencing, 50 µL of the second-round PCR product should be used.