

Pooled Genome-Scale CRISPR-Cas9 Knock-out Screens in Human Cells

This is a research protocol that describes a protocol to perform pooled genome scale gRNA depletion screens in human cells using CRISPR libraries

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1 Introduction:

CRISPR-Cas9 technology is an efficient approach for gene inactivation. Through a synthetic single gRNA (sgRNA), Cas9 nuclease can be programmed to induce loss of function mutations at the target site. We have developed a unique CRISPR-Cas9 gRNA lentiviral library, the Toronto KnockOut library v3 (TKOv3) that covers all ~18,000 human genes to enable genome-scale loss of function screens in mammalian cells. TKOv3 is available as a one-component library (LCV2::TKOv3, Addgene pooled library #90294), expressing Cas9 and sgRNAs on a single vector. This alleviates the need to generate stable Cas9 expressing cells, enabling genome-wide knockout across a broad range of mammalian cell types. TKOv3 is also available in a vector without Cas9 (pLCKO2::TKOv3, Addgene pooled library #125517), and can be utilized in cells that express Cas9.

Library description: The CRISPR TKOv3 pooled library consists of specific sgRNA for gene knock out in the human genome. The library contains 70,948 gRNAs targeting 18,053 protein coding genes (4 gRNAs/gene) with 142 control non-targeting guides against EGFP, LacZ and Luciferase for a total library size of 71,090 guides.

2 Biosafety practices:

- All work in procedures 3.2 to 3.5 will be performed in a Class IIa biosafety cabinet and 37°C, 5% CO₂ incubator.
- Aseptic techniques must be practiced to ensure sterility.
- **Lentiviral precautions:** When working with active lentiviral (production, MOI determination and primary infection steps), proper handling of lentivirus should be followed as outlined by your institutions' Environmental Health and Safety Office.

3 Procedures:

3.1 Toronto KnockOut version (TKOv3) CRISPR Library Amplification

1. Dilute the TKO plasmid DNA library to 50 ng/ μ L in TE.
2. Electroporate the library using Endura electrocompetent cells (Lucigen, 60242). Consult the manufacturer's protocol for extra details. Set up a total of 4 electroporations as follows:
 - a. Add 2 μ L of 50 ng/ μ L TKO library to 25 μ L of thawed Lucigen Endura electrocompetent cells to pre-chilled cuvettes (1.0 mm) on ice;
 - b. Electroporate according to the manufacturer's suggested conditions and protocol;
 - c. Within 10 seconds of the pulse, add 975 μ L of Recovery Medium (or SOC medium) to the cuvette;
 - d. Transfer cells to a culture tube with an additional 1 mL of Recovery Medium;
 - e. Place tubes in a shaking incubator at 250 rpm for 1 hour at 37°C.
3. Set up a dilution plate to titer the library and estimate transformation efficiency
 - a. Pool all 8 mL of recovered cells and mix well.
 - b. Transfer 10 μ L of the pooled cells to 990 μ L of Recovery Medium for an 800-fold dilution and mix well. Plate 20 μ L of the dilution onto a pre-warmed 10-cm LB + carbenicillin (100 mg/mL) agar plate. This results in a 40,000-fold dilution of the full transformation that will be used to calculate the transformation efficiency.
4. Plate the library by spreading the rest of the recovered cells on a total of 20 pre-warmed 15-cm LB agar plates containing carbenicillin (100 mg/mL). Spread 400 μ L of recovered cells evenly on each plate
5. Incubate the plates for 14-16 hours at 30°C. Growth at this lower temperature minimizes recombination between long-terminal repeats (LTR).
6. Calculate transformation efficiency.
 - a. Count the number of colonies on the 40,000-fold dilution plate (set up in step 4).
 - b. Multiply the number of colonies by 40,000 to obtain the total number of colonies plated.
 - c. Proceed if the total number of colonies represents a library coverage equivalent to minimum of 200X colonies per sgRNA (most optimal is 500-1000X). If using TKOv3 library, the minimal colony number is at least 1.4×10^7 . This is equivalent to 200X colonies per sgRNA in the 71,090 sgRNA TKOv3 library. Obtaining sufficient number of colonies will ensure full library representation is preserved.
 - d. If colony representation is insufficient, increase the number of electroporations in step 1.2 based of the number of colonies on your dilution plate to achieve the minimum library coverage.
7. Harvest colonies
 - a. Transfer 7 mL of LB + carbenicillin (100 μ g/L) medium to one 15-cm plate.
 - b. Scrape the colonies off with a cell spreader.

- c. Transfer the scraped cells into a sterile 1 L Erlenmeyer flask or bottle using a 10-mL pipet.
 - d. Rinse the scraped plate with an additional 5 mL of LB + carbenicillin medium and transfer to the bottle.
 - e. Repeat steps (a)-(d) for all plates. Pool all scraped cells from 20 plates to a sterile bottle.
 - f. Mix collected cells with a stir bar for 1 hour at room temperature to break up cell clumps.
 - g. Transfer cells to pre-weighed centrifuge bottles.
 - h. Centrifuge at 7,000 x g to pellet bacteria, then discard media.
 - i. Weigh the wet cell pellet and subtract the weight of the centrifuge bottle to determine the final weight of the wet pellet.
8. Purify the library plasmid pool.
- a. Purify plasmid DNA using a maxi- or mega-scale plasmid purification kit.
 - b. Perform multiple maxi or mega preps according to column capacity. Typically, a maxi column can process 1 g of wet cell pellet, and a mega column can process 2.5 g of wet cell pellet.

3.2 Large scale lentivirus production of TKOv3 in 15-cm TC plates

*Biosafety precautions: Proper handling of lentivirus should be followed as outlined by your institutions' Environmental Health and Safety Office.

Materials needed:

- 293T packaging cells (recommended: passage number < 15)
- Transfection quality plasmids: CRISPR library (TKOv3), psPAX (packaging plasmid Addgene plasmid #12260), pMD2.G (envelope plasmid, Addgene plasmid #12259)
- X-tremeGene 9 (Roche, 06 365 787 001)
- OPTI-MEM serum-free media (Invitrogen, #31985-070)
- Cell seeding media - Low-antibiotic growth media (DMEM + 10% FBS + *optional*: 0.1x Pen/Strep):
 - 500 mL DMEM (Dulbecco's Modification of Eagle's Medium)
 - 50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone #SH30071.03)
 - 0.5 mL 100x Pen/Strep (*optional*)
- Viral harvest media- Serum-Free, High-BSA 293T growth media (DMEM + 1.1 g/100 mL BSA + 1x Pen/Strep)

- 500 mL DMEM (Dulbecco's Modification of Eagle's Medium)
- 32 mL of 20 g/100 mL BSA stock (dissolved in DMEM, filter sterilized with 0.22-micron filter)
- 5 mL 100x Pen/Strep

Instructions:

1. Seed 293T packaging cells in low-antibiotic growth media. 8E6 cells per 15 cm plate in 20 mL media. For 500 mL virus production prepare ~30 plates
2. Incubate cells for 24 hours (37 °C, 5% CO₂), or until the following afternoon. The cells should be 70% -80% confluent and evenly spread at moment of transfection.
3. Transfect packaging cells:
 - a. Prepare a mixture of the three transfection plasmids (~1:1:1 molar ratio) in Opti-MEM as outlined for 15-cm plates below. Calculate the amount of plasmid needed for each transfection and make a mix of plasmids for the number of plates, plus one to be transfected.

Reagent	Amount per 15-cm plate	
	LCV2::TKOv3	pLCKO2::TKOv3
Opti-MEM	100 µL	100 µL
psPAX2	4.8 µg	7.0 µg
pMD2.G	3.8 µg	4.0 µg
TKOv3*	8.0 µg	5.0 µg

*Amount TKO plasmid based on CRISPR library vector backbone. LCV2 all-in-one vector =13 kb, non-Cas9
pLCKO2 vector = 7.6 kb

- b. Prepare a separate mixture of X-tremeGENE 9 as outlined below. Aliquot Opti-MEM into 1.5-mL microcentrifuge tubes for the number of plates to be transfected. Add X-tremeGENE 9 into Opti-MEM aliquots, mix gently and incubate for 5 minutes at room temperature.

Reagent	Amount per 15-cm plate
Opti-MEM	800 µL
XtremeGENE 9	48 µL

- c. Following 5-minute incubation, add appropriate amount of plasmid mix to the X-tremeGENE 9 mix for a 3:1 ratio of transfection reagent:µg of DNA complex. Mix gently and incubate for 30 minutes at room temperature.

- d. Repeat until all DNA:XtremeGene 9 complexes have been made. **Tip:** Prepare single XtremeGENE 9 dilutions in sets of 5 or less, wait 3 minutes before setting up the next set. Add DNA at the 5-minute mark to the first set and so forth. This will stagger the preparation of transfection mix without over incubation.
 - e. After 30-minute incubation, carefully transfer each transfection mix to each plate of packaging cells. Add entire mix using a 1 mL pipette tip, drop-wise in a circular, zigzag motion without disturbing cell monolayer. Place plates in incubator and making sure plates are level.
4. Incubate cells for 18 hours (37 °C, 5% CO₂)
 5. After 18 hours, change media to remove the transfection reagent and gently replace with viral harvest media (serum-free high-BSA growth media) for viral harvests (18-20mL/15cm plate). Note: Use biosafety precautions for handling lentivirus waste.
 6. Incubate cells for 24 hours (37 °C, 5% CO₂).
 7. After 24 hours, harvest media containing lentivirus at ~40 hours post-transfection. Transfer media to a polypropylene storage tube. Packaging cells should look abnormal and fused, as an indication of good virus production. For large volumes of virus collect supernatant from all plates into in large volume centrifuge tubes.
 8. Spin the media containing virus at 1000 rpm for 5-10 minutes to pellet any packaging cells that were collected during harvesting. Aliquot supernatant into sterile polypropylene storage tube.
 9. Virus may be stored at 4 °C for short periods (hours to days), but should be frozen at -80 °C for long-term storage. To reduce the number of freeze/thaw cycles, aliquot large-scale virus preps to smaller storage tubes prior to long-term storage.
 10. **Note:** Based on functional titration on a transduction efficient cell line, typical viral titers achieved for the all-in-one LCV2::TKOv3 library ranges from 10⁵ to 10⁶ TU/mL; the non-Cas9 pLCKO2::TKOv3 library ranges from 10⁷ to 10⁸ TU/mL. Productive virus titration is required for individual cell lines.

3.3 Cell Line Characterization

1. Select desired cell line. Stable Cas9 expression is not required for screening with an all-in-one Cas9 containing CRISPR sgRNA libraries (e.g. LCV2::TKOv3). For non-Cas9 CRISPR sgRNA libraries (e.g. pLCKO2::TKOv3), generation of cell lines stably expressing Cas9 is required.
2. Ensure cell line is mycoplasma free before starting screen.
3. Ensure that all media requirements (+/- serum, growth factors, etc.) are met.
4. Determine optimal cell plating density for culturing cells in vessel of choice. Choose vessel that can grow an optimal number of cells with cell passaging at every 3-4 doublings.
5. Measure and record the approximate doubling time of your cells.

6. Determine puromycin sensitivity of cell line by doing a kill curve. This can be done in 12-well plates, and can be measured either by cell counting or by trypan blue staining. Dilution range should span 0 $\mu\text{g}/\text{mL}$ to 10 $\mu\text{g}/\text{mL}$ in 0.5 $\mu\text{g}/\text{mL}$ increments. Determine the lowest concentration that kills 100% of uninfected cells in 48 hours. Use this concentration of puromycin for cell selection after infection during the screen. Sensitivity and response time to puromycin may vary per cell lines, it is important to determine the concentration that will select cells within 48-72 hours to minimize drop out of essential genes before starting the screen. For cell lines with longer doubling times, longer incubations with puromycin can be tolerated. In these situations, determine the kill curve for the incubation time required for <3 cell doublings.
7. Check cells for sensitivity to either polybrene (up to 8 $\mu\text{g}/\text{mL}$) or protamine sulphate (up to 5 $\mu\text{g}/\text{mL}$) by doing a dose response curve in the same method as used for measuring puromycin sensitivity. If cells are sensitive to either polybrene $< 8 \mu\text{g}/\text{mL}$ or protamine sulphate $<5 \mu\text{g}/\text{mL}$, do not use.

3.4 Lentivirus MOI determination

- The multiplicity of infection (MOI) must be determined under the same cell culture conditions used during the primary screen. This includes using the same tissue culture vessels, media constituents and volume, cell plating density, and pooled virus preps (without prior thaws) that will be used in the screen. Measurements made in different formats (e.g. 6-well plates) cannot be reliably scaled to the screening format.

Day 1

- a. Thaw a fresh aliquot of TKOv3 pooled lentivirus (keep on ice)
- b. Harvest cells for test infection, and measure number of cells/ml. (HAP1 requires $3\text{E}6\text{-}5\text{E}6$ cells/plate)
- c. Design dilution series of virus for MOI determination between 0 to 2 mL.
- d. Plate cells, media and polybrene (typically final concentration is 8 $\mu\text{g}/\text{mL}$) and then add your designed volume of virus to each plate. Mix plates thoroughly then transfer plates to a tissue culture incubator and ensure they sit level. Minimize final volume in each vessel, for example in 15-cm plates infections are done in 20 mL total volume.
- e. Add the same volume of virus to two vessels for each dilution point
- f. Mix plates thoroughly by tilting for 2 min.
- g. Transfer plates to incubator. Ensure that they sit level.

Day 2

- a. 24 hrs after addition of virus, cells should be infected and tightly adhered to plate.

- b. Remove media using pipettes (use lentivirus biosafety precautions).
- c. Optional: Gently wash plate with warm PBS to remove any extraneous virus (dispose of PBS and pipettes in 20% bleach solution).
- d. Add fresh media (20 mL for 15-cm plate) containing puromycin at the required concentration to vessels of one virus dilution series and fresh media without puromycin to the other virus dilution series
- e. Return plates to incubator

Day 4

- a. After 48 hrs, all uninfected cells should be dead. You must use a dose of puromycin that will kill all uninfected cells within 48 hrs.
- b. Harvest cells. Make sure that any clumps of cells were dispersed by repeated gentle pipetting.
- c. Count cells from all plates and graph results for the two series (+/- puromycin).
- d. Determine virus volume that gives 30-40% survival with puromycin selection vs. without puromycin. This is the volume of pooled virus that gives MOI of 0.3-0.4 with the tissue culture conditions that you used

3.5 Pooled gRNA depletion screens

- 3.5.1. Primary screen infection and cell passaging
- 3.5.2. Genomic DNA extraction
- 3.5.3. TKOv3 CRISPR sequencing library preparation
- 3.5.4. High-throughput DNA sequencing and analysis

3.5.1 Primary Screen Infection and Cell Passaging

(The screening format described here uses 15-cm tissue culture plates. Any vessel of choice can be used with proper scaling of cell seeding density, see protocol 3.3)

- Expand cells to approximately 80-90E6 cells for Day 1.

Day 1: Infection

- The total number of cells plated for infection should be such that with infection at MOI 0.3, puromycin selection and the growth rate of the cells, you will be able to harvest ~ 120E6 cells on T0.

- Set up infections in 15-cm TC plates at minimum 200-fold coverage of the CRISPR sgRNA library. For example, for 200-fold coverage of TKOv3 (71,090 guides), 1.5×10^7 infected cells are required to maintain this coverage. To achieve this at 30% infection efficiency (MOI 0.3) at least 5×10^7 cells is required for infection. Calculate the starting number of cells required for infection as follows:

$$\text{sgRNA library size} * \text{coverage} \div 0.3 \text{ MOI} = \text{starting cell number}$$

- Determine the number of plates required for infection as described below. Include extra plates to accommodate for MOI fluctuations and control plates.

	Treatment	Number of plates required for infection
Screening plates	Virus, + puromycin	$(\text{sgRNA library size} * 200\text{-fold}) \div 0.3 \text{ MOI} \div \text{cell density at infection} = \text{number of plates required}$
Control 1	No virus, + puromycin (0% survival control)	1
Control 2	Virus, + No puromycin (100% survival control)	1

- Harvest cells and pool into a sterile vessel. Seed required cell number to each plate.
- Add virus + polybrene 8 $\mu\text{g}/\text{mL}$ to plates.
- Mix thoroughly by titling the plates for 2 min.
- Alternatively, batch infections can be done by adding virus and polybrene to cells in suspension, mixing well, then plating.
- Place plates in the incubator, ensure they are level.

Day 2: Puromycin Selection

- 24 hours after addition of virus, cells should be infected and tightly adhered to plate.
- Remove media using pipette (dispose of media and pipettes in 20% bleach solution).
- Optional: Gently wash plate with warm PBS to remove any extraneous virus (dispose of PBS and pipettes in 20% bleach solution).
- Add fresh media containing puromycin at the required concentration to the cells. Add fresh media with no puromycin to control 2 plate.

Day 4: T0

- a. 48 hours after puromycin addition, all uninfected cells should be dead (control 1)
- b. Remove media, gently wash plate with warm PBS to dislodge remaining dead cells. It is important to remove all dead cells since their genomic DNA can contaminate the T0 prep.
- c. Trypsinize and collect cells from all plates into one sterile container. Make sure that all cell clumps are dispersed by gentle repeated pipetting when harvesting cells from plates/flasks.
- d. Count cells from pooled screening cells, control 1 and control 2 separately and calculate number of cells/mL.
- e. Harvest 3 replicates of pooled screening cells by centrifugation. Replicates should be collected at minimum 200-fold library coverage, 30E6 is recommended for T0.
- f. Spin at 1200 rpm for 5 minutes.
- g. Wash with PBS.
- h. Remove PBS, label tubes and freeze the cell pellets “dry” at -80°C. These are your time zero (T0) samples.
- i. From the pool, plate cells into three replicate groups. Do NOT use puromycin in this or in subsequent plating steps. Each replicate should contain in total 15E6 cells (200-fold library coverage) across the required number of vessels. Use exactly same number of cells for each replicate plate, and exactly same number of total cells between each replicate- example Replicate A – seed 3 plates at 5E6/plate for total 15E6 cells, repeat for Replicate B and C

**15E6 cells gives ~200-fold representation for each gRNA in TKOv3. If desired, you may use higher representation, 200-fold should be considered a minimum.

Day 5 Onward: (T1, T2,T18), cell passaging

- Most adherent cell lines require splitting every 3-8 days. The pool-infected cells should be passaged at the same density that you normally would split when expanding them. You may notice a transient slowing of cell growth after the infection, with a return to normal growth speed 5-15 days after infection. The length and severity of this effect is cell-line dependent.
- Each instance that you passage the cells out to the end-point (~3 weeks), do the following things:
 - a. Trypsinize and collect cells. Pool cells from all the vessels in each separate replicate group with each other. All the cells from replicate A are re-mixed together from separate plates, all the cells from replicate B are re-mixed together from separate plates, etc. This serves to minimize stochastic growth effects in random sub-populations in individual vessels within a replicate group.

- b. Re-plate 15E6 cells for each replicate group exactly as done at T0.
 - c. Freeze down 20E6 cells per pellet for each replicate group. Each pellet gets a time (T) number and a replicate designation. This number corresponds to the number of days post T0 that it was collected (eg. T3_A, T6_B, T_C, etc). If you chose to pellet more cells, make sure that ALL pellets collected in the screen have the same number of cells.
- Continue passaging cells to achieve 15-20 cell doublings. This is typically 15-18 days for cells that double every 24 hours.

* note: pelleting more cells (ie. 20E6) than the 15E6 cells representation of 200-fold – buffers for material loss during downstream genomic DNA processing procedures.

3.5.2 Genomic DNA (gDNA) Extraction and DNA precipitation

Genomic DNA extraction is performed using the Wizard Genomic DNA Purification Kit (Promega, A1120) and PureLink RNase A (Thermo Fisher Scientific, 12091021) as described in the kit manual. This protocol is optimized for cell pellets containing 20 to 50 million cells. To avoid contamination during purification of genomic DNA, use filtered pipette tips.

1. Thaw cell pellets at room temperature for 5-10 minutes.
2. Add 1.4 mL PBS to a 50-mL centrifuge tube containing cell pellet. Vortex for 20 seconds to resuspend cells and rest for 1 minute. If required, pipette 15 times with P1000, to break remaining cell clumps. If transferring cells from 15-mL or 1.5-mL tube, resuspend cells with 1 mL PBS, then transfer cells to a 50-ml tube, and rinse the original tube with 400 μ L PBS.
3. Add 5 mL Nuclei Lysis Solution to the resuspended cells. Using a 10-mL pipette, mix the sample by pipetting up and down 5 times.
4. Add 32 μ L RNase A (20 mg/mL), to a final concentration of 100 μ g/mL, to the nuclear lysate and mix the sample by inverting the tube 5 times. Incubate the mixture at 37 $^{\circ}$ C for 15 minutes, and allow sample to cool for 10 minutes at room temperature.
5. Add 1.67 mL Protein Precipitation Solution to the nuclear lysate, and vortex vigorously for 20 seconds. Small protein clumps may be visible after vortexing.
6. Centrifuge at 4,500 $\times g$ for 10 minutes at room temperature.
7. Using a 10-mL pipette, transfer the supernatant to a 50-mL centrifuge tube containing 5 mL isopropanol.
8. Gently mix the solution 10 times by inversion, until the white thread-like strands of DNA form a visible mass.

9. Centrifuge at $4,500 \times g$ for 5 minutes at room temperature. The DNA will be visible as a small white pellet.
10. Using a 10-mL pipette, carefully remove supernatant and avoid dislodging DNA pellet.
11. Add 5 mL room temperature 70% ethanol to the DNA. Gently rotate the tube to wash the DNA pellet and the sides of the centrifuge tube.
12. Centrifuge at $4,500 \times g$ for 5 minutes at room temperature.
13. Using a 10-mL pipette, carefully remove 70% ethanol and avoid dislodging DNA pellet. Air-dry genomic DNA for 10 minutes at room temperature.
14. Add 400 μ L DNA Rehydration Solution or TE to the tube, and let DNA dissolve by incubating at 65 °C for 1 hour. Mix DNA by gently flicking the tube every 15 minutes.
If DNA does not dissolve completely, incubate tube at 65 °C for an additional 1 hour, gently flicking the tube every 15 minutes, and leave it at 4 °C overnight.
15. Centrifuge at $4,500 \times g$ for 1 minute at room temperature and transfer genomic DNA to a 1.5-mL low-binding tube.
16. Quantitate and measure purity of genomic DNA on both NanoDrop (for total nucleic acid content) and Qubit (for double-stranded DNA content) using Qubit dsDNA BR Assay (Thermo Fisher Scientific, Q32853). Using both systems will ensure highest quality DNA and accurate quantitation of double-stranded DNA. Determine the purity of DNA spectrophotometrically on the NanoDrop. High quality DNA samples have an A260/280 ratio of 1.8 to 2.0, indicating the absence of contaminating proteins and an A260/230 ratio of >2.0, indicating the absence of other organic contaminants.

Genomic DNA Precipitation (optional)

This step is recommended if you experience issues with downstream PCR amplification of the guide-RNA.

1. Transfer 400 μ L genomic DNA into a 1.5-mL microcentrifuge tube.
2. Add 18 μ L of 5 M NaCl (final concentration of 0.2 M) and 900 μ L of 100% ethanol.
Do NOT use sodium acetate as residual acetate interferes with downstream PCR.
3. Invert tube 10 times until thoroughly mixed, then centrifuge at 13,000 rpm for 15 minutes at room temperature.
4. Carefully remove supernatant and avoid dislodging DNA pellet.
5. Wash DNA pellet with 500 μ L of 70% ethanol. Gently rotate the tube to wash the DNA pellet.
6. Centrifuge at 13,000 rpm for 15 minutes at room temperature.
7. Carefully remove supernatant and avoid dislodging DNA pellet.
8. Air-dry genomic DNA for 10 minutes at room temperature.

9. Add 300 μ L of TE to dissolve DNA as described in steps 14 and 15.

3.5.3 TKOv3 CRISPR Sequencing Library Preparation

- This sequencing library protocol is optimized for TKOv3 CRISPR libraries in both vector backbones.
- Perform two-step PCR to (1) enrich guide-RNA regions in the genome and (2) amplify guide-RNAs with Illumina TruSeq adapters with i5 and i7 indices. These indices are unique sequences that are added to DNA samples during library preparation and act as sample identifiers during multiplex sequencing. Combinatorial dual indices enable multiplexing a large number of samples without the high cost of ordering PCR primers with unique sample index. Alternatively, sequencing libraries can be generated in a single PCR protocol similar to the one described in¹. For one-step PCR approach, use the barcoded PCR2 primers described herein.
- Include a no-template negative control when setting up PCR. Set up PCR reactions in a dedicated PCR hood to minimize contamination from plasmids and other sequencing libraries. Treat all agarose gel equipment for purifying amplified products with 0.1 N HCl for 10 minutes prior to casting a gel.
- PCR 1 primers are regular desalted oligos. PCR 2 primers can be ordered from IDT (www.idtdna.com) as desalted Ultramer DNA oligos. Table 1 lists primer sequences for amplification of LCV2::TKOv3 sequencing libraries. Table 2 lists primer sequences for amplification of pLCKO2::TKOv3 sequencing libraries.

Instructions:

1. Set up PCR 1 as outlined below using a total of 100 μ g genomic DNA. Assuming a diploid genome is \sim 7.2 pg and one guide-RNA per genome, 100 μ g of genomic DNA yields \sim 200-fold coverage of the TKOv3 library. Add 3.5 μ g of genomic DNA per 50 μ L reaction. Set up identical 50 μ L reactions to achieve the desired coverage. Note: For positive selections (e.g. drug resistance screen) library coverage and read depth can be reduced to 50-100-fold representation since only a small cell population is expected to survive.

PCR 1.	1x
2x NEBNext Ultra II Q5 Master Mix	25 μ L
10 μ M PCR 1 LCV2 forward primer	2.5 μ L
10 μ M PCR 1 LCV2 reverse primer	2.5 μ L
Genomic DNA	3.5 μ g

¹Hart, T. et al. High-Resolution CRISPR Screens Reveal Fitness Genes and Genotype-Specific Cancer Liabilities. Cell. 163 (6), 1515-1526, (2015).

Water	up to 50 μ L
Total	50 μ L

2. Amplify reactions in a thermocycler using the following program

Step	Temperature	Time	
1	98°C	30 sec	
2	98°C	10 sec	25 cycles (step 2 – 4)
3	66°C	30 sec	
4	72°C	15 sec	
5	72°C	2 min	
6	10°C	Hold	

3. Run 2 μ L of PCR 1 product on a 1% agarose gel. Visualize the PCR product on a gel imager. PCR 1 yields a product of 600 bp.
4. Pool all individual 50 μ L reactions for each genomic DNA sample, mix by vortex.
5. Set up PCR 2 as outlined below. Use unique i5 and i7 index primer combinations for each individual sample to allow pooling of sequencing library samples. Set up one 50 μ L reaction for each sample. Use 5 μ L of the pooled PCR 1 product as template.

PCR 2.	1x
2x NEBNext Ultra II Q5 Master Mix	25 μ L
10 μ M i5 forward primer	2.5 μ L
10 μ M i7 reverse primer	2.5 μ L
PCR 1 product	5 μ L
Water	15 μ L
Total	50 μ L

6. Amplify PCR 2 reaction in a thermocycler using the following program

Step	Temperature	Time	
1	98°C	30 sec	
2	98°C	10 sec	10 cycles (step 2 – 4)
3	55°C	30 sec	

4	65°C	15 sec	
5	65°C	5 min	
6	10°C	Hold	

- Run 50 μ L of PCR 2 product on a 2% agarose gel. PCR 2 yields a product of 200 bp.
- Visualize the PCR product on a Dark Reader (blue light transilluminator). Excise the 200 bp band and purify DNA from agarose gel slice using a gel extraction kit.
- Quantitate and measure purity of sequencing library on both NanoDrop and Qubit.

Table 1. PCR primers for amplification of LCV2::TKOv3 sequencing libraries.

PCR 1 – Primer Sequences	
LCV2 forward primer	GAGGGCCTATTTCCCATGATTC
LCV2 reverse primer	GTTGCGAAAAGAACGTTACGG
PCR 2 – i5 and i7 Index Primer Sequences for Illumina Sequencer	
PCR 2 – i5 forward primers	
D501-F	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D502-F	AATGATACGGCGACCACCGAGATCTACAC ATAGAGGC ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D503-F	AATGATACGGCGACCACCGAGATCTACAC CCTATCCT ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D504-F	AATGATACGGCGACCACCGAGATCTACAC GGCTCTGA ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D505-F	AATGATACGGCGACCACCGAGATCTACAC AGGCGAAG ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
D506-F	AATGATACGGCGACCACCGAGATCTACAC TAATCTTA ACACTCTTT CCCTACACGACGCTCTTCCGATCT TTGTGGAAAGGACGAAACACCG
PCR 2 – i7 reverse primers	
D701-R	CAAGCAGAAGACGGCATAACGAGAT CGAGTAAT GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAC
D702-R	CAAGCAGAAGACGGCATAACGAGAT TCTCCGGA GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAC

D704-R	CAAGCAGAAGACGGCATAACGAGAT GGAATCT CGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAAC
D705-R	CAAGCAGAAGACGGCATAACGAGAT TTCTGAAT GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAAC
D706-R	CAAGCAGAAGACGGCATAACGAGAT ACGAATTC GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAAC
D707-R	CAAGCAGAAGACGGCATAACGAGAT AGCTTCAG GTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT ACTTGCTATTTCTAGCTCTAAAAC

Red sequence denotes i5 or i7 index

Blue sequence denotes annealing sequence

Table 2. PCR primers for amplification of pLCKO2::TKOv3 sequencing libraries.

PCR 1 – Primer Sequences	
pLCKO2 forward primer	GAGGGCCTATTTCCCATGATTC
pLCKO2 reverse primer	CAAACCCAGGGCTGCCTTGAA
PCR 2 – i5 and i7 Index Primer Sequences for Illumina Sequencer	
PCR 2 – i5 forward primers	
S501-F	AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
S502-F	AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
S503-F	AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
S504-F	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
S505-F	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
S506-F	AATGATACGGCGACCACCGAGATCTACACACTGCATAACACTCTTT CCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAGGTACCG
PCR 2 – i7 reverse primers	
D701-R	CAAGCAGAAGACGGCATAACGAGATCGAGTAATGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
D702-R	CAAGCAGAAGACGGCATAACGAGATTCTCCGGAGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
D704-R	CAAGCAGAAGACGGCATAACGAGATGGAATCTCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
D705-R	CAAGCAGAAGACGGCATAACGAGATTTCTGAATGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
D706-R	CAAGCAGAAGACGGCATAACGAGATACGAATTCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
D707-R	CAAGCAGAAGACGGCATAACGAGATAGCTTCAGGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC

Red sequence denotes i5 or i7 index

Blue sequence denotes annealing sequence

3.5.4. High-throughput DNA sequencing and analysis

- Sequencing libraries can be sequenced on Illumina HiSeq 2500 or NextSeq 500. For HiSeq 2500, use standard Single-Read (SR) 50-cycle chemistry with dual-index. For NextSeq 500, use standard Single-Read (SR) 75-cycle chemistry with dual-index.
- The following sequencing run parameter is recommended: Dark Cycle: 21; Read 1: 26; Index Read 1: 8; Index Read 2: 8. 21 dark cycles, base additions without imaging, are applied to get through the constant amplicon primer region that corresponds to the 3' end of the U6 promoter. The actual sequence read begins after the dark cycles.
- If the option of dark cycles is not available, modify i5 forward primers to include a staggered region of different lengths. The resulting amplified products will contain staggered regions to provide sequence diversity in the constant amplicon primer sequences. Introduce staggered sequence 5' immediate of the annealing sequence in the i5 forward primer as outlined below. We recommend varying the length of the staggered sequence from 0 to 5 bases. The i7 reverse primers do not need to be modified.

Example of staggered sequence design for i5 forward primer (N refers to the i5 barcode, S refers to the staggered region):

```
AATGATACGGCGACCACCGAGATCTACAC [NNNNNNNN] ACACTCTTTCCTACACGAC
GCTCTTCCGATCT [S] TTGTGGAAAGGACGAAACACCG
```

To sequence samples amplified with staggered primers, adjust the sequencing run parameter by increasing the number of cycles for Read 1 as follows: 26 bp + [length of the longest staggered region].

- Sequence reference T0 samples at higher read depth of 400-500-fold library coverage. Sequence experimental timepoint samples for drop out screens at a minimum read depth of 200-fold. For strong positive selection screens, a minimum of read depth of 50-fold coverage is sufficient for identification of enriched sgRNAs.
- It is critical to sequence the T0 sample to determine library representation for a particular screen and serve as the reference for the determining sgRNA fold changes over time.

Instructions:

1. To de-multiplex sequencing data, trim sequence reads to remove the last six bases and map reads to the reference sgRNA sequence library (provided for all ready-made libraries) using Bowtie with the following parameters: -v2 (allowing two mismatches) and -m1 (discarding any read that mapped to more than one sequence in the library)
2. Normalize read counts to ten million reads per sample.
3. Calculate log₂ fold change of each sgRNA for each replicate at each timepoint (T_n) compared to the T₀ sample (T_n/T₀). Add a pseudo count of 0.5 reads to all read counts to prevent discontinuities from zeros. Exclude sgRNAs with < 30 raw reads in the T₀ sample from fold-change calculation and downstream analysis.

4. Assess screen performance by calculating the fold change values for the gold-standard reference essentials and non-essentials gene sets.
5. Analyze fold changes with the Bayesian Analysis of Gene Essentiality (BAGEL) algorithm², using the essential and non-essential training sets defined in³ to calculate a Bayes Factor (BF) for each gene. BAGEL uses a Bayesian framework to compare the distributions of known essential and non-essential gene sets to the log-fold change of all guides targeting a gene. This method is described in detail in⁴ and available at github.com/hart-lab/bagel. In addition to BAGEL, other algorithms designed to identify both enriched and depleted sgRNAs, such as MAGeCK⁵ can also be used. For drug screens we recommend using the DrugZ algorithm developed by Wang et al⁶, to identify both synergistic and suppressor chemical genetic interactions, available at github.com/hart-lab/drugZ.

² Hart, T. & Moffat, J. BAGEL: a computational framework for identifying essential genes from pooled library screens. *BMC Bioinformatics*. 17 164, (2016).

³ Hart, T. et al. Evaluation and Design of Genome-Wide CRISPR/SpCas9 Knockout Screens. *G3: Genes/Genomes/Genetics*. 7 (8), 2719-2727, (2017).

⁴ Hart, T. et al. High-Resolution CRISPR Screens Reveal Fitness Genes and Genotype-Specific Cancer Liabilities. *Cell*. 163 (6), 1515-1526, (2015).

⁵Evers, B. et al. CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nature Biotechnology*. 34 (6), 631-633, (2016).

⁶ Wang, G. Z., M.; Mascall, K.; Lenoir, WF., Moffat J.; Angers, S.; Durocher, D.; Hart, T. Identifying drug-gene interactions from CRISPR knockout screens with drugZ. *bioRxiv*. <https://doi.org/10.1101/232736>, (2017).