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TITLE:

Pooled CRISPR-Based Genetic Screens in Mammalian Cells

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SUMMARY:

CRISPR-Cas9 technology provides an efficient method to precisely edit the mammalian genome in any cell type and represents a novel means to perform genome-wide genetic screens. A detailed protocol discussing the steps required for the successful performance of pooled genome-wide CRISPR-Cas9 screens is provided here.

ABSTRACT:

Genome editing using the CRISPR-Cas system has vastly advanced the ability to precisely edit the genomes of various organisms. In the context of mammalian cells, this technology represents a novel means to perform genome-wide genetic screens for functional genomics studies. Libraries of guide RNAs (sgRNA) targeting all open reading frames permit the facile generation of thousands of genetic perturbations in a single pool of cells that can be screened for specific phenotypes to implicate gene function and cellular processes in an unbiased and systematic way. CRISPR-Cas screens provide researchers with a simple, efficient, and inexpensive method to uncover the genetic blueprints for cellular phenotypes. Furthermore, differential analysis of screens performed in various cell lines and from different cancer types can identify genes that are contextually essential in tumor cells, revealing potential targets for specific anticancer therapies. Performing genome-wide screens in human cells can be daunting, as this involves the handling of tens of millions of cells and requires analysis of large sets of

data. The details of these screens, such as cell line characterization, CRISPR library considerations, and understanding the limitations and capabilities of CRISPR technology during analysis, are often overlooked. Provided here is a detailed protocol for the successful performance of pooled genome-wide CRISPR-Cas9 based screens.

INTRODUCTION:

CRISPR-Cas, short for clustered regularly interspaced short palindromic repeats and CRISPR-associated nuclease, consists of a single nuclease protein (e.g., Cas9) in complex with a synthetic guide RNA (sgRNA). This ribonucleoprotein complex targets the Cas9 enzyme to induce double-stranded DNA breaks at a specific genomic locus[1]. Double-stranded breaks can be repaired via homology directed repair (HDR) or, more commonly, through non-homologous end joining (NHEJ), an error prone repair mechanism that results in insertion and/or deletions (INDELS) that frequently disrupt gene function[1]. The efficiency and simplicity of CRISPR enables a previously unattainable level of genomic targeting that far surpasses previous genome editing technologies [i.e., zinc finger nucleases (ZNF) or transcription activator-like effector nucleases (TALENS), both of which suffer from heightened design complexity, lower transfection efficiency, and limitations in multiplex gene editing[2]].

The basic research application of CRISPR single-guide RNA-based genome editing has allowed scientists to efficiently and inexpensively interrogate the functions of individual genes and topology of genetic interaction networks. The ability to perform functional genome-wide screens has been greatly enhanced by use of the CRISPR-Cas system, particularly when compared to earlier genetic perturbation technologies such as RNA interference (RNAi) and gene trap mutagenesis. In particular, RNAi suffers from high off-target effects and incomplete knockdown, resulting in lower sensitivity and specificity compared to CRISPR[3-5], while gene trap methods are only feasible in haploid cells for loss-of-function screens, limiting the scope of cell models that can be interrogated[6]. The ability of CRISPR to generate complete gene knock-out provides a more biologically robust system to interrogate mutant phenotypes, with low noise, minimal off-target effects and consistent activity across reagents[5]. CRISPR-Cas9 sgRNA libraries that target the entire human genome are now widely available, allowing simultaneous generation of thousands of gene knock-outs in a single experiment[3,7-9].

We have developed unique CRISPR-Cas9 genome-wide sgRNA lentiviral libraries called the Toronto Knock-out (TKO) libraries (available through Addgene) that are compact and sequence-optimized to facilitate high resolution functional genomics screens. The latest library, TKOv3, targets ~18,000 human protein-coding genes with 71,090 guides optimized for editing efficiency using empirical data[10]. Additionally, TKOv3 is available as a one-component library (LCV2::TKOv3, Addgene ID #90294) expressing Cas9 and sgRNAs on a single vector, alleviating the need to generate stable Cas9-expressing cells, enabling genome-wide knock-out across a broad range of mammalian cell types. TKOv3 is also available in a vector without Cas9 (pLCKO2::TKOv3, Addgene ID# 125517) and can be utilized in cells that express Cas9[11].

A genome-wide CRISPR-Cas9 edited cell population can be exposed to different growth conditions, with the abundance of sgRNAs over time quantified by next-generation sequencing, providing a readout to assess drop-out or enrichment of cells with traceable genetic perturbations. CRISPR knock-out libraries can be harnessed to identify genes that, upon perturbation, cause cellular fitness defects, moderate drug sensitivity (e.g., sensitive or resistant genes), regulate protein expression (e.g., reporter), or are required for a certain pathway function and cellular state[12-14]. For example, differential fitness screens in a cancer cell line can identify both depletion or reduction of oncogenes and enrichment or an increase of tumor suppressors genes[3,14,15]. Similarly, using intermediate doses of therapeutic drugs can reveal both drug resistance and sensitization genes[16,17].

Provided here is a detailed screening protocol for genome-scale CRISPR-Cas9 loss-of-function screening using the Toronto Knock-out libraries (TKOv1 or v3) in mammalian cells from library generation, screening performance to data analysis. Although this protocol has been optimized for screening using the Toronto Knock-out libraries, it can be applied and become scalable to all CRISPR sgRNA pooled libraries.

PROTOCOL:

The experiments outlined below follows the institute's Environmental Health and Safety Office guidelines.

1. Pooled CRISPR sgRNA lentiviral library plasmid amplification

1.1 Dilute the ready-made CRISPR sgRNA plasmid DNA library to 50 ng/μL in TE (e.g., TKOv3).

1.2 Electroporate the library using electrocompetent cells. Set up a total of four electroporation reactions as described below.

1.2.1 Add 2 μL of 50 ng/μL TKO library to 25 μL of thawed electrocompetent cells to pre-chilled cuvettes (1.0 mm) on ice.

1.2.2 Electroporate using optimal settings suggested by the manufacturer's protocol. Within 10 s of the pulse, add 975 μL of Recovery Medium (or SOC medium) to the cuvette.

1.2.3 Transfer electroporated cells to a culture tube and add 1 mL of Recovery Medium. Incubate tubes in a shaking incubator at 250 rpm for 1 h at 37 °C.

1.3 Set up a dilution plate to titer the library and estimate transformation efficiency.

1.3.1 Pool all 8 mL of recovered cells and mix well. Transfer 10 μL of the pooled cells to 990 μL of Recovery Medium for an 800-fold dilution and mix well.

1.3.2 Plate 20 µL of the dilution onto a pre-warmed 10 cm LB + carbenicillin (100 µg/L) agar plate. This results in a 40,000-fold dilution of the transformants that will be used to calculate the transformation efficiency.

1.3.3 Plate 400 µL of recovered cells on each plate across a total of 20 pre-warmed 15 cm LB + carbenicillin agar plates. Incubate the plates for 14–16 h at 30 °C.

NOTE: Growth at this lower temperature minimizes the recombination between long-terminal repeats (LTR)[18].

1.3.4 To calculate the transformation efficiency, count the number of colonies on the 40,000-fold dilution plate (step 1.3.2). Multiply the number of colonies counted by 40,000 to obtain the total number of colonies on all plates. Proceed if the total number of colonies represents a library coverage equivalent to minimum of 200x colonies per sgRNA (most optimal is 500-1000x).

1.3.4.1. For example, the minimal colony number for TKOv3 library (71,090 sgRNA) is 1.4×10^7, which is equivalent to 200x colonies per sgRNA. If colony representation is insufficient, increase the number of electroporations in step 1.2 based on the number of colonies on the dilution plate to achieve the minimum library coverage.

1.4 Harvest the colonies as described below

1.4.1 To each 15 cm plate, add 7 mL of LB + carbenicillin (100 µg/L) medium, then scrape the colonies off with a cell spreader. With a 10 mL pipette, transfer the scraped cells into a sterile 1 L conical flask or bottle.

1.4.2 Once again rinse the plate with 5 mL of LB + carbenicillin medium and transfer the solution to the bottle.

1.4.3 Repeat for all plates to pool cells from 20 plates into a sterile bottle.

1.5 Mix collected cells with a stir bar for 1 h at room temperature (RT) to break up cell clumps. Transfer cells to pre-weighed centrifuge bottles and centrifuge at 7,000 x *g* to pellet bacteria, then discard media.

1.6 Weigh the wet cell pellet and subtract the weight of the centrifuge bottle to determine the final weight of the wet pellet. Purify plasmid DNA using a maxi- or mega-scale plasmid purification kit depending on the amount of bacterial pellet each column can process.

2. Large-scale CRISPR sgRNA library lentivirus production

NOTE: All steps in this section of the protocol are performed in a BSL2+ facility in a Class II, Type A2 biosafety cabinet.

2.1 Calculate the number of 15 cm plates required for virus production based on the estimate that 18 mL of virus is typically harvested from one 15 cm plate.

2.2 Prepare cells for transfection by seeding HEK-293T packaging cells in low-antibiotic growth media (DMEM + 10% FBS + 0.1x pen/strep) at 8 x 10^6 cells per 15 cm plate in 20 mL of media. Incubate cells overnight at 37 °C, 5% CO_2. Ensure that the plated cells are 70%–80% confluent and evenly spread at moment of transfection.

2.3 Prepare three transfection plasmids mixture as outlined in **Table 1** for 15 cm plates. Calculate the amount of plasmid needed for one transfection and make a mix of plasmids for the number of plates, plus one to be transfected.

2.4 Prepare a lipid-based transfection reagent for each transfection as outlined in **Table 2.** Aliquot reduced serum media into individual 1.5 mL microcentrifuge tubes for the number of plates to be transfected. Add transfection reagent, mix gently, and incubate for 5 min at RT.

2.5 Following 5 min incubation, add the amount of DNA required for one transfection to the transfection reagent for a 3:1 ratio of transfection reagent-to-µg of DNA complex. Mix gently and incubate for 30 min at RT.

NOTE: Subsequent transfections can be prepared in sets of five or less, with 5 min intervals to optimize for time and avoid over-incubation.

2.6 After 30 min of incubation, carefully transfer each transfection mix to each plate of packaging cells. Add the entire mix using a 1 mL pipette tip dropwise in a circular, zigzag motion without disturbing the cell monolayer. Incubate cells at 37 °C for 18 h at 5% CO_2.

2.7 Prepare viral harvest media: 500 mL of DMEM medium + 32 mL of BSA stock (20 g/100 mL, dissolved in DMEM, filter sterilized with 0.22 µm filter) + 5 mL of 100x pen/strep.

2.8 After 18 h, remove media (use proper handling of lentivirus waste such as incubation in 1% sodium hypochlorite for 30 min before disposal). Gently replace with 18 mL of viral harvest media to each plate. Incubate cells at 37 °C for 18 h at 5% CO_2.

2.9 After 24 h, check packaging cells for abnormal and fused morphology as an indication of good virus production. Then, harvest the lentivirus by collecting all supernatant and transferring into a sterile conical centrifuge tube.

2.10 Spin the media containing virus at 300 x *g* for 5 min and pellet the packing cells. Aliquot the supernatant into a sterile polypropylene tube without disturbing the pellet.

2.11 Store the virus at 4 °C for short periods (less than 1 week) or immediately at -80 °C for long-term storage. Aliquot large-scale virus preps to single use volumes for long-term storage to avoid freeze/thawing.

3. Cell line characterization for screening

3.1 Select the desired cell line.

3.1.1 Measure and record the approximate doubling time of the cells.

3.1.2 Determine optimal cell plating density for culturing cells every 3–4 cell doublings in a tissue culture vessel of choice (e.g., 15 cm tissue culture plates).

3.2 Determine the puromycin concentration to use in the desired cell line for selection of TKO libraries containing puromycin resistance markers as follows:

3.2.1 Seed cells in a 12 well plate at the density required to reach confluence after 72 h, then incubate overnight (37 °C, 5% CO_2).

3.2.2 The next day, change to a media containing a dilution range of puromycin concentrations from 0 μg/mL to 10 μg/mL, in 0.5 μg/mL increments. Incubate the cells for 48 h.

3.2.3 After 48 h, measure the cell viability by cell counting or alamarBlue staining.

3.2.4 Determine the lowest concentration that kills 100% of cells in 48 h. Use this concentration to select for CRISPR library transduced cell populations in steps 4.6 and 5.2.6.

NOTE: 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. Minimize the time for selection to avoid dropout of essential genes before the start of screening.

3.3 Check cells for sensitivity to hexadimethrine bromide (up to 8 μg/mL) by performing a dose response curve in the same method as used for measuring puromycin sensitivity (step 3.2). If toxicity is observed with <8 μg/mL of hexadimethrine bromide, do not use.

4. Functional titration of pooled CRISPR lentivirus library for determination of MOI

4.1 Thaw a fresh aliquot of pooled CRISPR gRNA library lentivirus (e.g., LCV2::TKOv3) and keep on ice.

4.2 Design a series of virus volumes to test between the ranges of 0–2 mL (i.e., 0 mL, 0.25 mL, 0.5 mL, 1 mL, and 2 mL).

4.3 Harvest target cells and seed cells in 15 cm plates at the density required to reach confluence in 72 h.

4.4 For each virus volume to be tested, prepare duplicate plates. Add cells, virus, hexadimethrine bromide (8 µg/mL), and media to a final volume of 20 mL. Mix plates thoroughly, sit plates level in incubator and incubate for 24 h (37 °C, 5% CO_2).

4.5 After 24 h, remove virus containing media and dispose (use biosafety precautions for handling of lentivirus waste). Optionally, gently wash the plate with warm PBS to remove extraneous virus.

4.6 For each virus condition, replace with 20 mL of media containing puromycin using the concentration determined to kill cells in section 3, to one replicate plate. To the other plate, add 20 mL of fresh media without puromycin. Incubate for 48 h (37 °C, 5% CO_2).

4.7 After 48 h, check that all uninfected cells (0 mL virus condition) treated with puromycin are dead. Harvest all plates individually and disperse cells by repeated gentle pipetting.

4.8 Count cells from all the plates and calculate the MOI for each virus volume by comparing cell counts with puromycin selection to cell counts without puromycin (i.e., +/- puromycin).

4.9 Graph results to determine the virus volume that leads to 30%–40% cell survival with puromycin selection versus without puromycin. Use this virus volume to achieve a MOI of 0.3–0.4 during the screen under the same tissue culture conditions.

5. Primary screen infection, selection, and cell passaging

5.1 Select the CRISPR sgRNA library coverage to be maintained throughout the screen (recommended minimum of 200-fold).

5.1.1 Based on the library coverage, determine the number of cells required to maintain this coverage per sgRNA and the number of cells required for infection at MOI 0.3 (**Table 3)**.

5.1.2 Determine the number of plates required to set up the infection (**Table 4)**.

5.2 Infecting the cells with CRIPSR library

5.2.1 Harvest cells and seed the required cell number to each 15 cm plate.

5.2.3 Add hexadimethrine bromide (8 µg/mL) to all plates.

5.2.4 Add the virus at the volume required for MOI 0.3 to screening and the Control 2 plates. For the Control 1, do not add virus, and replace that volume with media.

5.2.5 Mix plates thoroughly by tilting. Place plates in incubator, making sure they are level.

NOTE: Batch infections can be done by combining a master mix of virus, media, and hexadimethrine bromide to cells in suspension before plating.

5.2.6 Remove media and replace with fresh media containing puromycin at the concentration determined in step 3.2.4 to the screening and control 1 plates 24 h after virus infection. Add fresh media with no puromycin to the control 2 plate. Incubate cells for 48 h (37 °C, 5% CO_2).

5.2.7 48 h after puromycin addition, ensure that all uninfected cells are dead (control 1) to confirm puromycin activity, then harvest the infected cells.

5.3 Harvesting infected cell population and cell passaging

5.3.1 Harvest the puromycin-selected cells from all screening plates into one sterile container. Collect the cells from each control plate separately. Disperse cells by gentle repeated pipetting.

5.3.2 Count cells from pooled screening cells, control 1, and control 2 separately and calculate the number of cells per 1 mL.

5.3.3 Calculate MOI and fold coverage achieved as follows:

$$i)\ MOI = total\ cells\ per\ screening\ plate\ \div total\ cells\ in\ control\ 2\ plate$$
$$ii)\ Fold\ coverage = (number\ of\ cells\ infected \times final\ MOI) \div sgRNA\ library\ size$$

5.3.4 Collect three replicates of cell pellets from the pooled cells at the selected library coverage for genomic DNA extraction. Centrifuge the cells at 500 x *g* for 5 min. Wash with PBS. Label the tubes and freeze-dry the cell pellets at -80 °C (these are T0 reference samples).

5.3.5 Split the pool of infected cells into three replicate groups (e.g., replicate A, replicate B, replicate C), while maintaining library coverage within each replicate. Seed cells at the same seeding density as would normally be used when expanding them. Use the same number of cells for each replicate plate and same total number of cells between replicates.

5.3.6 Continue to passage cells and harvest three replicates of cell pellets from each replicate of pooled-infected cells as above, every 3–8 days depending on the cell line, for up to 15–20 cell doublings. At each passage, harvest the cells from all plates in each replicate group with each other (i.e., all cells from replicate A plates are re-mixed together, all cells from replicate B plates are re-mixed together, etc.).

5.3.7 Label each pellet with a time (T) and replicate designation. This corresponds to the number of days post-T0 the pellet is collected (e.g., T3_A, T6_B, T_C, etc).

5.4 For the negative selection drug screens, allow cells to recover for at least one passage after T0 before treatment. At T3 or T6, split the cells from each replicate group (A, B, C) into drug treatment and control populations, using the same seeding density used in step 5.3.5.

5.4.1 Separately pool the number of cells required for library coverage for each replicate in the drug treatment group. Add the drug at intermediate concentrations (IC_{20}-IC_{50}). Seed the cells and incubate (37 °C, 5% CO_2) until next passage.

5.4.2 Separately pool the number of cells required for library coverage for each replicate in the vehicle control group. Add the vehicle control using the same volume as the drug (<0.5% v/v). Seed the cells and incubate (37 °C, 5% CO_2) until the next passage.

5.4.3 Continue to passage the cells and harvest the cell pellets for genomic DNA every 3 days as described in step 5.3.5, while refreshing the drug or vehicle at each passage.

5.5 For the positive selection or drug resistance screens, split each replicate group according to the number of cells required for library coverage. Add IC_{90} drug concentrations to each replicate. At IC_{90}, a majority cells will be killed. Allow resistant populations to grow and collect cell pellets (1–2 x 10^7 cells) for genomic DNA extraction.

6. CRISPR sample preparation and sequencing

6.1 Genomic DNA purification

6.1.1 Incubate the frozen cell pellets for 5–10 min at RT for thawing.

6.1.2 Add 1.4 mL of PBS to a 50 mL centrifuge tube containing a cell pellet. Vortex for 20 s to resuspend the cells and rest for 1 min. If required, pipette 15x with P1000 to break up the remaining cell clumps. If transferring cells from a 15 mL or 1.5 mL tube, resuspend the cells with 1 mL of PBS, then transfer cells to a 50 mL tube and rinse the original tube with 400 µL of PBS.

6.1.3 Add 5 mL of Nuclei Lysis Solution to the resuspended cells. Using a 10 mL pipette, mix the sample by pipetting up and down 5x.

6.1.4 Add 32 µL of RNase A (20 mg/mL; to obtain a final concentration of 100 µg/mL) to the nuclear lysate and mix the sample by inverting the tube 5x. Incubate the mixture at 37°C for 15 min and allow sample to cool for 10 min at RT.

6.1.5 Add 1.67 mL of Protein Precipitation Solution to the lysate and vortex vigorously for 20 s. Small protein clumps may be visible after mixing.

6.1.6 Centrifuge at 4,500 x *g* for 10 min at RT.

6.1.7 Using a 10 mL pipette, transfer the supernatant to a 50 mL centrifuge tube containing 5 mL of isopropanol. Gently mix the solution 10x by inversion until the DNA is observed.

NOTE: DNA can be observed as white, thread-like strands that form a visible mass.

6.1.8 Centrifuge at 4,500 x *g* for 5 min at RT to pellet the DNA.

6.1.9 Using a 10 mL pipette, carefully remove the supernatant and avoid dislodging the DNA pellet. Add 5 mL of 70% ethanol at RT to the DNA. Gently rotate the tube to wash the DNA pellet and sides of the centrifuge tube.

6.1.10 Centrifuge at 4,500 x *g* for 5 min at RT.

6.1.11 Using a 10 mL pipette, carefully remove the 70% ethanol and avoid dislodging the DNA pellet. Air-dry genomic DNA for 10 min at RT.

6.1.12 Add 400 µL of TE solution to the tube and let the DNA dissolve by incubating at 65 °C for 1 h. Mix the DNA by gently flicking the tube every 15 min. If the DNA does not dissolve completely, incubate tube at 65 °C for an additional 1 h whiel gently flicking the tube every 15 min, and leave it at 4 °C overnight.

6.1.13 Centrifuge at 4,500 x *g* for 1 min at RT and transfer genomic DNA to a 1.5 mL low-binding tube.

6.1.14 Quantify and measure the purity of genomic DNA on both the spectrophotometer (for total nucleic acid content) and fluorometer (for double-stranded DNA content).

6.2 Optionally, precipitate genomic DNA if there are issues with downstream PCR amplification of the sgRNA as follows.

6.2.1 Transfer 400 µL genomic DNA into a 1.5 mL microcentrifuge tube.

6.2.2 Add 18 µL of 5 M NaCl (final concentration of 0.2 M) and 900 µL of 95% ethanol.

6.2.3 Invert tube 10x until thoroughly mixed, then centrifuge at 16,000 x *g* for 10 min at RT.

6.2.4 Carefully remove the supernatant and avoid dislodging the DNA pellet. Wash the DNA pellet with 500 µL of 70% ethanol. Gently rotate the tube to wash the DNA pellet.

6.2.5 Centrifuge at 16,000 x *g* for 5 min at RT.

6.2.6 Carefully remove supernatant and avoid dislodging DNA pellet. Air-dry genomic DNA for 10 min at RT.

6.2.7 Add 300 µL of TE to dissolve DNA as described in steps 6.1.12.

6.2.8 Quantify and measure the purity of genomic DNA as described in step 6.1.14.

6.3 CRISPR sequencing library preparation

6.3.1 Set up PCR 1 as outlined in **Table 5** using a total of 100 µg of genomic DNA. Add 3.5 µg of genomic DNA per 50 µL reaction and set up identical 50 µL reactions to achieve the desired coverage. **Table 6** lists examples of primer sequences for amplification of LCV2::TKOv3 sequencing libraries. **Table 7** lists examples of primer sequences for amplification of pLCKO2::TKOv3 sequencing libraries.

6.3.2 Amplify PCR 1 reactions in a thermocycler using the program outlined in **Table 8**.

6.3.3 Check PCR 1 amplification by running 2 µL of the PCR product on a 1% agarose gel. PCR 1 yields a product of 600 bp.

6.3.4 Pool all individual 50 µL reactions for each genomic DNA sample and mix by vortexing.

6.3.5 Set up one PCR 2 reaction (50 µL) for each sample as outlined in **Table 9** using 5 µL of the pooled PCR 1 product as a template. Use unique index primer combinations for each individual sample to allow pooling of sequencing library samples.

6.3.6 Amplify the PCR2 reaction in a thermocycler using the program outlined in **Table 10**.

6.3.7 Clean agarose gel equipment for purifying amplified products with 0.1 N HCl for 10 min prior to casting a gel. Prepare a 2% agarose gel containing DNA stain for purifying PCR 2 amplified products.

6.3.8 Run the PCR 2 product on the 2% agarose gel at low voltage (1.0–1.5 h run). PCR 2 yields a product of 200 bp.

6.3.9 Visualize the PCR products on a blue light transilluminator. Excise the 200 bp band and purify DNA from the agarose gel slice using a gel extraction kit. Quantify and measure the purity of the sequencing library on both the spectrophotometer and fluorometer.

NOTE: A typical gel-purified sequencing library concentration ranges from 5–10 ng/µL and a total yield of 150–300 ng.

6.4 High-throughput sequencing

6.4.1 Sequence the CRISPR sequencing libraries on next-generation sequencers.

6.4.2 Sequence reference T0 samples at higher read depth of 400- to 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.

NOTE: It is critical to sequence the T0 sample to determine library representation for a particular screen and serve as a reference for the determining sgRNA fold changes over time.

7. Data analysis

NOTE: Depending on the sequencing platform used, raw sequence reads may require pre-processing and trimming before they can be mapped to the reference sgRNA sequence library (provided for all ready-made libraries).

7.1 Align sequence using programs such as Bowtie to map sequence reads to the reference library using the following parameters: -v2 (allowing two mismatches) and -m1 (discarding any read that mapped to more than one sequence in the library).

7.2 Normalize the number of uniquely mapped reads for each sgRNA for a given sample to 10 million reads per sample as follows:

$$normalized\ reads\ per\ sgRNA = \frac{reads\ per\ sgRNA}{total\ reads\ for\ all\ sgRNA\ in\ sample}\ x10^7$$

7.3 Calculate the log2 fold change of each sgRNA for each replicate at each timepoint (Tn) compared to the T0 sample (Tn/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.

7.4 Analyze fold changes with the Bayesian Analysis of Gene Essentiality (BAGEL) algorithm <https://github.com/hart-lab/bagel>, using the core essential and non-essential training sets defined previously[19] for gene essentiality screens (**Supplementary Table S1**) or DrugZ <https://github.com/hart-lab/drugZ> for drug screens[20].

7.5 Calculate the precision and recall for screen performance assessment using BF scores. Use the essential set from step 7.4 as the true positive list for the precision_recall_curve function of the Scikit-learn library for Python, along with the above BF score subset. Alternatively, perform the same using the PRROC package in R.

7.6 Calculate the mean fold change of all guides for each gene. Generate density plots for the essential and non-essential genes (see step 7.4) in R or equivalent software. In R, if x.ess is a

vector containing the log fold change values of essential genes and x.nonEss contain non-essential genes, plot using the following command:

```
plot( density( x.ess ), xlab="mean logFC",col="red",lwd=2 )
lines( density( x.nonEss ), col="blue",lwd=2 )
```

NOTE: For Python version details and packages used, see scikit-learn v0.19.1: (published by Pedregosa et al.[21]).

REPRESENTATIVE RESULTS:

Overview of genome-scale CRISPR screening workflow

Figure 1 illustrates an overview of the pooled CRISPR screening work flow, starting with infection of target cells with CRISPR library lentivirus at a low MOI to ensure single integration events and adequate library representation (typically 200- to 1000-fold). Following infection, cells are treated with the antibiotic puromycin to select for transduced cells. After selection, a baseline T0 cell pellet is collected to assess library distribution at the start of screening. The remaining cells, comprised of a heterogeneous population of genetic perturbations, are passaged at desired library representation every 3–4 days for 15–20 doublings to allow gene editing and the resulting effects to manifest. Screens with drug treatments are typically added at T3 or T6 after the cells have recovered from virus infection and puromycin selection. Cells are harvested at the desired library representation at every passage for genomic DNA, to determine guide abundance by next generation sequencing at desired timepoints.

It is recommended to collect multiple samples in case of any failures that may occur in the downstream sequencing library preparation steps. Pooled screens are typically viability-based assays that are designed for either positive or negative selection of essential sgRNAs. Positive screens identify genes that show resistance or increase survival under specific selection pressure (e.g., drugs or mutant cell line). In this case, most cells will die from the selection, and cells that remain will be enriched for sgRNAs targeting genes that are resistant for the drug or condition being tested. Negative selection screens or "drop-out" screens identify gene knock-outs with increased sensitivity to or loss of survival under the screen selection pressure. To identify perturbations that have a phenotypic effect such as a growth defect, guide abundance at each timepoint is quantified by next-generation sequencing and compared to T0 to assess drop-out or enrichment of guides over the course of the screen. Using analysis platforms, log-fold changes are measured for guides, and algorithms such as the BAGEL can be applied to enable ranking of gene hits.

Library amplification and maintenance of library representation in pooled CRISPR screens

Figure 2 illustrates the expected distribution of guides after amplification of the plasmid library. TKOv3 library consists of 71,090 sgRNAs with four sgRNAs per gene, targeting ~18,000 protein coding genes[10]. An ideal library should have every single sgRNA represented at similar quantities. Therefore, it is recommended to confirm the distribution of guides in the amplified library by next-generation sequencing. Shown here is an amplified library with very tight

distribution of sgRNAs, confirming that >95% of all sgRNAs are within 4-fold distribution range (**Figure 2**). A wider distribution of sgRNAs will indicate that the abundance of library guides are not equally represented and can contribute to the noise in pooled screens.

Evaluation of screen performance

Figure 3 illustrates that the performance quality of a screen can be evaluated by assessing the fold change distribution of all sgRNA against a gold standard reference list of essential (684 genes) and nonessential genes (927 genes) and visualized as precision-recall curves[10]. Using the gold-standard reference sets, Bayes Factor (BF) scores are calculated for the screen endpoint, and precision-recall curves are plotted. BF scores are calculated by analyzing the log-fold change for all guides targeting a gene using a Bayesian framework (the BAGEL algorithm described previously[19]) to compare distributions of known essential and non-essential guide sets. False discovery rates (FDR) are derived empirically using the same gold standard reference sets. A high performing screen should recover a high number of essential genes at a threshold of BF >6 and FDR <5%, as evidenced by a sharp "elbow" in most curves and a straight line to the terminal point as shown by the blue line in **Figure 3A.** The dropout of guides targeting essential and nonessential genes should also be examined (**Figure 3B**). Guides targeting the reference nonessentials genes should show a largely symmetric distribution of log-fold changes centered at zero, as shown by the dashed line in **Figure 3B**. The fold change distribution of guides targeting essential genes shows a strong negative shift relative to the distribution of guides targeting nonessential genes, as shown by the solid line in **Figure 3B**.

Essential genes

One of the basic applications of pooled genome-wide drop-out screens is to identify essential genes. Essential genes, a subcategory of fitness genes, are genes whose perturbation causes cell lethality, also considered loosely as proliferation genes. In the context of cancer biology, it is possible to identify context-specific essentials in order to identify dependencies for a particular tumor cell line. **Figure 4**, shows the gene rank of essential genes using Bayes Factor scores, derived from the BAGEL algorithm. Bayes Factor (BF) represents a confidence measure that the gene knock-out results in a fitness defect. More positive scores indicate higher confidence that the perturbation causes a decrease in fitness.

Positive selection screen

Genome-wide knock-out pools can be cultured in the presence of excess drug agent to look for suppressor/resistance genes. Shown here is an example of HCT116 cells screened in the presence of thymidine to look for suppressors of G1/S arrest[3]. Details of this screen can be found in a previous publication[3]. Briefly, 6 days after selection of CRISPR library infected cells, cells were split into replicates maintaining library coverage and treated with thymidine. Cells were passaged in the presence of drug until ample resistant cells were recovered for genomic DNA sampling. Positive selections can be sequenced (read depth) at lower coverage than negative screens since only a small fraction of guides will remain due to the strong selective pressure. In this example, sequencing was obtained with a few million reads, and 11 of 12 sgRNAs targeting thymidine kinase (TK1) were recovered and enriched as expected (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Table 1: Recommended amount of plasmid for TKOv3 transfection.

Table 2: Lipid-based transfection reagent set-up.

Table 3L Determination of cell numbers required for TKOv3 CRISPR library infection and cell plating at various fold-coverage.

Table 4: Calculation for infection set-up.

Table 5: PCR 1 set-up.

Table 6: PCR primers for amplification of LCV2::TKOv3 sequencing libraries.

Table 7: PCR primers for amplification of pLCKO2::TKOv3 sequencing libraries.

Table 8: PCR 1 cycle parameters.

Table 9: PCR 2 set-up.

Table 10: PCR 2 cycle parameters.

Supplementary table S1. TKO reference gene sets

Figure 1: Schematic overview of pooled screening workflow. (**A**) Target cell population is infected with CRISPR library lentivirus at low MOI to ensure that most cells receive one viral integration and that library representation is maintained. The different colors represent different sgRNAs in each viral particle. Genetically modified cell pools are selected. Once selection is complete, cells are sampled for T0 reference and serially passaged. (**B**) At the first passage after T0, cells have recovered from infection and drug treatments can be added, if required. Following treatment, cell populations are serially passaged for several weeks. During each passage, cells are collected for genomic DNA and reseeded at the required fold coverage of the sgRNA library. (**C**) Two types of screens can be performed: 1) positive selection screens, which identify mutant cells that show resistance or increased survival under the specific selection pressure (e.g., drugs or mutant cell line), as they will be enriched during the screen; or 2) negative selection screens, which identify mutant cells with increased sensitivity to or loss of survival under the screen selection pressure, as they will be lost during the screen. (**D**) Genomic DNA is harvested and PCR-amplified to enrich for guide regions. (**E**) Guide abundance is quantified by next-generation sequencing and enriched, or depleted guides are determined for "hit" identification.

Figure 2: **Quality of amplified CRISPR sgRNA library.** Amplified library plasmids are analyzed by next-generation sequencing (recommended reads: 30 million reads, corresponding to ~400-fold

representation of the library). Shown here is a library with tight distribution of sgRNAs, with >95% of all sgRNAs within a 4-fold distribution range.

Figure 3: **Evaluation of drop-out screen quality using gold-standard essential gene reference sets**. (**A**) Precision recall analysis of screening results in recovering of essential genes at a threshold of BF >6 and FDR of 5%. High performing screen are represented by blue line and low performing screens are represented by red line. (**B**) Fold change distribution of sgRNA targeting essential genes (solid line) and nonessential genes (dotted lines).

Figure 4: Determination of gene essentiality. Bayes Factor ranking of gene essentially in a particular screen. Bayes Factor (BF) represents a confidence measure that the gene knock-out results in a fitness defect. Higher Bayes Factors indicate increased confidence that gene knock-out results in fitness defect, (red dots). Lower Bayes Factors scores suggest knock-out provides growth advantage (blue dots).

Figure 5: Positive selection screen for suppressor of thymidine block in HCT116 cells. Normalized read counts for all sgRNAs at T0 plotted against mean normalized read counts for thymidine treated samples. For positive selection screens (i.e., using an IC90 concentration of drug), the number of perturbations that will confer resistance to the drug is expected to be small. For this reason, read depth can be lower than what is needed for negative screens, in whch most of the library is expected to be represented. *TK1* sgRNAs are circled in red. This figure has been modified from a previous publication[3].

DISCUSSION:

Due to its simplicity of use and high pliability, CRISPR technology has been widely adopted as the tool of choice for precise genome editing. Pooled CRISPR screening provides a method to interrogate thousands of genetic perturbations in a single experiment. In pooled screens, sgRNA libraries serve as molecular barcodes, as each sequence is unique and is mapped to the targeted gene. By isolating the genomic DNA from the cell population, genes causing the phenotype of interest can be determined by quantifying sgRNA abundance by next generation sequencing. Massively parallel sequencing methods are utilized to quantify sgRNAs in samples, meaning that multiple independent cell populations can be pooled into the same sequencing lane to minimize cost.

Before embarking on a large-scale screening project, it is important to have a well-characterized and technically optimized model. Genetic background, growth rate, and transduction efficiency are important factors when choosing your cell lines for screening. For example, growth rates and editing efficiency will determine scalability and technical suitability of the model. In order to adequately represent large sgRNA libraries, tens of millions of cells are required, therefore cell number could be a limiting factor in screening feasibility for cell lines with slower doublings or ones that do not have good proliferative capacity (e.g., primary cells). Based on growth rates, cell culture conditions such as cell seeding density and plate size for screening should be selected accordingly. It is recommended to culture cells in the largest vessel that is practical and technically feasible for the screen.

Lentivirus transduction efficiencies vary between cell types, as cells differ in inherent infectivity. As a result, the volume of virus required to achieve sufficient infection in one cell type will not necessarily be the same in another. Therefore, it is critical to functionally titer each batch of lentivirus library produced in the cell line to be screened to ensure sufficient coverage of the library and mostly single transduction events per cell by transducing at lower MOIs around 0.3 (section 4). Transduction efficiencies can also be influenced by cell culture conditions; therefore, functional titers should be determined using the same cell conditions that will be used in the screen. That is, it is important to use the same tissue culture vessels, media constituents and volume, cell plating density, and virus preps without prior thaws. Measurements made in different formats or conditions will not reliably scale to the screening format.

Despite the advantage of using all-in-one CRISPR-Cas9 guide libraries such as LCV2::TKOv3, the gene encoding Cas9 is quite large, making it difficult to efficiently package into viral particles (10^5–10^6 TU/mL). Delivering lower lentiviral titers can be a limitation for cell lines that are difficult to transduce, as they will have even more difficulty with the all-one-CRISPR libraries. To mitigate this, Cas9 should be expressed in the cell line in advance, followed by delivery of CRISPR libraries only containing sgRNAs (e.g., pLCKO2::TKOv3), which can be made at much higher titers (10^7–10^8 TU/mL). The ploidy of a cell line is also important, as it determines the number of target loci that need to be modified. The ability to generate complete knock-outs in haploid cells is more efficient than in cells with multiple copies of a given gene. Therefore, screens in haploid cells may be more sensitive and yield higher quality data than screens performed in diploid or aneuploid cell lines[6]. Testing known genes that are linked to the phenotype will help determine the screen-ability of a cell line model. For example, for essentiality screens, guides targeting a subunit of the 26S proteasome, *PSMD1* (Addgene: plasmid #74180), a core essential gene, can be used to test editing efficiency and infectibility of cell lines, as perturbation of *PSMD1* will result in cell death.

The robustness of pooled screens highly depends on sgRNA representation. This is an important metric that determines library performance during a screen and the ability to identify hits. Library diversity is biased in the representation of each sgRNA; therefore, the population of cells to be screened and analyzed should be sufficiently large to ensure the capture of under-represented sgRNAs[6]. 200- to 1000-fold representation of each sgRNA is the typical coverage that has been used in published screens (i.e., 200–1000 cells per sgRNA)[10,15]. This representation should be maintained when amplifying the library plasmid (section 1) and throughout the screen by infecting and passaging the required cell number (section 5) to represent the desired library coverage and during sequencing library preparation (protocol 6), as described throughout the protocol. For example, to achieve ~200-fold coverage of the TKOv3 library requires selection and passaging of 15 million infected cells. During sequencing, assuming a diploid human genome contains ~7.2 pg of DNA and 1 sgRNA per genome, a total of 100 μg of genomic DNA is required to generate the sequencing library for 15 million sequence reads. The decision of coverage will depend on the size of the library, as coverage of larger

libraries will require culturing larger number of cells that can be difficult to maintain and not technically practical. A minimum of 200-fold coverage is recommend with TKOv3 libraries, as 200-fold provides an optimal balance between the logistics of screening large number of cells and maintaining sufficient dynamic range to detect true biological sgRNA drop-outs with limited noise from random depletions[22,23]. Higher fold library representations will result in improved reproducibility and ensure sufficient window for detection of changes in sgRNA abundance, especially for negative selections. A limiting feature of negative screens is that the perturbation is only depleted to the extent that it was present in the starting library[24]. In comparison, the dynamic range of positive selection screens is much larger, as they rely on enrichment of cells, and could enrich to 100% of the final population[23]. Therefore, for positive selection screens (e.g. drug resistance screens), library coverage and read depth can be reduced to 50- to 100-fold representation since only a small cell population is expected to survive.

The sequencing library protocol described here is a two-step PCR optimized for TKOv3 CRISPR libraries in both vector backbones and sequenced on the Illumina sequencing platform. These sequencing libraries can also be generated using a single PCR protocol, similar to that described in Hart et al.[3]. For other ready-made libraries, the primers and sequencing protocols provided for those libraries should be consulted. When preparing genomic DNA and PCR samples, it is essential to be considerate of contamination precautions. For example, a dedicated area for genomic DNA purification is highly recommended. It should also be physically distinct from bacterial plasmid preps, which are common contaminants found in genomic DNA samples. PCR reactions should be set up in a dedicated PCR hood, as this will minimize contamination from plasmids and other sequencing libraries. For good practice, a no-template negative control can be included to help monitor for PCR contamination.

Data analysis to translate sequencing reads from screens is a non-trivial task, given the size and diversity of these datasets. Once the sequence reads have been aligned and normalized, several bioinformatic tools are available to assist with evaluating screen performance (**Figure 3**) and hit identification (**Figure 4**). BAGEL is described in this protocol as the key tool for data analysis. 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 in Hart et al[3]. In addition to BAGEL, other algorithms designed to identify both enriched and depleted sgRNAs, such as MAGeCK[25] can also be used. For drug screens, it is recommended to use the DrugZ algorithm to identify both synergistic and suppressor chemical genetic interactions. DrugZ was designed to compare the relative abundance of sgRNA in a treated population to the relative abundance of sgRNA in an untreated population at the same timepoint (Wang BiorXvi REF).

A limitation of CRISPR screens is that Cas9 does not always lead to a knock-out, as there is always a possibility that the indels created are in-frame mutations, leaving the gene function intact[13]. This results in a mixed population, making the screen “noisy” and interpretation of data challenging. Using multiple independent sgRNAs targeting a gene can build-in redundancy, reducing the effect of sgRNAs with low activity. An additional caveat to CRISPR studies is the effect of the double strand breaks created by Cas9 nuclease, which can lead to cellular lethality

independent of the gene being targeted. This anti-proliferative effect increases with target site copy number, leading to false positive identification of genes within highly amplified regions[26]. Computational methods like CERES have been developed to correct for copy number effects[27]. These workflows consider the copy number effect to estimate gene dependency levels in knock-out-based essentiality screens. Careful examination of genomic locations of hit genes in amplified regions can help determine false positives that are due to multiplicity of cutting effects[13]. Primary screens can only identify potential hits. It is important to follow-up with a secondary screen or protocol to validate the hits and distinguish on-target from off-target effects, weeding out false positives and ensuring genes that those scored weakly due to ineffective perturbations are not left behind as false negatives[23].

This protocol focuses on viability-based screening approaches, in which the condition of study should lead to a proliferation defect or death of cells. For processes that do not lead to a change in cellular viability, the viability-based pooled screening method can be restrictive. An alternative is to perform screens using reporter or marker-based assays and enrichment by fluorescence activated cell sorting (FACS) approaches. In marker-based selection screens, the phenotype is based on mutations that regulate marker gene expression rather than cell health[13,23]. Arrayed CRISPR formats are also available for one-gene per well screening. Arrayed formats are more amenable to complex or microscopy-based read outs. However, arrayed formats require automated equipment and large amounts of reagents[28].

The screening protocol discussed here uses *S. pyogenes* Cas9 nuclease to create null alleles, which is the most widely used for genetic screens and for which many libraries are available (Addgene: Pooled Libraries). Alternative options to knock-out libraries are also available, which use a catalytically dead dCas9 tethered to chromatin modifier proteins to inhibit (CRISPRi) or activate (CRISPRa) transcription of genes. Similar to RNAi, CRISPRi offers the ability to study phenotypic effects at different gene doses and essential genes that cannot tolerate complete knock-out, while CRISPRa can be used to perform gain-of-function screens. Each of these technologies have their advantages, but in general, the CRISPR knock-out approach is the most developed. It has been proven to perform well with low noise, minimal off-target effects, and experimental consistency, especially in lethality-based essential gene screens, when compared to knock-down approaches using either CRISPRi and shRNAs[5]. Despite its extensive applicability to date, CRISPR screening technology remains in its early stages. New tools are continuing to be built from the basic components of CRISPR. These include combinatorial gene editing strategies that can target multiple genomic loci, optimization of orthogonal Cas enzymes, and modifications with chromatin functional domains to diversify Cas9 activities. As CRISPR technology continues to grow, its coupling to genetic screening approaches will serve as a powerful platform for functional discovery in genetics.

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The authors declare no competing financial interests.

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Figure1

Figure 1. Overview of pooled screening workflow

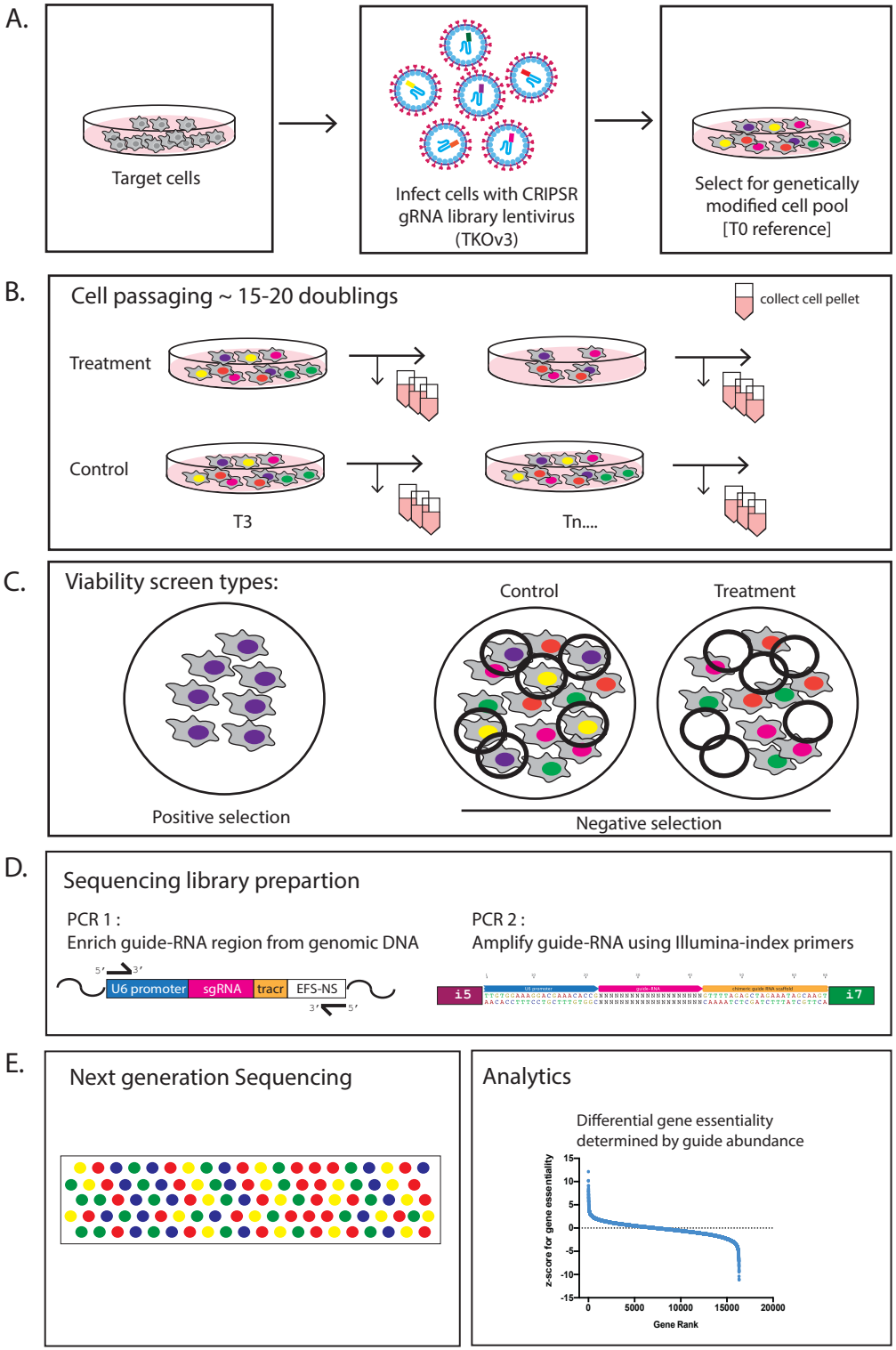


Figure 2.

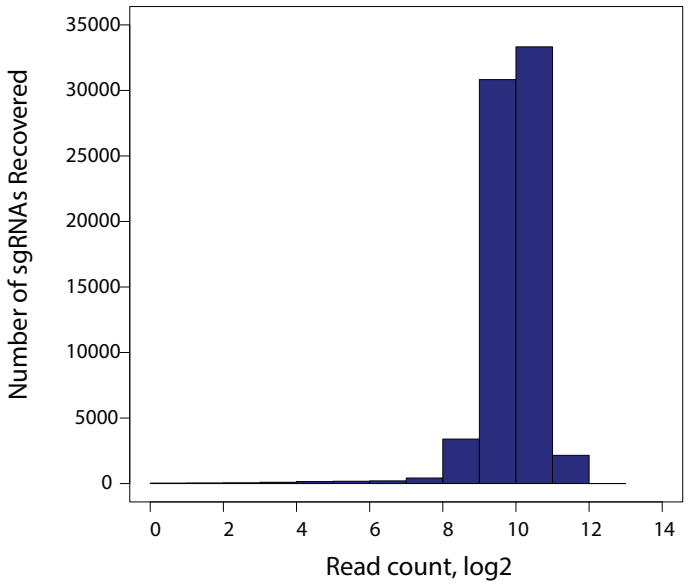
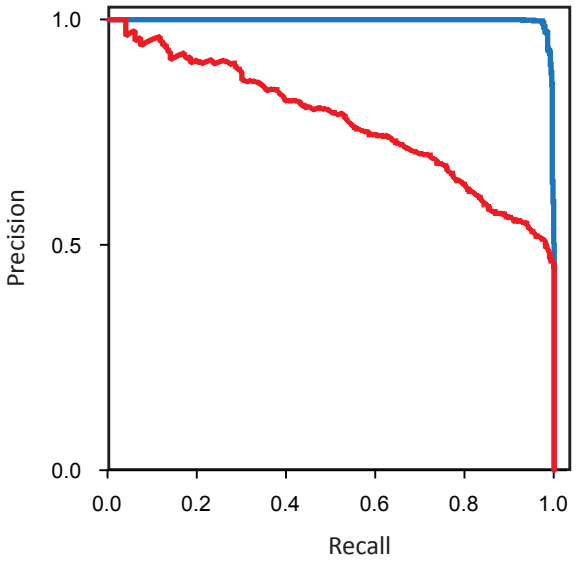


Figure 3.

A)



B)

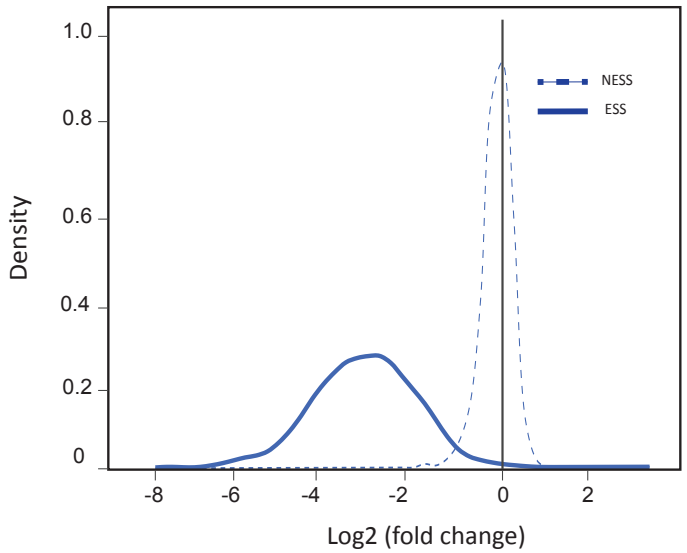


Figure4

Figure 4.

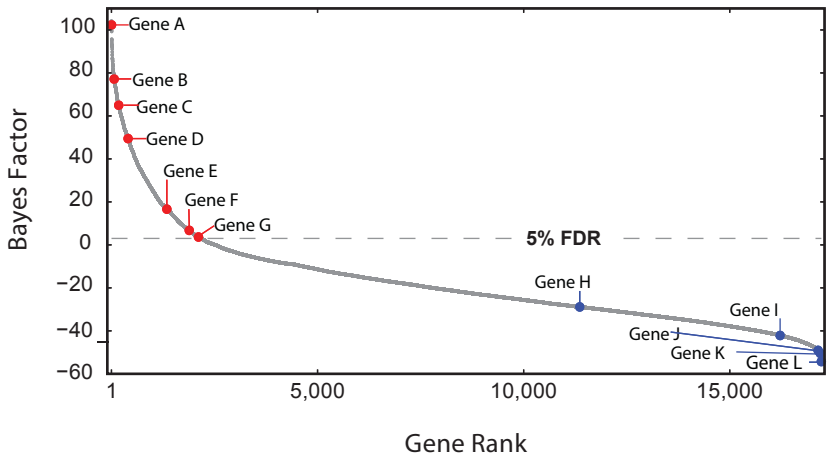
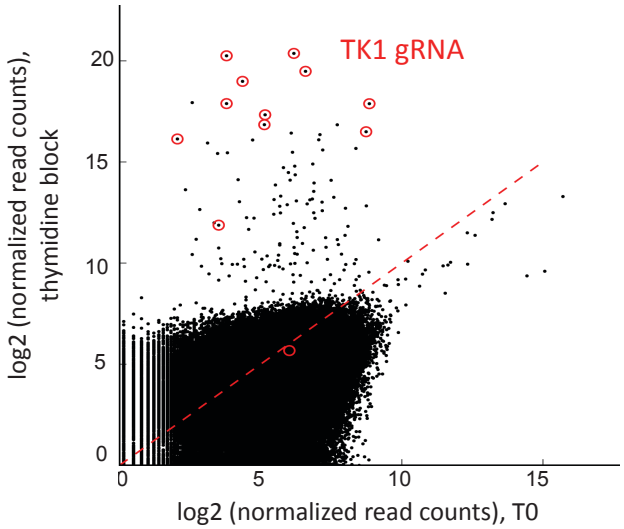


Figure5

Figure 5.



Tabel 1. Recommended amount of plasmid for TKOv3 transfection

Amounts were determined based on molar ratio of 1:1:1

Component	Amount per 15-cm plate [a]	
	LCV2::TKOv3	pLCKO2::TKOv3
psPAX2	4.8 µg	7.0 µg
pMD2.G	3.8 µg	4.0 µg
TKOv3[b]	8.0 µg	5.0 µg

[a]Amounts determined based on most productive plasmid combination for TKO library at 1:1:1 molar

[b]Amount TKO plasmid based on CRISPR library vector backbone. LCV2 all-in-one vector =13 kb, non-

r ratio

Cas9 pLCKO2 vector = 7.6 kb

Table 2. Lipid-based transfection reagent set up

Component	**Amount per 15-cm plate**
Opti-MEM	800 µL
Transfection reagent	48 µL

Table 3. Determination of cell numbers required for TKOv3 CRISPR

Fold-coverage	Number of cells per sgRNA[b] (sgRNA library size[a] × fold coverage)
200	1.5×10^7
500	3.6×10^7
1000	7.1×10^7

[a] Based on TKOv3 library size = 71,090 sgRNA

[b] Numbers are rounded up

library infection and cell plating at various fold-coverage

Number of cells required for infection[b] (sgRNA library size × fold coverage ÷ 0.3 MOI)
5×10^7
1.2×10^8
2.4×10^8

Table 4. Calculation for infection set up

	Treatment
Screening plates	Virus, + puromycin
Control 1	No virus, + puromycin (0% survival control)
Control 2	Virus, + No puromycin (100% survival control)

[a] Include extra plates to accommodate for MOI fluctuations and gr

Number of plates required for infection
(sgRNA library size × 200-fold) ÷ 0.3 MOI ÷ cell seeding density at infection = number of plates required[a]
1
1

owth rates

Table 5. PCR 1 set up

Reagents	Amount per 1x reaction
2x Master Mix	25 µL
10 µM PCR 1 LCV2 forward primer	2.5 µL
10 µM PCR 1 LCV2 reverse primer	2.5 µL
Genomic DNA	3.5 µg
Water	up to 50 µL
Total	50 µL

r amplification of LCV2::TKOv3 sequencing libraries

es
GAGGGCCTATTTCCCATGATTC
GTTGCGAAAAAGAACGTTCACGG

Primer Sequences for Illumina Sequencer
i5 or i7 index (see Table 3)
annealing sequence
ers
AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
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AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
ers
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC

Table 7. PCR primers for a

PCR 1 - Primer Sequences
pLCKO2 forward primer
pLCKO2 reverse primer
PCR 2 - i5 and i7 Index Pr
Red sequence denotes i5
Blue sequence denotes ar
PCR 2 - i5 forward primer
S501-F
S502-F
S503-F
S504-F
S505-F
S506-F
PCR 2 – i7 reverse primer
D701-R
D702-R
D704-R
D705-R
D706-R
D707-R

amplification of pLCKO2::TKOv3 sequencing libraries

GAGGGCCTATTTCCCATGATTC
CAAACCCAGGGCTGCCTTGGAA

imer Sequences for Illumina Sequencer
or i7 index
nealing sequence
s
AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGG AAAGGACGAGGTACCG
AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGA AAGGACGAGGTACCG
AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGGA AAGGACGAGGTACCG
AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGG AAAGGACGAGGTACCG
AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGG AAAGGACGAGGTACCG
AATGATACGGCGACCACCGAGATCTACACACTGCATAACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTGG AAAGGACGAGGTACCG
s
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTAT TTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTAT TTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTAT TTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTATT TCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTAT TTCTAGCTCTAAAAC
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTTGCTAT TTCTAGCTCTAAAAC

Table 8. PCR 1 cycle parameters

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	

Table 9. PCR 2 set up

Reagents
2x Master Mix
10 μM i5 forward primer
10 μM i7 reverse primer
PCR 1 product
Water
Total

Amount per 1x reaction
25 μL
2.5 μL
2.5 μL
5 μL
15 μL
50 μL

Table 10. PCR 2 cycle parameters

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	

Name of Material/ Equipment	Company	Catalog Number
0.22 micron filter		
30°C plate incubator		
37°C shaking incubator		
37°C, 5% CO_2 incubator		
5 M NaCl	Promega	V4221
50X TAE buffer	BioShop	TAE222.4
6 N Hydrochloric acid solution	BioShop	HCL666.500
95% Ethanol		
Alamar blue	ThermoFisher Scientific	DAL1025
Blue-light transilluminator	ThermoFisher Scientific	G6600
Bovine Serum Albumin,Heat Shock Isolation, Fraction V, Min. 98%	Bioshop	ALB001.250
Dulbecco's Modification of Eagles Medium	Life Technologies	11995-065
Electroporation cuvettes	BTX	45-0134
Electroporator	BTX	45-0651
Endura electrocompetent cells	Lucigen	90293
Fetal Bovine Serum	GIBCO	12483-020
HEK293T packaging cells	ATCC	CRL-3216
Hexadimethrine Bromide (Polybrene)	Sigma	H9268

Hexadimethrine Bromide (Polybrene)		
LB agar plates with carbenicillin		
LB medium with carbenicillin		
Low molecular weight DNA ladder	New England Biolabs	N3233S
Nanodrop spectrophotometer	ThermoFisher Scientific	ND-ONE-W
NEBNext Ultra II Q5 Master Mix	New England Biolabs	M0544L
Opti-MEM	Life Technologies	31985-070
Plasmid maxi purification kit	Qiagen	12963
pMD2.G (envelope plasmid)	Addgene	Plasmid #12259
psPAX2 (packaging plasmid)	Addgene	Plasmid #12260
Puromycin	Wisent	400-160-UG
QIAquick gel extraction kit	Qiagen	28704
Qubit dsDNA BR assay	ThermoFisher Scientific	Q32853
Qubit fluorometer	ThermoFisher Scientific	Q33226
RNAse A	Invitrogen	12091021
S.O.C recovery medium	Invitrogen	15544034
SYRB Safe DNA gel stain	ThermoFisher Scientific	S33102
Toronto KnockOut CRIPSR library (TKOv3) - Cas9 included	Addgene	Addgene ID #90203
Toronto KnockOut CRIPSR library (TKOv3) - non-cas9	Addgene	Addgene ID #125517
Tris-EDTA (TE) solution, pH8.0		

UltraPure agarose	ThermoFisher Scientific	16500500
Wizard genomic DNA purification kit	Promega	A1120
X-tremeGENE 9 DNA transfection reagent	Roche	06 365 809 001

Comments/Description
Cel culture media
recommend passage number <15
Cationic polymer to enhance transduction efficiency

Reduced serum media

lentiviral system

lentiviral system

Genome-wide CRISPR library , includes Cas9, 71,090 sgRNA

Genome-wide CRISPR library, non-Cas9, 71,090 sgRNA

Lipid based transfection reagent

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Author(s): Katherine Chan, Amy Tong, Kevin Brown, Patricia Mero, Jason Moffat

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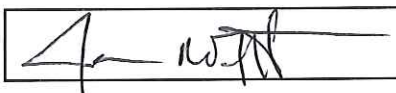
Signature:	
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March 20, 2019

Dear Vineeta,

Thank you for your review of this protocol. We have revised the manuscript as recommended by the editorial and reviewers' comments. Please find below answers and comments to the reviews in blue font.

Sincerely,
Jason Moffat

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 36-38, much of the protocol, and 580-584. Much of the protocol text has been previously published and we require novel text throughout for publication.
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7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material

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We have addressed the main issues in the editorial comments as follows:

- The protocol has been revised to use as much original language as possible to ensure that it is written in imperative tense. More detail is included throughout on the “how” question as well. The edits are tracked for the editor to review the changes.
- Discussions in the protocol are required pre-amble to help the user with the steps in the protocol and address the “how” and the “why”. We feel this is important for the protocol user to understand the steps. Safety procedures and use of hoods have been updated throughout each step.
- All other editing details have been corrected as directed: 1) Materials table sorted alphabetically, 2) removal of TM symbols, 3) All figures will be provided as .ai

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript "Pooled Genome Scale CRISPR-Cas9 Screens in Mammalian Cells" by Jason Moffat and colleagues describes a detailed protocol on how to do pooled CRISPR screens, from amplification of the library all the way through to sequencing and identification of hits. The protocol is very well written, very detailed and comprehensive and easy to follow. In my view, this would be the best guide to this technique that is currently out there, especially when combined with the video.

Major Concerns:
None

Minor Concerns:

I have some minor points, mostly for clarification:

Step 1.6: is this really at 30 and not 37C?

- Low growth temp (30C) is required to reduce recombination of lentivirus LTRs, this note has been added to the step 1.6.

Step 1.7c: what if the colony number is lower? Repeat?

- Instructions have been added (step 1.7d). In the event colony number is low, electroporations should be increased to produce sufficient coverage of the library.

Step 1.8c: might help to know what size bottle is required at this step

- Details regarding bottle size has been added to step 1.8c. We recommend using a sterile 1L Erlenmeyer flask or bottle.

Step 2.2 and throughout: instead of 8E6 use 8x106 ?

- We have revised the protocol to have numbers in this format, e.g. $8x10^6$

Step 2.2d: incubate for 30 min - RT, what temp?

- Incubation temperature has been added to this step. Transfection mix should be incubated at room temp (2.2f)

Step 5.1.3: should this be 15E6 here and not 5E6? Otherwise, I am not sure how this is calculated. Also, later it says 15E6.

- This is $50x10^6$, indicating that approximately 3.33x more cells are required for infection in order to have $15x10^6$ cells representing the library coverage at 200-fold after infection efficiency at MOI of 0.3. Extra details are included in this step to clarify.

Step 5.4: it might be helpful to explain what i5 and i7 are, what their purpose is.

- A description has been added about the purpose of the Illumina TruSeq adaptor primers. Briefly, these primers are unique sequences used to tag sequencing libraries. This allows large numbers of samples to be pooled and sequenced simultaneously for multiplexed NGS run.

Line 479: explain what BF and FDR are and how they are calculated.

- A brief explanation for calculation of Bayes factor (BF) and False Discovery Rate (FDR) is now included in step 7.5, as well as in the figure representation. A reference to this algorithm is also included for more detail in Hart and Moffat BMC bioinformatics 2016.

Figure 4B is probably not necessary, in my view. It is more distracting than helpful.

- We have removed this figure as recommend.

Figure 5 and its description for a positive selection screen are very short. Might be easier to understand if expanded a bit.

- Figure 5 legend is now more descriptive as suggested. We've included a description on how the values are determined and the sequence depth for positive selections.

Line 583: "representation should be maintained throughout the screen" - how? Referring to the steps in the protocol might be helpful or some comment here.

- We have revised this line to include more details, as well as referencing steps in the protocol as suggested. See starting at line 717.

Reviewer #2:

The manuscript describes the application of pooled CRISPR-Cas9 screening approaches to research in functional genomics. These technologies have been nothing short of transformative for biology over the last several years, and as such, whilst the field is crowded, it is important that descriptive and instructional texts are available for scientists in a variety of formats.

Overall, it is concise and precise and provides a very nice work flow that would be both easy to follow for beginners and provides some tips and tricks that seasoned screening professionals can appreciate. Some particular elements I was impressed to see in the manuscript is the notes on scalability (or lack thereof) for infection vessels and description of several NGS strategies to

overcome in-variance in the screening amplicon cassette. Some minor comments and suggestions are highlighted below, but I endorse and recommend publication if these are satisfied.

Minor Concerns:

I realise copy-editing will be done, but don't forget to weed out the occasional "CRIPSR", a classic typo.
The TKOv3 plasmid is referenced wrongly - should be #90294

- The above items have been corrected

Although it is intimated and touched on more in the discussion, it would be worth spelling out at the beginning that all of the protocol assumes that the Cas9-sgRNA all-in-one is used here Throughout, the protocol is designed for the TKOv3 library and in some places this is not cited. For example, in the transformation (1.1) and the cell expansion (5.1.1), this should explicitly state that scale here is contingent on library complexity and guide number. Although it is covered well in the cell biology section.
Representation (line 114) should be defined early on

- The protocol now includes more detail on the library being used. In general, the protocol can be used directly for either TKOv3 cas9 or non-cas9 libraries. It can also be adapted to other ready-made libraries available.

  Lenti protocol (2.1) is for a 3rd gen lenti - would it not be more appropriate for this to be fourth gen for safety?
- Currently, our experience and expertise is with using 3rd generation lenti. However, any generation is applicable following the protocol for that system.

  Comments on virus concentration would be welcome (line 189) - particularly given the all-in-one focus
- Expected range of virus concentration is provided based on functional MOI determinations, step 2.1n.

  Is there a citation to defend the coverage recommendation (200-fold)
- All our TKOv3 published screens are performed at 200-fold [Hart et al, G3 and cell]. We use 200-fold representation as it provides an optimal balance between the logistics of screening large number of cells and maintaining sufficient dynamic range to detect true biological sgRNA drop-outs with limited noise from random depletions. This note is now included in the discussion section, line 726.

  Discussion on the use of multi-layer flasks would be welcome for large scale screens
- In our experience multi-layer flasks are difficult to work with. We had many issues of contamination and skewed representation due to difficulty trypsinizing cells out of these flasks. However, any kind of vessel can be used as long as cells grow efficiently and users are confident their cell samples and cell counts are accurately represented.

  Very little discussion is made on protocol deviation for drug-gene interaction analysis, and since the protocol describes a very basic screen (most of which will soon be completed by the DepMap programme and others) this aspect would hold much more value for readers that that one described in detail.

- More detail is now included for drug screens throughout the protocol.

  Puromycin response time will vary per cell line (5.1.9) - this should be stated
- We have updated the protocol to include situations where puromycin response times vary. However, it is ideal to do selections within <3 doublings to reduce losing cells due to drop out of essential genes in the T0 sample. At T0, it is ideal to have a starting point for library representation before essential genes drop out due to editing and doubling of cells.

  Why do the authors recommend purification of gDNA followed by precipitation? Surely this is superfluous.
- A note is now added that this step is optional. However, we recommend ethanol precipitation of DNA sample if issues occur in PCR amplification steps due to presence of impurities. From our experience, precipitation removes some impurities in the DNA sample and increases efficiency of downstream PCR.

  The cycle number on the PCR is quite high - it is generally considered advisable to limit this to under 30 cycles in total across the two runs
- In a pooled CRISPR screen with majority of the infected cells with one integrant, and library representation of 200-fold, the total available number of templates for amplification is ~1.5 x 10^7 molecules. We optimized the PCR conditions to ensure efficient and robust amplification of sgRNAs from genomic DNA preparations across different cell types and conditions. The number of cycles we use is not significantly different than the Zhang lab: PCR1 – 18 cycles; PCR2 – 24 cycles (Shalem et al, Science, 343, 84 (2014).

  If the libraries are quantified by both nanodrop and quibit (5.4.12), which to trust (they will surely vary)
- The Qubit provides the most accurate quantification as the assay is based on fluorescent dye that binds specifically to double-stranded DNA. The Nanodrop is a spectrophotometer and the absorbance measurement of a DNA sample includes both DNA and RNA. The Qubit is recommended for accurate quantification of sequencing libraries and Nanodrop for indication of contaminants.

  Treated sample in Fig 5 shows a poor read coverage - maybe there is a better screen example you could use?
- Since this was a positive selection screen, read depth was reduced because only a small population of perturbations are expected to survive the selection. A more detailed description is included in the figure legend (Figure 5) and in the representative results section (line 633-644).

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ABCE1	6059	HGNC:69
ABCF1	23	HGNC:70
ACTB	60	HGNC:132
ACTL6A	86	HGNC:24124
ACTR10	55860	HGNC:17372
ACTR2	10097	HGNC:169
ADSL	158	HGNC:291
ADSS	159	HGNC:292
AHCY	191	HGNC:343
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ALG14	199857	HGNC:28287
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ANAPC4	29945	HGNC:19990
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ARIH1	25820	HGNC:689
ARL2	402	HGNC:693
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ATP5F1C	509	HGNC:833
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ATP6V1A	523	HGNC:851
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ERH	2079	HGNC:3447
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NAPA	8775	HGNC:7641
CIAO3	64428	HGNC:14179
NARS	4677	HGNC:7643
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APOA4	HGNC:602	337
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C10orf113	HGNC:31447	387638
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LINC01599	HGNC:27285	196913
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TEX45	HGNC:24745	374877
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C20orf173	HGNC:16166	140873
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SCP2D1	HGNC:16211	140856
TEX44	HGNC:28563	165100
STPG4	HGNC:26850	285051
PCARE	HGNC:34383	388939
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PRR27	HGNC:33193	401137
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CRX	HGNC:2383	1406
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CTCFL	HGNC:16234	140690
CTRB1	HGNC:2521	1504
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DGKK	HGNC:32395	139189
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DMRTB1	HGNC:13913	63948
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DUXA	HGNC:32179	503835
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EVX1	HGNC:3506	2128
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FIGLA	HGNC:24669	344018
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FRMD7	HGNC:8079	90167
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GBP7	HGNC:29606	388646
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GFRAL	HGNC:32789	389400
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GSX2	HGNC:24959	170825
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GUCY2F	HGNC:4691	2986
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HRG	HGNC:5181	3273
HRH3	HGNC:5184	11255
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IFNW1	HGNC:5448	3467
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ITIH6	HGNC:28907	347365
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LGALS14	HGNC:30054	56891
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LHX3	HGNC:6595	8022
LHX5	HGNC:14216	64211
LIM2	HGNC:6610	3982
LIN28A	HGNC:15986	79727
LIPM	HGNC:23455	340654
LOR	HGNC:6663	4014
LRIT1	HGNC:23404	26103
LRIT2	HGNC:23443	340745
LRRC10	HGNC:20264	376132
LUZP4	HGNC:24971	51213
LYZL1	HGNC:30502	84569
LYZL2	HGNC:29613	119180
LYZL6	HGNC:29614	57151
MAGEA10	HGNC:6797	4109
MAGEA11	HGNC:6798	4110
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MAGEC3	HGNC:23798	139081
MAS1	HGNC:6899	4142
MAS1L	HGNC:13961	116511
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MC3R	HGNC:6931	4159
MC5R	HGNC:6933	4161
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MEPE	HGNC:13361	56955
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MMP20	HGNC:7167	9313
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MYF5	HGNC:7565	4617
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NDST4	HGNC:20779	64579
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NOBOX	HGNC:22448	135935
NOTO	HGNC:31839	344022
NOX3	HGNC:7890	50508
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NPVF	HGNC:13782	64111
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NYX	HGNC:8082	60506
OC90	HGNC:8100	729330
OLIG2	HGNC:9398	10215
OLIG3	HGNC:18003	167826
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OPN5	HGNC:19992	221391
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OR10A4	HGNC:15130	283297
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OR10H1	HGNC:8172	26539
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OR10J1	HGNC:8175	26476
OR10R2	HGNC:14820	343406
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OR10X1	HGNC:14995	128367
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OR12D2	HGNC:8178	26529
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OR13C3	HGNC:14704	138803
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OR1A1	HGNC:8179	8383
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PBOV1	HGNC:21079	59351
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OOSP2	HGNC:26699	219990
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PROP1	HGNC:9455	5626
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RD3	HGNC:19689	343035
RDH8	HGNC:14423	50700
REG3A	HGNC:8601	5068
RESP18	HGNC:33762	389075
RETNLB	HGNC:20388	84666
REXO1L1P	HGNC:24660	254958
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RFPL4B	HGNC:33264	442247
RFX6	HGNC:21478	222546
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RTP1	HGNC:28580	132112
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RXFP2	HGNC:17318	122042
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S100G	HGNC:1436	795
SAGE1	HGNC:30369	55511
SAMD7	HGNC:25394	344658
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SERPINB12	HGNC:14220	89777
SHCBP1L	HGNC:16788	81626
SHOX	HGNC:10853	6473
SI	HGNC:10856	6476
SIGLECL1	HGNC:26856	284369
SIX6	HGNC:10892	4990
SLC10A2	HGNC:10906	6555
SLC13A1	HGNC:10916	6561
SLC34A1	HGNC:11019	6569
SLC17A6	HGNC:16703	57084
SLC18A3	HGNC:10936	6572
SLC22A12	HGNC:17989	116085
SLC22A13	HGNC:8494	9390
SLC22A24	HGNC:28542	283238
SLC22A25	HGNC:32935	387601
SLC22A6	HGNC:10970	9356
SLC22A8	HGNC:10972	9376
SLC22A9	HGNC:16261	114571
SLC25A2	HGNC:22921	83884
SLC25A31	HGNC:25319	83447
SLC2A2	HGNC:11006	6514
SLC2A7	HGNC:13445	155184
SLC32A1	HGNC:11018	140679
SLC34A1	HGNC:11019	6569

SLC36A3	HGNC:19659	285641
SLC39A12	HGNC:20860	221074
SLC6A18	HGNC:26441	348932
SLC6A5	HGNC:11051	9152
SLC6A7	HGNC:11054	6534
SLC7A13	HGNC:23092	157724
SLCO1B1	HGNC:10959	10599
SLCO6A1	HGNC:23613	133482
SLITRK1	HGNC:20297	114798
SOHLH1	HGNC:27845	402381
SOX1	HGNC:11189	6656
SOX14	HGNC:11193	8403
SP8	HGNC:19196	221833
SPACA1	HGNC:14967	81833
SPACA5	HGNC:31353	389852
SPACA7	HGNC:29575	122258
SPATA16	HGNC:29935	83893
SPATA21	HGNC:28026	374955
SPEM1	HGNC:32429	374768
SPHAR	HGNC:16957	10638
SPINK14	HGNC:33825	408187
SPO11	HGNC:11250	23626
SPPL2C	HGNC:28902	162540
SPRR4	HGNC:23173	163778
SSTR4	HGNC:11333	6754
SSX3	HGNC:11337	10214
SSX5	HGNC:11339	6758
SSX7	HGNC:19653	280658
SSX8P	HGNC:19654	280659
SSX9P	HGNC:19655	280660
STATH	HGNC:11369	6779
SULT6B1	HGNC:33433	391365
SUN5	HGNC:16252	140732
TBXT	HGNC:11515	6862
TAAR1	HGNC:17734	134864
TAAR2	HGNC:4514	9287
TAAR5	HGNC:30236	9038
TAAR6	HGNC:20978	319100
TAAR8	HGNC:14964	83551
TAAR9	HGNC:20977	134860
TAS1R2	HGNC:14905	80834
TAS2R1	HGNC:14909	50834
TAS2R13	HGNC:14919	50838

TAS2R16	HGNC:14921	50833
TAS2R39	HGNC:18886	259285
TAS2R40	HGNC:18885	259286
TAS2R41	HGNC:18883	259287
TAS2R42	HGNC:18888	353164
TAS2R43	HGNC:18875	259289
TAS2R46	HGNC:18877	259292
TAS2R50	HGNC:18882	259296
TAS2R60	HGNC:20639	338398
TAS2R7	HGNC:14913	50837
TAS2R8	HGNC:14915	50836
TAS2R9	HGNC:14917	50835
TBC1D21	HGNC:28536	161514
TBC1D29	HGNC:24509	26083
TBL1Y	HGNC:18502	90665
TBPL2	HGNC:19841	387332
TBR1	HGNC:11590	10716
TBX10	HGNC:11593	347853
ELOA2	HGNC:30771	51224
ELOA3	HGNC:24617	162699
TCHHL1	HGNC:31796	126637
TCP10L2	HGNC:21254	401285
TEDDM1	HGNC:30233	127670
TEX101	HGNC:30722	83639
TEX13A	HGNC:11735	56157
TEX28	HGNC:2563	1527
SPATA32	HGNC:26349	124783
TFAP2D	HGNC:15581	83741
TFDP3	HGNC:24603	51270
TGIF2LX	HGNC:18570	90316
TGIF2LY	HGNC:18569	90655
TGM6	HGNC:16255	343641
TKTL2	HGNC:25313	84076
TLX1	HGNC:5056	3195
TMEM132D	HGNC:29411	121256
TMEM174	HGNC:28187	134288
TMEM207	HGNC:33705	131920
TMEM225	HGNC:32390	338661
TMIGD1	HGNC:32431	388364
TMPRSS11A	HGNC:27954	339967
TMPRSS11B	HGNC:25398	132724
TMPRSS11F	HGNC:29994	389208
TMPRSS12	HGNC:28779	283471

TMPRSS15	HGNC:9490	5651
TNR	HGNC:11953	7143
TPD52L3	HGNC:23382	89882
TPH2	HGNC:20692	121278
TPRX1	HGNC:32174	284355
TPTE	HGNC:12023	7179
TREML4	HGNC:30807	285852
TRHR	HGNC:12299	7201
TRIM40	HGNC:18736	135644
TRIM42	HGNC:19014	287015
TRIM43	HGNC:19015	129868
TRIM48	HGNC:19021	79097
TRIM49	HGNC:13431	57093
TRIM51	HGNC:19023	84767
TRIM60	HGNC:21162	166655
TRIM67	HGNC:31859	440730
TRIML1	HGNC:26698	339976
TRPC5	HGNC:12337	7224
TRPC7	HGNC:20754	57113
TRPM1	HGNC:7146	4308
TRPV5	HGNC:3145	56302
TSGA13	HGNC:12369	114960
TSHB	HGNC:12372	7252
TSPAN16	HGNC:30725	26526
TSPO2	HGNC:21256	222642
TSPY1	HGNC:12381	7258
TSPYL6	HGNC:14521	388951
TSSK1B	HGNC:14968	83942
TSSK2	HGNC:11401	23617
TXNDC8	HGNC:31454	255220
TYR	HGNC:12442	7299
UBQLN3	HGNC:12510	50613
UMOD	HGNC:12559	7369
UROC1	HGNC:26444	131669
USP17L2	HGNC:34434	377630
USP26	HGNC:13485	83844
USP26	HGNC:18563	83844
UTS2R	HGNC:4468	2837
VAX1	HGNC:12660	11023
VCX3A	HGNC:18159	51481
VHLL	HGNC:30666	391104
VN1R2	HGNC:19872	317701
VN1R4	HGNC:19871	317703

VN1R5	HGNC:19870	317705
VPREB1	HGNC:12709	7441
VRTN	HGNC:20223	55237
VSX2	HGNC:1975	338917
WFDC10A	HGNC:16139	140832
WFDC11	HGNC:20478	259239
WFDC9	HGNC:20380	259240
XAGE2	HGNC:4112	9502
XAGE5	HGNC:30930	170627
XKR7	HGNC:23062	343702
ZAN	HGNC:12857	7455
ZCCHC13	HGNC:31749	389874
RTL4	HGNC:25214	340595
ZG16	HGNC:30961	653808
ZIC3	HGNC:12874	7547
ZIC3	HGNC:16366	7547
CBLL2	HGNC:26371	158506
ZNF648	HGNC:18190	127665
ZNF679	HGNC:28650	168417
ZNF804B	HGNC:21958	219578
ZNRF4	HGNC:17726	148066
ZP2	HGNC:13188	7783
ZP4	HGNC:15770	57829
ZSWIM2	HGNC:30990	151112