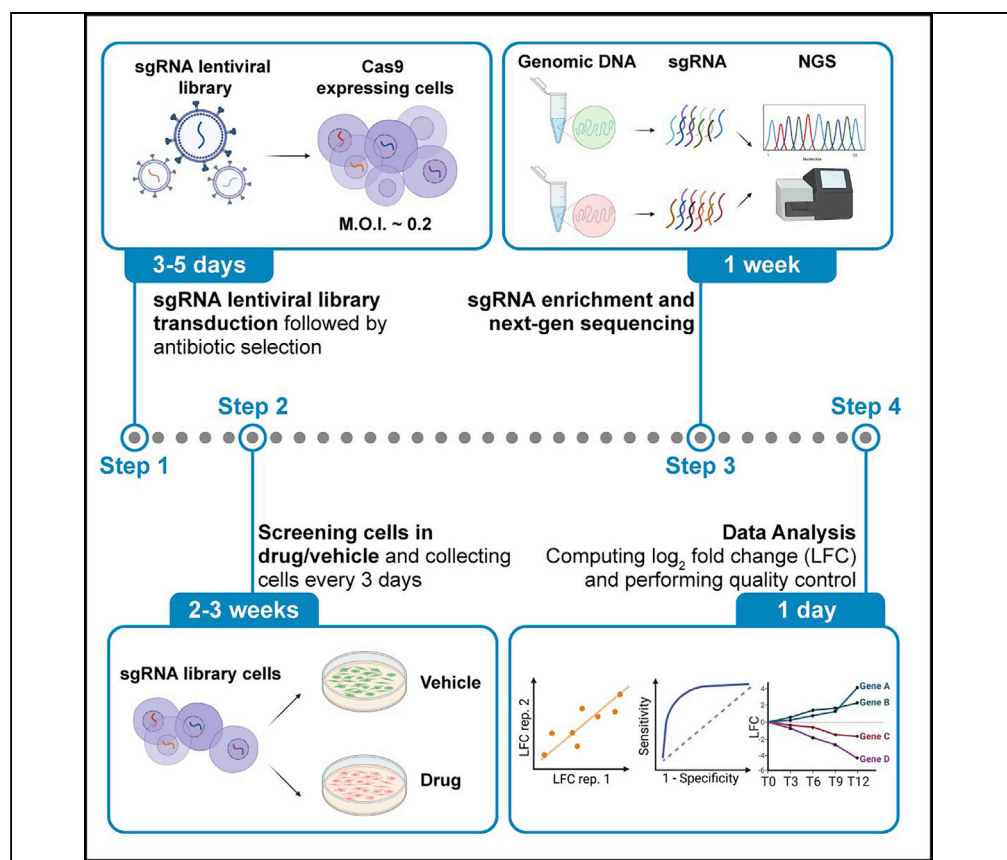


## Protocol

# Scalable CRISPR-Cas9 chemical genetic screens in non-transformed human cells



Pooled lentiviral CRISPR-Cas9 screens are utilized for assessing the differential sensitivity or resistance of many single-gene knockouts to a compound. Here, we present a scalable approach for high-throughput compound screening by utilizing a small custom library. We describe steps to perform a proof-of-principle chemical screen in non-transformed hTERT RPE-1 TP53<sup>-/-</sup> cells with higher coverage and greater timepoint resolution compared to genome-wide screens. This approach can be adapted for use in various cell lines, compounds, and other focused sgRNA libraries.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Kevin Lin, Ya-Chu Chang, Ezequiel Marron Fernandez de Velasco, Kevin Wickman, Chad L. Myers, Anja-Katrin Bielinsky

linx1048@umn.edu (K.L.)  
chan1285@umn.edu (Y.-C.C.)  
bieli003@umn.edu (A.-K.B.)

### Highlights

Protocol for scalable CRISPR-Cas9 chemical screens in a non-transformed human cell line

Lentiviral packaging of sgRNA library and compound dosage optimization for screens

Conducting scalable chemical screens with intermediate timepoints at 1000× coverage

Bioinformatics analysis of sgRNA enrichment or dropout with quality control plots

Lin et al., STAR Protocols 3, 101675  
December 16, 2022 © 2022  
The Author(s).  
<https://doi.org/10.1016/j.xpro.2022.101675>



## Protocol

## Scalable CRISPR-Cas9 chemical genetic screens in non-transformed human cells

Kevin Lin,<sup>1,4,5,\*</sup> Ya-Chu Chang,<sup>2,4,5,\*</sup> Ezequiel Marron Fernandez de Velasco,<sup>3</sup> Kevin Wickman,<sup>3</sup> Chad L. Myers,<sup>1</sup> and Anja-Katrin Bielinsky<sup>2,6,\*</sup>

<sup>1</sup>Department of Computer Science and Engineering, University of Minnesota, Minneapolis, MN 55455, USA

<sup>2</sup>Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455, USA

<sup>3</sup>Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, USA

<sup>4</sup>These authors contributed equally

<sup>5</sup>Technical contact

<sup>6</sup>Lead contact

\*Correspondence: [linx1048@umn.edu](mailto:linx1048@umn.edu) (K.L.), [chan1285@umn.edu](mailto:chan1285@umn.edu) (Y.-C.C.), [bieli003@umn.edu](mailto:bieli003@umn.edu) (A.-K.B.)  
<https://doi.org/10.1016/j.xpro.2022.101675>

## SUMMARY

Pooled lentiviral CRISPR-Cas9 screens are utilized for assessing the differential sensitivity or resistance of many single-gene knockouts to a compound. Here, we present a scalable approach for high-throughput compound screening by utilizing a small custom library. We describe steps to perform a proof-of-principle chemical screen in non-transformed hTERT RPE-1 *TP53*<sup>-/-</sup> cells with higher coverage and greater timepoint resolution compared to genome-wide screens. This approach can be adapted for use in various cell lines, compounds, and other focused sgRNA libraries.

## BEFORE YOU BEGIN

In this protocol, we describe how to perform scalable clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) chemical genetic screens in human cell lines. We use a custom Collecta library containing 3,033 single guide RNAs (sgRNAs) targeting 350 DNA damage response (DDR) genes. Additional genes are included for monitoring the performance of CRISPR-Cas9 screens in comparison to previously published screens. This protocol is established in human telomerase reverse transcriptase (hTERT)-immortalized retinal pigmented epithelial (RPE-1) cells. In addition, this protocol is optimized for screening chemical-genetic interactions with DNA replication and repair genes by knocking out the *TP53* gene, as its expression induces transient cell cycle arrest upon Cas9 cutting and thus masks the identification of essential genes in drop-out screens (Haapaniemi et al., 2018). The size of the custom sgRNA library is considerably small compared to genome-wide libraries and allows for higher coverage (e.g., 1000×) of sgRNAs and more technical replicates, providing greater dynamic range and more robust data analysis.

We first introduce how the custom sgRNA library is packaged into lentivirus with several modifications (Salmon and Trono, 2007; Chen et al., 2019). Secondly, we engineer hTERT-immortalized RPE-1 cells with constitutive Cas9 expression. After isolating the appropriate Cas9-expressing clones, the puromycin *N*-acetyltransferase (*PAC*, or puromycin-resistant gene (*PuroR*)), which was co-introduced with hTERT to immortalize RPE-1 cells, is knocked out. This allows for puromycin selection of the lenti-library. The *TP53* gene is also knocked out to enhance the sensitivity of detecting essential DNA replication and repair genes. Lastly, we introduce how to determine appropriate chemical concentrations used in the chemical-genetic screens.



Before performing the screens, several preparatory steps must be taken. (1) Determine the number of compounds and replicates to screen. We recommend screening three or fewer compounds (vehicle control must be included) at a time, and performing screens in triplicate. (2) Calculate the number of “samples” or pellets to be sequenced. This determines how many cells you need at T0 and how many plates you should initially seed. (3) Reach out to your institutional sequencing facility or third-party sequencing company to determine which next-generation sequencing instrument you will use, how many sequencing lanes you will need, and how many samples you can multiplex in one sequencing lane.

All of the manipulations involved in tissue culture must be handled with care to ensure consistency and avoid bacterial or fungal contamination. Additionally, many of the preparation steps require the production and usage of lentivirus. Therefore, Institutional Biosafety Committee (IBC) review and approval are required prior to conducting the experiments.

### Lentiviral packaging of library

⌚ Timing: 2–3 weeks

1. Day 1 (morning): plate 293T cells for transfection.

⚠ **CRITICAL:** All steps in which cell culture vessels or reagents are opened must be performed in a biosafety cabinet.

**Growth medium:** 500 mL of DMEM high glucose, 40 mL of FBS, 5.5 mL of penicillin and streptomycin (Pen/Strep), 5.5 mL of GlutaMAX™, 12.5 mL of 1 M HEPES pH 7.4.

**Transfection medium:** 500 mL of DMEM high glucose, 40 mL of FBS, 1 mL of penicillin and streptomycin (Pen/Strep), 5.5 mL of GlutaMAX™, 12.5 mL of 1 M HEPES pH 7.4.

- a. Before beginning this step, culture HEK293T cells in 15 cm plates using **growth medium**. Determine the number of cells needed from step 1.b.
  - b. Each prep requires three 15-cm plates. Seed 10 million cells (in 20 mL **transfection medium**) per 15 cm plate and place plates in the incubator for 18–24 h.
2. Day 2 (30 h later): Polyethylenimine (PEI) transfection.

**Note:** To increase transfection efficiency, the cells can be treated with 25  $\mu$ M chloroquine for 2–4 h before the transfection. Depending on the transfection agent and/or cell line used, this could increase transfection efficiency by 20-fold.

- a. Prepare the following PEI/DNA mixture for each prep in 15 mL conical tube(s):

PEI/DNA transfection mixture (for 3 $\times$ 15-cm plates, 10 million cells per plate)	
Reagent	Amount
psPAX2	64.8 $\mu$ g
pMD2G	7.2 $\mu$ g
Custom sgRNA library vector	72 $\mu$ g
150 mM NaCl	6 mL
16 mg/mL PEI solution*	18 $\mu$ L

**Note:** psPAX2 and pMD2G plasmid are used for 2nd generation lentiviral packaging system. See differences between the 2nd and 3rd generation lentiviral systems on [Addgene](#). This protocol is adapted from (Chen et al., 2019) and uses a 10:9:1 ratio of the transfer vector:psPAX2:pMD2G. This ratio is standard at our institutional Viral Vector Core. We recommend testing how different ratios may affect yields in your system.

- i. \*Since it is not possible to add the 18  $\mu$ L dropwise, pipette small droplets on the side of the 15 mL tube.
  - ii. Vortex the tube (1 s bursts, 10 times) immediately after adding the PEI solution.
  - iii. Incubate the PEI/DNA mixture for at least 10 min in the biosafety cabinet.
  - iv. Mix the PEI/DNA mixture by pipetting up and down a few times.
- b. Add 2 mL of the PEI/DNA mixture dropwise to each plate with a 5 mL serological pipette in a spiral motion in order to achieve maximal and even coverage across the plate surface.
  - c. Gently rock the plates to distribute the PEI/DNA mixture evenly. Return plates to the incubator.

**△ CRITICAL:** When adding the PEI/DNA mixture, do so as gently as possible to avoid disrupting cells. Ensure that plates sit flat and level on the incubator shelf.

### 3. Day 3 (18 h after transfection): Medium change.

**△ CRITICAL:** At this point infective lentivirus is likely present in the supernatant. Thus, wear safety sleeves, bleach all supplies that have come in contact with viral material, and follow thorough safety procedures.

- a. For each plate, aspirate the growth medium.
  - b. Add 20 mL pre-warmed growth medium to each plate, taking care not to disrupt the cells growing in the plate. Return plates to the incubator.
- ### 4. Day 4 (~40 h after the transfection): Harvesting the virus and virus purification.
- a. Collect medium from all plates into labeled 50 mL conical tubes (2 tubes/prep).
  - b. Centrifuge at 4,000  $\times$  g for 10 min at 4°C.
  - c. Attach a 0.45  $\mu$ m filter to a 60 mL syringe, removing the plunger before attaching the filter.
  - d. Pour the supernatant from a given prep into the 60 mL syringe. Put the plunger into the syringe and gently push it down into two 50 mL conical tubes.
    - i. Change the filter every 15 mL, or when the pressure needed to push the plunger increases considerably and the flow is slow.

**▢▢ Pause point:** The supernatant can be aliquoted into an appropriate volume and frozen at  $-80^{\circ}\text{C}$  until needed. The direct use of supernatant is possible; however, when the virus will be used in a sensitive cell line, primary culture or in vivo extra purification might be required.

- e. Transfer the viral supernatant into the ultracentrifuge tubes and underlay with 10 mL of 20% sucrose TNE cushion solution by using a 10 mL serological pipette.

#### 20% sucrose TNE cushion solution (store at 4°C up to one year if no precipitation or bacterial/fungal contamination)

Reagent	Final concentration	Amount
Sucrose	20%	100 g
Tris HCL	50 mM, pH 7.4	3.94 g
NaCl	100 mM	2.92 g
0.5 M EDTA	0.5 mM	500 $\mu$ L
Total	N/A	500 mL

- i. Guide the pipette tip down along the side of the tube wall, then release slowly, gradually pulling the pipette tip up along the wall.
- ii. When there is only a small amount of sucrose solution remaining in the pipette (~100  $\mu$ L), slowly pull the pipette out of the solution (this will prevent the creation of bubbles).

- f. Remove SW-32Ti rotor centrifuge buckets from the refrigerator and carefully place tubes inside of them.
- g. Using a small scale, balance the buckets by adding 1 × PBS to the lighter one(s). Tubes must be balanced to within 0.01 g.
- h. Centrifuge at 50,000 × g for 4 h at 4°C (accel at 5, decel at max) in a Optima XPN-80 ultracentrifuge (or similar) to pellet the virus.
5. Day 4 (~45 h after transduction): Virus concentration.
  - a. Following ultracentrifugation, retrieve the ultracentrifuge tubes from the buckets.
  - b. Carefully remove the medium and sucrose from each tube using a vacuum and leave tubes slightly tilted so that liquid pulls out of the pellet.
  - c. Hold each tube horizontally, then carefully wipe the inside with two sterile cotton tip swabs to absorb any remaining trace of liquid, taking care to avoid the virus pellet.
    - i. Start at the bottom of the tube (not touching the pellet), circling the tube with the swabs, and gradually moving your way to the top of the tube.
    - ii. Repeat with the second set of swabs (can be repeated with a third set if the second set still appears pink after swabbing).
  - d. Add 80 μL of ice-cold 1 × PBS + 5% glycerol to the ultracentrifuge tubes with a P200 pipette.
  - e. Set the P200 to 60 μL, then mix by gently pipetting.
    - i. Place the pipette tip on the side wall of the tube near the bottom, slightly above it. Do not put the pipette tip at the bottom as it will generate shear forces and damage the virus.
    - ii. Slowly move around the side walls periodically to cover the entire surface of the tube bottom. Avoid the formation of bubbles.
    - iii. Be careful not to touch the shaft of the pipette to the tube wall — if this happens, be sure to clean it thoroughly with 10% bleach then ethanol.
  - f. Put the tubes on the shaker in a cold room for 12–18 h at 4°C to fully resuspend the lentiviral pellet.
6. Day 5 (morning): Virus concentration.

**Note:** Highly concentrated virus titer is recommended as it avoids potential batch effects when multiple screens are needed.

- a. Retrieve tubes from the cold room and place them on ice.
- b. Perform a short spin at max speed to bring all the liquid down.
- c. Set a P200 to 120 μL, then carefully transfer the supernatant (~80 μL/ tube) of one prep to the ultracentrifuge tube of the same prep and repeat for the second tube.
  - i. Place the pipette tip on the side wall of the tube near the bottom, slightly above it, then pipette out all liquid.
  - ii. Be careful not to touch the shaft of the pipette to the tube wall. If this happens, be sure to clean it thoroughly with 10% bleach then ethanol.
- d. Centrifuge samples at 4,000 × g 10 min at 4°C.
- e. Carefully transfer the supernatant (~240 μL) to a labeled 1.5 mL screw-cap tube without disrupting the pellet.
- f. If the titer of the virus needs to be measured, prepare an aliquot of 10 μL of the purified virus.
- g. Store all the samples at –80°C.
7. Day 6 (morning): Viral infection of 293T cells for titer calculation.

**Note:** The day before the infection, a 6-well plate must be prepared with 100,000 cells per well in 2 mL of medium, using the same procedure described for Day 1.

**△ CRITICAL:** The number of cells is used in the calculation of the titer, so accuracy is critical.

- a. Take the 10 μL aliquot of purified virus out of the –80°C freezer and thaw it on wet ice.
- b. Once the virus is thawed, take ~500 μL of medium from one of the wells of the 6-well plate and use it to dilute the virus.

- c. Add all 10  $\mu\text{L}$  of the diluted virus dropwise back to the well of the 6-well plate.
  - d. Gently rock the plates to distribute the virus homogeneously. Return the plate back to the incubator.
8. Day 7 (morning/18 h post-infection): Add medium to the infected cells.
- a. Add 2 mL of growth medium to each of the infected wells of the 6-well plate without removing the previous 2 mL of medium/virus.

**Note:** By reducing the amount of medium per well during the infection, we are maximizing the interaction of the virus with the host cells.

9. Day 9 (morning/48 h post-infection): Genomic DNA extraction and quantitative PCR (qPCR).

**Note:** At this point, most infective viruses should have infected the 293T cells. However, it is recommended to continue following safety precautions since infectious material may still be present.

- a. Purify the genomic DNA of the infected 293T cells using the QIAGEN DNeasy Blood and Tissue genomic extraction kit following manufacturer's recommendations.
- b. Prepare a standard curve for human genomic DNA (Promega, see [key resources table](#)) doing serial dilutions. To calculate the number of copies of gDNA per volume the following formula is used:  $(\text{amount of DNA (in ng)} \times 6.023 \times 10^{23} \text{ molecules/mol}) / (\sim 3 \times 10^9 \text{ base pairs} \times 1 \times 10^9 \times 660 \text{ g/mol})$

**Note:** This titer determination protocol is based on the detection of integrated viral genomes into the genome of the host cells. To determine the lentiviral titer, two qPCR reactions are performed; the first, to detect the viral genome (GAG qPCR primers, see [key resources table](#)), and the second, to establish the number of host genomes (*b-Act* qPCR primer, see [key resources table](#)) present in the sample. We recommend determining the lentivirus titer before conducting the screens, as it helps calculate the lentivirus needed for achieving the desired coverage of the sgRNA library (see [step-by-step method details](#)). Alternatively, the multiplicity of infection (MOI) can be determined by transducing the target cell line with the lentivirus, followed by antibiotic selection and calculating the number of surviving cells. The MOI can then be used in estimating the lentivirus needed in the screens.

- c. Prepare a standard curve for viral genome DNA doing serial dilutions. Any lentivirus plasmid can be used for this purpose if the size is known, so that the number of copies per volume can be calculated.
- d. Set qPCR reaction as follows:

### qPCR reaction master mix

Reagent	Amount
Genomic DNA/Viral DNA/Sample	2.5 $\mu\text{L}$
20 $\mu\text{M}$ forward PCR primer	0.5 $\mu\text{L}$
20 $\mu\text{M}$ reverse PCR primer	0.5 $\mu\text{L}$
2 $\times$ Sybr Green mix	5 $\mu\text{L}$
PCR-Grade Water	1.5 $\mu\text{L}$
<b>Total Volume</b>	<b>10 <math>\mu\text{L}</math></b>

### PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	20 s	1
Denaturation	95°C	10 s	40 cycles
Annealing/Extension	60°C	60 s	
Hold	4°C	forever	

### Construction of hTERT RPE-1 Cas9, PuroR<sup>-/-</sup>, TP53<sup>-/-</sup> cells

⌚ Timing: ~6–8 weeks

10. Generating Cas9 overexpressing hTERT RPE-1 cells.
  - a. Day 1: Plate  $4 \times 10^6$  293T cells in a 10-cm dish so that they will be ~50–70% confluent the next day.
  - b. Day 2: Use Lipofectamine™ 3000 following [the manufacturer's instruction](#) with modifications listed below to transfect 293T with lentivirus packaging plasmids.
    - i. Prepare Transfection Tube 1 and Transfection Tube 2 in two 15 mL conical tubes. Incubate both tubes at 22°C for 5 min.

#### Transfection Tube 1 (for one transfection)

Reagent	Amount
Lipofectamine™ 3000	41 $\mu$ L
Opti-MEM™ Medium	to 1.5 mL total

#### Transfection Tube 2\* (for one transfection)

Reagent	Amount
psPAX2	7.2 $\mu$ g
pMD2G	4.8 $\mu$ g
Lenti-Cas9-2A-Blast	12 $\mu$ g
P3000™ reagent (2 $\mu$ L/ $\mu$ g DNA)	48 $\mu$ L
Opti-MEM™ Medium	to 1.5 mL total

**Note:** The recipe above uses a 10:7:4 ratio of the transfer vector:psPAX2:pMD2G. We recommend testing how different ratios may affect yields in your system.

- ii. \*Dilute DNA in Opti-MEM™ Medium first, then add P3000™ reagent. Mix well.
- iii. Add Transfection Tube 2 to Transfection Tube 1. Incubate at 22°C for 20 min.
- iv. Retrieve a plate of 293T cells from the incubator. Remove medium from the plate and add 9 mL of pre-warmed DMEM medium.
- v. Add the transfection mixture dropwise to the plate. Gently rock the plates to distribute the transfection mixture evenly. Return the plate to the incubator.
- c. Day 3: 18 h after the transfection or until the next morning, retrieve the plate from the incubator. Remove medium and add 10 mL of pre-warmed DMEM medium. Return the plate to the incubator.
- d. Day 4: Harvest lentivirus from the 293T medium.
  - i. 48 h post-transfection, collect all the medium from the lentivirus producing plate.
  - ii. Centrifuge the supernatant at  $4,000 \times g$  at 4°C for 10 min.
  - iii. Attach a 0.45  $\mu$ m filter to a 25 mL syringe, removing the plunger before attaching the filter.
  - iv. Pour the supernatant from a given prep into the 25 mL syringe. Put the plunger into the syringe and gently push it down into a 15 mL conical tube.
  - v. Lentivirus can be used fresh or aliquoted into 500  $\mu$ L/tube and stored at –80°C.
  - vi. Bleach and discard 293T cells.
- e. Day 5: Plate  $3 \times 10^5$  cells per well of hTERT RPE-1 cells in all wells of a 6-well plate. The growth medium for hTERT RPE-1 cells is DMEM:F12.
- f. Day 6: Lentivirus transduction.
  - i. Remove an aliquot of the lentivirus from the –80°C and thaw on ice.

- ii. Add 5  $\mu\text{L}$  of 1 mg/mL polybrene to each 500  $\mu\text{L}$  lentivirus aliquot to a final concentration of 10  $\mu\text{g}/\text{mL}$  (the recommended final concentration of polybrene for mammalian cells is 2–10  $\mu\text{g}/\text{mL}$ ).
- iii. Add 25, 50, 100, and 200  $\mu\text{L}$  of lentivirus to each well. Add 100  $\mu\text{L}$  of lentivirus to an additional well to serve as a “no-selection” control. Keep one well without any lentivirus as a “no-virus control”. Different amounts of lentivirus are added to find the appropriate transduction efficiency with limited cytotoxicity.
- g. Day 7: Remove lentivirus-containing medium and add fresh, pre-warmed DMEM:F12 medium.
- h. Day 8: Start blasticidin selection. The Lenti-Cas9-2A-Blast plasmid contains a blasticidin-resistant cassette as a selection marker.
  - i. Prepare blasticidin-containing medium. Dilute the 10 mg/mL blasticidin stock to a final concentration of 10  $\mu\text{g}/\text{mL}$ . The blasticidin concentration required for selection in different mammalian cells must be empirically determined (recommended 2–10  $\mu\text{g}/\text{mL}$  for mammalian cells).
  - ii. Retrieve lentivirus transduced cells from the incubator. Aspirate medium and add the blasticidin-containing medium into the wells, including the no-virus control well. For the no-selection control, replace the medium with fresh medium without blasticidin.
  - iii. Keep changing the blasticidin-containing medium every three days until the cells in the “no-virus control” die. Make sure the “no-selection cells” grow normally. If transduced cells reach confluency in the 6-well plate, trypsinize and re-plate cells in the blasticidin-containing medium in 6-cm or 10-cm plates.

**Note:** We recommend re-plating all cells from the initial transduction into larger plates to ensure a broad spectrum of Cas9 expression levels. Afterward, passage cells every couple of days when they reach ~70% confluence.

- iv. It takes about 14 days for the “no-virus control cells” to be killed by blasticidin.
- i. Day 22: Collect all blasticidin-resistant cells, freeze down a portion of the bulk population in DMSO, and subclone the bulk population into 96-well plates.
  - i. Aspirate medium and gently wash cells with 1 $\times$  PBS once. Add 2 mL of trypsin-EDTA to plates. Incubate the plates at 37°C for 5 min.
  - ii. Add 6 mL of pre-warmed medium to inactivate trypsin. Collect cell suspension into a 15 mL conical tube.
  - iii. Centrifuge at 300  $\times$  g for 5 min at 22°C.
  - iv. Aspirate supernatant and resuspend the cell pellet with 1 mL of pre-warmed medium. Pipette up and down approximately 20 times to make sure all cells are separated from each other.
  - v. Count cells using trypan blue with a hemocytometer or other automatic cell counter. Cells will be seeded at a very low density into 96-well plates (e.g., 1 cell per well). It is practical to determine cell count in a small volume first and then dilute (e.g., 1:100 dilution) the cell suspension to a lower concentration.

**Note:** If most wells have dead cells, repeat this step and seed up to 3 cells per well. Mark wells that have only a monoclonal population in step 10.j.

**Alternatives:** Cells can also be subcloned into 96-well plates using flow cytometry cell sorting to avoid excessive dilution.

- vi. Calculate the necessary volume of the cell suspension required for the desired seeding density (e.g., 250 cells in 50 mL of medium if aiming for 1 cell per well for two 96-well plates.) Add the calculated volume of the cell suspension into 50 mL of fresh medium in a conical tube. Mix thoroughly by inverting the 50 mL tubes 5–6 times.



- vii. Dispense cell suspension into 96-well plates with a multichannel pipette. Pipette 200  $\mu$ L of cell suspension in each well. Place plates back in the incubator.
- viii. For the remaining cell suspension, centrifuge at 300  $\times$  g for 5 min at 22°C.
- ix. Prepare the freezing solution.

**Freezing solution (prepare fresh before use)**

Reagent	Final concentration	Amount
DMSO	10%	0.5 mL
FBS	50%	2.5 mL
Fresh medium	40%	2 mL
Total	N/A	5 mL

- x. Remove supernatant. Resuspend the cell pellet in the freezing solution and transfer into cryogenic vials. Put cryovials into a freezing container (or other insulating containers) to achieve a rate of cooling of  $\sim$ –1°C /min and store them in –80°C at least 18–24 h. Transfer the frozen cells to liquid nitrogen for long-term storage.
- j. Day 29: for hTERT RPE-1 cells, monoclonal cells in the 96-well plate can be observed under the microscope after 7 days. Mark wells that have only a monoclonal population under the microscope. Mark at least 24 clones.
- k. Day 36: transfer the monoclonal population from the 96-well plates to 24-well plates.
  - i. Gently aspirate the medium and add 50  $\mu$ L of trypsin-EDTA to each well. Incubate at 37°C for 5 min.
  - ii. Prepare 24-well plates containing 1 mL of fresh medium in each well.
  - iii. Transfer the 50  $\mu$ L trypsinized cell suspension into 24-well plates. Place the plate back in the incubator.
- l. Day 39: When cells reach 70% confluency, prepare whole cell extracts and examine Cas9 expression levels by western blots (Baxley et al., 2021). The Cas9 contains an N-terminal 3 $\times$ Flag-tag so that the Cas9 can be detected with an anti-Flag antibody.
  - i. Cas9 cutting efficiency should be determined by transfecting cells with sgRNAs (see steps 11.a – 11.d).
- m. After identifying the monoclonal population with appropriate Cas9 expression levels, propagate cells and freeze down multiple vials (similar to steps 10.i.x – 10.i.xi) for future use.
- 11. Knocking out the *PuroR* and *TP53* genes: Sequentially knock out *PuroR* and *TP53* by transfecting Cas9-expressing cells with chemically modified sgRNA. 48 h post transfection, cells are collected and genomic DNA is extracted to examine the sgRNA cutting efficiency in the bulk population. Monoclonal populations can be isolated by subcloning the bulk population into 96-well plates, as described above. *PuroR* knockouts can be screened by selecting cells with puromycin (steps 11.g – 11.i), whereas *TP53* knockouts can be screened by PCR followed by restriction enzyme digestion (steps 11.j – 11.l).

**Note:** Dual transfection (i.e., transfecting two sgRNAs simultaneously) is not recommended, as it might increase the risk of gene translocation.

- a. Day 1: Transfect hTERT RPE-1 with Cas9 overexpression cells with sgRNAs targeting *PuroR* or *TP53* (see sgRNA sequences in [key resources table](#)) by Neon™ electroporation transfection 10  $\mu$ L system, following [the manufacturer's instructions](#) with modifications listed below.
  - i. Take one 10-cm plate of previously engineered healthy hTERT RPE-1 with Cas9 overexpression cells.
  - ii. Trypsinize and collect cells. Obtain cell counts.

- iii. Prepare a 6-well plate. Add 2 mL of fresh, pre-warmed medium without antibiotics into each well. The use of no-antibiotic medium avoids the reduced viability after transfection.
- iv. Calculate the necessary volume of the cell suspension for  $1 \times 10^6$  cells and transfer the cell suspension into 1.7 mL eppendorf tubes. Centrifuge at  $300 \times g$  for 5 min at 22°C.
- v. Aspirate medium in the tube. Resuspend cell suspension in 10  $\mu$ L of Resuspension Buffer R provided in the Neon™ transfection kit.

**Alternatives:** Resuspension Buffer R can be replaced with D-PBS.

- vi. Add 1  $\mu$ L of 1 ng/ $\mu$ L sgRNA into the cell suspension. For non-targeting control, add 1  $\mu$ L of nuclease-free water instead. Mix by flicking the tube 5–10 times.

**Note:** The chemically modified sgRNA comes as lyophilized powder. Resuspend sgRNA powder in nuclease-free water to a final concentration of 1 ng/ $\mu$ L. Use filter tips when handling RNA to avoid RNA degradation.

- vii. Set up a Neon™ Tube with 3 mL Electrolytic Buffer E into the Neon™ Pipette Station. Set the input pulse conditions as below.

<b>Neon™ electroporation input values</b>	
Input voltage	1530 mV
Input Pulse Width	10 ms
Input Pulse Number	3

**Note:** Input pulse conditions should be adjusted based on different cell types.

- viii. Aspirate the cell-sgRNA mixture into the Neon™ tip and perform the electric pulse following the manufacturer's instructions.

**△ CRITICAL:** Avoid air bubbles during pipetting as air bubbles cause arcing during electroporation, leading to lowered efficiency, failed transfection, or reduced cell viability.

After completing the electric pulse, immediately transfer the electroporated samples into the prepared culture plate containing pre-warmed medium. Evenly distribute cells in the well by gently rocking the plate. Put the plate back in the incubator.

- b. Day 2: Remove medium from the wells. Add fresh, pre-warmed complete medium into the wells.
- c. Day 3: Collect cell pellets for genomic DNA extraction and set up a PCR to test the sgRNA cutting efficiency. Genomic DNA can be extracted with a commercially available kit or as described below.
  - i. 48 h post-electroporation, collect cells by trypsinization followed by centrifugation.
  - ii. Aspirate the supernatant. Resuspend cell pellet in 1 mL of complete medium. Transfer part of the cell suspension into a new 10-cm plate containing fresh, pre-warmed complete medium.
  - iii. Transfer the remaining cell suspension into a 1.7 mL microcentrifuge tube. Centrifuge at  $300 \times g$  for 5 min at 22°C. Aspirate the supernatant and wash the cell pellet with 500  $\mu$ L of  $1 \times$  PBS. Centrifuge at  $300 \times g$  for 5 min at 22°C and aspirate the supernatant. Cell pellets can be processed immediately or stored at  $-80^\circ\text{C}$ .

**Alternatives:** Cells can also be frozen down as in step 10.i.x.

- iv. Add 450  $\mu\text{L}$  cell lysis solution to resuspend the pellet and pipet up and down 5–6 times to lyse cells.

Cell lysis solution (Store at 22°C for no more than 6 months)		
Reagent	Final concentration	Amount
1 M Tris HCl, pH 7.5	100 mM	1 mL
0.5 M EDTA	100 mM	2 mL
20% SDS	0.5%	0.25 mL
ddH <sub>2</sub> O	N/A	6.75 mL

**Note:** The amount of cell lysis solution should be adjusted based on the size of the pellet to ensure sufficient lysis. The solution will become viscous after cells are lysed.

- v. Add 150  $\mu\text{L}$  protein precipitation Solution (or  $\frac{1}{3}$  of the volume of the cell lysis solution).

Protein precipitation solution (Store at 22°C for no more than 6 months)		
Reagent	Final concentration	Amount
Ammonium acetate	6 M	4.625 g
ddH <sub>2</sub> O	N/A	To 10 mL

- vi. Vortex the tube vigorously for 20 s. Place tubes on ice for 5 min. Centrifuge at 17,000  $\times g$  for 10 min at 4°C.
- vii. Prepare a 1.7 mL eppendorf tube with 600  $\mu\text{L}$  of isopropanol for each sample.
- viii. Transfer the supernatant into the prepared 1.7 mL eppendorf tube containing isopropanol.

**△ CRITICAL:** Do not disturb the protein precipitation pellet when transferring the supernatant.

- ix. Invert the tube 50 times to mix and precipitate the DNA.
- x. Centrifuge at 17,000  $\times g$  for 10 min at 4°C.
- xi. Carefully discard the supernatant. Add 500  $\mu\text{L}$  of 70% ethanol to remove excess salt from the DNA pellet.
- xii. Centrifuge at 17,000  $\times g$  for 10 min at 4°C.
- xiii. Carefully discard the supernatant. Invert the tube on a clean Kimwipe and allow the DNA pellet to dry for 5–10 min.

**△ CRITICAL:** DNA pellets should be semi-transparent but not completely dried. If DNA pellets are overdried, they become less soluble.

- xiv. Add 25  $\mu\text{L}$  of nuclease-free water or TE. Place tubes in a 65°C heat block for 5 min. Determine DNA concentration using NanoDrop or other DNA quantification methods. DNA can be stored at 4°C for short term or –20°C for long term storage.
- xv. Set up PCR reaction as follows:

PCR reaction master mix	
Reagent	Amount
Genomic DNA (100 ng)	x $\mu\text{L}$
10 $\mu\text{M}$ forward PCR primer	1 $\mu\text{L}$
10 $\mu\text{M}$ reverse PCR primer	1 $\mu\text{L}$
10 mM dNTP Mix	1 $\mu\text{L}$

(Continued on next page)

### Continued

Reagent	Amount
25 mM MgCl <sub>2</sub>	3
10× Bullseye Taq polymerase buffer	5 μL
PCR-Grade Water	38.8 - x μL
Bullseye Taq Polymerase	0.2 μL
<b>Total Volume</b>	<b>50 μL</b>

### PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	45 s	35 cycles
Annealing	55°C	30 s	
Extension	74°C	45 s	
Final extension	74°C	10 min	1
Hold	4°C	Forever	

- xvi. Perform PCR cleanup with a commercially available kit. Submit purified PCR product for Sanger sequencing.
- d. Day 4: Examine the sgRNA cutting efficiency. Once the sequencing results are available, check the sgRNA cutting efficiency using the tracking of indels by decomposition (TIDE) algorithm (Brinkman et al., 2014) or other similar tools. The sgRNA cutting efficiency serves as a good tool in evaluating the Cas9 activity and provides an estimate of how many clones need to be screened.
  - i. For TIDE analysis, inputs are the sgRNA sequence and two trace files from non-targeted control and targeted samples.
- e. Day 5: The targeted population can be subcloned into 96-well plates and frozen down as step 10.i.x.
- f. Day 12: Mark wells that have only a monoclonal population under a microscope. Mark at least 24 clones.

**Optional:** More monoclonal population should be marked if the targeting efficiency of the sgRNA is low.

**Note:** Proceed with steps 11.g –11.i for *PuroR* knockout. Proceed with steps 11.j –11 for *TP53* knockout.

- g. *PuroR* knockout - Day 19: transfer monoclonal population from the 96-well plates and split into two 24-well plates.
  - i. Gently aspirate medium and add 50 μL of trypsin-EDTA to each well. Incubate at 37°C for 5 min.
  - ii. Prepare two 24-well plates containing 1 mL of fresh medium in each well. One plate (Plate 1) will be treated with puromycin while the other (Plate 2) will not.
  - iii. Transfer 25 μL trypsinized cell suspension into Plate 1 and Plate 2. Place the plate back in the incubator.
- h. *PuroR* knockout - Day 20: remove medium from Plate 1. Add 1 mL of pre-warmed complete medium containing 1 μg/mL of puromycin into each well. For Plate 2, remove medium and add regular medium (without puromycin). Select cells with puromycin for 3–5 days.
- i. *PuroR* knockout - Day 23: Clones that have *PuroR* successfully knocked out should be completely dead in Plate 1 after selection. Propagate *PuroR* knocked out clones from Plate 2.

- j. TP53 knockout - Day 19: Transfer monoclonal population from the 96-well plates and into new 96-well plates.
  - i. Gently aspirate medium and add 50  $\mu$ L of trypsin-EDTA to each well. Incubate at 37°C for 5 min.
  - ii. Prepare 96-well plates containing 150  $\mu$ L of fresh medium in each well.
  - iii. Transfer 50  $\mu$ L of trypsinized cell suspension into the new plate.
- k. TP53 knockout - Day 26: Crude genomic DNA extraction.
  - i. Monitor cell growth in the 96-well plates. If most of the cells are near confluent, proceed with crude genomic DNA extraction.
  - ii. Gently remove the medium from the 96-well plate. Add 50  $\mu$ L of trypsin-EDTA to each well. Incubate at 37°C for 5 min.
  - iii. Add 150  $\mu$ L of sterile 1  $\times$  PBS to each well in an independent, v-bottom 96-well plate.
  - iv. Transfer 40  $\mu$ L of trypsinized cells to the PBS-containing, 96-well, v-bottom plate.
  - v. Centrifuge cells in the v-bottom plate at 300  $\times$  g for 10 min.
  - vi. Add 190  $\mu$ L of growth medium to the original 96-well cell culture plate and return it to the incubator to regrow cells.
  - vii. Remove PBS/trypsin from the v-bottom plate.
  - viii. Add 40  $\mu$ L of DirectPCR lysis reagent containing 0.2 mg/mL proteinase K into each well.
  - ix. Cover plate with microplate sealing film tape and wrap in plastic wrap. Incubate at 55°C for 18–24 h.
- l. TP53 knockout - Day 27.
  - i. Do a short spin of the v-bottom plate and wrap it in new plastic wrap. Inactivate proteinase K by incubating the plate at 85°C for 1.5–2 h.

**△ CRITICAL:** Complete inactivation of proteinase K is required for downstream PCR and/or restriction enzyme digestion. If the downstream PCR does not work, see [troubleshooting section](#) for additional information.

- ii. Use 2  $\mu$ L of crude genomic DNA as the PCR template. Set up PCR reactions in the 96-well plates.

#### PCR reaction master mix

Reagent	Amount
Crude genomic DNA	2 $\mu$ L
10 $\mu$ M forward PCR primer	1 $\mu$ L
10 $\mu$ M reverse PCR primer	1 $\mu$ L
10 mM dNTP Mix	1 $\mu$ L
25 mM MgCl <sub>2</sub>	3 $\mu$ L
10 $\times$ Bullseye Taq polymerase buffer	5 $\mu$ L
PCR-Grade Water	36.8 $\mu$ L
Bullseye Taq Polymerase	0.2 $\mu$ L
<b>Total Volume</b>	<b>50 <math>\mu</math>L</b>

#### PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	45 s	35 cycles
Annealing	55°C	30 s	
Extension	74°C	45 s	
Final extension	74°C	10 min	1
Hold	4°C	Forever	

- iii. Transfer 20  $\mu\text{L}$  of the PCR reaction to a new 96-well v-bottom plate. Cas9 induced double strand breaks at the *TP53* sgRNA locus will disrupt the endogenous *PfI*M1 restriction enzyme cut site. Perform restriction enzyme digestion following manufacturer's instructions.

<b><i>PfI</i>M1 restriction enzyme digestion master mix</b>	
Reagent	Amount
10 $\times$ NEBuffer™3.1	5 $\mu\text{L}$
PCR product	20 $\mu\text{L}$
PCR-Grade Water	24.5 $\mu\text{L}$
<i>PfI</i> M1 enzyme	0.5 $\mu\text{L}$
<b>Total Volume</b>	<b>50 <math>\mu\text{L}</math></b>

**△ CRITICAL:** If a different sgRNA is used to target *TP53*, identify the appropriate restriction enzyme cut site for screening purposes.

- iv. Incubate the plate at 37°C for 1 h, followed by heat inactivation at 65°C for 20 min.
- v. Run 20  $\mu\text{L}$  of digestion product on 2% agarose gel. WT (non-targeted) allele will show two fragments at 283 bp and 181 bp, whereas targeted allele will show a single 464 bp product.
- m. *TP53* knockout - Day 34: Propagate successfully targeted monoclonal population. Confirm clonality by PCR followed by Sanger sequencing and TIDE analysis. Verify *TP53* is knocked out by western blot.

**Note:** To test potential knockout clones, we recommend using Sanger sequencing and subsequent TIDE analysis or deep sequencing to determine the genotype of the clone in question. TIDE analysis will provide the nature of the detected indels and their frequencies in comparison to the wildtype allele. In a TIDE diagram, position "0" denotes unedited wildtype alleles. Any negative numbers represent deletions, and any positive numbers mark insertions. For example, a clonal, homozygous knockout population with a single base pair insertion will register at the +1 position with a near 100% frequency, whereas a clonal, compound heterozygous knockout population with a one base pair insertion on one allele and a one base pair deletion on the other allele will register at the +1 and -1 positions with near 50% frequencies. The absence of unedited wildtype alleles is indicated by the lack of signal at position zero. Lastly, a mixed population can be distinguished by the various frequencies at multiple positions in the TIDE analysis (Brinkman et al., 2014).

### Determine compound concentration for screen

⌚ Timing: 1–2 weeks

To determine an optimal compound concentration for the screen, dose-response curves for each compound are generated. Using a CellTiterGlo® luminescent cell assay, generate dose-response curves after 72 h of continuous compound treatment and calculate an  $\text{IC}_{20}$  value (20% inhibition relative to no treatment). We reason that an  $\text{IC}_{20}$  value provides a large enough window to capture both sgRNA enrichment and dropout throughout the length of the screen. A lower dose (e.g.,  $\text{IC}_{20}$ ) is more suitable in identifying sensitizers than a higher dose (e.g.,  $\text{IC}_{50}$ ) in chemical genetic screens. Treating cells with a lower dose of compound allows for higher cell survival, which helps to maintain high coverage of the sgRNA library throughout the screen. We recommend verifying the  $\text{IC}_{20}$  dosage in the same tissue culture conditions (15-cm plates) before starting the screen.

△ **CRITICAL:** Too high of a compound dosage will result in too much cell death (not enough cells for collecting pellets at 1000× coverage). Too low of a compound dosage may make it difficult to see differences between the vehicle control and compound screen. It is important to generate these compound inhibition curves for each combination of cell line and compound that you plan to screen.

12. Day 1: Seed cells in 96-well plates.

- a. Take one 10-cm plate of healthy, previously engineered hTERT RPE-1 (Flag-Cas9, *PuroR*<sup>-/-</sup>/*TP53*<sup>-/-</sup>) cells. Trypsinize and count cells.
- b. For a 3-day compound sensitivity assay, the RPE-1 cells should be seeded at 1,000–2,000 cells per well in a 96-well plate. The seeding density needs to be adjusted based on the length of compound treatment and different cell types. The goal is for the untreated control cells to reach confluency on the day of assessing luminescent intensity. Cells should be seeded at least in triplicate for each compound concentration. To build compound sensitivity curves, use asymmetrical (five-parameter) logistic dose-response models with multiple (at least 5) compound concentrations.
  - i. Calculate the necessary volume of the cell suspension for 1,000–2,000 cells per well. 200 µL of cell suspension is needed per well in a 96-well plate. Add the calculated volume of the cell suspension into fresh medium in a conical tube. Mix thoroughly by inverting the conical tubes 5–6 times.
  - ii. Dispense cell suspension into opaque-walled 96-well plates with a multichannel pipette. Pipette 200 µL of cell suspension in each well. Place plates back in the incubator.

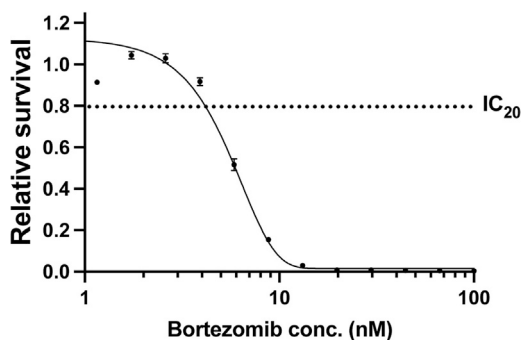
△ **CRITICAL:** Opaque-walled 96-well plates are needed for accurate luminescent reading.

13. Day 3: Treat cells with compounds.

- a. Prepare different doses of compound by serial dilution. The range of compound concentrations is determined empirically. Generally, more than 5 concentrations spanning several orders of magnitude are recommended. If available, you may search primary literature for the IC<sub>20</sub> value of the compound in the cell line you are using.
- b. Prepare several tubes containing fresh medium in conical tubes.
- c. Perform a serial dilution with a fixed dilution factor (e.g., 2 or 4). Prepare the highest concentration from the stock solution in the first conical tube. Mix well by inverting tubes several times or vortexing. Transfer the required volume (according to the dilution factor) to the second tube. Mix well in the second tube. Repeat the same procedure until the lowest concentration is made. Also prepare a tube for untreated control by adding the same volume of vehicle solution (i.e., H<sub>2</sub>O or DMSO) for making the highest concentration tube.
- d. Retrieve the cells from the incubator. Discard the medium by gently aspirating or pipetting with a multichannel pipette.
- e. Dispense compound-containing medium into each well (200 µL per well) with a multichannel pipette. Return the plates to the incubator.

14. Day 6: Assess cell survival 3 days after compound treatment with Promega CellTiterGlo<sup>®</sup> luminescent assay following the manufacturer's instructions with modifications listed below. The kit determines the number of metabolically active cells in culture based on the quantitation of the ATP present in the cells.

- a. Mix CellTiterGlo<sup>®</sup> substrate and CellTiterGlo<sup>®</sup> buffer in a 1:1 ratio to make the CellTiterGlo<sup>®</sup> reagent.
- b. Prepare enough volume of the CellTiterGlo<sup>®</sup> reagent with pre-warmed medium in a 1:1 ratio. For 96-well plates, a total of 100 µL of the mixture (50 µL reagent + 50 µL medium) per well is sufficient.
- c. Retrieve the cells from the incubator. Discard the medium by gently aspirating or pipetting with a multichannel pipette.
- d. Dispense 100 µL of the mixture into each well with a multichannel pipette.



**Figure 1. Compound dose-response inhibition curve**

A compound (bortezomib) inhibition curve was generated using an ATPase cell viability assay (CellTiterGlo®) in a 96-well plate format. Bortezomib concentrations range from 1-100 nM. Each data point is represented as the average of triplicates (bars represent standard deviation). All data points are normalized to three DMSO replicates (relative survival = 1.0). Curves and IC<sub>20</sub> values were generated using the PRISM software.

- e. Mix contents for 2 min on an orbital shaker to induce cell lysis. Incubate at 22°C for another 10 min to stabilize the luminescent signal.
  - f. Read luminescence on a Promega GloMax® Microplate Reader instrument (or similar).
15. Data analysis:
- a. Divide the luminescent read for each concentration by the average luminescent read for the untreated cells. Express the relative survival as the percentage of the untreated control.
  - b. Generate compound dose-response curves using Prism with an asymmetrical (five-parameter) logistic dose-response model (Figure 1).
  - c. After getting the IC<sub>50</sub> and Hill Slope values from Prism, calculate the IC<sub>20</sub> value using Prism's [online calculator](#).

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-Flag® M2 antibody (1:1,000 dilution for western blotting)	MilliporeSigma	Cat#F3165; RRID: AB_259529
<b>Chemicals, peptides, and recombinant proteins</b>		
pRSG16-U6- HTS6C-UbiC-TagRFP-2A-Puro	Collecta	Cat#SVCRU616-L
Polybrene	MilliporeSigma	Cat#TR-1003-G1
Blasticidin S HCL	Thermo Fisher Scientific	Cat#R21001
Puromycin	MilliporeSigma	Cat#P8833
Proteinase K	MACHERY-NAGEL	Cat#740506
Polyethylenimine	Polysciences	Cat#24765-1
Chloroquine diphosphate	MilliporeSigma	Cat#C6628-50G
Dulbecco's Modified Eagle Medium (DMEM) High Glucose (1×)	MilliporeSigma	Cat#D6429-500ML
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12) (1×)	Thermo Fisher Scientific	Cat#11320033
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985062
GlutaMAX™	Gibco	Cat#35050061
Penicillin and streptomycin	Gibco	Cat#15140-122
Trypsin-EDTA (0.05%)	Gibco	Cat#25300-062
DPBS (1×)	Gibco	Cat#14190-144
DirectPCR lysis reagent	Viagen	Cat#301-C
<b>Critical commercial assays</b>		
Lipofectamine™ 3000	Thermo Fisher Scientific	Cat#L3000-008
Neon™ transfection system starter pack	Thermo Fisher Scientific	Cat#MPK5000S
Neon™ transfection system 10 µL kit	Thermo Fisher Scientific	Cat#MPK1096
Bullseye Taq DNA polymerase	MIDSCI	Cat#BETAQ-1000

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CellTiter-Glo®	Promega	Cat#G75752
Wizard genomic DNA purification kit	Promega	Cat#A1120
Human genomic DNA	Promega	Cat#G3041
NGS prep kit for sgRNA libraries in pRSG16/17 (KOHGW)	Cellecta	Cat#LNGS-120
Supplementary primer set	Cellecta	Cat#LNGS-120-SP
QIAquick PCR purification kit	QIAGEN	Cat#28104
QIAquick gel extraction kit	QIAGEN	Cat#28704
DNeasy blood & tissue kit	QIAGEN	Cat#69504

**Deposited data**

Sample dataset and analysis code	This paper	<a href="https://zenodo.org/badge/latestdoi/520248222">https://zenodo.org/badge/latestdoi/520248222</a>
----------------------------------	------------	---

**Experimental models: Cell lines**

Human 293T cell line	ATCC	Cat#CRL-3216™
Human hTERT RPE-1 cell line	ATCC	Cat#CRL-4000™

**Oligonucleotides**

GAG qPCR forward primer: GGAGCTAGAACGATTTCGCAGTTA	(Salmon and Trono, 2007)	N/A
GAG qPCR reverse primer: GGTGTAGCTGTCCCAGTATTTGTC	(Salmon and Trono, 2007)	N/A
b-Act qPCR forward primer: TCCGTGTGGATCGGCGGCTCCA	(Salmon and Trono, 2007)	N/A
b-Act qPCR reverse primer: CTGCTTGCTGATCCACATCTG	(Salmon and Trono, 2007)	N/A
sgRNA targeting <i>PuroR</i> : CGTCGGGCTCGACATCGGCA	This paper	N/A
<i>PuroR</i> PCR forward primer: ATGACCGAGTACAAGCCAC	This paper	N/A
<i>PuroR</i> PCR reverse primer: GAGGCCTCCATCTGTTGCT	This paper	N/A
sgRNA targeting <i>TP53</i> : AGCAATGGATGATTGATGC	This paper	N/A
<i>TP53</i> PCR forward primer: GACCTATGGAACTGTGAGTGG	This paper	N/A
<i>TP53</i> PCR reverse primer: GGGAAGGGACAGAAGAT	This paper	N/A

**Recombinant DNA**

psPAX2 plasmid	A gift from Didier Trono	Addgene plasmid #12260; RRID: Addgene_12260
pMD2.G plasmid	A gift from Didier Trono	Addgene plasmid #12259; RRID: Addgene_12259
Lenti-Cas9-2A-Blast	(Hart et al., 2015)	Addgene plasmid #73310; RRID: Addgene_73310
Cellecta custom DDR library	This paper	N/A

**Software and algorithms**

Prism 9	GraphPad Software	<a href="https://www.graphpad.com">https://www.graphpad.com</a> ; RRID: SCR_002798
Microsoft Excel	Microsoft	<a href="https://www.microsoft.com/en-gb/">https://www.microsoft.com/en-gb/</a> ; RRID: SCR_016137
Tracking of Indels by decomposition (TIDE)	(Brinkman et al., 2014)	<a href="https://tide.nki.nl">https://tide.nki.nl</a>
Cellecta NGS demultiplexing and alignment software	Cellecta	<a href="https://manuals.cellecta.com/ngs-prep-kit-for-sgrna-shrna-dna-barcode-libraries/v1/en/topic/demultiplex-and-align-sequencing-reads">https://manuals.cellecta.com/ngs-prep-kit-for-sgrna-shrna-dna-barcode-libraries/v1/en/topic/demultiplex-and-align-sequencing-reads</a>
R	<a href="https://www.r-project.org/">https://www.r-project.org/</a>	<a href="https://www.r-project.org/">https://www.r-project.org/</a>
RStudio	<a href="https://www.rstudio.com/products/rstudio/download/">https://www.rstudio.com/products/rstudio/download/</a>	<a href="https://www.rstudio.com/products/rstudio/download/">https://www.rstudio.com/products/rstudio/download/</a>

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
GloMax® Discover Microplate Reader	Promega	Cat#GM3000
Agilent High Sensitivity D1000 ScreenTape System	Agilent	Cat#G2964AA
AMPure XP	Beckman Coulter	Cat#A63880
Quant-iT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific	Cat#P7589
Kapa SYBR Fast qPCR	Roche	Cat#KK4600

## STEP-BY-STEP METHOD DETAILS

### Pooled lentiviral CRISPR-Cas9 chemical genetic screens

⌚ Timing: ~2–3 weeks

This step involves transduction of the CRISPR-Cas9 library into a Cas9-expressing cell line of choice and screening these cells in the context of treated (compound) vs. untreated conditions. Before beginning the screen, an appropriate number of cells are seeded to ensure >1000× coverage of each sgRNA in the library. Lentiviral infection of the cell line is performed at a multiplicity of infection (MOI) of 0.2 (~1 in 5 cells will have one guide RNA). The library plasmid vector contains a puromycin resistance cassette, enabling puromycin selection of cells that have integrated a guide RNA. At T0, cells are split into treated vs. untreated conditions, with 3 technical replicates per condition. From T0 to T18, cells are pelleted every 3 days and passaged with fresh compound/medium.

⚠ **CRITICAL:** The length of the screen and the frequency of cell passages depends on the doubling time of your cell line and the aimed representation of the sgRNA library. Adjust the timeline for collecting cell pellets accordingly. For example, if you have a rapidly dividing cell line, you may want to harvest cells at earlier timepoints.

1. Calculate number of cells to seed for screen.
  - a. Determine number of cells required at T0.
    - i. Multiply number of library **guides**, desired **coverage**, number of **replicates**, and number of **conditions**.
    - ii. Example: Library of ~3,000 **guides** \* 1000× **coverage** \* 2 “experiments” (one DMSO control, one compound) \* 3 technical **replicates** = **18 million cells required at T0**.
  - b. Determine number of cells needed at the lentiviral transduction step.
    - i. The calculation from step 1.a.ii. denotes number of cells present **AFTER** puromycin selection.
    - ii. At a MOI of 0.2, 1 in 5 cells will have a library plasmid with a puromycin resistance cassette. Multiply the result from step 1.a.ii. by 5.
    - iii. Example: 18 million cells \* 5 = **90 million cells required at lentiviral transduction step**.
  - c. Using the doubling rate of the cell line, approximate the seeding density of cells ~24 h before lentiviral transduction (~**48 million cells**).

⚠ **CRITICAL:** We reason that seeding 3 million RPE-1 cells per 15-cm plate and propagating them every three days will provide us sufficient coverage (1000×) while also avoiding cell overgrowth. Overgrowing non-transformed cells will lead to contact inhibition and potential cell cycle arrest, which will significantly confound the identification of gene dropouts, especially when screening with a DDR-focused sgRNA library. Carefully determining the cells needed, seeding density, and the duration of propagation will ensure the robustness of the screen.

△ **CRITICAL:** Use a low multiplicity of infection, or MOI, to decrease the likelihood of cells getting more than one sgRNA integration.

2. Day -T4: Seeding Cells.
  - a. Seed total number of cells (48 million cells) determined in step 1. For this cell line, aim for a seeding density of 3 million cells per 15-cm cell culture plate with 20 mL DMEM:F12 medium (16 plates total).
    - i. The seeding density can vary here; however, aim to never exceed 70% confluence by the time of lentiviral transduction of the library.
  - b. Seed 3 million cells in a separate plate, which will act as a control for the puromycin selection.
3. Day -T3: Lentiviral Transduction of CRISPR library.
  - a. Calculate the **total** volume of lentivirus stock needed to deliver the packaged lentiviral library at a MOI of 0.2 using the following titer formula:
    - i.  $TU/mL = (\text{number of cells at transduction}) \times [\text{MOI} / (\text{mL of lentiviral stock used at transduction})]$
    - ii. Divide the **total** volume of lentivirus stock by the number of **total** plates to obtain the volume of lentivirus stock needed for **each** plate.
  - b. Prepare conical tubes containing 40 mL of fresh medium for every 2 plates of cells you plan to infect. Add the volume of lentivirus stock for 2 plates and polybrene to a final concentration of 10  $\mu\text{g/mL}$ . Mix by inverting tubes.
  - c. Retrieve plates from the incubator. Gently remove the medium.
  - d. Gently add 20 mL of the lentivirus mixture prepared in step 3.b into each plate. Do not disturb cells on the plate. For the control plate, add fresh medium (without lentivirus).
4. Day -T2: Puromycin Selection.
  - a. Remove medium 24 h after transduction. Wash cells with PBS twice. Add 3  $\mu\text{g/mL}$ \* puromycin to the transduced plates and to the control plate in fresh medium. Cells in the control plate should all die over the next 48–72 h. See [troubleshooting](#) section for additional information.
    - i. \*Perform a puromycin experiment prior to the screen with the cell line of choice to determine the dosage required to kill all cells in 48–72 h. It is important to perform this experiment in the same tissue culture conditions (15-cm plates) as what will be used in the screen.
5. Day T0: Split cells into control vs. compound treatment screens. Collect T0 pellets.
  - a. Remove the medium from each plate. Gently wash plates with warm PBS.
  - b. Trypsinize and pool cells from all plates into one sterile container.
  - c. Spin cell pellet down at 300  $\times$  g for 5 min.
  - d. Determine the number of “conditions”, or screens being performed. Each batch of compound screens requires at least one vehicle control screen (DMSO).
    - i. For example, to screen one compound and one DMSO control, we have 2 screens \* 3 technical replicates = 6 “conditions”
  - e. Start screen: Split the cell pellet from step 5.c. into “conditions”.
    - i. For each “condition”, seed 3 million cells per 15 cm-plate to maintain 1000 $\times$  representation of the library.
  - f. **T0 sample collection:** Split leftover cell pellet from step 5.c. into T0 cell pellets.
    - i. Collect 3 $\times$  T0 cell pellets in 1.5 mL eppendorf tubes, each containing 4 million cells. Centrifuge at 300  $\times$  g for 5 min. Remove supernatant and wash the cells with 1 $\times$  PBS once. Aspirate PBS and leave pellet half-covered in PBS (so pellet does not dry out). Store at  $-80^{\circ}\text{C}$ .

△ **CRITICAL:** Aim to collect more cells than needed to account for the downstream loss.

- g. Add the appropriate volume of vehicle control (DMSO) or compound to each plate.
  - i. Compound dosage is determined above in the “Determine compound concentrations” section.

- ii. Add the equivalent volume of DMSO for the control screen. Ensure that the concentration of DMSO does not exceed 0.5% w/v.
- iii. Transfer volume of cell suspension for 3 million cells into 50 mL conical tubes and bring up the volume to 20 mL with fresh medium. Add the compound/DMSO. Mix well by inverting 5×. Transfer to a 15 cm plate.

△ **CRITICAL:** If the compound dosage is too high, you may have difficulty collecting enough cells per pellet. Think about expanding how many plates are used per condition.

6. Day T3-T18: Collect cell pellets every 3 days.
  - a. Passage cells every 3 days and add compound/DMSO as in the previous step.
    - i. Trypsinize cells from each replicate. Do not pool all replicates together (i.e., keep each replicate as an independent lineage).
    - ii. Seed 3 million cells in compound/DMSO containing medium as in step 5.g.
  - b. Collect 4 million cells and freeze pellets at  $-80^{\circ}\text{C}$  at each passage.
    - i. Pelleting more cells (4 million instead of 3 million) than the 1000× representation buffers for loss during downstream genomic DNA processing.
    - ii. Wash the cells with 1× PBS once. Spin down the cell pellet and aspirate 1× PBS. Store at  $-80^{\circ}\text{C}$ .
    - iii. If possible, collect more pellets than needed.
    - iv. We empirically found that T12 and T18 results do not differ by much. Sequencing pellets from T3-T12 allows us to differentiate between acute and chronic responses to compounds.

### Genomic DNA extraction

⌚ **Timing:** ~1–2 days

In this step, genomic DNA is extracted from frozen cell pellets using the [Wizard Genomic DNA Purification Kit](#) (Promega, see [key resources table](#)). The extracted DNA will be used as template DNA in the subsequent two-round PCR step. This protocol is adapted from the Promega kit protocol and optimized for  $0.5\text{--}5 \times 10^6$  cells.

7. Before starting this step, prepare the following:
  - a. Ensure a sufficient amount of all buffers for the number of samples being processed.
  - b. Make 70% ethanol solution using nuclease-free water.
  - c. Set the heat block to  $65^{\circ}\text{C}$ .
  - d. Pre-warm nuclease-free water to  $65^{\circ}\text{C}$ .
  - e. All centrifugation steps are performed at  $4^{\circ}\text{C}$ .
  - f. Use filtered pipette tips for all steps to avoid contamination of genomic DNA.
8. Genomic DNA Extraction using Promega Wizard Genomic DNA Purification Kit.
  - a. Thaw cell pellets at  $22^{\circ}\text{C}$  for 5 min.
  - b. Add 200  $\mu\text{L}$  PBS to a 1.5 mL microfuge tube containing the cell pellet. Vortex for 20 s to resuspend cells and rest for 1 min. If required, pipette 15× with P200 pipette to break remaining cell clumps.
  - c. Add 600  $\mu\text{L}$  Nuclei Lysis Solution to the resuspended cells. Pipette mixture 5× using a P1000 pipette to mix.
  - d. Add 4  $\mu\text{L}$  RNase A (20 mg/mL), to a final concentration of 100  $\mu\text{g}/\text{mL}$ , to the nuclear lysate and mix the sample by inverting the tube 5×. Incubate the mixture at  $37^{\circ}\text{C}$  for 15 min and cool to  $22^{\circ}\text{C}$  (~2 min).
  - e. Add 200  $\mu\text{L}$  Protein Precipitation Solution to the nuclear lysate and vortex vigorously for 20 s. Incubate the mixture on ice for 5 min.
  - f. Centrifuge at 13,000 × g for 10 min at  $4^{\circ}\text{C}$ .

- i. The supernatant layer containing DNA should separate from the solid white protein layer. If not, centrifuge again at 13,000 × g for 10 min at 4°C.
- g. Using a P1000 pipette, transfer the supernatant (~900 μL) to a 1.5-mL microfuge tube containing 600 μL isopropanol.
  - i. Avoid transfer of protein. If necessary, use a P200 pipette, aim the tip against the side of the tube wall, and carefully go down the wall to pick up supernatant from the top.
- h. Gently mix the solution 50× by inversion until the white thread-like strands of DNA form a visible mass.
- i. Centrifuge at 13,000 × g for 10 min at 4°C. The DNA will be visible as a small white pellet. See [troubleshooting](#) section for additional information.
- j. Using a P1000 pipette, carefully remove supernatant, leaving ~10 μL. Remove remaining supernatant with a P20 pipette to avoid dislodging pellets.
- k. Add 600 μL 70% ethanol to the DNA. Gently rotate the tube to wash the DNA pellet and the sides of the microfuge tube.
- l. Centrifuge at 13,000 × g for 10 min at 4°C.
- m. Using a P1000 pipette, carefully remove 70% ethanol. Spin tube and remove remaining ethanol with P20 pipette. Air-dry genomic DNA for 10 min at 22°C under the fume hood.
- n. Add 50 μL–100 μL nuclease-free water to the tube, depending on the number of cells used in the purification. Let DNA dissolve by incubating at 65°C for 1 h, rotating at 300 rpm.
  - i. Alternatively, pre-warm nuclease-free water at 65°C, add to DNA pellets and leave at 22°C for 18–24 h.
- o. Quantitate DNA and measure purity by spectrophotometry (using a Nanodrop), and store at –20°C.

**△ CRITICAL:** If genomic DNA is too concentrated and too viscous, add more nuclease-free water to dilute it. A highly concentrated and non-homogenous DNA solution will result in inaccurate DNA concentration when using Nanodrop. If the DNA concentration is too diluted for downstream applications, concentrate DNA by ethanol precipitation and dissolve DNA in a smaller volume of nuclease-free water. Genomic DNA concentrations ranging from 500–1000 ng/μL are needed for downstream PCR amplification.

**Optional:** Ethanol precipitation of genomic DNA.

- p. Bring the volume of genomic DNA to 100 μL by adding nuclease-free water.
- q. Add 4 μL 5 M NaCl and 200 μL of 100% ethanol to DNA.
- r. Mix the sample by inverting the tube 5×.
- s. Centrifuge at 13,000 × g for 10 min at 4°C.
- t. Using a P1000 pipette, carefully remove supernatant, leaving ~10 μL. Remove remaining supernatant with P20 pipette to avoid dislodging the pellet.
- u. Add 200 μL 70% ethanol to the DNA. Gently rotate the tube to wash the DNA pellet and the sides of the microfuge tube.
- v. Centrifuge at 13,000 × g for 10 min at 4°C.
- w. Using a P1000 pipette, carefully remove 70% ethanol. Spin tube and remove remaining ethanol with P20 pipette. Air-dry genomic DNA for 10 min at 22°C under the fume hood.
- x. Calculate genomic DNA yield based on Nanodrop measurement from the previous step and determine the optimal concentration and volume to resuspend the DNA.
- y. Let DNA dissolve by incubating at 65°C for 1 h. Mix DNA by gently rotating at 300 rpm.
- z. Quantitate DNA by Nanodrop, and store at –20°C.

## Two-round PCR

⌚ Timing: ~1–2 days

In this step, two-round PCR is performed on the genomic DNA template for each sample. The first round PCR enriches the sgRNA regions in the genome. The second round amplifies the sgRNA, reduces genomic DNA carryover into the next-generation sequencing (NGS) step, adds multiplex identifier barcodes, and adds p5/p7 sequences that are complementary to the immobilized primers in the NGS Illumina Flow Cells.

This protocol is adapted from the Cellalecta “NGS Prep Kit for sgRNA, shRNA, and DNA Barcode Libraries” manual.

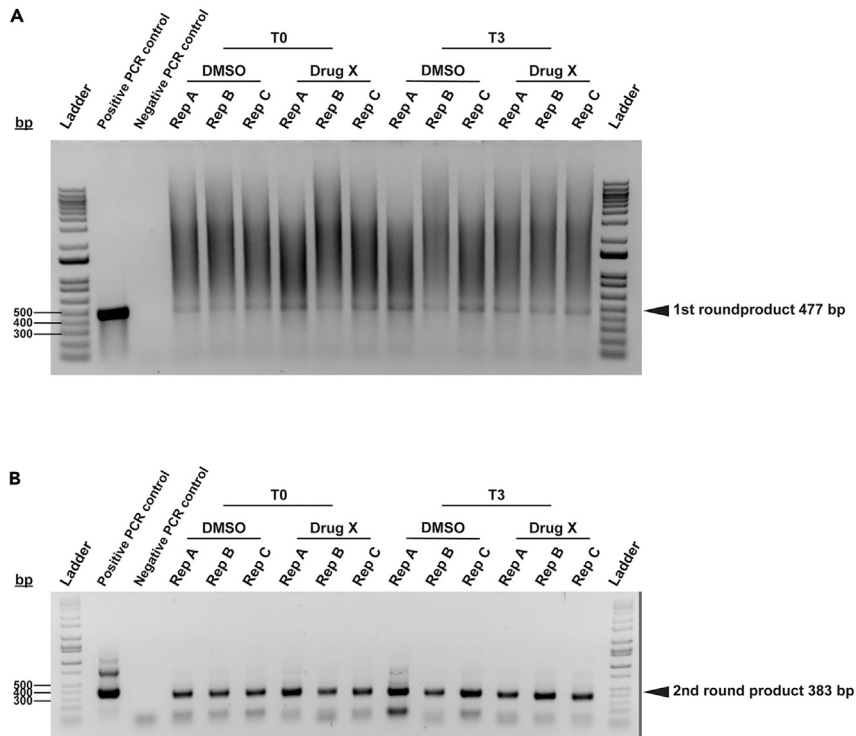
9. Before starting:
  - a. Order the NGS prep kit for sgRNA libraries (see [key resources table](#)) from Cellalecta.
    - i. In this kit, 12 NRev Index primers (reverse 2nd round primers) each contain a different index sequence which will be used to deconvolute multiplex sequencing results for samples mixed together in the same sequencing lane.
    - ii. If more index primers are needed, order a supplemental kit (see [key resources table](#)), which contains an additional 12 NRev Index primers. With this kit, a maximum of 24 samples can be sequenced on the same lane.
    - iii. Please refer to the Cellalecta manual that comes with these kits to find the primer/index sequences.
  - b. Wipe the workbench with 10% bleach to prevent contamination.
10. First Round PCR.
  - a. Assuming the diploid genome is ~6.5 pg, 20 µg of genomic DNA yields 1000-fold coverage of our custom library.
  - b. For each sample, set up a 1st round PCR as follows. Include the original plasmid library (1 ng) as a positive control. Include a negative control without genomic DNA to ensure no DNA contamination.

PCR1 reaction master mix	
Reagent	Amount
DNA template (20 µg genomic DNA*)	x µL
Forward 1st Round PCR Primer <sup>#</sup>	1.5 µL
Reverse 1st Round PCR Primer <sup>#</sup>	1.5 µL
dNTP Mix	1 µL
Taq Polymerase Buffer	5 µL
PCR-Grade Water	40 - x µL
Taq Polymerase	1 µL
<b>Total Volume</b>	<b>50 µL*</b>

**Note:** \*We have empirically determined that a 50 µL PCR reaction is sufficient to amplify 20 µg of genomic DNA. This PCR reaction requires genomic DNA concentration to be higher than 500 ng/µL (see step 8.o). If more genomic DNA is needed to achieve coverage for different sizes of sgRNA library, split genomic DNA into multiple PCR reactions for one sample. Do not exceed 50 µg of genomic DNA in a 100 µL PCR reaction.

**Note:** <sup>#</sup>The primer sequences can be found in the Cellalecta NGS prep kit manual.

**Optional:** To check the quality of genomic DNA before 1st round PCR, run 1 µL of the genomic DNA for each sample on a 1% agarose gel. The presence of smears on the gel may point to sample contamination or degradation and reduce the yield of each subsequent PCR reaction.



**Figure 2. First and second round PCR products. PCR products obtained from timepoints T0 and T3 are resolved on 2% agarose gels**

At each timepoint, three replicates are shown for the DMSO vehicle control and Compound X screen. Positive (1 ng of plasmid library) and negative (no template DNA) PCR controls are included.

(A) First round PCR. Note that all of the genomic DNA samples show an expected band at 477 bp with smears. Smears are likely due to the degradation of genomic DNA during PCR.

(B) Second round PCR. Note that genomic DNA smear is not observed in the second round PCR. The two-step PCR improves the specificity of the expected product at 383 bp for downstream NGS analysis.

- c. Amplify reactions in a thermocycler using the following cycling conditions. Visualize the first round PCR products (~477 bp) on a 2% agarose gel before proceeding to the second round PCR (Figure 2A).

△ **CRITICAL:** The first round PCR product may appear as a smear on the gel and may or may not show a specific band. The smear is likely due to the degradation of the template DNA, rather than non-specific annealing of the primers. Continue using the first round PCR product as a template for the second round PCR.

PCR1 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	30 s	22 cycles
Annealing	65°C	30 s	
Extension	68°C	2 min	
Final extension	68°C	5 min	1
Hold	4°C	Forever	

11. Second Round PCR.
  - a. Set up the second round PCR as follows:

PCR2 reaction master mix	
Reagent	Amount
1st Round PCR Product*	5 $\mu$ L
NFwd Primer <sup>#</sup>	2.5 $\mu$ L
NRev Index Primer <sup>##</sup>	2.5 $\mu$ L
dNTP Mix	1 $\mu$ L
Taq Polymerase Buffer	5 $\mu$ L
PCR-Grade Water	33 $\mu$ L
Taq Polymerase	1 $\mu$ L
Total Volume	50 $\mu$ L

**Note:** <sup>#</sup>The primer sequences can be found in the Collecta NGS prep kit manual. The 6-bp index barcode sequences can also be found in the manual.

- i. Include the original plasmid library (1 ng) as a positive control. Include a negative control without genomic DNA to ensure no DNA contamination.
  - ii. \*Master Mix is prepared without these components. Each sample must use a different Index Primer to enable multiplexing in the single next-generation sequencing lane.
- b. Amplify reactions in a thermocycler using the following cycling conditions.

PCR2 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	45 s	12 cycles*
Annealing	65°C	15 s	
Extension	72°C	30 s	
Final extension	72°C	2 min	1
Hold	4°C	Forever	

- c. Run 5  $\mu$ L of PCR2 product on a 2% agarose gel. Visualize the PCR product on a gel imager. PCR2 yields a product of 383 bp (Figure 2B). All samples should have similar yields, if not, see [troubleshooting](#) section.
- d. DNA samples can be stored at  $-20^{\circ}\text{C}$  at this stage.

### Preparation for next-generation sequencing (NGS)

⌚ Timing: ~1 day

To maximize sequencing of sgRNAs and minimize background sequences, purify the amplified PCR products by preparative gel electrophoresis.

12. PCR Purification.
  - a. Purify and concentrate the PCR products from each sample using the QIAquick PCR Purification Kit (see [key resources table](#)) following the manufacturer's [protocol](#). Samples should be eluted in a volume of ~30  $\mu$ L.
  - b. After purifying/concentrating, run each purified PCR product with a 6 $\times$  loading buffer on a 2% agarose-1XTAE gel.



- c. Use a blue transilluminator and scalpel to excise the narrow band corresponding to the correct target sgRNA amplicon size. Be sure to use UV safety glasses to protect your eyes.

13. Gel Extraction.

- a. After excision, purify each PCR product from the gel fragment using a QIAquick Gel Extraction Kit (see [key resources table](#)) following the manufacturer's [protocol](#). Samples should be eluted in a volume of ~20  $\mu$ L.

**Note:** Centrifuge QIAquick columns at maximum speed for >3 min before eluting DNA to avoid ethanol contamination in the purified PCR product.

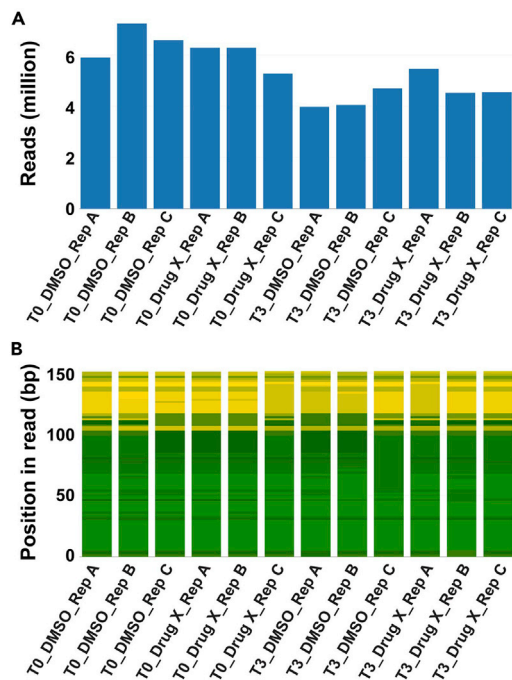
- b. Quantify extracted DNA by NanoDrop and adjust concentrations in all samples to 10 nM. Samples can be stored at  $-20^{\circ}\text{C}$  at this stage.
- c. Run eluted samples in a final 2% agarose-1XTAE gel to check if product size is correct.
- d. Samples are now ready for next-generation sequencing.

14. Guidelines for Sample Submission for Sequencing.

- a. Resulting samples can be sequenced on the Illumina NextSeq 550 (standard Single-Read (SR) 50-cycles). Other sequencing instruments can be used. Contact a sequencing facility to determine if the sequencer meets your requirements.
- b. Contact a sequencing facility to inquire about sample preparation (including PhiX spike-in, library denaturation, etc.).
- c. Submit all samples (10 nM each), as well as the "Seq NGS Primer" and "Index NGS Primer" from the Collecta kit.

15. NGS Quality Control.

- a. The following quality control steps (or similar methods) are performed by a core sequencing facility and will ensure high quality library preparations before committing to next-generation sequencing.
- b. **DNA quantification** – Use the Quant-iT PicoGreen dsDNA assay to quantify sample library preparations (see [key resources table](#)). Samples must be above 2 nM to proceed with sequencing.
- c. **High resolution electrophoresis** – Use an Agilent Bioanalyzer (see [key resources table](#)) to produce Agilent traces. These traces provide sample intensity peaks to ensure that samples are at the correct amplicon size. If adapter dimers are discovered, see [troubleshooting](#) section for additional sample cleanup.
- d. Pool equimolar amounts of all samples to be sequenced in one sequencing lane for a final pooled sample concentration of 2.5 nM.
- e. **Quantitative real-time PCR (qRT-PCR) validation** – Perform Kapa qPCR (see [key resources table](#)) on pooled samples. Concentration should exceed 2 nM. If lower than 2 nM, consider re-pooling samples or submitting individual library preps at higher concentration for re-pooling.
- f. Each pool was clustered at 1.5 pM with 10% Phi-X spike for diversity.
- g. Each pool was then sequenced in a lane of the Illumina NextSeq550, Mid-output 1  $\times$  150-bp run.
- h. NGS data quality control.
  - i. After the sequencing run, the sequencing facility will generate quality control plots ([Figure 3](#)).
  - ii. At 1000 $\times$  coverage, we would expect 3 million reads per sample for this custom sgRNA library. Our collected samples exceeded this coverage (3.8–8 million reads, [Figure 3A](#)).
  - iii. The mean base quality score is defined as  $Q = -10 \cdot \log_{10}(e)$ , where  $e$  is the estimated probability of the base call being wrong. This score is plotted for each sample along each position in the 150 bp read ([Figure 3B](#)). The sgRNA sequence is located between 20–40 bp. Across all samples, the quality score at the sgRNA position is extremely high (above Q28).



**Figure 3. Next-generation sequencing data quality control (QC) plots**

Representative Illumina NextSeq 550 QC data. Three replicates of the DMSO vehicle control and Compound X screen from T0 and T3 are shown.

(A) The number of reads per sample. Note that each sample has more than 3 million reads, which is the expected read counts for the 1000 $\times$  coverage of the custom sgRNA library.

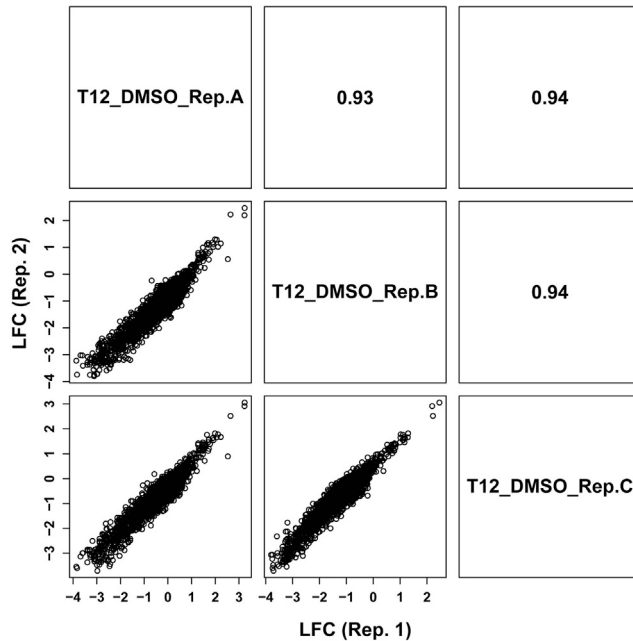
(B) The mean base quality score for each position. The higher the score, the better the base call. Note that the sgRNA position is between 20-40 bp, which show very good quality in all samples. Green: very good quality (>Q28); yellow: reasonable quality (Q20-Q28); red: poor quality (<Q20).

### Generate read count data and screen quality control

⌚ Timing: 1 h

In this section, we describe a step-by-step approach to generating raw read counts, depth-normalized read counts, and log<sub>2</sub>-fold change values for each sgRNA in the custom library. We also describe quality control metrics for these screens, including evaluation of replicate correlation and essential gene dropout. The code used to run these steps can be found [here](#). Example data for one screen (Bortezomib) is provided to illustrate this example.

16. Generating raw read counts (performed by institutional informatics experts).
  - a. Next-generation sequencing was performed using an Illumina NextSeq 550 instrument at an institutional genomics center. The resulting sequencing data (base call files, or .bcl files) were demultiplexed and converted into zipped fastq files using bcl2fastq v2.0.
  - b. Next, the *Collecta NGS Demultiplexing and Alignment Software* was used to extract raw read counts from fastq files. Detailed instructions can be found [here](#). The software requires 3 input files:
    - i. FASTQ files (in .fastq or .fastq.gz format).
    - ii. Sample Description File (matching multiplex tag to sample ID).
    - iii. Library Configuration File (describing guide sequences).
  - c. The software outputs a guide abundance table for each sample (column) and each sgRNA (row).
17. Converting to depth-normalized read counts.
  - a. The following steps can be run using the “analysis.Rmd” file found [here](#). All raw reads are converted to normalized reads by performing the following:
    - i. Each raw read count is normalized by the sequencing depth (total number of reads) for each sample/barcode (column).
    - ii. Next, the value from the previous step is multiplied by a scaling factor (1 million).



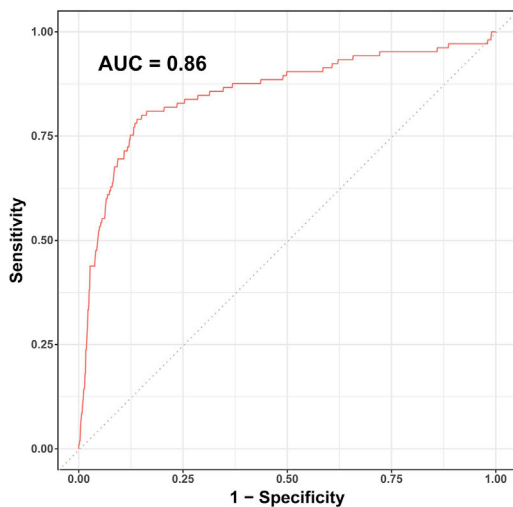
**Figure 4. Replicate correlation plots**

Replicate correlation plots were generated for T12 DMSO replicates (A, B, C). Representative scatterplots of log<sub>2</sub>-fold changes (LFCs) between two replicates are shown on the lower panels (left of diagonal). Pearson's correlation coefficients (PCC) are displayed on the upper panel (right of diagonal).

- iii. Finally, we add a pseudo-count of 1 to the value from the previous step and take log base 2 to produce the final log-normalized read count.
18. Calculating log<sub>2</sub>-fold change (LFC) values.
  - a. To measure whether a guide has been enriched or depleted at a certain time point relative to T<sub>0</sub>, we first average the T<sub>0</sub> replicate log-normalized read counts.
  - b. Next, we subtract the log-normalized T<sub>0</sub> counts from the log-normalized counts at each timepoint. This produces a log<sub>2</sub>-fold change (LFC) value for each guide at each timepoint (relative to T<sub>0</sub>).
19. Screen quality control.
  - a. Replicate correlation: by manually selecting a desired screen of interest (e.g., DMSO) and time point (e.g., T12), we can generate scatterplots of LFCs between replicates and calculate a Pearson's correlation coefficient (PCC). [Figure 4](#) shows an example scatterplot and PCC values for the T12 DMSO sample. PCC values above 0.8 are typically expected.
  - b. Essential gene dropout recovery: using a core essential gene standard ([Hart et al., 2015](#)), we identified 35 essential genes in this custom targeted library. Using a receiver operating characteristic (ROC) curve, we can quantify essential gene recovery in these screens ([Figure 5](#)). An area under the curve (AUC) should range from 0.5 (random classification) to 1 (perfect classification of essential genes as true positive dropouts). AUC values should generally be higher at later time points.

## EXPECTED OUTCOMES

Data for a bortezomib screen in hTERT RPE-1 cells using a custom Cellesta sgRNA library targeting 350 DNA damage response genes can be found [here](#). Raw read counts are generated for DMSO and bortezomib-treated samples at the following time points: T<sub>0</sub>, T<sub>3</sub>, T<sub>6</sub>, T<sub>9</sub>, T<sub>12</sub>, and T<sub>18</sub>. Downstream analysis and quality control metrics can be generated using the "analysis.Rmd" file.



**Figure 5. ROC curve for essential gene dropout recovery**

35 core essential genes (Hart et al., 2015) were found in the custom library. To determine if these essential genes drop out as expected, we generate a receiver operating characteristic (ROC) curve representing classification of core essential genes. Along varying thresholds of LFC values, we generate false positive rates (1-specificity) and true positive rates (sensitivity) for this classification problem. This example ROC curve for the T12 DMSO sample has an area under the curve (AUC) value of 0.86.

## LIMITATIONS

This protocol can be adapted for various compounds, cell lines, small sgRNA libraries, and different Illumina sequencers. However, changing these variables may impact the timing and amount of necessary reagents. Before starting this protocol, collect all reagents that you expect to use. Also, contact sequencing facilities to explore your options with multiplexing samples and sequencing depth.

## TROUBLESHOOTING

### Problem 1

Insufficient inactivation of proteinase K will interfere with downstream PCR and/or restriction enzyme digestion during the screening process ([before you begin](#) step 11.i.i).

### Potential solution

If the downstream PCR does not work, put the v-bottom plates back to 85°C for another hour to inactivate the remaining proteinase K.

### Problem 2

Puromycin selection for cells that have been transduced with a sgRNA construct may not be complete after 48 h. This is evident when the control plate of non-transduced cells (control plate) still has live cells after 48 h. If we continue with the screen, we would not be able to distinguish between transduced vs. non-transduced cells in the cell pellets, which may affect representation of sgRNAs in downstream next-generation sequencing (step 4.a).

### Potential solution

If control plate cells are not completely dead after 48 h, split all plates, add fresh puromycin (possibly at higher dosage) and medium, and monitor every 24 h until all control plate cells are dead. It is important to split the cells to avoid overgrowth. Keep in mind that essential genes will have already begun to dropout and will continue dropping out throughout this step.

### Problem 3

The protein precipitation and isopropanol DNA precipitation steps need to be handled with care to ensure maximal DNA extraction. For protein precipitation, the protein may come loose instead of settling into the bottom layer. This is problematic when attempting to transfer the supernatant

with a P1000 to isopropanol. After isopropanol precipitation and inversion of tubes, DNA may not be visible (step 8.i).

#### Potential solution

For either step, spinning down at maximum centrifuge speed at 4°C for at least 30 min can help with separating the protein layer or visualizing a white pellet.

#### Problem 4

All samples should have similar yields in the second round PCR. Inconsistent band intensity points to inconsistent PCR product amplification across samples (step 11.d).

#### Potential solution

Quantify the amount of the amplified product for each sample by the gel densitometry to ensure similar yield of PCR products (less than 2-fold difference). If there is a greater than 2-fold difference, put the samples with lower yield back into the thermocycler for additional cycles. \*We recommend testing the cycle number starting from 8 cycles and increase in an increment of 2 cycles. Overcycling may cause non-specific products, interfering with downstream sequencing efficiency.

#### Problem 5

Quantitative PCR quality control before next-generating sequencing may reveal adapter dimers (peaks at amplicon size: ~150–170 bp). These are formed when the flow cell adapter sequences (found in the 2<sup>nd</sup> round PCR primers) dimerize. Adapter dimers can bind and cluster more efficiently on the flow cell due to their small size. They should be removed so as not to affect next generation sequencing results (step 15.c).

#### Potential solution

Use AMPure XP (see [key resources table](#)) or SPRI bead cleanup to remove adapter dimers. Rerun quantitative PCR to ensure removal; then, proceed with sequencing.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anja-Katrin Bielinsky ([bieli003@umn.edu](mailto:bieli003@umn.edu)).

### Materials availability

Custom Collecta sgRNA library (3,033 sgRNAs targeting 350 DNA damage response genes) is available upon request.

### Data and code availability

The datasets supporting the current study have not been deposited in a public repository because the publication using this data is under review. However, datasets are available from the corresponding author on request. The analysis code and sample dataset used for this protocol has been deposited at Zenodo and is publicly available [here](#) as of the date of publication. DOIs are listed in the [key resources table](#). Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

## ACKNOWLEDGMENTS

We would like to thank the Bielinsky -, Myers -, and Jason Moffat's laboratories for contributing to the development of this protocol. K.L. is supported by the NIH/NCATS TL1 TRACT award (UL1TR002494, TL1R002493) through the University of Minnesota Clinical and Translational Science Institute (UMN CTSI) and by the NIH/NCI Ruth L. Kirschstein National Research F30 award (5F30CA257227-02). This work was also supported by NIH R35 GM141805 to A.K.B. and by a grant from the National Science Foundation (MCB 1818293) to C.L.M. We would also like to acknowledge

Aaron Becker (UMN Genomics Center), who consulted on next-generation sequencing options; John Garbe (UMN Genomics Center) as the informatics lead that converted bcl files to fastq files; and Juan Abrahante Lloréns (UMN Informatics Institute) as the genomics analyst who we consulted on converting fastq files to raw read count data.

## AUTHOR CONTRIBUTIONS

K.L. and Y.C.C. contributed equally to writing and collating this protocol. E.M. and K.W. contributed to the lentiviral library packaging as part of the UMN Viral Vector and Cloning Core (VVCC). A.K.B. and C.L.M. made conceptual contributions to the protocol and oversaw all screening work.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

- Baxley, R.M., Leung, W., Schmit, M.M., Matson, J.P., Yin, L., Oram, M.K., Wang, L., Taylor, J., Hedberg, J., Rogers, C.B., et al. (2021). Bi-allelic MCM10 variants associated with immune dysfunction and cardiomyopathy cause telomere shortening. *Nat. Commun.* 12, 1626. <https://doi.org/10.1038/s41467-021-21878-x>.
- Brinkman, E.K., Chen, T., Amendola, M., and van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 42, e168. <https://doi.org/10.1093/nar/gku936>.
- Chen, S.-H., Haam, J., Walker, M., Scappini, E., Naughton, J., and Martin, N.P. (2019). Production of viral constructs for neuroanatomy, calcium imaging, and optogenetics. *Curr. Protoc. Neurosci.* 87, e66. <https://doi.org/10.1002/cpns.66>.
- Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., and Taipale, J. (2018). CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response. *Nat. Med.* 24, 927–930. <https://doi.org/10.1038/s41591-018-0049-z>.
- Hart, T., Chandrashekhar, M., Aregger, M., Steinhart, Z., Brown, K.R., MacLeod, G., Mis, M., Zimmermann, M., Fradet-Turcotte, A., Sun, S., et al. (2015). High-resolution CRISPR screens reveal fitness genes and genotype-specific cancer liabilities. *Cell* 163, 1515–1526. <https://doi.org/10.1016/j.cell.2015.11.015>.
- Salmon, P., and Trono, D. (2007). Production and titration of lentiviral vectors. *Curr. Protoc. Hum. Genet. Chapter 12. Unit 12.10*. <https://doi.org/10.1002/0471142905.hg1210s54>.