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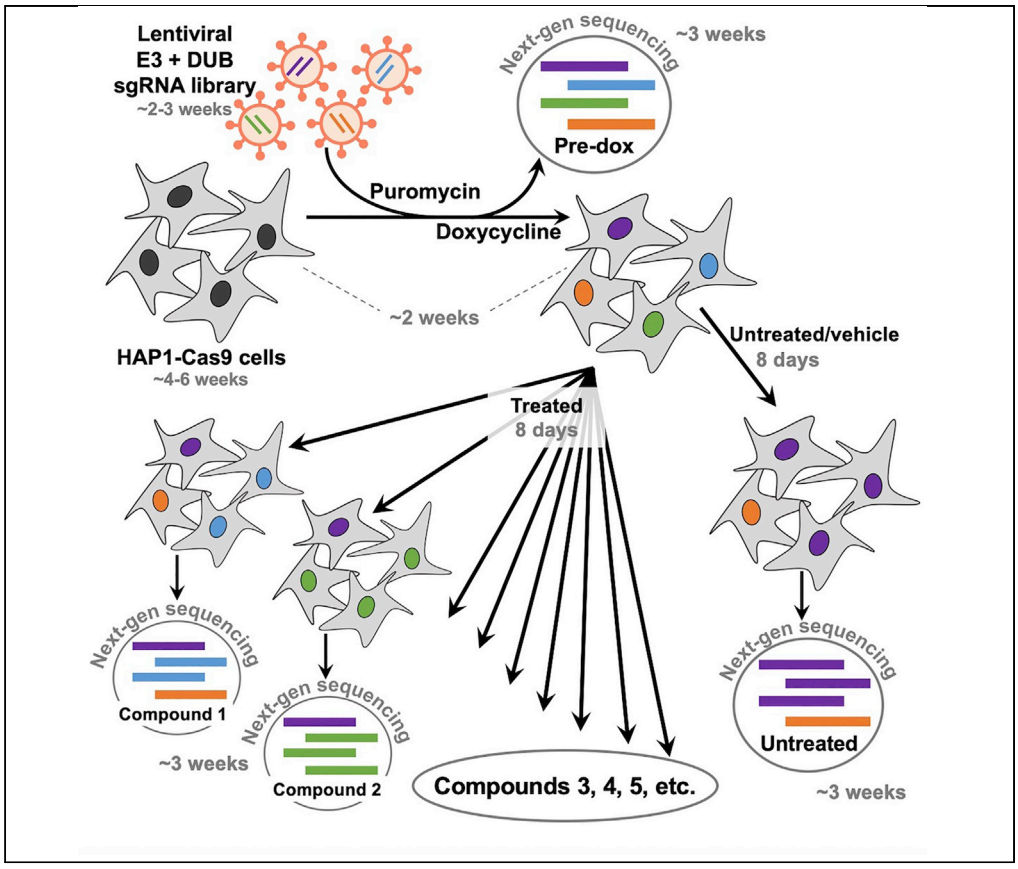
Chemical-genetic CRISPR-Cas9 screens in human cells using a pathway-specific library



The development of CRISPR-Cas9 screening techniques coupled with chemical inhibition of specific biological processes enables high-throughput investigation into many areas of molecular biology. We present a protocol to conduct ubiquitin proteasome system-specific chemical-genetic CRISPR-Cas9 screens in the human HAP1 cell line. This protocol can be adapted for use in other cell lines, with other compounds and types of treatments, and with any other sgRNA library.

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Highlights

Protocol for chemical-genetic CRISPR-Cas9 screens in human cells

Determination of chemical compound concentrations and infection conditions

Preparation of samples for next-generation sequencing

Bioinformatic analysis of sequencing results

Hundley & Toczyski, STAR Protocols 2, 100685
September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100685

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Chemical-genetic CRISPR-Cas9 screens in human cells using a pathway-specific library

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https://doi.org/10.1016/j.xpro.2021.100685

SUMMARY

The development of CRISPR-Cas9 screening techniques coupled with chemical inhibition of specific biological processes enables high-throughput investigation into many areas of molecular biology. We present a protocol to conduct ubiquitin proteasome system-specific chemical-genetic CRISPR-Cas9 screens in the human HAP1 cell line. This protocol can be adapted for use in other cell lines, with other compounds and types of treatments, and with any other sgRNA library.
For complete details on the use and execution of this protocol, please refer to Hundley et al. (2021).

BEFORE YOU BEGIN

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In this protocol, we describe how to perform chemical-genetic CRISPR-Cas9 screens using a pathway-specific or sub-genomic library. This protocol is adapted from numerous other CRISPR-Cas9 screening papers (e.g., Hart et al., 2015; Wang et al., 2015). We use a library containing 6,306 sgRNAs targeting 685 components of the human ubiquitin proteasome system (UPS) and 41 compounds targeting a broad range of biological processes. This protocol can be adapted for use in other cell lines, with other compounds and types of treatments, and with any other sgRNA library. Although this protocol is highly adaptable, alterations may significantly change the number of personnel required and may change the amount of time and reagents needed, so estimates must be made in advance to determine whether such an undertaking is feasible.

To increase the chance of biologically meaningful and reproducible results, care must be taken throughout to ensure consistency and to avoid cross-contamination and bacterial or fungal contamination. Consistent culture conditions and reagents are necessary for successful screens. We recommend purchasing reagents and plasticware before beginning the screens.

sgRNA library synthesis

Timing: 2–3 weeks

In this protocol, we use a pooled, partially custom-designed CRISPR-Cas9 library of 6,306 sgRNAs targeting 685 human UPS components and containing targeting and non-targeting control sgRNAs (Hundley et al., 2021). For each UPS gene, the library contains nine sgRNAs, seven selected from a previously published genome-wide essentiality screen (Wang et al., 2015) and two designed to

target key catalytic or binding residues (Hundley et al., 2021). As more CRISPR-Cas9 screens are performed in numerous laboratories and optimal sgRNAs are determined empirically, robust screens can be performed with fewer sgRNAs per gene. A smaller library has several advantages including the ability to perform more screens in parallel with the same number of cells and same amount of effort. This protocol can be adapted for use with any other sub-genomic or genome-wide sgRNA library. Numerous genome-wide and process-specific sgRNA libraries are available commercially and through the plasmid repository Addgene. If using a previously published library, use the provided instructions for library amplification and preparation. If generating a custom library, the following instructions can be used to amplify sufficient quantities of sgRNA expression plasmids to conduct the screens.

1. Prepare ~20–30 15 cm LB agar plates containing carbenicillin (100 μg/mL final). The number of plates needed for the sgRNA library depends on the size of the library and the desired coverage during library cloning. For this step, library coverage of ≥200-fold is generally considered sufficient, although for small libraries (<9,000 sgRNAs), higher coverage (e.g., ~500-fold) is preferable. For our UPS sgRNA library with 6,306 sgRNAs, at least 1.26×10^6 bacterial colonies are needed to achieve 200-fold coverage and this requires ~20–30 15 cm LB agar plates. If bacterial colonies grow unequally or in a lawn, sgRNA coverage will be highly biased. Therefore, growth should not be allowed to continue more than is necessary. Note that it is also possible to grow the sgRNA library in liquid culture, and similar considerations apply.

2. Digest lentiGuidePuro vector: we use a modified version of lentiGuide-Puro (Addgene, plasmid #52963) (Sanjana et al., 2014) containing Sbf1 restriction sites flanking the sgRNA region (Sbf1 restriction sites not used in this protocol).
   a. Assemble the digestion reaction on ice: combine reagents, mix gently by pipetting, and divide into six tubes with 50 μL each. Incubate at 55°C on a thermocycler for 2.5 h. Combine all reactions back together (~300 μL).

lentiGuidePuro digestion (to digest 15 μg of vector)	
10× reaction buffer	30 μL
lentiGuidePuro plasmid	15 μg
BsmBI restriction enzyme	12 μL
MilliQ H_2O (autoclaved) or other ultra-pure water	to 300 μL total

   b. Gel purify the digested plasmid: run the digested vector on an 0.8% agarose gel with a lane large enough to accommodate the sample volume. Select run time to achieve good separation between the desired ~8,300 bp band and the ~1,800 bp band. TAE buffer is preferred over TBE for preparative gels.
   c. Using a clean razor blade, excise the gel band containing the vector backbone (~8,300 bp). Remove as much excess gel as possible. Work as quickly as possible to limit UV damage to the sample.
   d. Using a Zymoclean Gel DNA recovery kit and following the manufacturer's protocol, extract the digested plasmid from the gel slice(s). Split over three to six columns (each has a maximum binding capacity of 5 μg), and elute with 10 μL MilliQ H_2O (autoclaved) or other ultra-pure water per column.
   e. Pool all six eluates and determine DNA concentration by Nanodrop or similar. Yield is typically 5–8 μg when starting with 15 μg of plasmid DNA.

3. Amplify the sgRNA oligos (we ordered our pooled sgRNA library from CustomArray Inc.). General sgRNA oligo design (with sgRNA sequence underlined) and universal forward and reverse primers are shown below.

UPS sgRNA library oligos and primers	
General sgRNA oligo	5′-TACATCCTGGTTACTTGGCCGCATGAGCAGGCGTCTCTCACCg nnnnnnnnnnnnnnnnnnnnGTTTGGAGACGGTACGACA-3′
Universal forward primer	5′-TACATCCTGGTTACTTGGC-3′
Universal reverse primer	5′-TGTCGTACCGTCTCCAAAC-3′

a. Assemble pooled sgRNA library PCR on ice, using the following recipe.

sgRNA library PCR components	
5× Phusion HF buffer (Thermo)	60 μL
dNTPs (25 mM each)	2.4 μL
Universal forward primer (10 μM)	15 μL
Universal reverse primer (10 μM)	15 μL
Phusion HS II polymerase	1 μL
sgRNA library	7.5 ng (~1.8 × 10^{11} copies)
MilliQ H_2O (autoclaved) or other ultra-pure water	to 300 μL total

b. Divide the 300 μL reaction into six tubes with 50 μL each before running thermocycler program, shown below. Also assemble a control reaction that does not contain template DNA. Note that it is important to avoid too many rounds of amplification by PCR since this can introduce sgRNA representation bias into the pool.

PCR cycling conditions			
Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	15 s	
Annealing	57°C	15 s	
Extension	72°C	15 s	go to step 2 (14x)
Final extension	72°C	10 min	1
Hold	4°C	∞	1

4. Perform PCR cleanup with Qiagen MinElute PCR Purification Kit (or similar) following manufacturer's instructions with these modifications:
   a. Combine all ~300 μL of PCR product into one sample for purification.
   b. Add 4× sample volume (~1200 μL) of Buffer PB.
   c. Add 1/100th volume 3M sodium acetate (~15 μL).
   d. Perform two wash steps, each with 750 μL of Buffer PE.
   e. Perform one dry spin after final wash.
   f. For elution, pipette 15 μL of Buffer EB directly onto column, incubate for 5 min at RT (~22°C), then spin as described.
   g. Determine DNA concentration by NanoDrop or similar. The yield of the 82 bp PCR product from the UPS sgRNA library should be ~2.5–4 μg. If the yield is low, optimize PCR conditions before proceeding (see the troubleshooting section for PCR optimization suggestions).
5. Esp3I digestion of PCR product: the PCR product of the pooled sgRNA oligos must be digested to produce sticky ends for ligation into the digested lentiGuidePuro vector. We use Esp3I, an isoschizomer of BsmBI, because in our hands, Esp3I more efficiently digests the 82 bp PCR products. Assemble digestion reaction on ice using the following recipe. Incubate at 37°C for 2 h.

Esp3I digestion of sgRNA oligo PCR product	
10× Tango buffer	5 μL
20 mM DTT (made fresh)	2.5 μL
Esp3I	3 μL
Cleaned PCR product from step (4)	2 μg
MilliQ H_2O (autoclaved) or other ultra-pure water	to 50 μL total

6. Polyacrylamide gel purification of digested sgRNAs.
   a. Make a 20% polyacrylamide gel. Note that volumes will vary depending on type of gel casting equipment available. Pour gel with wells large enough to accommodate entire Esp3I digestion reaction product plus desired loading buffer.

20% polyacrylamide gel	
10xTBE	1.2 mL
30% acrylamide solution	8 mL
10% Ammonium persulfate solution	200 μL
TEMED	10 μL
MilliQ H_2O (autoclaved) or other ultra-pure water	2.8 mL

   b. Use caution when working with acrylamide prior to polymerization and follow institutional safety protocols and disposal guidelines.
   c. Load sample alongside un-digested sgRNA PCR product and a DNA ladder of appropriate size. Markers of the desired sizes can be made by designing, ordering, and annealing top and bottom strands of partially complementary oligos that mimic the dsDNA and ssDNA structure of the expected Esp3I digestion product.
   d. Run gel long enough to achieve sufficient separation between all expected fragments.
   e. Stain gel with ethidium bromide.
   f. Using a clean razor blade, excise the gel band containing the sgRNA fragment with Esp3I-generated sticky ends. With the UPS sgRNA library, the partially single-stranded product runs at ~27 bp. Remove as much excess gel as possible. Work as quickly as possible to limit UV damage to the sample.
   g. Pierce the bottom of a 0.5 mL low retention tube with an 18.5-gauge needle and place inside a 1.5 mL low retention tube.
   h. Place gel slice inside the upper 0.5 mL tube.
   i. Spin tube at 20,000 × *g* for 3 min to force the gel slice through the small opening made by the needle. After spin, ensure that all of the gel slice is in the 1.5 mL tube.
   j. Remove upper 0.5 mL tube. Add 300 μL TE to the fragmented gel slice.
   k. Vortex gel + TE slurry for 5–10 s.
   l. Incubate slurry at 37°C overnight (12–16 h).
   m. Vortex gel slurry for 5–10 s.
   n. Using a pipette tip with the end cut off to make the opening wider, transfer the gel slurry to a Costar Spin-X column.
   o. Spin at 20,000 × *g* for 3 min.
   p. Transfer eluate (~250–300 μL) to a new low retention tube.
   q. Isopropanol precipitation of digested sgRNA fragments.
      i. Pre-chill 80% ethanol to −20°C.
      ii. Add 3M sodium acetate to ~375 mM final (31.25–37.5 μL).
      iii. Mix by inverting 10–15 times.
      iv. Add isopropanol to ~75% final and mix by inverting 10–15 times.
      v. To precipitate DNA, incubate at −80°C for 30 min.
      vi. Spin at 20,000 × *g* for 30–60 min at 4°C.
      vii. Remove supernatant.
      viii. Wash pellet twice at 20,000 × *g* for 4 min with ~500 μL ice cold 80% ethanol.
      ix. After removing final wash, leave tube open and allow to air dry for ~30 min.
      x. Resuspend DNA pellet in 15 μL MilliQ H_2O.
      xi. Determine DNA concentration by NanoDrop or similar. Yield should be approximately 150–200 ng.
      xii. Optional: glycoblue can be added at the same time as 3M sodium acetate to aid in visualizing the DNA pellet.

xiii. Note: do not heat sample because the melting temperature of small fragments is quite low in H_2O.

7. Ligate the digested and gel purified vector and sgRNA library.
   a. Assemble ligation reactions on ice using the following recipe and using a 1:2 vector:insert molar ratio. Assemble at least two of the following reaction with vector and insert (for a total of 40 μL). Also, assemble a "vector only" control ligation containing all reagents listed below except the insert DNA (use MilliQ H_2O to make up to difference in volume).

Ligation reaction (for one ligation)	
T4 DNA ligase buffer	2 μL
T4 DNA ligase	2 μL
Digested and gel purified vector	500 ng
Digested and gel purified insert	2.5 ng
MilliQ H_2O (autoclaved) or other ultra-pure water	to 20 μL total

   b. Incubate at 16°C for 16 h.
   c. Concentrate the ligation reactions using a Zymo DNA clean and concentrator kit, following the manufacturer's instructions. Combine the two ligation reactions onto one column and use a second column for the "vector only" control ligation. Elute the combined reaction with 13 μL MilliQ H_2O (autoclaved), and elute the control reaction with 9 μL MilliQ H_2O (autoclaved). Yield should be approximately 300–500 ng.

8. Perform a small test transformation in DH5α *E. coli* with 50 ng of DNA from the previous step. Make sure this transformation yields at least a few dozen colonies the day after the transformation before proceeding to electroporation of electrocompetent MegaX cells. If the test transformation is inefficient, optimize the ligation reaction and/or earlier steps (see troubleshooting section for additional information).

9. MegaX electrocompetent transformation.
   a. Pre-warm ~40 mL SOC recovery media to 37°C.
   b. Pre-warm 15 cm plates with LB agar + carbenicillin to 37°C.
   c. For each of three transformations (two of the vector and insert ligation, and one "vector only" control ligation), pre-chill 1.5 mL tubes and electroporation cuvettes. Pre-chill ~100 μL 10% glycerol.
   d. Thaw ~60–70 μL MegaXDH10B $T1^R$ Electrocomp Cells on ice (20 μL per reaction).
   e. In pre-chilled 1.5 mL tubes, assemble transformation reactions with 100 ng of purified ligation reaction and 20 μL MegaXDH10B $T1^R$ Electrocomp Cells per reaction. Mix by flicking very gently several times.
   f. Incubate on ice for 30 min.
   g. Add 20 μL chilled 10% glycerol to each tube, and mix by flicking very gently several times.
   h. Transfer mixtures to chilled electroporation cuvettes.
   i. Working quickly, electroporate each at 1800V. Time constants should be ~5.1–5.5 ms.
   j. Quickly add 300 μL warm SOC to each cuvette.
   k. Transfer to new 1.5 mL tubes at RT (~22°C).
   l. Add another 300 μL of SOC to each cuvette to recover remaining cells. Use a long and thin gel loading tip to recover as many cells as possible from cuvette.
   m. Incubate tubes at 37°C with rotation for 1 h.
   n. Plate entire "vector only" control on one 15 cm plate. To enable even spreading across the plate, add 1000 μL warm SOC.
   o. Plate two dilutions of one vector plus insert ligation transformation to enable quantification of transformation efficiency. Make serial dilutions in SOC to achieve the following dilutions.

i. Plate the equivalent of 1 μL of one transformation, or about 640-fold dilution (0.16%), adding SOC to dilute and enable even spreading across the plate).
ii. Plate the equivalent of 0.1 μL of one transformation, or about 6,400-fold dilution (0.016%), adding SOC to dilute and enable even spreading across the plate).

p. Combine the remaining two vector plus insert ligation transformations (~1270 μL). Dilute in ~20 mL warm SOC and plate 1 mL of this onto each of 22 15 cm plates. Spread as evenly as possible to reduce sgRNA amplification bias.
q. Allow plates to dry at 37°C before inverting and incubating at 37°C overnight (12–15 h).

10. Determine the transformation efficiency and sgRNA coverage.
    a. The "vector only" ligation plate may have several thousand distinct colonies, representing up to ~6% background in the library ligation reactions.
    b. If possible, count colonies on the dilution plates from the vector plus insert transformation. If the colonies on these plates are too dense, the transformation may need to be repeated with lower dilutions plated for counting purposes and the rest of the transformation spread across more plates to ensure distinct colonies can grow. Estimate the number of colonies on the undiluted plates based on the colonies counted on the two dilution plates, then calculate the estimated sgRNA coverage based on the estimated total colonies across all 22 15 cm plates. At this point, sgRNA coverage should be ≥200-fold. For the 6,306 UPS sgRNA library, to achieve ≥200-fold coverage, there should be approximately 1,000 colonies on the 640-fold dilution plate and about 100 on the 6,400-fold dilution plate. This means that one of the two vector plus insert ligation transformations produced approximately 6.4×10^5 colonies, and both ligation transformations combined produced approximately 1.28×10^6 colonies, representing 200-fold coverage. If coverage is below 200-fold, optimize the electroporation and/or ligation until the desired coverage is achieved. There should be distinct colonies on the 22 undiluted 15 cm plates. If growth is unequal or too dense (lawn), the transformation must be repeated and plated at a higher dilution so that individual colonies can grow.
11. Preliminary quality control of the sgRNA library.
    a. Select 20–40 individual colonies from one of the dilution plates. Use these colonies to inoculate 20–40 overnight (12–16 h) cultures in LB plus carbenicillin. The next day, isolate plasmid DNA from the cultures using a standard DNA miniprep kit, and send for Sanger sequencing using primer FH167 (key resources table).
    b. On average, ~85% of the sgRNAs should be a perfect match to an sgRNA in the library, and ~15% will have one or more incorrect base compared to sgRNAs in the library. Statistically, sequencing 20–40 isolates from the 6,306 sgRNA UPS library will most likely yield all unique sgRNAs.
    c. More thorough sgRNA library quality control is done in a later step (see "next generation sequencing library preparation").
12. Harvest the sgRNA library from the *E. coli*.
    a. Pre-warm ~250 mL LB plus carbenicillin.
    b. Pipette 10 mL LB + carbenicillin onto each of the 22 15 cm plates.
    c. Using a sterilized spreading implement, scrape each plate. Divide the ~220 mL of *E. coli* from 22 plates into three 750 mL LB + carbenicillin cultures (each inoculated with 70–80 mL of scraped cells).
    d. Grow at 37°C and 220 rpm in an orbital shaker for 1–2 h. Do not grow longer than 2 h.
    e. Pellet cells in 12 50 mL conical tubes in a swinging bucket centrifuge at maximum speed for 10–20 min (or until supernatant is clear) at RT (~22°C). Wet pellet mass should be approximately 0.5–1 g per 50 mL conical tube. Note that pellet mass may differ from the pellets of typical overnight culture growths.
    f. Remove supernatant. Flash freeze pellets and store at −80°C or proceed to the next step.
    g. Isolate plasmid DNA from the pelleted *E. coli* using the Genelute HP Plasmid Maxiprep Kit (which removes endotoxins), following the manufacturers' instructions. Use one column for

each of the 12 cell pellets. For each column, elute with 3 mL of freshly filter sterilized MilliQ H_2O into sterile tubes. Determine DNA concentration by NanoDrop or similar. Note that care must be taken in the final stages of the maxiprep to keep plasmids sterile for later transfection into 293T cells. Total yield from the twelve columns should be approximately 1.2–2 mg of plasmid DNA. Higher yield can be achieved with a faster elution spin, but resulting DNA concentration will be lower.

HAP1-Cas9 cell line generation

Timing: ~4–6 weeks

In this protocol, we use three independent stable clones of HAP1 cells (an adherent cell line) expressing doxycycline-inducible Cas9 (referred to as "HAP1-Cas9") for each replicate of the screen. HAP1 cells have been used extensively in the last decade—including in CRISPR-Cas9 screens—since their derivation from the chronic myeloid leukemia line KBM7 (Carette et al., 2011), in part due to their ease of infection and relatively rapid growth. While one can conduct a CRISPR-Cas9 screen with an all-in-one library containing both Cas9 and the sgRNA in the same plasmid, we chose to use HAP1-Cas9 clones infected with an sgRNA library in order to increase reproducibility and to reduce the size of the lentiviral insert. Using HAP1-Cas9 clones also allows initial characterization of independent clones for Cas9 induction upon treatment with doxycycline, Cas9 activity, and silencing of Cas9, which may occur depending on the site of genomic integration. Doxycycline induction of Cas9 expression enables tighter control of the screen.

To make HAP1-Cas9 clones, we use pAAVS1-PDi-CRISPRn (Mandegar et al, 2016), which we modified to contain a Blasticidin S deaminase gene (conferring resistance to blasticidin) rather than Puromycin N-acetyltransferase (PAC; conferring resistance to puromycin) so that sgRNAs could be marked with PAC and selected with puromycin. pAAVS1-PDi-CRISPRn expresses doxycycline-inducible 3xFlag-Cas9 and rtTA. The plasmid can be randomly integrated into the genome by transfection. Always use endotoxin-free plasmid preps for transfections as bacterial endotoxin can affect transfection efficiency and cell health.

13. Day 1: Plate relatively low passage, healthy HAP1 cells in two 10 cm dishes so that they will be ~60%–70% confluent the next day.

14. Day 2: Transfect HAP1 cells.
    a. Check HAP1 cells and ensure they are healthy and ~60%–70% confluent.
    b. Pre-warm transfection reagent (FuGENE HD) to RT (~22°C).
    c. Pre-warm ~1 mL of serum-free media (e.g., Opti-MEM) to RT (~22°C).
    d. Assemble transfection mix: first add the Opti-MEM and DNA, mix thoroughly by vortexing or pipetting, then add the FuGENE HD and mix gently by pipetting. The amount of DNA may need to be optimized, and some cell lines may need to be infected rather than transfected if transfection efficiency is very low. Note that 3 μL of FuGENE HD should be used for every 1 μg of DNA.

pAAVS1-PDi-CRISPRn transfection mix (for one 10 cm dish of HAP1 cells)	
pAAVS1-PDi-CRISPRn (with Blasticidin S deaminase gene)	1–5 μg
FuGENE HD	3–15 μL
Opti-MEM serum-free media	to 200 μL total

    e. Incubate transfection mix at RT (~22°C) for 10 min.
    f. Retrieve one 10 cm dish of HAP1 cells from incubator.

g. In hood, gently add 200 μL transfection mix dropwise to dish. Gently swirl dish in a circular motion several times.
h. Return dish to incubator.
i. 5–6 h after transfection, replace the media on the transfected HAP1 cells with 10 mL pre-warmed IMDM (4 mM L-glutamine) + 10% FBS. The second dish of HAP1 cells will serve as the negative/un-transfected control for subsequent blasticidin selection. Gently aspirate the media from the un-transfected dish. Replace media with 10 mL pre-warmed IMDM (4 mM L-glutamine) + 10% FBS. Return dishes to incubator.

15. Day 3.
    a. Check HAP1 cells. They will likely be very dense (90%–100% confluent).
    b. Split each dish to a new 10 cm dish, seeding ~2–3 mL of cells in 10 mL total volume.
16. Day 4: Begin selection for pAAVS1-PDi-CRISPRn integration with blasticidin.
    a. Check HAP1 cells and record confluency.
    b. Replace media on both dishes (transfected and un-transfected) with fresh media plus blasticidin S HCL (10 μg/mL final). Note that this dose of blasticidin works well with HAP1 cells in our hands, but the best dose should be determined empirically.
17. Days 5 through 9: Continue selection with blasticidin.
    a. Check cells every day and record confluency.
    b. Replace media and give cells fresh blasticidin every 2–3 days.
    c. Given the low efficiency of random integration, it is unlikely that the transfected dish will become dense enough to require splitting during the selection process.
18. Day 9 or day 10: End blasticidin selection.
    a. Check negative/un-transfected control dish. Once this dish is completely dead, the selection is complete. Discard un-transfected dish.
    b. Check transfected dish. It may be very sparse, but there should be at least a few cells growing. Replace the media with fresh IMDM (4 mM L-glutamine) + 10% FBS (without blasticidin).
19. Days 9 or 10 through 42: Isolate clones & evaluate for Cas9 expression and activity.
    a. Check transfected dish every day and record estimated colony size.
    b. Replace media every 2–3 days until colonies begin to grow and are large enough to isolate single cells to generate clones.
    c. Isolate clones by picking colonies, by sorting single cells into 96-well plates, or by counting and diluting to single cells in 96-well plates. Because Cas9 silencing is a frequent problem in "stable" cell lines, isolating at least 12 clonal lines for further analysis is advised. Because these are clonal populations of random integrations, it is important to use a different, carefully characterized clone for each replicate of the screen.
    d. After isolating single cells, begin growing cells in the presence of blasticidin again, and continue to grow in blasticidin for all subsequent steps.
    e. Once clones have grown sufficiently, freeze a subset of each clone and seed clones in 6-well plates for Cas9 induction with doxycycline. Be sure to freeze many vials (e.g., ≥8) of each early passage HAP1-Cas9 clone to return to for later experiments.
    f. Test clones grown in 6-well plates for inducible Cas9 expression by treating with doxycycline for three days (1 μg/mL final, fresh each day) alongside untreated controls, and harvest for analysis by Western blot using a standard anti-Flag or anti-Cas9 antibody.
    g. Test clones for sustained inducible Cas9 expression by growing for several weeks in the presence of blasticidin, then repeating step (f). Some clones may silence Cas9 over time, which can be detected by Western blot comparing the extent of Cas9 induction in step (f) versus step (g).
    h. For clones that do not appear to silence Cas9, the Cas9 activity can be tested by infecting these clones with a single sgRNA construct in lentiGuidePuro (or similar), following a protocol similar to that described below, and checking for target protein reduction by Western blot and/or genome modification by PCR of the region surrounding the sgRNA target site

followed by Sanger sequencing. Note that during the infection with an sgRNA, the HAP1-Cas9 cells must be taken off blasticidin.

Note: Because doxycycline-inducible promoters are also activated by tetracycline, it is important to use either guaranteed tetracycline-free FBS or a lot of FBS that has undetectable tetracycline.

Note: HAP1 cells grow quite quickly with a doubling time of approximately 17 hr in our hands. Significant adjustments to the timelines in this protocol will be needed for other cell types with slower doubling times.

Note: If haploid HAP1 cells are desired, live cell ploidy analysis (e.g. with Hoechst 33342) should be performed prior to Cas9 integration and selection and prior to beginning the CRISPR-Cas9 screen. HAP1 cells diploidize rapidly and the diploids have a growth advantage (Olbrich et al., 2017), so frequent sorting for haploid cells (~every two weeks) needs to be performed to maintain a haploid population. Overgrowing HAP1 cells and other stresses that can occur during tissue culture work should be avoided as these stresses tend to increase the rate of diploids taking over the population. In contrast to gene-trap mutagenesis screens which require haploid cells, we and others have found that haploid cells are not necessary to achieve efficient cutting of target genes in CRISPR-Cas9 screens (see for example Wang et al., 2015).

Determine optimal lentiviral titer

Timing: 8–9 days

Before beginning the screen, it is essential to determine the amount of sgRNA library to infect each 15 cm plate of HAP1-Cas9 cells to achieve an infection efficiency of close to 30% (or an MOI of 0.3). An MOI of close to 0.3 maximizes the number of cells that have a single sgRNA integration. Significantly higher MOIs may result in a high percentage of cells containing more than one sgRNA integration. While studying genetic interactions is useful (see for example Horlbeck et al., 2018), multiple sgRNA integrations cannot be detected by this next-generation sequencing method and will impact the reproducibility and usefulness of the results. The following describes one method for determining functional lentiviral titer by comparing the number of infected cells with and without puromycin selection. Alternative methods can be used. Note that we have noticed that estimating the infection efficiency on a smaller scale (e.g., in 6-well tissue-culture treated plates) does not accurately scale to a larger scale (e.g., 15 cm dishes), so it is best to test lentiviral infection efficiency in 15 cm dishes (or whichever types of dish will be used for the screen).

Our UPS library is in a modified version of lentiGuide-Puro (Addgene, plasmid #52963) (Sanjana et al., 2014) containing Sbf1 restriction sites flanking the sgRNA region (Sbf1 restriction sites not used in this protocol). To generate lentivirus containing the UPS library to infect HAP1-Cas9 cells, use the pooled library along with psPAX2 (Addgene, plasmid #12260) and pCMV-VSV-G (Addgene, plasmid #8454), or equivalent. Always use endotoxin-free plasmid preps for transfections as bacterial endotoxin can affect transfection efficiency and cell health.

20. Day 1: Plate 293T cells.
    a. Seed 293T cells in four 15 cm dishes so that cells will be ~60% confluent the next day for transfection.

21. Day 2: Transfect 293T cells & plate HAP1-Cas9 cells.
    a. Check 293T cells and ensure they are healthy and ~60% confluent.
    b. Pre-warm transfection reagent (FuGENE HD) to RT (~22°C).
    c. Pre-warm 8–12 mL of serum-free media (e.g., Opti-MEM) to RT (~22°C).

d. Assemble transfection mix: first add the Opti-MEM and DNA, mix thoroughly by vortexing or pipetting, then add the FuGENE HD and mix gently by pipetting. These amounts work well using the UPS sgRNA library and HAP1-Cas9 cells, but this will likely need to be optimized for a different sgRNA library or cell line. Note that the transfer vector (UPS sgRNA library) to psPAX2 to pCMV-VSV-G ratio should be maintained if altering the total amount of DNA. Note that 3 μL of FuGENE HD should be used for every 1 μg of DNA.

sgRNA library transfection mix (for one 15 cm dish of 293T cells)	
UPS sgRNA library (Hundley et al., 2021)	11.25 μg
psPAX2	6.75 μg
pCMV-VSV-G	3.375 μg
FuGENE HD	64.125 μL
Opti-MEM serum-free media	to 2000 μL total

e. Incubate transfection mix at RT (~22°C) for 10 min.
f. Retrieve the four 15 cm dishes of 293T cells from incubator.
g. In hood, gently add transfection mix dropwise (2 mL per dish). Gently swirl dishes in a circular motion several times.
h. Return dishes to incubator.
i. Seed HAP1-Cas9 cells in nine 15 cm dishes so that cells will be ~70% confluent the next day.
j. 5–6 h after the transfection, replace the media on the 293T cells with pre-warmed IMDM (4 mM L-glutamine) + 10% FBS (normal growth media for HAP1 cells), using 10 mL per plate (1/2 the volume usually used for a 15 cm dish). Starting at this step, treat all materials as potentially contaminated with lentivirus.

22. Day 3: Harvest lentivirus & infect HAP1-Cas9 cells.
    a. Check 293T and HAP1-Cas9 cells to ensure the former are healthy and dense (~80%–90% confluent), and the latter are healthy and ~70% confluent.
    b. 24 h after transfection, harvest the lentivirus from the 293T media:
        i. Using a 60 mL plastic syringe, slowly collect the media from the four 15 cm dishes of 293T cells.
        ii. Slowly filter the lentiviral media with a 0.45 μm SFCA filter into a conical tube. This step should filter out any 293T cells that came off the plate.
        iii. Gently aliquot the filtered lentiviral media as follows to four pre-labeled conical tubes: Tube 1: aliquot ~3.34 mL (1/12 of the total); Tube 2: aliquot ~6.66 mL (1/6 of the total); Tube 3: aliquot ~10 mL (1/4 of the total); Tube 4: aliquot ~20 mL (1/2 of the total)
        iv. Add pre-warmed IMDM (4 mM L-glutamine) + 10% FBS to bring the total volume in each tube to 20 mL.
        v. Add polybrene to each tube to 8 μg/mL final. Gently mix by inverting conical tubes several times.
        vi. Bleach and discard 293T dishes. Alternatively, add 10 mL fresh, pre-warmed IMDM (4 mM L-glutamine) + 10% FBS to each dish, return dishes to incubator, and harvest more virus 24 h later. Note that lentiviral titer from the second 24 h after transfection is usually lower than from the first 24 h.
    c. Infect HAP1-Cas9 cells:
        i. HAP1-Cas9 dishes 1 & 2: gently aspirate the media from the HAP1-Cas9 dishes. Gently pipette 10 mL of the lentiviral media from tube 1 onto the wall of each dish so as not to dislodge the HAP1 cells. Return dishes to incubator.
        ii. HAP1-Cas9 dishes 3 & 4: gently aspirate the media from the HAP1-Cas9 dishes. Gently pipette 10 mL of the lentiviral media from tube 2 onto the wall of each dishes so as not to dislodge the HAP1 cells. Return dishes to incubator.

iii. HAP1-Cas9 dishes 5 & 6: gently aspirate the media from the HAP1-Cas9 dishes. Gently pipette 10 mL of the lentiviral media from tube 3 onto the wall of each dish so as not to dislodge the HAP1 cells. Return dishes to incubator.
iv. HAP1-Cas9 dishes 7 & 8: gently aspirate the media from the HAP1-Cas9 dishes. Gently pipette 10 mL of the lentiviral media from tube 4 onto the wall of each dish so as not to dislodge the HAP1 cells. Return dishes to incubator.
v. HAP1-Cas9 dish 9/negative control: The ninth and final 15 cm dish of HAP1-Cas9 cells is not infected and serves as the negative control for subsequent puromycin selection. Gently aspirate the media from the HAP1-Cas9 dish. Replace media with 10 mL pre-warmed IMDM (4 mM L-glutamine) + 10% FBS. Return dish to incubator.

23. Day 4: Change media.
    a. Check HAP1-Cas9 cells. They should be more confluent than the day before (~80%–90% confluent), but often grow more slowly in the presence of lentivirus than without. Some cell death may be observed due to the infection and/or polybrene. Use dish 9/negative control for comparison.
    b. 24 h after infecting HAP1-Cas9 cells, gently aspirate the lentiviral media from each dish (or the negative control media from dish 9), and add 20 mL fresh, pre-warmed IMDM (4 mM L-glutamine) + 10% FBS per dish.
24. Day 5: Begin selection for sgRNA integration with puromycin. Odd-numbered dishes (1, 3, 5, and 7) will be treated with puromycin, while corresponding even-numbered dishes (2, 4, 6, and 8) will not.
    a. Check HAP1-Cas9 cells. They should be very confluent (~90%–100% confluent).
    b. 24 h after removing lentiviral media: split cells from dishes 1, 3, 5, and 7 into puromycin (1 μg/mL final). Cells should be split quite dense (1/3 of resuspended cell volume split into a new 15 cm dish) since the infection efficiency is not yet known. Dishes 2, 4, 6, and 8 should be split in an identical manner into media without puromycin. Split dish 9/negative control in the same manner. Note that 1 μg/mL puromycin works well for HAP1-Cas9 cells in our hands, but the best dose should be determined empirically.
25. Day 6: Puromycin selection, start of day 2.
    a. Check dish 9/negative control. 24 h of puromycin treatment should begin to kill the negative control cells, indicated by many cells rounding up on the dish and some beginning to detach.
    b. Check dishes 1–8. Observe the confluency and extent of rounded and detached cells. For odd-numbered dishes treated with puromycin, this should be correlated with the amount of lentivirus used for each dish.
    c. Dish 9/negative control: change the media and give fresh puromycin (1 μg/mL final).
    d. Dishes 1–8: For odd-numbered dishes that seem somewhat similar to the negative control, change the media and give fresh puromycin (1 μg/mL final). Give fresh media without puromycin to the corresponding even-numbered dishes. If cells in an odd-numbered dish appear more resistant to puromycin than the negative control and are dense ($\geq$80% confluent with few rounded or floating cells), count cells (using Countess Automated Cell Counter with trypan blue or similar) and split cells into puromycin (1/3 of resuspended cell volume split into a new 15 cm dish). Cells from duplicate infections (e.g., dishes 1 & 2) should be split in an identical manner in terms of volume of resuspended cells seeded in new plates. At this point the number of cells between duplicate infection dishes (e.g., dishes 1 & 2) will likely be different.
26. Day 7: Puromycin selection, start of day 3.
    a. As on day 2 of puromycin selection, observe confluency and observe number of cells rounding up on the dish and floating. Dish 9/negative control should be very sparse at this point (~5%–10% confluent) with many detached, floating cells. Infected dishes will have a range of densities and number of floating cells.
    b. Dish 9/negative control: change the media and give fresh puromycin (1 μg/mL final).
    c. Dishes 1–8: For odd-numbered dishes that seem somewhat similar to the negative control, change the media and give fresh puromycin (1 μg/mL final). Give fresh media without puromycin to the corresponding even-numbered dishes. If cells in an odd-numbered dish appear

more resistant to puromycin than the negative control and are dense (≥80% confluent with few rounded or floating cells), count cells and split cells into puromycin (1/3 of cells split into a new 15 cm dish). Cells from duplicate infections (e.g., dishes 1 & 2) should be split in an identical manner in terms of volume of resuspended cells seeded in new plates.

27. Day 8: Puromycin selection complete OR puromycin selection, start of day 4 (if needed).
    a. As on day 2 of puromycin selection, observe confluency and observe number of cells rounding up on the dish and floating. Dish 9/negative control should be very sparse at this point (0%–5% confluent) with some detached, floating cells. Infected dishes will have a range of densities and number of floating cells.
    b. If dish 9/negative control is completely dead, puromycin selection is complete. If even a small number of cells are still growing on this dish, treat all dishes with puromycin for one more day, following what was done on puromycin day 3.
    c. Dishes 1–8: record final observations (unless selecting with puromycin for one more day), and count the number of cells remaining in each dish. Determine which amount of lentiviral media gave ~30% infection efficiency by comparing the final number of cells on the infected dishes treated with puromycin (dishes 1, 3, 5, and 7) to the corresponding infected dishes not treated with puromycin (dishes 2, 4, 6, and 8). If cells from duplicate infections (e.g., dishes 1 & 2) were split in an identical manner throughout the puromycin selection, the final relative cell counts will indicate the infection efficiency. For example, if dish 1 has 1.5×10^6 cells and dish 2 has 1.5×10^7 cells, the infection was approximately 10% efficient.
    d. If needed, repeat the lentiviral titer process with a new range of lentivirus. In our hands with the UPS sgRNA library and HAP1-Cas9 cells, the lentivirus from 1/6 of one 15 cm dish of 293T cells (like dishes 1 & 2) yields a roughly 30% infection efficiency in HAP1-Cas9. Different lentiviral inserts will affect the lentiviral titer, and there is a large range in infectability in human cell lines. We have found HAP1 cells to be relatively easy to infect, requiring less lentivirus than many other cell lines.
28. Day 9 (if needed): Puromycin selection complete.
    a. As on day 4, observe confluency and observe number of cells rounding up on the dish and floating. Dish 9/negative control should be very sparse at this point (0%–5% confluent) with some detached, floating cells. Infected dishes will have a range of densities and number of floating cells. As described above, count the number of cells remaining on dishes 1–8 and determine the infection efficiency.
    b. Dish 9/negative control should be completely dead at this point, and the puromycin selection is complete. If dish 9/negative control is not completely dead, a higher puromycin dose should be used and the experiment should be repeated with a higher dose.

⚠ CRITICAL: When working with lentivirus, proper safety measures must be followed, including bleaching all surfaces, solutions, and plasticware after using and before disposing. Please also refer to specific university or institutional guidelines for lentiviral safety measures. These safety measures will also help prevent cross-contamination between samples and prevent lentiviral contamination of other experiments in the same laboratory. If possible, use a dedicated incubator or incubators for plates infected with lentivirus and routinely decontaminate these incubators.

Note: For optimal and consistent lentiviral production, use relatively low passage 293T, never allow 293T cells to overgrow, and transfect 293T cells at a consistent cell density.

Optional: For greater consistency and to save time, a large batch of sgRNA library lentivirus can be made by transfecting 293T cells, harvesting the virus as usual, and storing the virus in aliquots at -80°C. Before using for a screen, test the functional titer of a batch of aliquots by infecting two 15 cm dishes with one aliquot and comparing cell numbers with and without puromycin, as described above.

Compound concentration determination

Timing: 2–4 weeks (depending on the number of compounds)

In Hundley et al. (2021), we performed UPS CRISPR-Cas9 screens with 41 compounds targeting a broad range of biology, including cell cycle progression, gene expression, genome integrity, cytoskeletal integrity, intracellular transport, and metabolism. Some of these compounds, such as Palbociclib and cisplatin, are well-established cancer treatments. Others, such as JG-231, which inhibits HSP70, were developed relatively recently and are useful tools in the laboratory (Shao et al., 2018). This protocol can be adapted for use with essentially any compound of interest or other types of treatment that have an effect on cell growth and/or death, such as viral infection or heat shock.

The effective dose and time course of the treatment will affect the results, so consideration must be taken before beginning as to what sort of biology one wishes to uncover. For example, an acute treatment with a very high affective dose of a DNA damaging agent may uncover genes that are critical for surviving acute DNA damage and may reveal genes whose deletion renders cells resistant to acute DNA damage. A longer, chronic exposure to a lower dose may uncover a different set of genes. Chronic exposures of different lengths (e.g., eight days versus 21 days) may also yield different results. Note that CRISPR-Cas9 screens using either acute or chronic treatments can yield both sensitive and resistant genes, but an extremely high dose acute treatment will most likely reveal exclusively resistant genes.

The following steps describe how to identify compound concentrations appropriate for an eight-day screen in HAP1 cells where cells are treated every two days with a dose that results in a cumulative growth reduction to 50%–70% that of control cells (i.e., 30%–50% reduction over eight days). Adjust the following protocol to match the desired screen length and type. For example, if performing a 21-day screen looking for sensitivity to a particular compound, test compound concentrations for 21 days and identify a concentration that results in a cumulative reduction in cell growth to 50%–70% that of control cells (i.e., 30%–50% reduction over 21 days). Using a dose that has too strong of an effect too quickly will likely result in excessive cell death before completion of the desired time course. Using cell lines with longer or shorter doubling times will require different lengths of screens, and cell lines with particularly long doubling times will likely need to be split and treated less frequently than described here.

Keep in mind that sufficient sgRNA coverage (e.g., 1,000-fold) must be maintained throughout, so acute doses that significantly decrease cell number may require plating more cells initially.

See troubleshooting section for additional information.

29. For each compound, seed HAP1-Cas9 cells in 6 to 12 wells in 6-well tissue culture-treated plates at a density of $\sim 5 \times 10^5$ cells per well.
    a. Leave one well untreated (or add the appropriate vehicle if compound is not dissolved in H_2O).
    b. With the remaining wells, treat cells with a range of five to eleven doses of the compound. At the upper end of the dose range, use a published dose (if available) that has been used for acute treatment of tissue culture cells. At the lower end of the dose range, use a published dose (if available) that has been used for chronic treatment of tissue culture cells. If there are no published chronic exposure doses for the compound of interest, the lowest dose should be at least one order of magnitude lower than an acute dose. For novel compounds or those for which no published dosing information is available, it may be more efficient to initially perform a broader dosing test (e.g., a semi-log dilution series) in 96-well plate format using a standard cell viability assay, such as PrestoBlue. Using a standard plate reader to measure the effect on cell viability 48–96 h after treatment with a range of doses will help identify a

narrower range of doses to test in the 6-well plate format as described here. Keep in mind that repeated treatment with a compound every two days will likely have a cumulative effect, and therefore the ideal screening dose will likely be at the lower end of doses that have the desired effect in the shorter 96-well plate assay.

c. Every two days, observe cell confluency and death and split into fresh compound (or untreated/vehicle), seeding cells differentially according to their density and death. Some compounds may alter cell shape and/or size, which should be taken into consideration when deciding how to seed cells.

d. Continue for a total of eight days. For HAP1 cells, an eight-day treatment (approximately 12 doublings) is sufficient for a CRISPR-Cas9 screen, but longer chronic exposures may be needed for cell lines with longer doubling times.

e. Select a dose in the dose range that results in an apparent inhibition of cell growth (or apparent reduction in doubling time) to 50%–70% that of the untreated/vehicle control (i.e., 30%–50% reduction). Doubling time can be estimated by counting cells at each split using a standard cell counting method (e.g., Countess Automated Cell Counter with trypan blue) and taking into account how many cells are seeded and treated at each new time point. For an example, refer to Hundley et al. (2021) Figure 1C.

f. New dose ranges may need to be tested if initial doses are all either too low or too high to achieve the desired growth reduction over eight days.

30. We have observed that for some compounds, the same dose will have a slightly different effect on cells grown in small areas (such as 6-well plates) and on cells grown in large plates (such as the 15 cm dishes used for the screen). While it is not feasible to test a range of doses on cells grown in 15 cm dishes, once a dose has been selected through the above small-scale process, it may be beneficial to confirm that the dose affects cells in the same way when grown in a 15 cm dish before beginning the screen.

⚠ CRITICAL: Cell seeding density can affect how the cells respond to certain compounds, so it is essential to keep careful records of how cells were split and seeded during the dosing experiments so that this can be replicated as closely as possible on a larger scale during the screen.

⚠ CRITICAL: For consistency, it is important to use the same lot and preparation of each compound throughout the dosing experiments and the screens. For many compounds, low dose chronic exposure can be quite sensitive to slight changes in compound concentration that can result from lot-to-lot variability in potency or variability in compound preparation in solvent.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemical, peptides, and recombinant proteins		
Puromycin	Thermo Fisher Scientific	Cat# A1113803
Blasticidin	Thermo Fisher Scientific	Cat# A1113902
Doxycycline hyclate	Sigma-Aldrich	Cat# D9891
Hexadimethrine bromide (Polybrene)	Sigma-Aldrich	Cat# H9268
RNase A (Lyophilized)	MACHEREY-NAGEL	Cat# 740505
Bacterial and virus strains		
DH5α subcloning competent cells	This study	N/A
MegaX DH10B T1R Electrocomp Cells	Thermo Fisher Scientific	Cat# C640003
Critical commercial assays		
NucleoSpin Blood L kit	MACHEREY-NAGEL	Cat# 740954.20
NucleoSpin Gel and PCR Clean-up, Mini Kit	MACHEREY-NAGEL	Cat# 740609

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zymoclean Gel DNA Recovery Kit	Zymo Research	Cat# D4007
DNA Clean & Concentrator-5	Zymo Research	Cat#4013
E.Z.N.A. Endo-free Plasmid Mini Kit II	Omega Bio-Tek	Cat# D6950-02
MinElute PCR Purification Kit	QIAGEN	Cat# 28004
FuGENE HD Transfection Reagent	Promega	Cat# E2311
GenElute HP Plasmid Maxiprep Kit	Sigma-Aldrich	Cat# NA0310-1KT
Experimental models: cell lines		
HAP1	Laboratory of Joseph Puglisi, Stanford University	N/A
Oligonucleotides		
Sequencing primer FH167: 5′-GGGCAAGTTTGTGGAATTGG-3′	This study	N/A
Universal forward primer for amplification of sgRNA library: 5′-TACATCCTGGTTACTTGGC-3′	This study	N/A
Universal reverse primer for amplification of sgRNA library: 5′-TGTCGTACCGTCTCCAAAC-3′	This study	N/A
Recombinant DNA		
pCMV-VSV-G	Addgene	Cat# 8454
psPAX2	Addgene	Cat# 12260
Software and algorithms		
mhorlbeck/Screen processing	Horlbeck et al., 2016	https://github.com/mhorlbeck/ScreenProcessing
Canopy v.2.1.3.3542	Enthought Inc.	https://store.enthought.com/downloads/
TextWrangler v.5.5.2	Bare Bones Software	https://www.barebones.com/products/textwrangler/download.html
Fiji (ImageJ)	Schindelin et al., 2012	https://imagej.net/Fiji

STEP-BY-STEP METHOD DETAILS

Pooled chemical-genetic CRISPR-Cas9 screen

Timing: ~3–4 weeks

Before beginning the screens, it is important to estimate the amount of each reagent and plasticware that will be needed and order ahead of time, and these depend in large part on the desired sgRNA coverage. Picking an sgRNA coverage is a balance between maintaining the highest possible sgRNA coverage to obtain reliable data, and an sgRNA coverage that is feasible given a particular laboratory's constraints. When using smaller, pathway-specific sgRNA libraries such as the UPS library, it should be feasible to maintain at least 1,000-fold coverage of each sgRNA throughout the screen. For genome-wide libraries, it is common practice to maintain 300-fold coverage. When conducting a screen to look specifically for resistance to a particular compound or treatment, less than 1,000-fold coverage is generally sufficient. Based on the desired sgRNA coverage, calculate how many 15 cm plates are needed for each of three replicates for each condition (untreated/vehicle, compound 1, compound 2, etc.). For one replicate of one compound and with the UPS sgRNA library in HAP1 cells, one 15 cm plate is more than enough to maintain over 1,000-fold coverage of each sgRNA throughout the screen, taking into account the number of cells per plate when the cell number is at its lowest (e.g., when splitting a plate and taking a fraction of it forward to seed a new plate). This also leaves enough cells to keep a fraction of a plate every two days as a sample to prepare for next-generation sequencing (e.g., T2, T4, etc. in Figure 1). With two people and using the UPS sgRNA library and HAP1-Cas9 cells, it is possible to do three replicates of 20-30 different compounds/treatments (including untreated/vehicle), or a total of 60-90 plates, at one time. The replicates can be on the exact same timeline, or replicates can be staggered on alternating days such

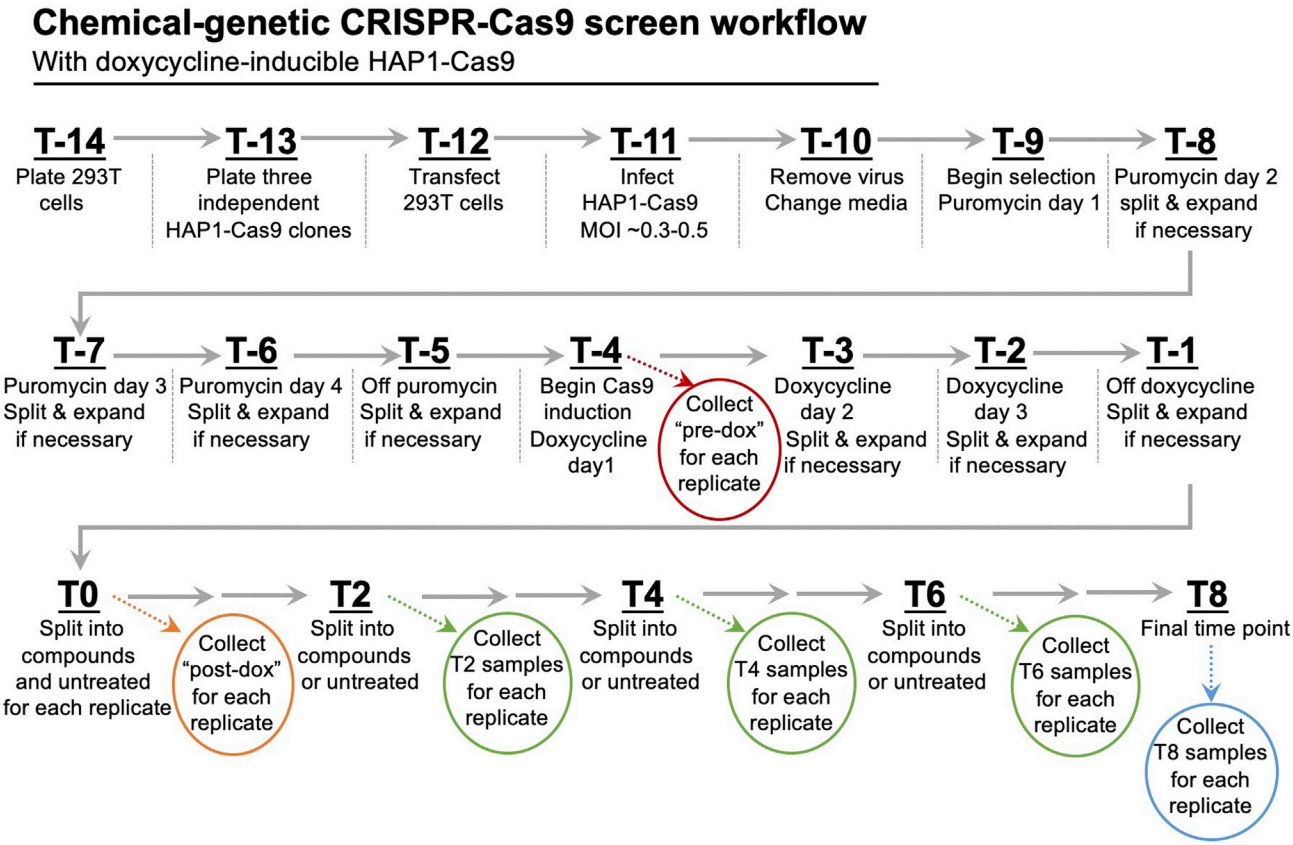

Figure 1. General workflow of a chemical-genetic CRISPR-Cas9 screen

Can be adapted for use with another sgRNA library or cell type. Length of selection and treatment with compounds of interest will vary with the cell line, as will the number of days between time points during compound treatment. Sufficient sgRNA coverage must be maintained at each split and for each collection. "Pre-dox", samples with integrated sgRNAs but before induction of Cas9. The "pre-dox" samples are essential for later data analysis. "Post-dox", samples with integrated sgRNAs and after three days of Cas9 induction with doxycycline. Collecting the "post-dox" samples is optional.

that replicates one and two are split on one day (40–60 plates) and replicate three is split on the next day (20–30 plates). It is not recommended to do the three replicates at completely different times as this will introduce more variability.

Please refer to Figure 1 for an outline of the experimental timeline, where time point zero (T0) indicates the day cells are initially split into desired compounds (or other desired treatment is initiated). Time points before T0 are indicated with a minus sign, e.g., T-14, T-13, etc.

1. T-14 through T-11: Generate sgRNA library lentivirus.
   a. Using the results from the "determine optimal lentiviral titer" section, calculate how many 15 cm dishes of 293T cells are needed to infect three independent clones of HAP1-Cas9 (one for each replicate) at an MOI of approximately 0.3, and with enough HAP1-Cas9 cells that $\geq$1,000-fold sgRNA coverage will be maintained. Also take into account how many HAP1-Cas9 cells will be needed by T0 to split into each compound or treatment of interest (including the untreated/vehicle controls) and to collect some of each replicate as the "post-dox" samples. In our hands, two 15 cm dishes of 293Ts will produce enough UPS sgRNA library lentivirus to infect three 15 cm dishes of each of the three independent HAP1-Cas9 clones. Because HAP1-Cas9 grow quite quickly, there will be several opportunities to split and expand the cells between the infection on T-11 and the initiation of treatment on T0.

b. One to two days before transfecting 293T cells (T-14 or T-13), seed 293T cells in 15 cm dishes such that they will be ~60% confluent on T-12.
c. One to two days before transfecting 293T cells (T-13 or T-12), seed three independent clones of HAP1-Cas9 cells (representing three replicates) in four 15 cm dishes each such that they will be ~70% confluent on T-11. The fourth 15 cm dish of each HAP1-Cas9 clone will serve as uninfected/negative control dishes for later puromycin selection. Grow HAP1-Cas9 cells in blasticidin (10 μg/mL final) until the infection.
d. On T-12, transfect 293T cells.
    i. Check 293T cells and ensure they are healthy and ~60% confluent.
    ii. Pre-warm transfection reagent (FuGENE HD) to RT (~22°C).
    iii. Pre-warm 4–6 mL of serum-free media (e.g., Opti-MEM) to RT (~22°C).
    iv. Assemble transfection mix: first add the Opti-MEM and DNA, mix thoroughly by vortexing or pipetting, then add the FuGENE HD and mix gently by pipetting. These amounts work well using the UPS sgRNA library and HAP1-Cas9 cells, but this will likely need to be optimized for a different sgRNA library or cell line. Note that the transfer vector (UPS sgRNA library) to psPAX2 to pCMV-VSV-G ratio should be maintained if altering the total amount of DNA. Note that 3 μL of FuGENE HD should be used for every 1 μg of DNA.

sgRNA library transfection mix (for two 15 cm dishes of 293T cells)	
UPS sgRNA library (Hundley et al., 2021)	22.5 μg
psPAX2	13.5 μg
pCMV-VSV-G	6.75 μg
FuGENE HD	128.25 μL
Opti-MEM serum-free media	to 4000 μL total

    v. Incubate transfection mix at RT (~22°C) for 10 min.
    vi. Retrieve two 15 cm dishes of 293T cells from incubator.
    vii. In hood, gently add transfection mix dropwise (2 mL per dish). Gently swirl dishes in a circular motion several times.
    viii. Return dishes to incubator.
    ix. 5–6 h after the transfection, replace the media on the 293T cells with pre-warmed IMDM (4 mM L-glutamine) + 10% FBS (normal growth media for HAP1 cells), using 10 mL per plate (1/2 the volume usually used for a 15 cm dish). Starting at this step, treat all materials as potentially contaminated with lentivirus.

2. T-11 through T-5: Infect HAP1-Cas9 cells with sgRNA library and select for integration. Throughout the infection and selection process, check the functional titer of the virus (as described in "Determine optimal lentiviral titer") to ensure that the MOI is close to 0.3.
    a. On T-11, check 293T and HAP1-Cas9 cells to ensure the former are healthy and dense (~80%–90% confluent), and the latter are healthy and ~70% confluent.
    b. 24 h after transfection on T-11, harvest the lentivirus from the 293T media:
        i. Using a 30 mL plastic syringe, slowly collect the media from the two 15 cm dishes of 293T cells (~20 mL total).
        ii. Slowly filter the lentiviral media with a 0.45 μm SFCA filter into a sterile, sealable bottle (≥200 mL capacity). This step should filter out any 293T cells that came off the plate.
        iii. Add pre-warmed IMDM (4 mM L-glutamine) + 10% FBS to bring the total volume to 120 mL. Note that the lentiviral dilution should be adjusted according to the results obtained in the "determine optimal lentiviral titer" section.
        iv. Add polybrene to 8 μg/mL final. Gently mix by inverting bottle several times.
        v. Bleach and discard 293T dishes.

vi. For each HAP1-Cas9 clone, gently aspirate the media from three of the 15 cm dishes. Gently pipette 10 mL of the diluted lentiviral media from (iv) onto the wall of each dish so as not to dislodge the HAP1 cells. Return dishes to incubator.

vii. One dish of each HAP1-Cas9 clone will serve as the un-infected/negative control. For each negative control dish, gently aspirate the media and replace the media with 10 mL pre-warmed IMDM (4 mM L-glutamine) + 10% FBS. Return dishes to incubator.

c. On T-10, remove virus and change media.
   i. Check HAP1-Cas9 cells. They should be more confluent than the day before (~80%–90% confluent), but often grow slower in the presence of lentivirus than without. Some cell death may be observed due to the infection and/or polybrene. Use the un-infected/negative control dishes for comparison.
   ii. 24 h after infecting HAP1-Cas9 cells, gently aspirate the lentiviral media from each dish (or the negative control media), and add 20 mL fresh, pre-warmed IMDM (4 mM L-glutamine) + 10% FBS per dish.

d. On T-9, begin selection for sgRNA integration with puromycin day 1.
   i. Check HAP1-Cas9 cells. They should be very confluent (~90%–100% confluent).
   ii. 24 h after removing lentiviral media: split cells into puromycin (1 μg/mL final). Cells should be split quite dense (1/2 of cells split into a new 15 cm dish). If expansion is necessary at this point, use all of the cells and seed two new 15 cm dishes for every original 15 cm dish (or six 15 cm dishes per HAP1-Cas9 clone). Split the un-infected/negative control dishes in the same manner. Note that 1 μg/mL puromycin works well for HAP1-Cas9 cells in our hands, but the best dose should be determined empirically.

e. On T-8, begin puromycin day 2.
   i. Check un-infected/negative control dishes. 24 h of puromycin treatment should begin to kill the negative control cells, indicated by many cells rounding up on the dish and some beginning to detach.
   ii. Check infected dishes. Observe the confluency and extent of rounded and detached cells. The infected cells should appear more resistant to puromycin than the negative controls, but significant cell death should occur in puromycin if MOI was close to 0.3.
   iii. Un-infected/negative controls: change the media and give fresh puromycin (1 μg/mL final).
   iv. Infected dishes: change the media and give fresh puromycin (1 μg/mL final).

f. On T-7, begin puromycin day 3.
   i. As on T-8, observe confluency and observe number of cells rounding up on the dish and floating. The un-infected/negative controls should be very sparse at this point (~5%–10% confluent) with many detached, floating cells. Infected dishes should be relatively dense (~60%–90%) and have fewer floating cells than the negative controls.
   ii. Un-infected/negative controls: change the media and give fresh puromycin (1 μg/mL final).
   iii. Infected dishes: change the media and give fresh puromycin (1 μg/mL final).

g. On T-6, begin puromycin day 4.
   i. Observe confluency and observe number of cells rounding up on the dish and floating. The un-infected/negative controls should be almost completely dead at this point (<5% confluent) with some detached, floating cells. Infected dishes should be relatively dense (~70%–100% confluent), depending on the precise MOI.
   ii. Un-infected/negative controls: change the media and give fresh puromycin (1 μg/mL final).
   iii. Infected dishes: split cells into puromycin (1/3 of cells split into a new 15 cm dish). If necessary, expand each infected HAP1-Cas9 clone by seeding more than one new 15 cm dish per starting dish.

h. On T-5, end puromycin selection.
   i. Observe confluency and observe number of cells rounding up on the dish and floating. The un-infected/negative controls should be completely dead at this point (if not, a higher puromycin dose is likely needed), indicating that puromycin selection is complete. Infected dishes should be very dense (~80%–100% confluent).
   ii. Un-infected/negative control dishes: discard.

iii. Infected dishes: split cells into media without puromycin (1/5 to 1/3 of cells split into a new 15 cm dish). If necessary, expand each infected HAP1-Cas9 clone by seeding more than one new 15 cm dish per starting dish. Note that one 15 cm dish (or enough to achieve ≥1,000-fold sgRNA coverage) per HAP1-Cas9 clone must be collected the following day (T-4) to serve as "pre-dox" samples for next-generation sequencing. The "pre-dox" samples are critical for analyzing the screen data as they represent the initial sgRNA distribution before treatment with compounds and before the deletion of essential genes begins to affect cell growth.

3. T-4 through T-1: Collect "pre-dox" samples & induce Cas9 expression.
   a. On T-4, collect a "pre-dox" sample for each HAP1-Cas9 clone. Collect sufficient cells for ≥1,000-fold sgRNA coverage. One 15 cm plate of HAP1-Cas9 cells infected with the UPS sgRNA library is more than enough for 1,000-fold coverage since one ~90% confluent 15 cm of HAP1-Cas9 cells has approximately 6×10^7 cells (6×10^7 cells/6,306 sgRNAs = 9,500-fold coverage). Collecting excess cells at this point is recommended since some loss may occur at later steps.
      i. Pre-chill 1× PBS with 5 mM EDTA to 4°C.
      ii. Pre-chill 1× PBS to 4°C.
      iii. Pre-label "pre-dox" 15 mL conical tubes for sample collection.
      iv. Fill an ice bucket with ice.
      v. Gently aspirate the media from the "pre-dox" plates to be collected.
      vi. Dispense 10 mL chilled PBS with 5 mM EDTA per plate.
      vii. Allow to sit ~2–3 min.
      viii. Gently collect cells by pipetting up and down across the dish surface several times. Note that HAP1 cells lift off of plates easily and do not require scraping or extended incubations. Transfer cells in PBS-EDTA to labeled conical tubes on ice.
      ix. Gently pellet cells at 800 × *g* for 5 min at 4°C.
      x. Aspirate supernatant.
      xi. Add 5 mL chilled PBS to each pellet.
      xii. Gently pellet cells at 800 × *g* for 5 min at 4°C.
      xiii. Aspirate supernatant.
      xiv. Flash freeze pellets and store at −80°C until ready to perform "genomic DNA extraction" step.
   b. Also on T-4, begin Cas9 induction with doxycycline day 1.
      i. On remaining HAP1-Cas9 dishes, change the media and add doxycycline hyclate to 1 μg/mL final.
   c. On T-3 and T-2, continue Cas9 induction with doxycycline days 2 and 3.
      i. Observe confluency and number of cells rounding up and floating. Some cell death may begin to occur during treatment with doxycycline as essential genes are deleted by some sgRNAs in the library.
      ii. If dishes are very confluent on T-3 or T-2 (≥80% confluent), split into new 15 cm dishes with doxycycline hyclate at 1 μg/mL final (1/4 to 1/3 of cells split into a new 15 cm dish). If necessary, expand each HAP1-Cas9 clone by seeding more than one new 15 cm dish per starting dish. As T0 approaches, keep in mind the number of cells that will be needed per replicate per compound (including untreated/vehicle controls). If desired, a "post-dox" sample can be collected for each replicate on T0, which would require having another 15 cm dish per replicate ready by T0.
      iii. If dishes are less confluent on T-3 or T-2 (<80% confluent), change the media and give fresh doxycycline hyclate (1 μg/mL final).
   d. On T-1, end doxycyline induction of Cas9.
      i. Observe confluency and number of cells rounding up and floating. Some cell death may occur as essential genes are deleted by some sgRNAs in the library.

ii. If dishes are very confluent (≥80% confluent), split into new 15 cm dishes (1/4 to 1/3 of cells split into a new 15 cm dish). If necessary, expand each HAP1-Cas9 clone by seeding more than one new 15 cm dish per starting dish.
iii. If dishes are less confluent (<80% confluent), leave until T0.

4. T0: Split cells into compounds to begin screen.
   a. Pre-warm sufficient IMDM (4 mM L-glutamine) + 10% FBS, PBS, and trypsin.
   b. Label new 15 cm dishes for all compounds (including untreated/vehicle) for each replicate.
   c. If required, thaw aliquots of compounds to be used.
   d. Prepare reagents needed to collect "post-dox" samples for each replicate (optional) as described above for collecting "pre-dox" samples.
   e. Observe confluency for all plates of each HAP1-Cas9 clone.
   f. Based on confluency and earlier dosing experiments, seed cells in each desired compound plus untreated/vehicle control dishes.
      i. Remember to always maintain ≥1,000-fold sgRNA coverage for each compound for each replicate, taking into account the expected growth reduction and/or death caused by each compound or treatment. If using the UPS sgRNA library, seed ≥1×10^7 HAP1-Cas9 cells per dish.
      ii. When adding compounds to dishes, be sure to mix thoroughly by gently swirling dish before placing in the incubator.
      iii. Work in batches so that cells are not are out of the incubator for too long (≤20 min).
      iv. If collecting "post-dox" samples, follow the same protocol described above for collecting "pre-dox" samples. It is helpful to have a second person spin and wash the cell pellets while the first person continues splitting and seeding cells.
5. T1: Check cells after one day in compounds/treatments.
   a. Record confluency and other observations for all plates of each HAP1-Cas9 clone in each compound/treatment and untreated/vehicle controls.
   b. Effects should be similar to that observed during the dosing trial.
6. T2, T4, and T6: Continue the screen.
   a. Every second day (T2, T4, T6), observe confluency, death, and cell morphology for all plates of each HAP1-Cas9 clone in each compound/treatment and untreated/vehicle controls.
   b. Based on confluency, determine how to split and seed each dish to maintain sufficient sgRNA coverage. Each replicate for the same compound/treatment and untreated/vehicle control should be treated similarly to the other replicates.
   c. Split and seed cells in fresh compound or untreated/vehicle control, working in batches so that cells are not out of the incubator for too long (≤20 min).
   d. Excess cells can be collected at T2, T4, and T6 for next-generation sequencing. Collect these samples in a similar manner to the "pre-dox" samples, with the following alteration: if collecting a subset of a 15 cm dish and using the rest to seed a new plate, transfer the excess trypsinized and quenched cells to pre-labeled 15 mL conical tubes on ice (no PBS+EDTA required), then proceed to pelleting and washing as described. It is helpful to have a second person spin and wash the cell pellets while the first person continues splitting and seeding cells.
7. T8 or later: Complete screen & collect final samples.
   a. On the final day of the screen (e.g., T8), record confluency and other observations for all plates of each HAP1-Cas9 clone in each compound/treatment and untreated/vehicle controls.
   b. Collect all remaining cells as T8 samples. Collect these samples in a similar manner to the T2, T4, and T6 samples, using trypsin to remove cells from dishes rather than PBS+EDTA.
   c. If desired, screen can be continued for longer than eight days.

⚠ CRITICAL: Sufficient sgRNA coverage must be maintained throughout the screen when splitting and seeding cells, and sufficient sgRNA coverage must be maintained when collecting samples for next-generation sequencing. Note that data quality will be improved with higher coverage, so if possible, higher coverage is better. An advantage of working with a small sgRNA library is that maintaining higher coverage is feasible.

⚠ CRITICAL: Care must be taken throughout the screen to treat all replicates consistently and to prevent cross-contamination and bacterial or fungal contamination.

⚠ CRITICAL: "Pre-dox" and T8 (or other endpoint) samples must be collected for each replicate. "Post-dox" and T2, T4, and T6 samples are optional.

Note: HAP1 cells are normally seeded much more thinly to account for their rapid growth and to avoid overcrowding. In contrast, during the screen where sgRNA coverage must be maintained throughout puromycin selection, Cas9 induction, and exposure to toxic compounds, HAP1-Cas9 cells should be seeded at a higher density.

Genomic DNA extraction

Timing: 1–3 days

In this step, genomic DNA is extracted from the cell pellets stored at −80°C. This genomic DNA will then be used as the templates for PCRs to amplify the integrated sgRNAs and to append Illumina sequencing adapters and indexes. Genomic DNA extraction is performed with a Nucleospin blood L midi kit (Macherey Nagel), using the following protocol adapted from the kit manual. Note that the following steps assume each cell pellet contains approximately 2-6 × 10^7 HAP1 cells, and adjustments must be made if using a different number of cells.

8. Before starting the genomic DNA extraction:
   a. Prepare all kit solutions according to manufacturer's instructions (e.g., add anhydrous ethanol to Wash Buffer BQ2, and dissolve the lyophilized Proteinase K in Proteinase Buffer PB).
   b. Pre-warm the elution buffer (Buffer BE) to 70°C.
   c. Set a water bath to 56°C.
   d. Midi kit columns should be stored at 4°C until use.
   e. Use a swinging bucket centrifuge that can reach the required speeds.
   f. Prepare a designated "pre-PCR" area and set of pipettes to conduct the genomic DNA extraction and to set up the subsequent PCRs (described in "next generation sequencing library preparation" section). This area must be separate from any work done after sequencing library prep PCRs (denoted as "post-PCR" work). Thoroughly clean the area and pipettes with 10% bleach followed by 70% ethanol.
   g. Use filter tips and DNase/RNase free plastics.
   h. Care must be taken throughout to avoid cross-contaminating samples from different replicates and different compound treatments.
9. Genomic DNA extraction using Nucleospin blood L midi kit.
   a. Thaw cell pellets at RT (~22°C).
   b. Resuspend each pellet in 2 mL of PBS by gently pipetting up and down 5–10 times.
   c. Add 150 µL of Proteinase K solution to resuspended cells. Mix by gently swirling tube.
   d. Add 20 µL of a 20 mg/mL RNase A solution. Mix by gently swirling tube.
   e. Add 2 mL of Buffer BQ1. Vortex for 10 s.
   f. Incubate in 56°C water bath for 15 min, vortexing twice during incubation. If sample is still very viscous at the end of the incubation, extend incubation time an additional 5–10 min at 56°C.
   g. Place on ice for 5–10 min to cool samples to RT (~22°C).
   h. Add 2 mL anhydrous 96%–100% ethanol. Mix immediately by inverting tube 10 times.
   i. Place nucelospin blood L column in provided collection tube and add 3 mL of the lysate solution. Avoid moistening the rim of the column.
   j. Centrifuge at 4,500 × *g* for 5 min at RT (~22°C).
   k. Remove the column and discard the flow through. If some lysate remains in the column, centrifuge at 4,500 × *g* for an additional 5 min or until all of the lysate passes through the column. Discard the flow through.

l. To wash the silica membrane, carefully replace the column in the collection tube and add 2 mL of Buffer BQ2. Avoid moistening the rim of the column.
m. Centrifuge at 4,500 × *g* for 2 min.
n. Remove the column and discard the flow through.
o. Perform a second wash by carefully replacing the column in the collection tube and adding 2 mL of Buffer BQ2. Avoid moistening the rim of the column.
p. Centrifuge at 4,500 × *g* for 10 min.
q. Remove the column and discard the flow through.
r. To ensure the silica membrane is completely dry, perform another spin at 4,500 × *g* for 5 min.
s. Transfer the column to a new collection tube.
t. Pipette 170 μL pre-warmed Buffer BE (70°C) directly onto the center of the membrane.
u. Incubate for 5 min at RT (~22°C).
v. Centrifuge at 4,500 × *g* for 2 min.
w. To obtain a high yield of DNA, perform a second elution using the same column and collection tube. Pipette 170 μL pre-warmed Buffer BE (70°C) directly onto the center of the membrane, incubate for 5 min at RT (~22°C), and centrifuge at 4,500 × *g* for 2 min.
x. Collect flow through and transfer to a microcentrifuge tube.
y. Quantify genomic DNA using a fluorometric assay (e.g., Qubit dsDNA assay).
z. Store samples at −20°C or proceed directly to next generation sequencing library preparation PCR.

Note: Using a Nanodrop or similar method to quantify genomic DNA concentration will vastly overestimate DNA yield due to contamination of carry-over RNA.

Next generation sequencing library preparation

Timing: 1–3 days

In this step, one round of PCR is used to amplify the sgRNAs integrated into the genome and to append Illumina sequencing adapters and indexes. Unlike typical PCRs, these reactions use a large quantity of template genomic DNA in order to maintain sufficient coverage of the sgRNA library. We found that with a small sgRNA library—such as our 6,306 sgRNA UPS library—and with approximately 2-6 × 10^7 HAP1 cells per sample only one round of PCR, rather than two sequential PCRs as described previously (Han et al., 2017), is needed to prepare samples for sequencing. Larger libraries and/or more cells per sample may require two sequential PCRs to prepare samples for next generation sequencing, and whether one or two PCRs are needed may need to be determined empirically.

Before assembling PCRs, determine the number of samples that can fit in one lane of the Illumina sequencer that you will use. This number depends on both the size of your sgRNA library and the desired sgRNA coverage (e.g., 1,000-fold). This number will dictate the number of unique Illumina indexes (and therefore unique 5′ adaptor primers) that you need. In this protocol, we use an Illumina HiSeq 4000 for sequencing, which yields an average of 3.9 × 10^8 pass-filter reads and 20,000 MB per lane. Typically, approximately 85% of the reads are useable and align exactly with the sgRNA library. With our UPS library of 6,306 sgRNAs we can combine up to 45 samples in one lane, with each sample amplified with a unique 5′ adaptor primer containing a unique hexamer barcode. This yields sequencing results with an average sgRNA coverage over 1,100-fold. For sufficient sequence diversity at the start of the reads, our sequencing core recommends sequencing about one half of the samples per lane in the opposite orientation. For a diagram explaining the two orientations and for the complete list of primers containing Illumina adapters and indexes used in this step, see Hundley et al. (2021) Table S2.

⚠ CRITICAL: To avoid contamination of PCRs with highly concentrated amplified PCR products, PCRs must be set up in a designated "pre-PCR" area and using "pre-PCR" pipettes that are distinct from the "post-PCR" area and pipettes. Use filter tips, pre-PCR-dedicated reagents (e.g., ultra-pure water and PCR tubes), and clean all surfaces and pipettes with 10% bleach followed by 70% ethanol. Note that many labs routinely use various CRISPR-Cas9 plasmids, such as lentiGuide-Puro (a modified version of which was used to generate our UPS library), and these plasmids could also be contaminants when preparing the next generation sequencing library by PCR. To determine whether contamination is an issue, always include two types of negative control reactions when preparing a batch of PCRs for next generation sequencing: 1) no template control/no DNA sample, and 2) template from HAP1-Cas9 cells without integrated sgRNAs. Use at least one set of primers for the negative control reactions. The control genomic DNA samples (2) should yield no product. The no template control (1) may have a trace of a band, and this is the more stringent but less "accurate" control.

⚠ CRITICAL: To obtain valid and interpretable sequencing results, care must be taken throughout PCR set up to avoid cross-contamination of PCRs. Work with one template/sample at a time, and bleach surfaces and pipettes in between templates/samples. Gel purification should be performed with clean gel casting and running components and with fresh running buffer. A fresh razor blade or similar cutting implement should be used to excise each gel band from a unique pooled sample.

10. PCR to append Illumina adapters and indexes.
    a. Determine how much input genomic DNA should be used as a template for the sequencing library preparation PCRs. A rough calculation can be performed as follows to estimate the amount of genomic DNA to use. Assuming 6×10^9 bases per diploid human genome and 660 Daltons per base, one human cell contains roughly 6.6×10^{-12} g or 6.6 pg of genomic DNA. For our UPS library containing 6,306 sgRNAs, to maintain at least 1,000-fold coverage of each sgRNA, use (6.6 pg genomic DNA per cell) × (6.306×10^6 cells) or 41.6 μg. Note that this calculation does not take into account mitochondrial DNA or genomic variations (e.g., ploidy) that may be present in different cell lines. Because this is a rough estimate, overcompensate and use 50 μg of genomic DNA as template for these PCRs.
    b. Assemble PCRs with the correct amount of template DNA and with the correct 3′ adaptor primer and 5′ adaptor primer with unique index. Use no more than 10 μg of genomic DNA per 100 μL reaction. For more details on potential primers and indexes that can be used, see Hundley et al. (2021), Table S2. As described above, assemble at least two negative control reactions, one containing no DNA and the other containing template DNA from cells lacking integrated sgRNAs.
        i. Make a reaction master mix on ice containing all reagents except the variable 5′ adaptor primer and template genomic DNA. Mix gently by pipetting up and down several times.

PCR components	
10× Taq DNA polymerase PCR buffer (Thermo)	50 μL
dNTPs (25 mM each)	4 μL
3′ adaptor primer (10 μM)	25 μL
5′ adaptor primer with unique index (10 μM)	25 μL
Homemade Taq polymerase, OneTaq polyermase (NEB), or similar	10 μL
Genomic DNA	50 μg
MilliQ H2O (autoclaved) or other ultra-pure water	to 500 μL total

        ii. Aliquot master mix to PCR tubes on ice.

iii. Add the correct template genomic DNA and 5′ adaptor primer with unique index (and, if necessary, more ultra-pure water to 500 μL total). Mix gently by pipetting up and down several times. When using a thermocycler that fits 500 μL tubes, put no more than 100 μL in each tube, splitting one reaction up into five tubes.

iv. Run the PCRs using the following cycling conditions. Only a fraction of the PCR products will be sequenced, and it is important to avoid introducing bias. Therefore, it is best to use as few cycles as possible, and the optimal number of cycles should be determined empirically.

PCR cycling conditions

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	
Annealing	58°C	60 s	
Extension	68°C	15 s	go to step 2 (19–29x; as few as possible)
Final extension	68°C	5 min	1
Hold	4°C	∞	1

11. Agarose gel electrophoresis.
    a. Once PCRs are complete, re-combine the five 100 μL reactions that amplified the same genomic DNA sample. Run a small fraction (e.g., ~1%–3%) of each sample on an agarose gel to determine whether the reaction worked. A larger fraction of the negative control reactions should be run (e.g., ~10%) for more sensitive detection of possible contamination. Run alongside a DNA ladder of known concentration to enable quantification of PCR product bands. See Figure 2 for an example.
    b. If any reactions have substantially lower yield than the others, repeat and consider using a different 5′ adaptor primer with unique index if possible.
12. Pool PCR products for next generation sequencing.
    a. Estimate concentration of PCR products relative to DNA ladder of known concentration using Fiji or another gel quantification tool.
    b. If reaction yields are within two-fold of one-another, proceed to pooling samples. If not, repeat and optimize reactions that were significantly less efficient before proceeding to pooling (see troubleshooting section for additional information).
    c. Pool all samples that will be sequenced in one lane of an Illumina HiSeq 4000 (or similar). For samples with yields within two-fold of one-another combine an equal volume of each reaction into a low retention tube. For our UPS library, a 300 μL reaction generally contains approximately 10 μg of the 265 bp product which is about 3.5×10^{13} copies. To maintain 1,000-fold coverage and to account for potentially significant loss during gel purification, use approximately 20 μL of each 300 μL PCR (roughly 800–900 ng of DNA or 3×10^{12} copies) to make the pooled sample.
13. Gel purification of pooled PCR products.
    a. Prior to running the gel, ensure that all gel casting and running equipment is thoroughly cleaned and be sure to use fresh running buffer.
    b. Run the pooled sample in desired sample loading buffer on an agarose gel with large lanes to accommodate the large sample. Select the appropriate agarose percentage and gel run time to achieve good separation between the sgRNA cassette band and any background bands. TAE buffer is preferred over TBE for preparative gels.
    c. Gel purify the pooled sample using the Macherey-Nagel NucleoSpin Gel and PCR clean-up mini kit (or another DNA gel purification kit).
        i. Prepare NucleoSpin kit components according to manufacturer's instructions (e.g., add anhydrous 96%–100% ethanol to wash butter NT3).

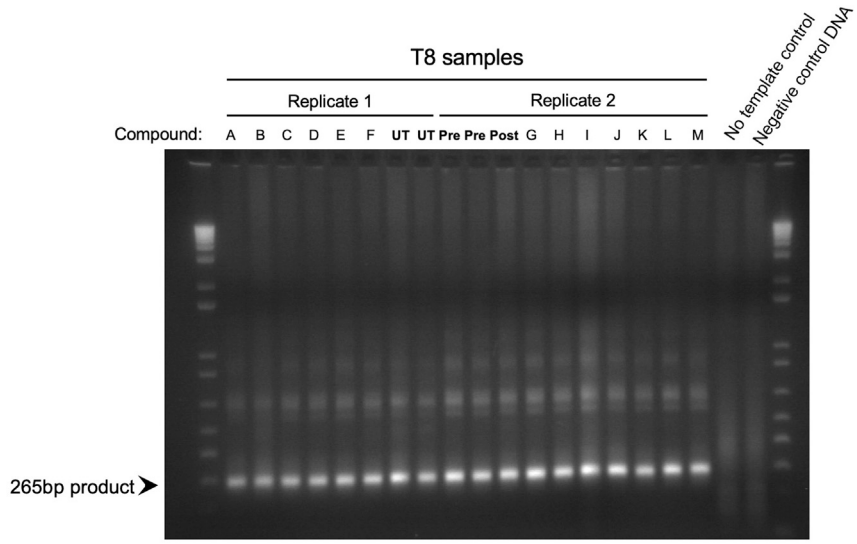

Figure 2. Example PCR products for sequencing library preparation

1.4% agarose gel of a sub-set of PCR products from replicates 1 and 2 treated with one of several compounds (A-M) for 8 days. UT, untreated. Pre, pre-dox. Post, post-dox. Each lane has 5 μL of a 300 μL reaction that began with 30 μg of genomic DNA, and each was amplified with a unique 5′ adaptor primer with unique index. "No template control" and "Negative control DNA" lanes each contain 10 μL of a 100 μL reaction. "Negative control DNA" reaction used 10 μg of genomic DNA from HAP1-Cas9 cells not infected with the sgRNA library.

ii. Pre-warm the elution buffer (Buffer NE) to 70°C.
iii. Set a water bath to 56°C.
iv. Using a clean razor blade, excise the gel band containing the sgRNA cassette. Remove as much excess gel as possible. Work as quickly as possible to limit UV damage to the sample.
v. Determine the weight of the gel slice(s) and record.
vi. Add 200 μL Buffer NTI for every 100 mg of agarose gel (if using <2% agarose).
vii. Incubate sample for 30–60 min in a 56°C water bath with occasional vortexing to ensure the gel slice completely dissolves.
viii. After gel slice is completely dissolved, add 3M sodium acetate to 30 mM final. Mix by inverting tube several times.
ix. Use one NucleoSpin Gel column per 10 μg of starting DNA. With 44 pooled samples from our UPS library, we start with approximately 40 μg of DNA (~900 ng per sample), so we split the sample over four columns. Place NucleoSpin Gel columns in provided collection tubes and load 700 μL of dissolved gel slice per column.
x. Centrifuge at 11,000 × *g* for 30 s at RT (~22°C).
xi. Remove the columns and discard flow through.
xii. Replace columns in collection tubes. Load 700 μL of dissolved gel slice per column and repeat spin.
xiii. Repeat until all dissolved gel slice solution has been loaded onto the columns.
xiv. To wash the silica membranes, replace the columns in the collection tubes and add 700 μL of Buffer NT3.
xv. Centrifuge at 11,000 × *g* for 30 s.
xvi. Remove the columns and discard flow through.
xvii. Perform a second wash step by replacing the columns in the collection tubes, adding 700 μL of Buffer NT3 per column, and centrifuging at 11,000 × *g* for 30 s.
xviii. Remove the columns and discard flow through.

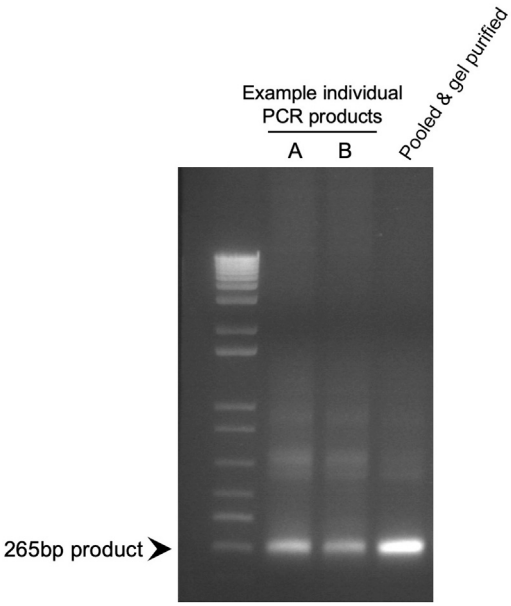

Figure 3. Example of pooled and gel-purified PCR products for sequencing

1.4% agarose gel of two example individual PCR reactions (as in Figure 2) and a pooled then gel-purified sample containing the products of 44 individual PCRs. Individual PCR reaction lanes contain 5 μL of a 300 μL reaction that began with 30 μg of genomic DNA. Pooled and gel purified lane contains 2 μL (or ~450 ng) of approximately 110 μL recovered from gel purification. Note that despite careful gel purification, bands at larger sizes begin to appear over time, possibly from annealing of PCR products. This does not seem to interfere with sequencing.

xix. To ensure the silica membrane is completely dry, replace the columns in the collection tubes and spin at 11,000 × *g* for 4 min.

xx. Place the columns in new 1.5 mL collection tubes.

xxi. Pipette 30 μL of pre-warmed Buffer NE directly unto each column.

xxii. Incubate for 5 min at RT (~22°C).

xxiii. Centrifuge at 11,000 × *g* for 1 min.

xxiv. If sample was split over several columns, combine eluates from same initial sample in a low retention tube (e.g., for a pool of 44 samples from our UPS library screens which were split over four columns, pool all four eluates for a final volume of approximately 110 μL).

xxv. Determine DNA concentration by NanoDrop or similar method.

d. To check for successful removal of background bands, run a small fraction of the pooled and gel purified sample on an agarose gel alongside a PCR product that has not been pooled or gel purified. See Figure 3 for an example.

⚠ CRITICAL: When using a custom sgRNA library for the first time, you should also sequence the library itself using a unique Illumina indexing primer. This will enable evaluation of the library for relative sgRNA distribution, which may be unequal if the library was not amplified properly or may indicate unequal distribution in synthesized oligonucleotides from the oligo synthesis company (the latter of which is less likely to be a problem when ordering pooled oligonucleotides from a company that performs rigorous QC). When we sequenced our UPS library, 248 sgRNAs out of 6,306 (or ~3.9%) had aligned counts below 100, while the average sgRNA coverage was 1,100 and the median was 1,063. These 248 sgRNAs were removed from later analysis, as is typically done in most CRISPR-Cas9 screen analysis pipelines.

Optional: The pooled, gel purified sample can also be checked for concentration and size distribution by Bioanalyzer High Sensitivity DNA assay or similar. Some sequencing cores require this analysis prior to next generation sequencing. However, unlike whole genome sequencing and other related methods where sample shearing is involved and generates fragments of variable lengths, a pooled and purified PCR product from this type of CRISPR-Cas9 screen should

yield DNA fragments of uniform length. Therefore, a diagnostic agarose gel is usually sufficient.

Note: For more robust data analysis, it may be beneficial to sequence additional reactions of samples that form the basis for key calculations (e.g., untreated/vehicle and "pre-dox" samples). This can be accomplished by preparing multiple PCRs for these samples using several different 5′ adaptor primers, each with different indexes.

Sequencing & analysis

Timing: ~2–3 weeks (depending on length of queue at sequencing facility)

Next generation sequencing is performed using an Illumina HiSeq 4000 with single end, 50 base reads, and using common 3′ and 5′ sequencing primers and an indexing primer provided by the core facility [UCSF Center for Advanced Technology (CAT)]. Alternative sequencing technologies can be used with adjustments.

To trim and align raw sequencing reads in Hundley et al. (2021), we used Max Horlbeck's Screen Processing code available on GitHub (Horlbeck et al., 2016) and the Canpoy platform for Python. Note that Canopy has since been phased out and was replaced by Enthought Deployment Manager (EDM). Other methods exist to process raw sequencing reads from CRISPR-Cas9 screen data, including the MAGeCK pipeline and its derivatives (Li et al., 2014). Several methods exist for downstream data analysis, including that presented in Hundley et al. (2021), drugZ (Colic et al., 2019), and BAGEL (Hart and Moffat, 2016). The user should decide which method(s) to use based on the screen particulars, and note that adjustments to code may be necessary depending on sgRNA library, sequencing method, and other factors.

14. Submit samples for sequencing.
    a. Dilute pooled and gel purified sample to 3–10 nM (~1–3 ng/ μL) in 15 μL in a low retention tube.
    b. Combine 3′ and 5′ sequencing primers in 10 μL at 100 μM in a low retention tube.
        i. 3′ sequencing primer:
        5′-CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT
        AGCTCTAAAAC-3′
        ii. 5′ sequencing primer:
        5′-TGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAA
        GGAC-3′
    c. Sequencing core will likely provide the standard Illumina indexing primer.
    d. Submit sample and 3′ and 5′ sequencing primer mixture.
15. Preliminary analysis once raw sequencing reads are available from sequencing facility.
    a. Install TextWrangler or similar on computer
    b. Go to: https://github.com/mhorlbeck/ScreenProcessing
    c. Follow directions in "ScreenProcessing_tutorial.pdf" to count sgRNAs in raw sequencing files (Step 1: fastqgz_to_counts.py) with necessary modifications for specific sgRNA library, cell line, etc. If processing a large number of files, do not run the code on all files at once as this will crash most standard computers.
        i. The code will trim raw sequencing reads down to the 20 nucleotide sgRNA sequence (removing the rest of the 50 base raw reads).
        ii. The code will align trimmed raw sequencing reads to your RefLib and RefLib_revcomp files, which can be made in TextWrangler or similar.
        iii. The code will generate count files of the number of trimmed sequencing reads that align with the RefLib files, as well as counts of unaligned reads. These output files can be transferred from TextWrangler to Microsoft Excel or other spreadsheet software. Note that it is

normal for ~85% of reads to align with RefLib or RefLib_revcomp (and for ~15% of reads to not align).

d. Further data analysis considerations including exclusion of sgRNAs with low counts, normalization, and statistical analysis is discussed in Hundley et al. (2021).

EXPECTED OUTCOMES

An example of expected outcomes with 41 compounds in HAP1-Cas9 cells and our UPS sgRNA library can be found in the original article Hundley et al. (2021). The supplementary tables in Hundley et al. contain: raw sequencing counts after read trimming and aligning to the reference sgRNA library file (Table S4); sgRNAs normalized to pre-dox samples (Table S5); and final, analyzed screen data showing the effect of growth in 41 compounds compared to untreated controls (Table S6). The final results of these screens are also available on the open database BioGRID ORCS.

LIMITATIONS

This protocol can be adapted for use in other cell lines, with other compounds, and with other sgRNA libraries. However, these changes may significantly impact the length of the screen, the number of personnel required, and the amount of space and reagents needed. It is important to make detailed estimates before beginning to determine whether it will be feasible to perform a particular screen given a particular laboratory's constraints.

To obtain accurate results, it is essential that great care be taken throughout the process to maintain the necessary sgRNA coverage, to avoid cross-contaminating samples, to avoid microbial contamination, and to treat all samples in a consistent manner. Failure to do so may result in uninterpretable and/or invalid results.

TROUBLESHOOTING

Problem 1

Amplifying the sgRNA oligos by PCR is generally very efficient since the template pool consists of short, low-complexity oligos. However, the yield from the PCR may be low if the reaction conditions are not optimal. Downstream cloning steps may not work if the yield is low or if the incorrect product band is extracted from the polyacrylamide gel after Esp3I digestion.

Potential solution

If yield is less than expected, attempt alternative annealing temperatures during the PCR (e.g., lower annealing temperature) or other standard PCR troubleshooting approaches. It may be useful to run the PCR product on a 20% polyacrylamide gel after the PCR (rather than waiting until after the Esp3I digest as described in the protocol above) to help diagnose the problem. A PCR cleanup kit designed for short products must be used since standard PCR cleanup kits may remove the short, desired product. If adjusting the PCR conditions does not solve the problem, if the correct PCR cleanup method is used, and if primer and oligo design are correct, it is possible that oligo synthesis is the problem, which can sometimes occur with certain pooled oligo synthesis companies.

Problem 2

Before electroporating the sgRNA library, a small test transformation in chemically-competent DH5α *E. coli* should be performed. Depending on the competency of the specific *E. coli* used, if very few colonies grow post-transformation, the ligation reaction may not have been efficient. Proceeding to electroporation with the product of an inefficient ligation reaction is unlikely to produce sufficient sgRNA coverage, so the ligation should be optimized before proceeding.

Potential solution

A DNA ligase capable of ligating sticky ends—such as T4 DNA ligase—is required for ligation of the Esp3I-digested sgRNA oligos into the BsmBI-digested vector. The DNA fragments should be

concentrated enough to fit into the suggested 20 μL reaction volume, and if they are too dilute, previous steps should be repeated to obtain more concentrated DNA fragments. Additionally, the ligation reaction may require a different insert-to-vector ratio, such as 1:1 or 1:3. Increasing the insert-to-vector ratio above 1:3 should be avoided as sgRNA concatemers may form at relatively high insert concentrations.

Problem 3

Most compound doses optimized in the "compound concentration determination" section will perform as expected during the screen. However, some compounds may have a stronger or weaker effect on cells during the screen than they did in dosing trials. If a compound has too weak of an effect, very few, if any, gene deletions will render cells significantly sensitive or resistant to the compound. Conversely, if a compound has too strong of an effect, sufficient sgRNA coverage may not be maintained and the results may unreliable. Differences in how a single dose affects cells may be due to slight variability in how compounds affect cells at different densities, human error, the compound mechanism of action (e.g., covalent inhibitors), or lot-to-lot variability of the compound.

Potential solution

The likelihood of this problem occurring can be minimized by practicing accurate and consistent pipetting and by preparing one batch of each compound which is used both to determine the optimal dose and to perform the screen. If the problem still occurs, altering the compound dose during the screen time course (e.g., lowering the dose if cells are dying too quickly) is acceptable as long as the same new dose is given to all replicates at the same time point. Performing all replicates for a particular compound at the same time is preferable for consistency. In some cases, it may be necessary to repeat the screen for a particularly finicky compound.

Problem 4

When appending the Illumina sequencing adapters and indexes by PCR, product yield may be low. To maintain sufficient coverage and avoid significant sample-to-sample variation, low-yield PCRs should be optimized before proceeding to sample pooling and submitting for next-generation sequencing.

Potential solution

As with other PCRs, low yield may be improved by slightly lowering the annealing temperature during cycling or other standard PCR troubleshooting approaches. Ensure that input genomic DNA is of good quality relative to other samples in the same set and that the different Illumina sequencing adapter and index primers are the same concentration and resuspended in the same manner (if ordered in lyophilized form). If unused unique primers are available (e.g., when sequencing only 45 samples in one lane of the Illumina HiSeq 4000 using a subset of 96 unique indexes), using an alternative primer may be the most efficient option to improve PCR yield.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Toczyski (david.toczyski@ucsf.edu).

Materials availability

This study did not generate new unique reagents. All reagents listed here can be found in the main article Hundley et al. (2021).

Data and code availability

For a complete example of aligned sequencing read counts and analysis of screen results, please see Hundley et al. (2021). CRISPR-Cas9 screen data analysis code by Max Horlbeck is available on GitHub: https://github.com/mhorlbeck/ScreenProcessing (Horlbeck et al., 2016).

ACKNOWLEDGMENTS

We thank members of the Toczyski lab for contributing to the development of this protocol and for assistance scanning notebook pages for F.V.H. after she graduated. We thank members of the Gilbert and McManus labs (at UCSF) for advice in the early stages of optimizing CRISPR-Cas9 screening methods. While at UCSF, F.V.H. was a Tetrad graduate student and is now a postdoctoral research fellow in the laboratory of J. Wade Harper at the Blavatnik Institute of Harvard Medical School. Work in the D.P.T. lab on CRISPR screens is supported by an NIH grant (R35 GM118104), funding from the UCSF RAP Program, and the UCSF Program for Breakthrough Biomedical Research (funded in part by the Sandler Foundation). The UCSF CAT performed Illumina sequencing.

AUTHOR CONTRIBUTIONS

F.V.H. developed and wrote this protocol with contributions from D.P.T., and D.P.T. acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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