Chapter 19

Dead-Seq: Discovering Synthetic Lethal Interactions from Dead Cells Genomics

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Abstract

Pooled genetic screens have revolutionized the field of functional genomics, yet perturbations that decrease fitness, such as those leading to synthetic lethality, have remained difficult to quantify at the genomic level. We and colleagues previously developed "death screening," a protocol based on the purification of dead cells in genetic screens, and used it to identify a set of genes necessary for mitochondrial gene expression, translation, and oxidative phosphorylation (OXPHOS), thus offering new possibilities for the diagnosis of mitochondrial disorders. Here, we describe *Dead-Seq*, a refined protocol for death screening that is compatible with most pooled screening protocols, including genome-wide CRISPR/Cas9 screening. *Dead-Seq* converts negative-selection screens into positive-selection screens and generates high-quality data directly from dead cells, at limited sequencing costs.

Key words Genome-wide screening, Synthetic lethality, Drop-out screen, Mitochondria, sgRNA, shRNA, RNAi, ORFeome, Annexin V, MACS, Auxotrophy, Systems genetics, Metabolism, Galactose, Apoptosis, Necroptosis, Mitochondrial translation

1 Introduction

The complete sequencing of the human genome and the development of high-throughput gene perturbation technologies, such as RNA interference and CRISPR/Cas9, have transformed the field of functional genomics. These new tools have significantly advanced the discovery of genes and helped assign new gene functions in response to environmental and drug challenges. Many screening methodologies have taken advantage of the synthetic lethal interactions that occur between gene pairs [1, 2], between genes and the environment [3, 4], or between genes and drugs [5–8] (Table 1, *see* **Note 1**). Yet, and somewhat paradoxically, the most common screening modalities used for studying gene essentiality have been based on cell growth [4, 9–11]. In this experimental setting, genes whose depletion confers a growth advantage are enriched in the population after several days of selection (positive-

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Table 1
Examples of synthetic lethal interactions resulting in phosphatidylserine exposure

	1st hit	2nd hit	Reference
Gene vs. gene	*BCL2L1*, *BLC2*	*MCL1*	[2, 23]
	EP300	*CREBBP*	[24]
Gene vs. environment	OXPHOS inhibition	Glucose deprivation	[3, 19, 25]
	AKT2	*Salmonella* infection	[26]
Gene vs. drug	*IDH1* mutation	Triptolide (NRF2 inhibitor)	[27]
	MYC overexpression	Tigecycline (mitochondrial translation inhibitor)	[28]

selection), whereas genes whose depletion decreases fitness are lost (negative-selection, or drop-out). Identifying drop-out genes however can be challenging given their low abundance in the cell population, or when the fitness difference is modest. Consequently, high-sequencing coverage is required for drop-out screens, with attendant high costs and often low efficiency [12].

With this in mind, we and colleagues previously developed "death screening," a pooled screening methodology based on the selection of dead cells [3]. Death screening relies on the Annexin V-mediated purification of phosphatidylserine-positive cells, characteristic of certain forms of cell death such as apoptosis and necroptosis [13–15]. Death screening has enabled the identification of a set of genes necessary for mitochondrial energy production, with among them 68 bona fide OXPHOS complexes subunits and 108 genes involved in the transcription and translation of the mitochondrial genome. This genetic screen also led to the discovery of a protein module involved in mitochondrial 16S rRNA maturation and translation [3], as well as novel nuclear pre-mRNA splicing mechanisms involved in energy metabolism [16].

We now present *Dead-Seq*, a refined protocol of death screening to study synthetic lethality at the genomic level (*see* Fig. 1). *Dead-Seq* is a versatile tool for converting negative-selection screens into positive-selection screens, thus improving the identification of drop-out genes with lower sequencing costs. It can be applied to a wide variety of genetic, environmental, or drug-induced genetic interactions which result in exposure of phosphatidylserine on the cell surface, and can be used in combination with all types of genetic libraries (e.g., CRISPR-based, shRNA, ORFeome). In this protocol, we provide a detailed procedure for *Dead-Seq*, using as an example a genome-wide CRISPR/Cas9 screen applied to the synthetic lethality between OXPHOS inhibition and reduced glycolysis.

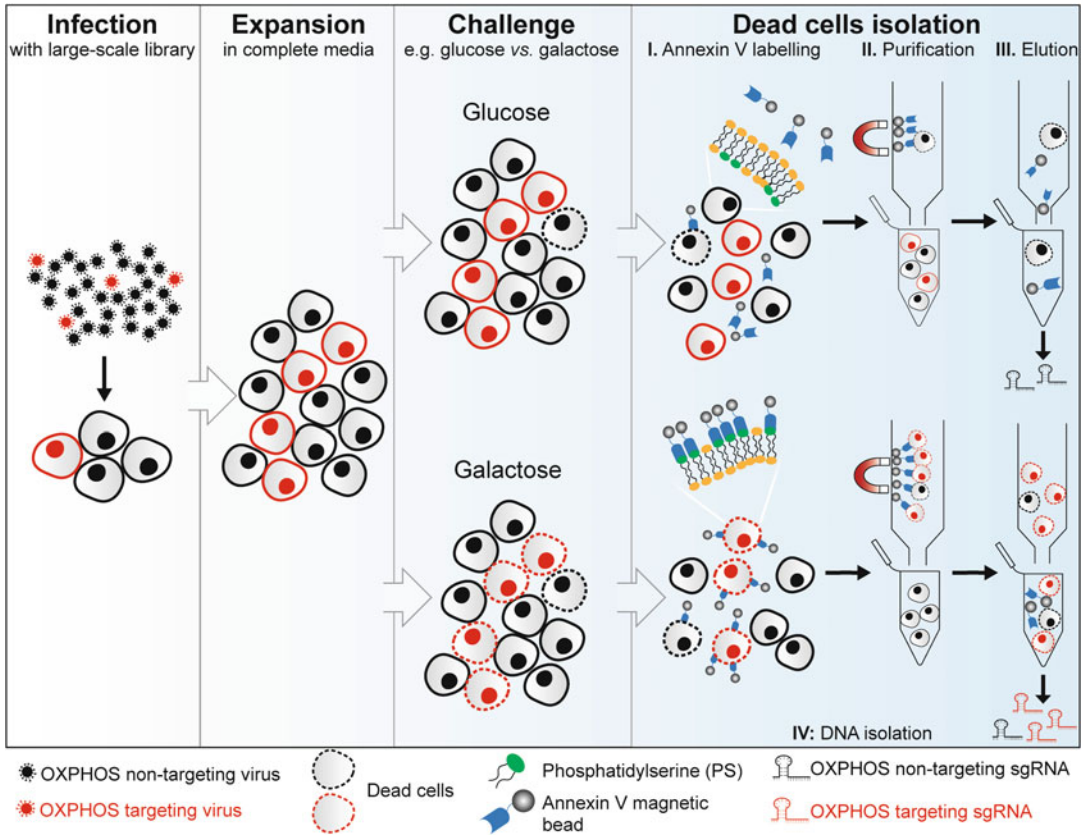

Fig. 1 Schematic overview of *Dead-Seq*. A large number of cells are infected with a genome-scale library carrying sgRNAs, shRNAs, or any barcoded constructs. After several days of expansion, the cells are divided into control and treatment groups (in our example, in glucose- and galactose-containing media). Shortly after treatment, dead cells from the population are purified using Annexin V-conjugated magnetic microbeads in a magnetic field. Total genomic DNA is then isolated from each condition and the sgRNAs/shRNAs/barcodes from the libraries are identified by next-generation sequencing. PS: phosphatidylserine, OXPHOS: oxidative phosphorylation

2 Materials

All solutions are prepared in a laminar flow cabinet using sterile milli-Q water and tissue culture-grade reagents.

2.1 Cell Culture

1. A cell line of interest. For genome-wide screening, we routinely use K562 cells (ATCC CCL-243).
2. Tissue culture (TC) flasks (25–300 cm^2) and multi-well plates (6- and 12-well format).
3. Disposable conical tubes (15–500 mL).
4. Phosphate-buffered saline (PBS).

5. Freezing solution: 10% dimethyl sulfoxide (DMSO) in fetal bovine serum (FBS). Aliquot and store at −20 °C.
6. Cell counter. We use the Vi-CELL BLU Cell Viability Analyzer (Beckman Coulter).
7. Centrifuge with a swinging-bucket rotor and adaptors for conical tubes and plates, a temperature range of at least 4–37 °C and a relative centrifugal force of at least 2000 × *g*.
8. Base media: DMEM containing 25 mM glucose and 4 mM L-glutamine supplemented with 10% FBS, 1 mM sodium pyruvate, 50 μg/mL uridine, 100 U/mL penicillin/streptomycin (*see* **Note 2**).
9. Sugar-free media: Glucose-free DMEM containing 4 mM L-glutamine supplemented with 10% dialyzed FBS (dFBS), 1 mM sodium pyruvate, 50 μg/mL uridine, 100 U/mL penicillin/streptomycin (*see* **Note 3**).

2.2 Drugs

1. Antimycin A: 10 mM (100,000×) stock dissolved in DMSO. Aliquot and store at −20 °C (*see* **Note 4**).
2. Puromycin: 10 mg/mL (5000×) stock dissolved in water. Aliquot and store at −20 °C.

2.3 Annexin V Labeling

1. FACS buffer: PBS supplemented with 2% FBS and 5 mM ethylenediaminetetraacetic acid (EDTA). Filter, sterilize, and store at 4 °C.
2. Annexin V-FITC.
3. Annexin V binding buffer: PBS containing 10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$ (pH 7.4).

2.4 Genetic Perturbation Library

1. Genetic perturbation library of interest. We have obtained good results using the human Avana [17], Brunello [17] and ORFeome [18] libraries. Several libraries are available at Addgene as ready-to-use lentiviral preparations carrying the puromycin resistance gene.
2. Polybrene (1000×) stock: Commercially available; or alternatively dissolve polybrene in water to 10 mg/mL. Aliquot and store at −20 °C.

2.5 Annexin V-Based MACS Purification

1. LS columns (Miltenyi Biotec).
2. Annexin V-coated microbeads (Miltenyi Biotec).
3. 20× binding buffer stock solution (Miltenyi Biotec).
4. QuadroMACS Separator (Miltenyi Biotec).

3 Methods

Perform all procedures under sterile conditions at room temperature. The protocol described is based on the use of K562 cells. It can be readily adapted to other cell lines, including adherent cells, using appropriate modifications to media and culture conditions. Follow local institute biosafety guidelines when handling viruses.

3.1 Cell Line Maintenance

1. Thaw cells in base media.
2. Maintain in flasks in an incubator at 37 °C and 5% CO_2.
3. Passage the cells every 3–4 days by dilution to 0.1×10^6 cells/mL.
4. Periodically check for the presence of mycoplasma.

3.2 Prescreen: Validating a Suitable Synthetic Lethal Interaction

Before performing a screen, it is essential to confirm phosphatidylserine exposure in the model of choice (*see* examples in Table 1). In the example below, we investigate the synthetic lethality conferred by OXPHOS inhibition during glucose deprivation [19].

1. Dilute antimycin A stock 1:100 in DMSO (*see* **Note 4**).
2. Prepare 4× 15 mL tubes each containing 4 mL of sugar-free media.
3. Add 180 μL of 1.11 M glucose or galactose, and 8 μL of DMSO or diluted antimycin A from **step 1** to obtain the following four conditions:
   (a) Glucose + DMSO
   (b) Glucose + Antimycin A
   (c) Galactose + DMSO
   (d) Galactose + Antimycin A
4. Centrifuge 40×10^6 K562 cells for 3 min at 300 × *g*.
5. Wash cells twice in PBS (*see* **Note 5**).
6. Resuspend cells in 20 mL of sugar-free media.
7. Add 4 mL of cells to each of the four media prepared in **step 3**. At this point, the final concentration of cells is 10^6 cells/mL, glucose and galactose are at 25 mM, and antimycin A, when present, is at 100 nM.
8. From each condition, transfer 3 × 2 mL cells into 3 wells of a 6-well plate (technical triplicates) and transfer to the incubator.
9. After 24 h, harvest the cells from each well into a 15 mL tube.
10. Centrifuge the cells for 3 min at 2000 × g (*see* **Note 6**).
11. Wash the pellets in FACS buffer.
12. Repeat **steps 10** and **11**.

13. Resuspend each pellet in 1 mL Annexin V binding buffer.
14. For each condition, transfer 100 μL of cells into a FACS-compatible tube and add 5 μL of Annexin V-FITC (*see* **Note 7**).
15. Protect the cells from light and incubate for 15 min at room temperature.
16. After 15 min of incubation, add a further 400 μL of annexin V binding buffer to each tube.
17. Measure FITC fluorescence by flow cytometry (*see* **Note 8**).

3.3 Screen

Perform all procedures under sterile conditions at room temperature. In this example, we use K562 cells and the genome-wide Brunello CRISPR/Cas9 library with 76,441 sgRNAs targeting 19,114 coding genes and 1000 noncoding sites [17]. The Brunello library carries Cas9 and the puromycin resistance gene for selection in mammalian cells and is available at Addgene as ready-to-use lentiviral supernatants (73179-LV). The protocol below is fully compatible with other libraries with appropriate modification of the resistance marker selection conditions.

3.3.1 Library Titration for the Cell Line Used

1. Centrifuge 21×10^6 cells for 3 min at 300 × *g*.
2. Discard the supernatant and resuspend the pellet in 7 mL of base media supplemented with 14 μL of polybrene.
3. Plate 1 mL of cells in 6 wells of a 12-well plate. Each well contains 3×10^6 cells.
4. Quickly thaw an aliquot of the viral library in a 37 °C water bath (*see* **Note 9**).
5. Label and prepare and 6 × 1.5 mL Eppendorf tubes as follows:
   (a) V0: 1 mL of base media.
   (b) V25: 975 μL of base media + 25 μL of lentiviral supernatant.
   (c) V50: 950 μL of base media + 50 μL of lentiviral supernatant.
   (d) V100: 900 μL of base media + 100 μL of lentiviral supernatant.
   (e) V200: 800 μL of base media + 200 μL of lentiviral supernatant.
   (f) V400: 600 μL of base media + 400 μL of lentiviral supernatant.
6. Add the contents of each tube prepared in **step 5** to one well of cells from **step 3**.
7. Centrifuge the plate at 1000 × *g*, 30 °C for 2 h using a swing-out rotor (spinfection).

8. Carefully aspirate all the media from the wells.
9. Add 2 mL of base media to each well and transfer the cells to the incubator.
10. 24 h postinfection, collect and count the cells from each well.
11. Centrifuge 1.5×10^6 cells from each of the six conditions for 3 min at $300 \times g$.
12. Resuspend the cell pellets in 3 mL of base media.
13. For each condition, add 1 mL of cells to two wells of a 12-well plate.
14. Prepare the selection buffer in a 15 mL tube by combining 7 mL of base media with 2.8 μL of puromycin.
15. To one well of each replicate pair, add 1 mL of base media alone; to the other add 1 mL of selection buffer. The final concentration of cells in each well is 0.25×10^6 cells/mL, and the final concentration of puromycin is either 0 or 2 μg/mL.
16. Transfer cells to the incubator.
17. After 72 h, determine the concentration of viable cells in each well. From the viral concentrations from **step 5**, determine the amount of virus required to obtain ~25% infection (*see* **Note 10**). This corresponds to a multiplicity of infection (MOI) of ~0.3 (*see* **Notes 11** and **12**).

3.3.2 Infection

Before starting the infection, it is important to determine the number of cells that need to be infected based on the library size, the desired infection rate per sgRNA, and the MOI. In this example, we use the Brunello library (76,441 sgRNAs) to target 500 cells/sgRNA at an MOI of 0.3 (*see* **Note 13**). In this case, we will infect 127×10^6 cells (*see* **Note 14** for the calculation). All steps are described for a single sample, but the screen should be performed in at least two infection replicates.

1. *Day 0*: Centrifuge 135×10^6 K562 cells for 5 min at $300 \times g$ (*see* **Note 15**).
2. Resuspend the cell pellet in 45 mL of base media (3×10^6 cells/mL).
3. Add 90 μL of polybrene to the cells.
4. Plate 44 × 1 mL of cells in 12-well plates. 42 wells will be used for the screen and 2 wells will serve as controls.
5. Quickly thaw the viral library in a 37 °C water bath (*see* **Note 9**).
6. In a 50 mL conical tube, add the volume of virus calculated to obtain the desired infection rate (MOI ~0.3) and add base media to a final volume of 45 mL.

7. Add 1 mL of the diluted virus to the 42 screening wells and to one of the control wells (+virus control).
8. Add 1 mL of base media alone to the remaining control well (−virus control).
9. Centrifuge the plates for 2 h at 1000 × *g*, 30 °C (spinfection).
10. Carefully remove all the supernatant and add 2 mL of base media to each well.
11. Transfer cells to the incubator.

3.3.3 Selection with Puromycin and Confirmation of the MOI

1. *Day 1*: 24 h postinfection, collect and pool the cells from the 42 screening wells (screen cells).
2. Collect the cells from each of the two control wells (+virus and −virus controls).
3. Count cells in all three conditions.
4. For the +virus and −virus controls: spin down 1.5×10^6 cells and resuspend in 3 mL of base media. The final cell concentration is 0.5×10^6 cells/mL. For each condition, add 1 ml of cells to 2 wells of a 12-well plate (there will be 4 wells in total).
5. For the screen cells: dilute to 0.5×10^6 cells/mL.
6. For selection, prepare a sufficient amount of base media containing puromycin at a 2× concentration (4 μg/mL).
7. For +virus and −virus controls: add 1 mL of base media to one replicate from each condition, and 1 mL of puromycin selection buffer to the other. The final concentration of cells is 0.25×10^6 cells/mL, and the final concentration of puromycin is 0 or 2 μg/mL.
8. For screen cells: Add a volume of puromycin-containing media equal to the volume of screen cells resuspended at 0.5×10^6 cells/mL from **step 5** and transfer to large TC flasks (e.g., 300 cm^2) to ensure adequate oxygenation. The final concentration of cells is 0.25×10^6 cells/mL, and the final concentration of puromycin is 2 μg/mL.
9. Transfer cells to the incubator.
10. *Day 4*: After 72 h, count the cells from the screen and the two controls with and without puromycin selection.
    - The concentration of the screen cells should be equal to the concentration of cells in the +virus control cells treated with puromycin, and ~25% of the +virus controls cells not treated with puromycin (for MOI ~0.3, *see* **Notes 10** and **16**).
    - The −virus control cells treated with puromycin should be mostly dead.

11. Centrifuge the screen cells for 5 min at 300 × *g* (*see* **Note 17**). Resuspend the cells at 0.1 × 10^6 cells/mL in base media. Use several large TC flasks to ensure adequate oxygenation.
12. Transfer cells to the incubator.

3.3.4 Cell Expansion, Early Time Point, and Preparation of Cell Stocks

1. *Day 7*: Count cells in each preparation.
2. "Day 7" sample: Centrifuge 40 × 10^6 viable cells for 5 min at 300 × *g*. Label the tube "day 7." Remove the supernatant and freeze the cell pellet at −80 °C for DNA isolation. This step is optional (*see* **Note 18**).
3. Screen: Centrifuge another 40 × 10^6 viable cells for 5 min at 300 × *g* and resuspend the pellet in 400 mL of base media. The concentration of cells is 0.1 × 10^6 cells/mL. Transfer to large 300 cm^2 flasks to ensure adequate oxygenation and return to the incubator.
4. Freezing cells for future use: At this point, it is also possible to freeze cells for future screens. We recommend freezing as many aliquots as possible, with a minimum of 1000–1500 cells/guide (80–120 × 10^6 cells) (*see* **Note 19**) in 10 mL of cell freezing solution (*see* **Note 20**).

3.3.5 Glucose/Galactose Treatment

1. *Day 10*: For each replicate, count the cells.
2. "Day 10" sample: Centrifuge 40 × 10^6 viable cells for 5 min at 300 × *g*. Label the tube "day 10." Remove the supernatant and freeze the cell pellet at −80 °C for DNA isolation. This step is optional (*see* **Note 18**).
3. Screen: For each replicate, centrifuge 360 × 10^6 viable cells for 5 min at 300 × *g*.
4. Wash the pellets twice in PBS by centrifugation for 5 min at 300 × *g* (*see* **Note 5**).
5. Resuspend cells in a total of 720 mL sugar-free media.
6. Prepare two tubes each with 320 mL of cells and add either 7.2 mL of 1.11 M glucose or 7.2 mL of 1.11 M galactose stock solutions. The final concentration of glucose or galactose is 25 mM, the cells are at a concentration of 0.5 × 10^6 cells/mL and there are 2000 cells/sgRNA.
7. Transfer cells to several large TC flasks to ensure oxygenation and place the flasks in the incubator.
8. Incubate for 24 h before harvesting the cells (*see* **Note 21**).

3.3.6 Select the Annexin V Positive (Dead) Cells by MACS for DNA Isolation

1. Prepare 120 mL of 1× Annexin V-microbead binding buffer by diluting 6 mL of the 20× stock solution into 114 mL of water.
2. Count the glucose and galactose cells and centrifuge both cell preparations for 5 min at 2000 × *g*.

3. Resuspend the cell pellets for each condition in 80 μL of 1× Annexin binding buffer per 10^7 cells. Transfer cells to a 50 mL tube.
4. Save a 10 μL aliquot from each condition labelled as "input."
5. Add 20 μL of Annexin V microbeads per 10^7 total cells from each condition.
6. Mix well and incubate 15 min at 4 °C.
7. Add 1 mL of 1× Annexin V-microbeads binding buffer per 10^7 cells to each condition.
8. Centrifuge cells for 5 min at 2000 × *g*.
9. Resuspend the cell pellet in Annexin V-microbead binding buffer at 2×10^8 cells/mL, or in a minimum volume of 0.5 mL.
10. Keep cells on ice.
11. Assemble a separate LS column for each condition in the magnetic field of a suitable MACS Separator (e.g., QuadroMACS).
12. Equilibrate the columns using 3 mL of 1× Annexin V-microbeads binding buffer.
13. Apply the cell suspension to the column, making sure that the maximal column capacity (2×10^9 cells/column) is not exceeded. In case the maximal column capacity is reached, split the sample in multiple columns.
14. Collect the flow-through containing the unlabeled cells.
15. Wash the columns 4 times with 3 mL 1× Annexin V-microbeads binding buffer. Collect the unlabeled cells from all the washes and combine with the flow-through from **step 14**. Label the tube as "viable cells." Keep on ice.
16. Remove the columns from the magnetic separator and fit them to a suitable collection tube.
17. Add 5 mL of 1× binding buffer to each column and recover the retained cells by applying firm pressure to a plunger inserted into the column.
18. If the number of cells reached the maximal column capacity in 13, pool the cells from similar columns.
19. Label the tubes as "dead cells" and keep on ice.
20. Transfer 10 μL from the "input", "viable cells" and "dead cells" tubes to 1.5 mL tubes and proceed to FACS-based analysis as in Subheading 3.2, **steps 10–17**. The viability (i.e. Annexin V-negative cells) is expected to be >90% in the "input" and "viable cells," and <10% in "dead cells" (*see* **Note 22**).
21. Spin down the "dead cells" for 5 min at 2000 × *g*.
22. Remove the supernatant and freeze the pellets at −80 °C for DNA isolation.

3.4 DNA Isolation and Data Analysis

We recommend contacting the local institute genomics platform regarding the preferred methods for gDNA isolation, PCR amplification, and next-generation sequencing. In our hands, good results have been obtained using the NucleoSpin Blood Kit from Macherey Nagel, and the Herculase polymerase for PCR amplification. We recommend sequencing all the samples, including "day 7" and "day 10" for comparative analysis. For data analysis, we have obtained highly consistent results using both MAGeCK [20] and Z-score-based methods [16, 21]. Comparisons can be made between both arms of the screen (e.g., glucose vs. galactose), or between each arm and corresponding earlier time points.

4 Notes

1. Synthetic lethality is defined as "a type of genetic interaction where the co-occurrence of two genetic events results in organismal or cellular death" [22]. *See* Table 1 for examples.
2. OXPHOS-deficient cells are auxotrophic for glucose, pyruvate, and uridine [19].
3. Normal FBS contains glucose and other nutrients which need to be excluded in glucose-free media. Using dFBS provides greater control over media composition.
4. Antimycin A is an inhibitor of the mitochondrial respiratory chain complex III. Mitochondrial protein synthesis inhibitors, such as chloramphenicol, may also be used. It is also possible to use genetic models of OXPHOS deficiency, such as those resulting from the genetic ablation of respiratory chain or mitoribosomal subunits.
5. PBS washes are important to remove any cell debris and left-over media from the previous passage.
6. Apoptotic cells are not completely recovered at 300 × *g*. Centrifugation at 2000 × *g* increases the collection of all cells without significantly reducing viability.
7. It may be necessary to titrate the amount of Annexin V-FITC used. Refer to the instructions from the supplier.
8. K562 cells in glucose should show low Annexin V staining (<10% Annexin V positive cells). Before analyzing Annexin V levels, cells can be counted in trypan blue to control for their viability.
9. Thaw at 37 °C and for no longer than necessary as lentiviruses are heat-sensitive. Viral supernatants from Addgene are usually supplied with a small 2 mL aliquot for titration.
10. Use the following formula to calculate the infection rate:

$$\text{Infection rate} = \frac{\text{Infected cells in selection media}}{\text{Infected cells in normal media}} \times 100$$

11. We use a MOI of ~0.3 to minimize the risk of a cell being infected with two different gRNAs.
12. The MOI varies between cell lines. Libraries need to be titrated for each cell line.
13. A high cells/sgRNA ratio will lead to a better signal-to-noise ratio.
14. In this example, about 127×10^6 cells need to be used with the Brunello library (76,441 sgRNA), targeting 500 cells/sgRNA and at an MOI of ~0.3:

$$\text{Guides in the library} \times \text{desired}\frac{\text{cells}}{\text{sgRNA}} \times \frac{1}{\text{MOI}} = \text{Number of cells for infection.}$$

$$76{,}441 \text{ guides} \times 500\frac{\text{cells}}{\text{sgRNA}} \times 3.33 = 127 \times 10^6 \text{cells}$$

15. Centrifugation time needs to be slightly longer for large volumes.
16. An infection rate of up to 50% is acceptable. Make sure that at least 500 cells/sgRNA are infected.
17. It is important to always centrifuge cells to avoid the presence of dead cells.
18. Cells can be used for DNA isolation to provide a baseline for guide representation after selection.
19. To obtain the number of cells required for a specific cells/sgRNA value, use the following formula:

$$\text{Desired}\frac{\text{cells}}{\text{sgRNA}} \times \text{guides in the library} = \text{Number of cells needed}$$

20. Freezing a batch of cells after selection provides a stock population of cells infected with the chosen library.
21. The duration of the synthetic lethality treatment depends on the experiment and the biological question addressed. In K562 cells, 24 h is sufficient for comparing viability in glucose and galactose.
22. For our synthetic lethal interaction, we expect more dead cells in the input of the galactose condition than in the glucose.

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