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Protocol

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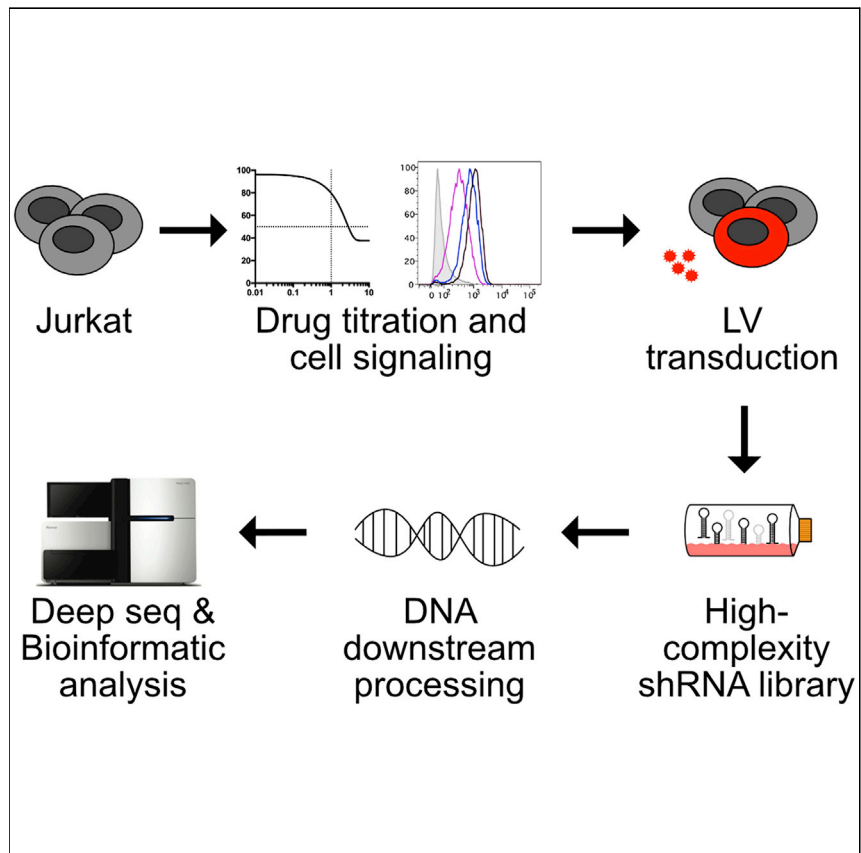
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HIGHLIGHTS

A protocol for synthetic lethality screens in non-adherent cells

Considerations and background information to consider before starting

Step-by-step protocol details with example images for easy application

Here, we provide a detailed protocol for synthetic lethality screens in a Jurkat T cell leukemia line using cell death as the readout measuring the combinatorial effect of a pan-PI3K inhibitor (GDC0941) with specific gene depletion by shRNA. We describe the use of an ultra-complex shRNA library, coverage considerations, time frames, protocol details, and bottlenecks with images to facilitate similar approaches. We discuss how this protocol resource can be readily adapted by investigators.

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Protocol for Comprehensive Synthetic Lethality Screens

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SUMMARY

Here, we provide a detailed protocol for synthetic lethality screens in a Jurkat T cell leukemia line using cell death as the readout measuring the combinatorial effect of a pan-PI3K inhibitor (GDC0941) with specific gene depletion by shRNA. We describe the use of an ultra-complex shRNA library, coverage considerations, time frames, protocol details, and bottlenecks with images to facilitate similar approaches. We discuss how this protocol resource can be readily adapted by investigators.
For complete details on the use and execution of this protocol, please refer to (Mues et al., 2019).

BEFORE YOU BEGIN

Here, we describe the use of a non-adherent, human Jurkat T cell leukemia cell line that we can easily expand to 1.6×10^8 cells, which can be efficiently transduced with 1.1×10^8 lentiviral infectious units to achieve a MOI (multiplicity of infection) of 0.7 (In general, a MOI of 0.3 -0.9 is usually recommended for screens).

1. Thaw Jurkat cell line from N2 in a 37°C water bath.
2. Resuspend cells at 2×10^5 cells/ml with Jurkat media

△ CRITICAL: Keep cell density between 10^5 -10^6 cells/ml, this ensures efficient transduction.

Figure 1A

Alternatives: Other cell lines could be used, if the lentiviral infection rate is established before use. Identification and validation of a cell platform that allows you to consider the previous parameters are critical. Can you obtain large numbers of cells? Do these cells die faithfully in your assay? Can you routinely and consistently infect your cells to efficiently introduce your shRNA- or sgRNA- guides? In our Mues *et al.* study (Mues et al., 2019) we validate one specific synthetic lethality combination in 28 different cancer cell lines, which demonstrated that this Jurkat line is, in principal, suitable to identify synthetic lethality that also impacts other cancer types and adherent cells.

Alternatives: We describe the use of cell death as a screening tool, but fluorescent reporters can work very well too. You should design an appropriate screening assay to answer your specific biological question.
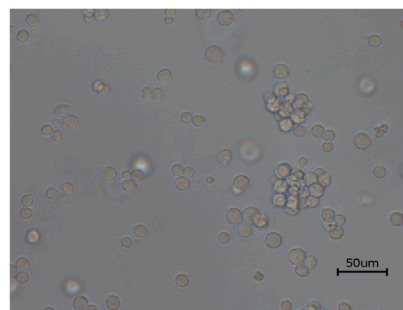
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Figure 1. Jurkat

(A) Representative image of healthy Jurkat cell line under the microscope.

Alternatives: We describe the use of FACS-based assays, but microscopy could also be used. Keep in mind that the quality of experimental read-out and its discriminating power is very important and depends strongly on the method selected. For example, we favor FACS-based assays over microscopy because we can apply well-defined gates with FACS. High throughput is also an important consideration.

Alternatives: In our screen, we used a high coverage shRNA library with 2000X coverage. There are several alternatives available (e.g., either shRNA or more novel sgRNA, such as for CRISPRi or CRISPRa platforms, which can be used to inhibit (CRISPRi) or activate (CRISPRa) gene expression respectively). The key issue in choosing a library is being able to work with high coverage. This avoids population-skewing, over- or under- estimating single target genes, and off-target complication. Many publications use at least 1000X coverage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
pAkt (Ser473), h	Cell Signaling Tech.	CST #4058
pS6 (Ser235/236), h	Cell Signaling Tech.	CST #2211
Annexin V-APC	eBioscience	BMS306APC-20
PE Donkey Anti-Rabbit IgG	Jackson Immuno	7711-116-152
Chemicals, Peptides, and Recombinant Proteins		
GDC-0941 (Pictislib)	Selleckchem	S1065
Sodium Azide (NaN3)		
calcium chloride (CaCl2 x 2H2O)	Sigma-Aldrich	C8106
Methanol	Fisher Chemical	A412
32% PFA	VWR	15714-S
DMSO	Fisher Chemical	BP321
2-propanol	Sigma-Aldrich	190764
Propidium Iodide	Molecular Probes	P3566
Acridine Orange	Sigma-Aldrich	235474
Puromycin Dihydrochloride	MP Biomedicals	0210055225

(Continued on next page)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
QIAamp DNA Blood Maxi Kit	QIAGEN	51192
Buffer AW1	QIAGEN	19081
Buffer AW2	QIAGEN	19072
QIAquick Gel Extraction Kit	QIAGEN	28115
QIAquick PCR Purification Kit	QIAGEN	R28104
PstI-HF	New England Biolabs	R3140T
HiFi Phusion polymerase	New England Biolabs	M0530L
Experimental Models: Cell Lines		
Human: JURKAT cells	Lab of Jeroen Roose	N/A
Oligonucleotides		
TruSeq Index Primers (The barcoding primers are proprietary to Illumina and were shared with the UCSF Genomics Core with the constraint not to publish the individual sequences.)	Illumina	N/A
Recombinant DNA		
UCSF EXPANDed RNAi library	Lab of Michael McManus, UCSF ViraCore	Bassik et al., 2009
Other		
Flow cytometer	N/A	N/A
Automatic or manual cell counter	N/A	N/A
Water bath	N/A	N/A
Centrifuge for 1.5, 15, 50ml tubes and 96 well plates	N/A	N/A
Vacuum system with manifold	N/A	N/A
Thermocycler	N/A	N/A
Agarose/DNA gel station	N/A	N/A
Cold room (4°C)	N/A	N/A
HyClone™ RPMI 1640 Media with L-Glutamine	GE Healthcare LifeScience	SH30027.02
1M HEPES, 100 ml	UCSF Cell culture Facility	INVZR929
1.2ml Microtiter tubes	ThermoFisher Scientific	3492
123count™ eBeads Counting Beads	Invitrogen	01-1234
Roller Bottle	Corning	430849
GeneRuler 1kbP Plus DNA ladder	ThermoFisher Scientific	SM1333

MATERIALS AND EQUIPMENT
Jurkat Media

RPMI with
L-Glutamine
10% FBS
1%P/S
1% HEPES

Drug Reconstitution
Reconstitute 1mg of GDC0941 at 10mM with 194 μl of DMSO

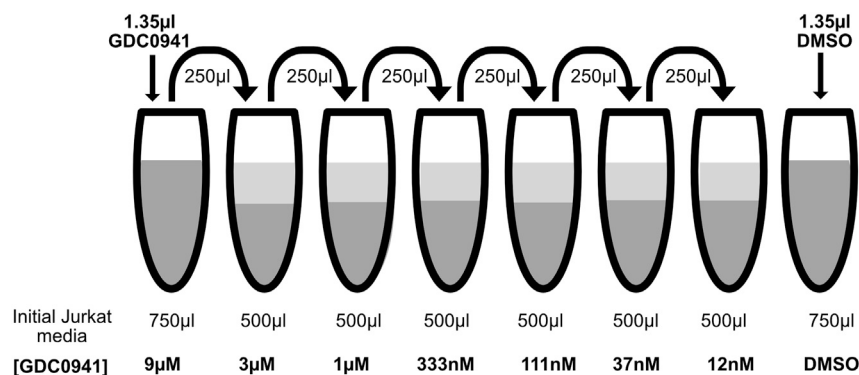Store at -20°C for long term storage.

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Figure 2. Drug Preparation

(A) Cartoon of serial dilution to prepare 3-fold drug dilution.

⚠ CRITICAL: Avoid freeze/thaw cycles

FACS Buffer

PBS
2mM EDTA
2% FBS
0.09% NaN$_3$

QIAamp DNA Blood Maxi Kit

Resuspend Protease according to manufacturer's protocol.

Make 500μl Protease aliquots with H2O and store at -20°C

Note: We recommend you order more wash buffers AW1 and AW2, which will be used for the vacuum protocol.

STEP-BY-STEP METHOD DETAILS

Titer Drug Concentration

⏱ TIMING: 30 min (Figure 2)

This step allows you to determine what drug concentration (GDC0941) to use in your screen.

1. Prepare 3-fold serial dilution (9μM, 3μM, 1μM, 333nM, 111nM, 37nM, 12nM, DMSO) at 2X
   a. Thaw a small aliquot of GDC0941 while you prepare tubes
   b. Set up 2 tubes with 750μl of Jurkat media and 6 tubes with 500μl of Jurkat media
   c. Add 1.35μl of 10mM GDC0941 to one tube containing 750μl and mix. This will be 9 μM
   d. Transfer 250 μl from 9 μM tube to a tube containing 500 μl and mix. This will be 3 μM.
   e. Transfer 250 μl from 3 μM tube to a tube containing 500 μl and mix. This will be 1 μM.
   f. Repeat previous dilution until 12nM tube.

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Table 1. CaCl2 and Acridine Orange Solutions

[Stock]	
1M CaCl2	1.47 g CaCl2 x2 H2O in 10ml ddH20
1mM Acridine Orange (AO)	2.65 mg AO in 10ml ddH2O

  g. For DMSO control, add 1.35μl DMSO to the last tube containing 750μl
  h. Transfer 200μl of each inhibitor concentration into two adjacent wells (for duplicates) of a 48 well plate

Analyzing Cell Proliferation with Flow Cytometry

⏱ TIMING: 2 h

This step describes how to obtain the cell counts with flow cytometry with live/dead/apoptotic discrimination

2. Prepare Jurkat cell line.
   a. Count cells according to your preferred method
   b. Resuspend $2x10^6$ cells at $400x10^3$ cells/ml with Jurkat media
   c. Add 200μl of cells to 48 well plate containing inhibitors
   d. Let the cells proliferate in the incubator for 3 days at 37°C
3. Set up reagents for 20 samples. Final volume will be 200μl.
   a. 10X CaCl2 in PBS (20mM). Table 1
   b. 10X Acridine Orange (AO, 200nM) and Propidium Iodide (PI, 2μM). Add 20μl of 10μM AO and 20μl of 100μM PI stocks to 1ml PBS. Table 1
   c. 10X Annexin V–APC in FACS Buffer. 1μl of Annexin V + 19μl of FACS Buffer for each sample.
   d. 123count eBeads Counting Beads. Remember manufacturer exact counts (∼10^6beads/ml)
4. Prepare a pre-mix transferring 200μl of each of the reagents (10X CaCl2 in PBS (20mM), 10X AO/PI (200nM/2μM), 10X Annexin-V in FACS Buffer (1:19), eBioscience 123 count Beads) into 1ml tube.
5. Add 80μl of the previous pre-mix into 16 1.2ml Micro titer tubes
6. Add 120μl of each well of cell proliferation experiment seeded 3 days ago into previous 1.2ml Micro titer tubes containing the pre-mix. Keep tubes on ice.
7. Run the samples in a Flow cytometer machine (Figure 3A) with the appropriate Lasers and filters. E.g. AO in FITC, PI in mCherry, Annexin V in APC, Beads in Pacific Blue and AmCyan

Flow Cytometry Analysis

⏱ TIMING: variable

This step describes a proposal of gating strategy in order to reduce variability. (Figure 3B)

8. No gating in FCS/SSC
9. All events in Pacific Blue and AmCyan. Gate double positive beads
10. All events in FITC and mCherry. Gate on AO+ (nucleated cells)
11. AO+ events in mCherry and APC. Make quad gate: live cells (PI-Annexin V-), apoptotic cells (PI- Annexin V+) dead cells (PI+ Annexin V+)
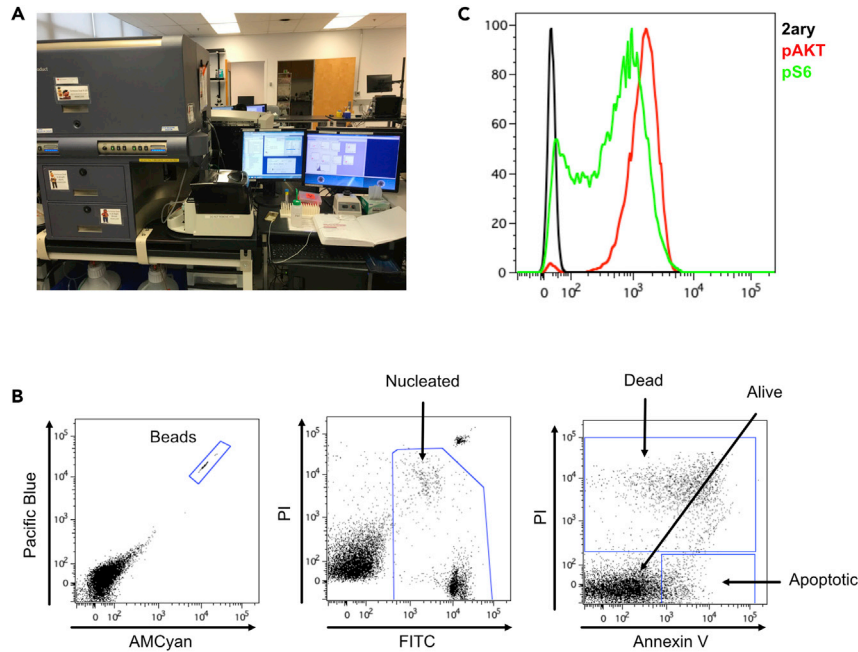12. Get counts of each parameter

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Figure 3. Flow Cytometry Analysis

(A) Picture of a flow machine suitable for the analysis.

(B) Representative flow cytometry panel of Live/Dead proliferation experiment. Beads are gated on Pacific Blue vs AMcyan. Nucleated cells are gated on Acridine Orange (FITC$^+$). Apoptotic cells are gated on Annexin V$^+$ Propidium Iodide (PI)$^-$. Dead cells are gated Propidium Iodide (PI)$^+$.

(C) Representative phospho-flow analysis of baseline pAKT (red) and pS6 (green) levels on Jurkat.

13. Cell concentration (cells/ml) = bead concentration x (cell count x bead volume) / (bead count x cell volume). For example, Cell concentration (cells/ml) = $\sim$10^6 beads/ml x (cell count x 20μl) / (beads count x 120μl)

Signaling Experiment

⏱ TIMING: 2 days

This step describes how to analyze the effect of 1h of GDC0941 in PI3K signaling by measuring levels of pAKT and pS6

14. Prepare Drug concentration as above.
15. Resuspend 8x10^6 Jurkat cells with 4ml of Jurkat media (2x10^6 cells/ml)
16. Add 400μl of each GDC0941 concentration (9μM, 3μM, 1μM, 333nM, 111nM, 37nM, 12nM, DMSO) in a different well of a 12 well plate
17. Add 400μl of Jurkat cells into each well containing GDC0941. Mix well and incubate for 1h at 37°C.
18. Add 200μl of 10% PFA and incubate at RT for 5 min. Transfer cells to 1.5ml tube
19. Centrifuge 5 min at 500G, remove supernatant, resuspend cells by flicking.
20. Add 300μl of 90% ice-cold methanol and incubate at -20°C at least 12-16h.

⏸⏸ PAUSE POINT: Samples here can be stored at -20 for up to 2 months.

21. Add 1ml of FACS buffer and incubate 5 min to rehydrate
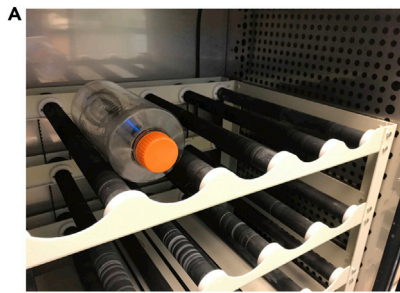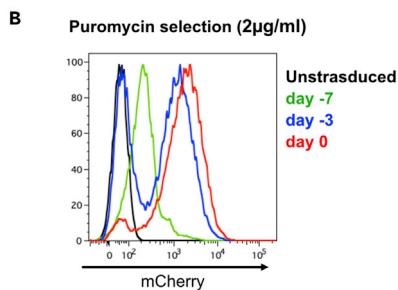   a. Prepare primary antibody

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Figure 4. Jurkats Containing the shRNA Library Are Resistant to Puromycin after Treatment
(A) Picture of a roller bottle in the roller bottle specific incubator.
(B) Representative flow cytometry histogram of Puromycin/mCherry selection from untransduced cells, day -7, day -3 and day 0.

    i. pAKT S473: 495µl of FACS buffer + 5µl of pAKT antibody
    ii. pS6 S235/236: 499µl of FACS buffer + 1µl of pS6 antibody
    iii. Control: 500µl of FACS buffer

22. Centrifuge cells for 2 min at 2500G, remove supernatant, resuspend cells by flicking
23. Add 150µl of FACS buffer
24. Split each condition (50µl) in 3 wells of a U-shaped 96 well plate
25. Add 150µl of FACS buffer
26. Centrifuge for 2 min at 2500G, remove supernatant, resuspend cells by flicking
27. Add 50µl of each antibody mix (pAKT, pS6 and control) to each condition.
28. Incubate 30 min at RT
    a. Prepare 2ary antibody
    b. Donkey anti-rabbit-PE: 1386µl of FACS buffer + 14ul of antibody
29. Wash cells twice with 200µl of FACS buffer
30. Add 50µl of 2ary antibody to each well
31. Incubate 15 min in the darkness at RT
32. Wash twice with 200µl of FACS buffer at 2500G for 2 min
33. Resuspend the cells with 150µl of FACS buffer and acquire on a flow cytometer.
34. Analyze your data e.g. in histograms that depict the levels of pAkt or pS6 (Figure 3C).

Lentiviral Transduction

⏱ TIMING: 10–12 days

This step describes the infection of Jurkat cell line containing the shRNA library with at least 2000X coverage. The shRNA-encoding vector has mCherry reporter gene and Puromycin selection gene.

35. Jurkat cell line is expanded in regular flasks until desired cell number is achieved
36. Day -9: shRNA library contains ~1,800 genes with 55,000 shRNAs. To use a 2000X coverage 1.1×10^8 lentiviral infectious units are seeded with 1.6×10^8 Jurkat cells to achieve a MOI of 0.7 in 80ml total in a roller bottle (Figure 4A).
37. Leave cells with lentivirus 12-16h in the incubator.
38. Day -8: Next morning add 90ml of fresh Jurkat media.

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39. Day -7: Analyze mCherry expression by flow cytometry and start treatment with 2μg/ml of Puromycin (previously tittered: protocol not described). Split cells when necessary adding 2μg/ml of Puromycin keeping cell density between 10^5 and 10^6 cells/ml to ensure they are in exponential growth.

   ⚠ CRITICAL: Do not use less than 1.1×10^8 cells. Coverage might be compromised

40. Day -3: Analyze mCherry expression by flow cytometry.
41. Day 0: Analyze mCherry expression by flow cytometry. Cells should be >90% positive. (Figure 4B).

   Note: If less than 90% of the cells are mCherry positive, continue the puromycin selection.

42. Cryopreserve at least 1.1×10^8 cells at T0.

Cell Growth and Selection Screen

   ⊙ TIMING: 3–4 weeks

Expand Jurkat cell line with GDC0941 or DMSO for ~3 weeks to allow for shRNA-induced cell death (synthetic lethality). Note that enrichment for shRNA species can also be picked up.

43. Day 0: Add 1.1×10^8 cells into 2 different roller bottles and fill up to 250ml of fresh Jurkat media with 1μg/ml of Puromycin. One bottle will have GDC0941 at 1.4μM, the concentration at which cell growth is reduced by 20% and phospho-signaling is reduced according to drug titration. 35μl of GDC0941 at 10mM will be added to the bottle. Same amount of DMSO will be added to the control bottle
44. Day 1: Split the 250ml of each bottle of GDC0941 and DMSO into 2 roller bottles (125 each bottle), to ensure there is not cell saturation. Add 125ml of new fresh media to each bottle with 17.5μl of GDC0941 at 10mM or DMSO and 1μg/ml of Puromycin.

   Note: Puromycin can be reduced at 0.5-1μg/ml to maintain the selection

45. Day 3: Count and check cell viability. Split cells down to 5.5×10^7 cells each bottle, add fresh Jurkat media up to 250ml with 1μg/ml of Puromycin and 1.4μM of GDC0941 or DMSO.
46. Repeat previous step every two or three days according to your cell growth. Keeping them under 2×10^6 cells/ml
47. On day 22, snap freeze. Count cells, centrifuge 1.2×10^8 cells 5min at 1500rpm, discard supernatant, transfer them into a 5ml cryovial, centrifuge and discard supernatant again. Resuspend cells in remaining liquid and snap freeze at liquid N2 for 1 min before you keep it at -80 for long term storage.

   Note: we chose a 22-day screening period. This is somewhat arbitrary and could be shortened. The reason to allow for a longer selection period here was two-fold. First, we use a very modest concentration of PI3K inhibitor as backdrop. Second, we wanted to be sure to catch synthetic lethality that was not instantly but could take several days.
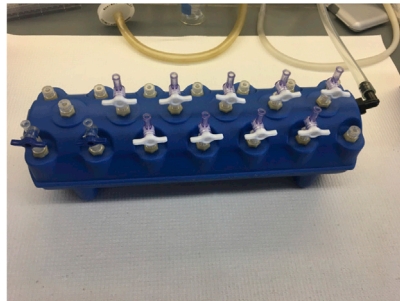
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Figure 5. Vacuum Manifold for DNA Extraction
(A) Vacuum manifold connected to the vacuum system for DNA extraction.

⊓⊔ PAUSE POINT: You can cryopreserve cells during the splitting days if you would like to preserve material from time points while the screen is ongoing.

gDNA Extraction

⊙ TIMING: 2–3 h

This step describes the extraction of gDNA from frozen pellet.

48. Use the QIAamp DNA Blood Maxi Kit. Protocol is slightly modified from the manufacturer. Before to start, warm water bath to 70°C, thaw an aliquot of Protease and set up the vacuum manifold connectors (Figure 5).
49. Thaw cell pellet at RT.
50. Resuspend cells completely when thawed.
51. Add 8.5ml of PBS and mix while transferring solution to a 50ml tube.
52. Add 500µl of Protease and mix.
53. Add 12 ml of buffer AL.
54. Invert 15 times to mix, then shake vigorously for 1 min. DO NOT VORTEX.
55. Close tube tightly and incubate 15min at 70°C, shake every 5 min.
56. Add 10ml of 100% EtOH.
57. Invert 15 times to mix, then shake vigorously for 1 min. DO NOT VORTEX.

   Note: Each sample is divided in 2 columns.

58. Add half of volume (∼15ml) to each column. Taking care not moisten the rim.
59. Open the vacuum ad let the lysate pass through the column.
60. Wash with 15ml of AW1 – 30-60min.
61. Wash with 15ml of AW2 – 15-30 min.
62. Dry each membrane individually opening the valve for 10s.
63. Wipe rim with dry clean tissue.
64. Dry all columns for 30 min with vacuum.
65. Transfer columns into 50ml tubes.
66. Add 600µl of AE buffer, incubate for 5 min, centrifuge 5min at 3500g.
67. Add 600µl of AE buffer, incubate for 5 min, centrifuge 5min at 3500g.
68. Add 400µl of AE buffer, incubate for 5 min, centrifuge 10min at 3500g.
69. Final volume ∼1100-1200µl.
70. Transfer elution into a 1.5ml tube.
71. Determine DNA concentration.

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gDNA Digestion

⊙ TIMING: 16–18 h

This step describes the digest of whole gDNA to excise the band of interest.

72. In a 2ml tube add the following:
    1100μl of gDNA.
    123μl of CutSmart Buffer.
    8μl of PstI-HF restriction enzyme.
73. Incubate 12-16h at 37°C in a rotation.
74. Estimate digestion by running 2μl of digested DNA and 1μl of undigested in a 1.5% agarose gel.
75. You are expecting a smear on the digested gDNA (Figure 6A).

DNA Gel Extraction

⊙ TIMING: 2 days

This step describes how to excise the gel portion to extract the size of interest.

76. Prepare a 0.6% agarose gel in a large caster. Make sure the gel has 3 lanes. 1 big lane in the middle which will hold 1400μl and 1 to each side for the ladder.
77. Once the gel is solidified, transfer it to the cold room (4°C).
78. Carefully pull out the combs and check for integrity.
79. Add 130μl of 10X DNA loading dye to digested DNA.
80. Load along with 1kb ladder and run the gel at 130V in the cold room (4°C) - 1-2h.
81. Cut band of interest out of the gel. In this protocol is 2.2kb (Between 1.5 and 4kb markers).
82. Weight the fragment of gel. From this moment we will assume the gel's weight is 10g.
83. Place the fragment of the gel in a 10ml syringe.
84. Push the gel slurry down using the syringe into a 50ml tube (Figure 6B).
85. Place it at -20°C for at least 12-16h.

⏸⏸ PAUSE POINT: Sample can be stored at -20°C for longer if desired.

86. Thaw it at RT – 30-60 min.
87. Use QIAquick Gel extraction kit using vacuum Manifold. Little modifications of the protocol are made.
88. Add 30 ml of QC buffer to the 50ml tube.
89. Incubate the tube at 50°C for 5-10 min or until the gel is melted. Mix to accelerate the process.
90. After the gel has dissolved completely, check that the color of the mixture is yellow. If the mixture is orange or violet add 10 μl of 3M sodium acetate pH 5 until the solution turns yellow (Figure 6C).
91. Use 2 columns for each sample. Take a new clean 15ml tube cut the bottom part of them with a clean blade. Making a hole. Place the tube inside the column (Figure 6D).
92. Seal the 15ml tube and the column with parafilm in order to add the solution in fewer steps (Figure 6E).
93. Add half of the volume (~20ml) to each 15ml tube (Figure 6E).
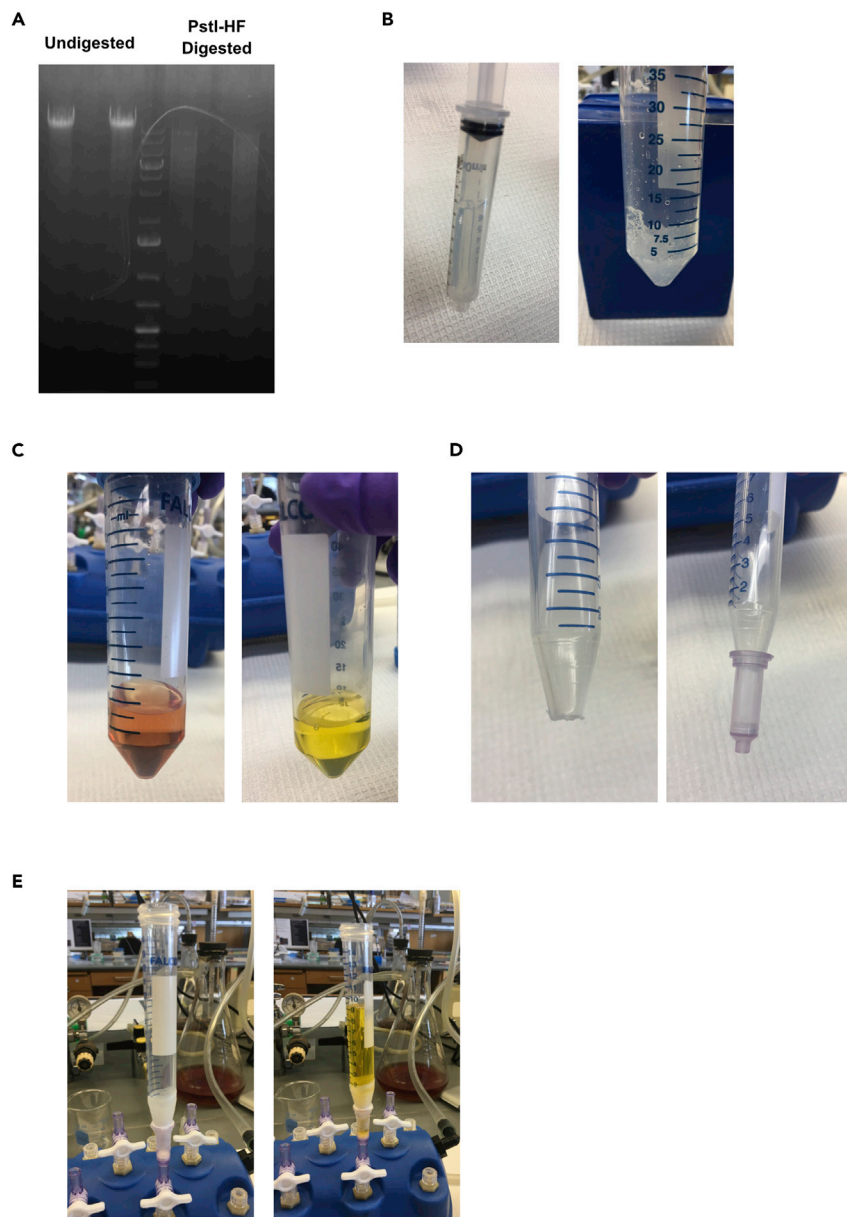94. Let the solution go through the column using the vacuum.

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Figure 6. Illustrated Steps for gDNA Extraction

(A) Representative agarose gel with undigested DNA (left) and PstI-HF digestion 12-16h.

(B) An excised piece of agarose inside 10ml syringe (left) and same piece slurred down (right).

(C) Representative picture of an orange gel extraction buffer (left) and yellow gel extraction buffer (right).

(D) 15ml tube with the end cut (left) and same tube inside columns (right).

(E) 15ml tube with the cut end inside the columns sealed with parafilm and ensembled in the vacuum manifold (left) same but with yellow extraction buffer (right).

95. Remove the 15ml tubes.
96. Wash twice with 500μl of QG buffer.
97. Wash 3 times with 800μl of PE buffer.
98. Vacuum for 5 min to dry the membrane.
99. Transfer the column to a 2ml collection tube.
100. Centrifuge at Max speed for 5 min and discard the collection tube.

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Table 2. PCR Optimization Calculations for Temperature

	Volume (μl)
H20	44
GC buffer	20
10mM dNTPs	2
10μM TrueSeqIndex forward	5
10μM TrueSeq reverse	5
DMSO	3
Phusion polymerase	1
DNA	20 (160ng/reaction)

101. Place the column to a new 1.5ml low DNA bind tube.
102. Elute each column twice with 55μl of EB and combine to one tube.
103. Final volume ~200μl.
104. Determine DNA concentration.
105. Run 2μl of the elution in a 1.5% agarose gel to confirm DNA extraction. A smear is a good sign.

DNA Amplification and Barcoding

⊙ TIMING: variable

This step describes the amplification of shRNA from the proviral DNA and barcoded for sequencing.

106. DNA concentration is adjusted to 40ng/μl
107. PCR optimization for temperature and cycles
    a. Prepare small amount of primers at 10μM for optimization. TrueSeqIndex forward and a common revers primer are used for barcode
    b. PCR are performed using NEB HiFi Phusion polymerase
    c. PCR pre-mix for 5 well to test temperature. Prepare on ice and add in the following order. Table 2
    d. Split 20μl to 4 different 0.2ml PCR tubes. Pre-heat the thermocycler at 98°C prior to add the tubes. Skip this step once the tubes are in the thermocycler (Figure 7A)
    e. Cycling protocol (Figure 7B)
        Stage 1 - 98°C - Forever
        Stage 2 - 98°C – 3 min
        Stage 3; step 1 - 98°C – 15s
        Stage 3; step 2 - Four gradients of temperatures according to your primers – 30s
        Stage 3; step 3 - 72°C – 30 s
        Repeat stage 3, 29 times
        Stage 4; step 1 - 72°C – 5min
        Stage 4; step 2 - 4°C – Forever
    f. Run samples at 1% agarose and choose the temperature that show the neat 270bp band
    g. PCR pre-mix for 10 well to test cycles. Prepare on ice and add in the following order. Table 4

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Figure 7. Indexing PCR
(A) Thermocycler with a gradient of temperatures used to choose the appropriate annealing temperature and number of cycles for indexing.
(B) Photo of the PCR cycle used to assess the right temperature of annealing (left & right).

h. Split 20µl to 9 different 0.2ml PCR tubes. Pre-heat the thermocycler at 98°C prior to add the tubes. Skip this step once the tubes are in the thermocycler

i. Cycling protocol
Stage 1 - 98°C – Forever
Stage 2 - 98°C – 3 min
Stage 3; step 1 - 98°C – 15s
Stage 3; step 2 - Previously step chosen temperature – 30s
Stage 3; step 3 - 72°C – 30 s
Repeat stage 3, 30 times

⚠ CRITICAL: Remove from the thermocycles one tube at cycles 10, 12, 14, 16, 18, 20, 22, 24, 28 and put them on ice until the cycle reach 30. Then add all the tubes again to the thermocycles to finish the last elongation step

Stage 4; step 1 - 72°C – 5min
Stage 4; step 2 - 4°C – Forever

108. Run samples at 1% agarose and choose the cycle that first shows a single 270bp band before higher and lower band form.
109. Now you are ready to scale up reaction. Multiply previous volumes for 75x.
Table 3
110. Divide into twelve 0.2ml tubes (120µl each). Pre-heat the thermocycler at 98°C prior to add the tubes. Skip this step once the tubes are in the thermocycler

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Table 3. Scale Up PCR Calculations

	Volume (μl)
H20	660
GC buffer	300
10mM dNTPs	30
10μM TrueSeqIndex forward	75
10μM TrueSeq reverse	75
DMSO	445
Phusion polymerase	15
DNA	300 (160ng/reaction)

111. Cycling protocol
     Stage 1 - 98°C - Forever
     Stage 2 - 98°C – 3 min
     Stage 3; step 1 - 98°C – 15s
     Stage 3; step 2 - Temperature – 30s
     Stage 3; step 3 - 72°C – 30 s
     Repeat stage 3, "n" times
     Stage 4; step 1 - 72°C – 5min
     Stage 4; step 2 - 4°C – Forever
112. Combine all PCR products from previous 12 tubes and run 15μl in a 1% agarose gel
113. Confirm there is a neat 270bp band

PCR Product Concentration

⏱ TIMING: 30 min

This step reduces the volume of the PCR reaction. QIAquick PCR purification kit is used.

114. Mix 1.5ml of PCR reaction + 7.5ml of Buffer PB
115. If the color of the mixture is orange or violet add 10μl of 3M sodium acetate pH 5 and mix. Repeat until color of the mix will turn yellow (Figure 6C)
116. Run each sample into 2 columns. Place a column into a 2ml collection tube
117. Add 750μl of solution into each column
118. Centrifuge for 30-60s at Max speed and discard flow-through
119. Add 750μl of solution again and centrifuge until all solution run through the columns
120. Wash twice with 1ml of PE buffer.
121. Centrifuge for 30-60s at Max speed.
122. Place the column into a new 2ml collection tube and centrifuge for 1 min to remove residual wash buffer.
123. Place each column in a clean low-DNA binding tube.
124. Add 55μl EB buffer to each column.
125. Incubate for 1 min at RT and centrifuge for 1 min at max speed.
126. Combine elution from both columns. Final volume will be 110μl.

PCR Purification from Agarose Gel

⏱ TIMING: 4–6h

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Table 4. PCR Optimization Calculations for Cycles

	Volume (μl)
H20	88
GC buffer	40
10mM dNTPs	4
10μM TrueSeqIndex forward	10
10μM TrueSeq reverse	10
DMSO	6
Phusion polymerase	2
DNA	40 (160ng/reaction)

This step describes the isolation of PCR product from agarose gel.

> *Note:* Different techniques can be used to isolate index DNA band from a gel. Mues et al. (Mues et al., 2019) described a purification using polyacrylamide gel and isolation from a gel with electro-elution. We have since had better success with agarose gel purification, which we describe here.

127. Prepare a 0.6% agarose gel.
128. Once the gel is solidified, transfer it to the cold room (4°C)
129. Add 11μl of 10X loading dye to the 110μl eluted previously
130. Load along with a ladder (i.e. GeneRuler 1kb Plus DNA Ladder) and run the gel at 130V in the cold room (4°C)
131. Cut band of interest out of the gel. In this protocol it is 270 bp fragment and safe for extraction.
132. Weigh the fragment of gel. From this moment we will assume the gel weight is 1g.
133. Use QIAquick Gel extraction kit using microcentrifuge protocol. Slight modifications are made.
134. Add 3 ml of QC buffer to the 15ml tube.
135. Incubate the tube at 50°C for 5-10 min or until the gel is melted. Mix to accelerate the process.
136. After the gel has dissolved completely, check that the color of the mixture is yellow. If mixture is orange or violet add 10 μl of 3M sodium acetate pH 5 until the solution turns yellow (Figure 6C).
137. Add 1ml of 2-propanol to increase the yield. Only works for DNA fragments <400bp and >4kb.
138. Place the column into 2ml collection tube.
139. Add 800μl of the solution and centrifuge for 1 min at max speed.
140. Discard flow-through and place the column back to the collection tube.
141. Add 800μl more of the solution and centrifuge for 1 min. Repeat as much as you need.
142. Wash once with 500μl of QG buffer.
143. Wash twice with 800μl of PE buffer.
144. Centrifuge at max speed for 2 min and discard the collection tube.
145. Place the column to a new 1.5ml low DNA bind tube.
146. Elute each column twice with 50μl of EB and combine.
147. Determine DNA concentration.

EXPECTED OUTCOMES

This protocol is meant to be used as a reference to design other screenings. We described the detailed methodological steps, drug titration, lentiviral

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transduction, DNA extraction and amplification to inform the reader what to expect. The "Before you begin" section contains considerations that are important when one aims to make adaptations to the described screen. If the considerations of cell line of choice, rigorous screening assay (e.g. cell death), and higher than 1000x coverage are followed properly, the research can expect to successfully identify synthetic lethality pairs, as we did in our Mues et al. study (Mues et al., 2019).

LIMITATIONS

This specific protocol cannot be adapted to all cell lines. The use of adherent cell lines will need a different approach for expansion of cell numbers, lentiviral transduction, and/or monitoring cell growth. Cell doubling times, confluency of adherent cells, available growth factors, and frequency of drug administration should be considered for each individual cell type of choice.

Library coverage is always a limitation; the higher the level of coverage, the lower the noise to data ratio, and the more confident the generated data sets become. Usually 1000X coverage is assumed as sufficient for experiments using libraries. In our Mues et al. study (Mues et al., 2019) we used 2000X coverage to increase the number of shRNA in the screening and obtain more statistical power. A screen should be designed where aspects like manageable set-up and affordable experimentation are rationally weighed against the highest possible coverage. A popular solution to this dilemma is to perform screen for families of genes, a selection of signaling pathways, or a collection of cell biological processes instead of whole genome screens.

AUTHOR CONTRIBUTIONS

Writing – Original Draft, D.R.-M.; Writing – Review & Editing, D.R.-M. and J.P.R.; Funding Acquisition, J.P.R.

DECLARATION OF INTERESTS

J.P.R. is a co-founder and scientific advisor of Seal Biosciences, Inc. and on the scientific advisory committee for the Mark Foundation for Cancer research. The other author has no financial interests to declare.

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