# **STAR Protocols**

### Protocol

# Protocol for Comprehensive Synthetic Lethality Screens



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.

Damia Romero-Moya, Jeroen P. Roose

jeroen.roose@ucsf.edu

### 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

Romero-Moya & Roose, STAR Protocols ■ ■, 100016 ■ ■, 2020 © 2020 The Authors. https://doi.org/10.1016/j.xpro.2020.100016

# **STAR Protocols**

# Protocol Protocol for Comprehensive Synthetic Lethality Screens

Damia Romero-Moya<sup>1</sup> and Jeroen P. Roose<sup>1,2,3,\*</sup>

<sup>1</sup>Department of Anatomy, University of California San Francisco (UCSF), 513 Parnassus Avenue, Room HSW-1326, San Francisco, CA 94143-0452, USA

<sup>2</sup>Technical Contact

<sup>3</sup>Lead Contact

\*Correspondence: jeroen.roose@ucsf.edu https://doi.org/10.1016/j.xpro.2020.100016

#### **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  $1.6 \times 10^8$  cells, which can be efficiently transduced with  $1.1 \times 10^8$  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  $37^{\circ}C$  water bath.
- 2. Resuspend cells at  $2x10^5$  cells/ml with Jurkat media
  - $\triangle$  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.

# **STAR Protocols**

**Cell**Press



#### 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)

# **STAR Protocols**



#### 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<br>primers are proprietary to Illumina and were<br>shared with the UCSF<br>Genomics Core with the constraint not to<br>publish the individual sequences.) | Illumina                                 | N/A                 |
| Recombinant DNA   |  |                     |
| UCSF EXPANDed RNAi library  | Lab of Michael McManus,<br>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-<br>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  $\mu l$  of DMSO

Store at -20°C for long term storage.

# **STAR Protocols**

## **Cell**Press



#### 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<sub>3</sub>

#### **QIAamp DNA Blood Maxi Kit**

Resuspend Protease according to manufacturer's protocol.

Make 500 $\mu$ l 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**

(9 TIMING: 30 min (Figure 2)

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

- 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  $750\mu l$  of Jurkat media and 6 tubes with  $500\mu l$  of Jurkat media
  - c. Add 1.35  $\mu l$  of 10mM GDC0941 to one tube containing 750  $\mu l$  and mix. This will be 9  $\mu M$
  - d. Transfer 250  $\mu l$  from 9  $\mu M$  tube to a tube containing 500  $\mu l$  and mix. This will be 3  $\mu M.$
  - e. Transfer 250  $\mu l$  from 3  $\mu M$  tube to a tube containing 500  $\mu l$  and mix. This will be 1  $\mu M.$
  - f. Repeat previous dilution until 12nM tube.

# **STAR Protocols**

**CellPress** 

#### Table 1. CaCl2 and Acridine Orange Solutions

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

- g. For DMSO control, add  $1.35\mu l$  DMSO to the last tube containing  $750\mu l$
- h. Transfer  $200\mu l$  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<sup>6</sup> cells at 400x10<sup>3</sup> cells/ml with Jurkat media
  - c. Add  $200\mu$ l 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,  $2\mu$ M). Add  $20\mu$ I of  $10\mu$ M AO and  $20\mu$ I of  $100\mu$ M PI stocks to 1ml PBS. Table 1
  - c. 10X Annexin V–APC in FACS Buffer. 1 $\mu$ l of Annexin V + 19 $\mu$ l of FACS Buffer for each sample.
  - d. 123<br/>count eBeads Counting Beads. Remember manufacturer exact counts<br/>  $({\sim}10^6 \text{beads/ml})$
- 4. Prepare a pre-mix transferring  $200\mu$ l of each of the reagents (10X CaCl2 in PBS (20mM), 10X AO/PI (200nM/2 $\mu$ M), 10X Annexin-V in FACS Buffer (1:19), eBioscience 123 count Beads) into 1ml tube.
- 5. Add  $80\mu$ l 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

# **STAR Protocols**

## **Cell**Press



#### 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<sup>+</sup>). Apoptotic cells are gated on Annexin V<sup>+</sup> Propidium Iodide (PI)<sup>-</sup>. Dead cells are gated Propidium Iodide (PI)<sup>+</sup>.
 (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<sup>6</sup> Jurkat cells with 4ml of Jurkat media (2x10<sup>6</sup> cells/ml)
- 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 400 $\mu$ l of Jurkat cells into each well containing GDC0941. Mix well and incubate for 1h at 37°C.
- 18. Add 200 $\mu l$  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 300 $\mu l$  of 90% ice-cold methanol and incubate at -20°C at least 12-16h.

**III 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

# **STAR Protocols**





Figure 4. Jurkats Containing the shRNA Library Are Resistant to Puromycin after Treatment (A) Picture of a roller bottle in the roller bottle

(B) Representative flow cytometry histogram of Puromycin/mCherry selection from

untransduced cells, day -7, day -3 and day 0.

B Puromycin selection (2µg/ml)



- 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: 500 $\mu$ l of FACS buffer
- 22. Centrifuge cells for 2 min at 2500G, remove supernatant, resuspend cells by flicking
- 23. Add 150 $\mu l$  of FACS buffer
- 24. Split each condition (50 $\mu$ l) in 3 wells of a U-shaped 96 well plate
- 25. Add 150 $\mu l$  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  $200\mu l$  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 200 $\mu l$  of FACS buffer at 2500G for 2 min
- 33. Resuspend the cells with  $150\mu$ l 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 1.1x10<sup>8</sup> lentiviral infectious units are seeded with 1.6x10<sup>8</sup> 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.

# **STAR Protocols**

- 39. Day -7: Analyze mCherry expression by flow cytometry and start treatment with  $2\mu$ g/ml of Puromycin (previously tittered: protocol not described). Split cells when necessary adding  $2\mu$ g/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  $1.1 \times 10^8$  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  $1.1 \times 10^8$  cells at T0.

#### **Cell Growth and Selection Screen**

#### © TIMING: 3-4 weeks

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

- 43. Day 0: Add  $1.1 \times 10^8$  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 5.5x10<sup>7</sup> cells each bottle, add fresh Jurkat media up to 250ml with 1µg/ml of Puromycin and 1.4µM of GDC0941 or DMSO.
- Repeat previous step every two or three days according to your cell growth. Keeping them under 2x10<sup>6</sup> cells/ml
- 47. On day 22, snap freeze. Count cells, centrifuge 1.2x10<sup>8</sup> 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.

## **Cell**Press

# **STAR Protocols**

**CellPress** 



Figure 5. Vacuum Manifold for DNA Extraction (A) Vacuum manifold connected to the vacuum system for DNA extraction.

**II 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 500 $\mu$ l 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  $600\mu$ l 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  $400\mu$ l of AE buffer, incubate for 5 min, centrifuge 10min at 3500g.
- 69. Final volume  ${\sim}1100{\text{-}}1200{\mu}\text{l}.$
- 70. Transfer elution into a 1.5ml tube.
- 71. Determine DNA concentration.

# **STAR Protocols**



### **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.

 $123 \mu l$  of CutSmart Buffer.

 $8\mu$ l of PstI-HF restriction enzyme.

- 73. Incubate 12-16h at  $37^{\circ}C$  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  $1400\mu$ l 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  $in the cold room (4^{\circ}C) 1-2h$ .
- 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.

II 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).
- 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.

# **STAR Protocols**







С





в



Е



### 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  $500\mu$ l of QG buffer.
- 97. Wash 3 times with  $800\mu l$  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.

# **STAR Protocols**



#### 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  $55\mu l$  of EB and combine to one tube.
- 103. Final volume  ${\sim}200\mu l.$
- 104. Determine DNA concentration.
- 105. Run  $2\mu$ l 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 $40 ng/\mu l$

- 107. PCR optimization for temperature and cycles
  - a. Prepare small amount of primers at  $10 \mu M$  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

# **STAR Protocols**









#### 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 20 $\mu$ l 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

# **STAR Protocols**



#### 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  $15\mu$ l 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  $10\mu$ l 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 750 $\mu l$  of solution into each column
- 118. Centrifuge for 30-60s at Max speed and discard flow-through
- 119. Add  $750\mu l$  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  $55\mu$ l 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  $110\mu$ l.

#### PCR Purification from Agarose Gel

© TIMING: 4-6h

# **STAR Protocols**



#### 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  $11\mu l$  of 10X loading dye to the  $110\mu l$  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  $50^{\circ}$ 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  $\mu$ l 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  $800\mu$ l 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  $800\mu l$  more of the solution and centrifuge for 1 min. Repeat as much as you need.
- 142. Wash once with  $500\mu l$  of QG buffer.
- 143. Wash twice with  $800\mu l$  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  $50\mu$ l 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

# **STAR Protocols**

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.

### ACKNOWLEDGMENTS

We thank the members of the Roose lab and particularly Marsilius Mues for excellent record keeping. Research support for our activities came from a Gabrielle's Angel Foundation grant, an Alex' Lemonade Stand Foundation Innovator Award, the NIH/NCI (R01 CA187318), and the NIH/NHLBI (R01 HL120724) (all to J.P.R.).

#### **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.

Received: July 31, 2019 Revised: November 15, 2019 Accepted: January 16, 2020 Published: March 6, 2020

### REFERENCES

Bassik, M.C., Lebbink, R.J., Churchman, L.S., Ingolia, N.T., Patena, W., LeProust, E.M., Schuldiner, M., Weissman, J.S., and McManus, M.T. (2009). Rapid creation and quantitative monitoring of high coverage shRNA libraries. Nat Methods *6*, 443–445. Mues, M., Karra, L., Romero-Moya, D., Wandler, A., Hangauer, M.J., Ksionda, O., Thus, Y., Lindenbergh, M., Shannon, K., Mcmanus, M.T., and Roose, J.P. (2019). High-complexity shRNA libraries and PI3 kinase inhibition in cancer: high-fidelity synthetic lethality predictions. Cell Rep. 27, 631–647.e5.

## CellPress