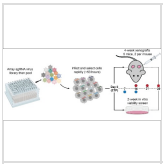

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In vivo Nanopool: Pooled sgRNA competition assays to evaluate in vivo cancer dependencies

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1 *Works for me* This protocol is published without a DOI.

Cancer Dependency Map Target Validation
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ABSTRACT

sgRNA competition assays with constitutive Cas9 systems allow for rapid de-risking of targets in a more representative in vivo microenvironment. Though not a full in vivo validation, the assay is useful as an “invalidation” strategy to discern artifacts of in vitro cultre from promising bona fide targets. This document describes a protocol to select an appropriate cell line, design and generate an sgRNA library for targets of interest, and perform parallel in vitro/in vivo experiments to assess in vivo dependency of the selected genes.

PROTOCOL CITATION

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GUIDELINES

Small library design guidelines:

We design a small library of guides (100-200 sgRNAs) to include a standard set of positive controls, negative controls, experimental guides, and genes that are known dependencies for that cell line as benchmarks. When designing a small sgRNA library, we aim for about 50% negative control sgRNAs to ensure enough viable cells to initiate tumors and persist during dropout. We also prefer to use two unique sgRNA sequences per gene that have been confirmed to inactivate the gene of interest, when possible. We include both “cutting” and “non-cutting” controls as negative controls. Non-cutting controls target genes not found in the human genome such as LacZ, and should have no effect, while cutting controls target genes in safe regions of the genome to control for the effect of DNA damage from double-strand breaks associated with Cas9 nuclease activity. For example, sgChr2 is a cutting control that targets a ‘gene desert’ region on the least copy-number altered chromosome across cancer, chromosome 2. Positive controls should make up approximately 20% of the guide library and should target common essential genes with very strong viability effects involved in essential cellular processes. We often use sgRNAs targeting POLR2D, a subunit of RNA polymerase II, KIF11, a microtubule motor gene that plays an essential role in mitosis, and SF3B1, a pre-mRNA splicing factor. The remainder of the library should be made up of experimental guides targeting the gene of interest, related genes, or known cancer targets as benchmarks.

MATERIALS

NAME	CATALOG #	VENDOR
Taq DNA Polymerase with Standard Taq Buffer - 400 units	M0273S	New England Biolabs
QIAgen DNeasy Blood and Tissue Kit, 50 rxn	69504	Qiagen
Qiagen Hi-Speed MidiPrep kit	12643	Qiagen
Phosphate Buffered Saline	28374	Thermo Fisher Scientific
Buffer ATL	19076	Qiagen
psPAX2	12260	addgene
Qubit		Invitrogen - Thermo Fisher
QIAamp DNA Mini kit		Qiagen
Cas9 2NLS nuclease (S. pyogenes)	Cas9 2NLS nuclease	Synthego
Surgical scissors		
Caliper		
Scale		
Illumina P5 and P7 primers 5uM combined; P5: 5' AATGATACGGCGACCACCGAGATCT P7: 5' CAAGCAGAAGACGGCATACGAGAT		
QIAquick PCR purification kit		Qiagen
Fetal Bovine Serum, qualified, heat inactivated, USDA-approved regions	10438018	Thermo Fisher
RPMI 1640 Medium	21875042	Thermo Fisher
Safety Scalpels, Scalpel No. 20	22079720	Thermo Fisher
Opti-MEM™ Reduced Serum Medium, powder	22600050	Thermo Fisher
Puromycin Dihydrochloride	A1113802	Thermo Fisher
Blasticidin S HCl (10 mg/mL)	A1113903	Thermo Fisher
TransIT®-LT1 Transfection Reagent	MIR 2304	Mirus Bio
Polybrene	32160801	Sigma Aldrich
VSV.G Plasmid #14888	14888	addgene
Chr2_2 CRISPR cutting control	GGTGTGCGTATGAAGCAGTG	
AAVS1	AGGGAGACATCCGTCGGAGA	
SF3B1 Positive control	AAGGGTATCCGCCAACACAG	
KIF11 Positive control	CAGTATAGACACCACAGTTG	
POLR2D Positive control	AGAGACTGCTGAGGAGTCCA	

Develop cell line model 4w

1 Choose dependent cell lines using the Cancer Dependency Map portal. Search the literature to determine whether the cell lines proliferate subcutaenously *in vivo.*

2 Plate and infect cell lines of interest to assess Cas9 expression. 4d

2.1 Plate 400,000 cells per well in 6-well plate.

2.2 Infect with 100µL Cas9 nuclease lentivirus and add 4µg/mL polybrene. Add RPMI with 10% FBS (R10) to a final volume of 2mL. 1d

2.3 Lift cells the next day and split into two 6-well plates per cell line. Select one with R10 + 10µg/mL blasticidin (10B). We use high concentrations of blasticidin to select for high levels of expression of the integrated transgenes. 2d

2.4 Calculate infection efficiency by lifting all cells, counting live cells with a ViCell Coulter counter, and dividing counts in plus puro (selected) by minus puro wells. No Infection Control (NIC, 0µL virus) should be close to zero to pass quality control check.

2.5 Expand selected (plus puro) cells and maintain 10B in media. Also expand unselected NIC as a negative control. 3d

2.6 Collect pellets from both conditions and rinse twice in cold PBS. Run a western to confirm robust Cas9 expression. As Cas9 is a large protein, it transfers best by wet transfer protocols.

3 Expand Cas9-expressing cells for *in vivo* model development in potential dependent cell lines. The exact cell counts will vary according to cell line growth rate. Standard subcutaneous tumor sizes are 4-10 million cells per 100-200 µL injected.

4 Initiate 2 subcutaneous tumors bilaterally per mouse at variable tumor sizes. Record mouse weight and tumor size every 3 days. 3w

5 Euthenize mice at humane endpoint. Note tumor latency and initial cell count that leads to tumor growth for 3-4 weeks.

6 Choose cell line with strong dependency, expression of Cas9, and robust *in vivo* growth kinetics to carry forward to next steps.

Generate and quality control small library of guides 2w

7 Design library of single guide RNAs (sgRNA). See "Guidelines" section for library considerations.

8 Prepare plasmid for each sgRNA and accurately quantify DNA concentration following midi- or maxiprep. Accurate quantification of the plasmid virus is essential to achieve equal representation in the pool. We recommend using fluorescent DNA quantification (like Qubit Broad Range Assay) instead of UV-based spectroscopy (like NanoDrop).

9 Prepare lentivirus in arrayed format using HEK293T cells. Pool to generate about 75mL of virus. Freeze down 4 x 10mL aliquots for future *in vivo* experiment and the rest in 1mL aliquots at -80C. 3d

10 Quality control and titrate the pooled virus by infecting cell line of interest.

10.1 Thaw one aliquot to infect one 6-well plate per cell line of interest with a range of viral titers along with 4µg/mL polybrene. Aim for 500x representation, or 500 cells per guide, to ensure adequate representation. We suggest 0µL, 25µL, 50µL, 100µL, 500µL of pooled virus. 1d

10.2 The next day, split each well into 2 equivalent wells of a plus selection and minus selection 6-well plate. Add RPMI + 2µg/mL puromycin to plus selection plate, normal RPMI to the other. 2d

10.3 After two days, calculate infection efficiency by lifting all cells, dividing cell counts in plus puro

(selected) by minus puro wells. Determine the viral titer at which infection efficiency is 30-50%. At these infection percentages, each cell is likely infected with only one sgRNA. Pellet two or more replicates of cells at 500 times the number of guides in the pool.

11 Extract gDNA from cell lines using QIAGEN DNA Mini Kit.

12 PCR-amplify 250 ng (100ng minimum - 10µg maximum) gDNA in a 100µL reaction. Lower concentrations of gDNA will benefit from additional cycles, up to 32. P5 and P7 primers amplify barcode regions for Illumina sequencing.

12.1 Per reaction, assemble the following on ice: 10µL 10x reaction buffer, 8µL dNTP, 0.5µL P5 primer and P7 primer (100µM), 1.5µL Takara ExTaq polymerase (Clontech). Up to 100µL with water.

12.2 Run PCR with following thermocycler conditions: (i) 95°C for 1 minute, (ii) 95°C for 30s, (iii) 58°C for 30s, (iv) 72°C for 30s. (v) Repeat steps ii-iv for 28 cycles. (vi) 72°C, 10 minutes, (vii) 4°C hold.

12.3 For troubleshooting, try a range of annealing temperatures 54-62°C, more cycles, or a range of input gDNA concentrations.

13 Run 10µL of 100µL PCR product on DNA gel to confirm amplification. Visualize by agarose gel electrophoresis, looking for crisp single band of about 400 nucleotides.

13.1 Clean up PCR products with QIAGEN PCR Purification kit, elute into 50µL water or EB.

14 Submit DNA for CRISPR Illumina sequencing.

15 Analyze data from read counts.

15.1 Extract read counts from Fastq files using open source MaGECK software.

15.2 Analyze representation of each guide in the pool, checking for representation of about 50% negative control guides, 20% positive controls, and 30% experimental guides. This will prevent excessive tumor regression early in the *in vivo* arm.

15.3 If the library passes this quality control check, proceed to the next section with the infected cell line of interest.

Parallel in vitro/in vivo experiments 3d

16 Spinfect cells following Broad's standard protocol. This puromycin optimization step requires about 30 million cells of interest, confirmed to be mycoplasma-free. Puromycin concentration optimization is designed to minimize time in 3d

tissue culture before inoculation, as the constitutive CRISPR/Cas9 system will start cutting DNA immediately upon infection with lentiviral guides.

16.1 After expansion and spinfection in 6-well plates, add 2mL R10 plus 0µg/mL (0P), 4µg/mL (4P), or 8µg/mL (8P) puromycin on top of existing 2mL R10. A total of five 6-well plates will be necessary from spinfection for 3 0P control plates harvested at 24, 48, and 72 hours from selection.

16.2 After 24 hours, visually inspect plates. When selection is complete, lift and assess infection efficiency by comparing cells counts of 2P and 4P to minus puro condition. Overselection is likely if infection efficiency is much lower for higher concentrations of puromycin at constant viral titer. Aim for the viral titer and puromycin concentration that confers 30-50% infection efficiency.

16.3 Calculate how many cells need to be infected to have enough for in vitro/in vivo experiments at this infection efficiency.

Infection efficiency and desired cell count will be known (for example, 7 million x 10 tumors + 30 million for in vitro = 100 million cells). Solve for total initial cells.

17 Expand cell line of interest expressing Cas9 to "total initial cells" value calculated above.

18 Lift and pool all cells together. Concentrate or dilute into 200 mL of media, along with 100µL polybrene and titrated amount of virus from **10.3**. We typically use about 20mL pooled virus. 1d

18.1 Distribute 3 million cells/2mL media in each well of 12-well plates (about four plates for 150 million cells) for infection.

18.2 Spinfect for 2 hours at 931g.

18.3 After spin, add 2 mL R10 and incubate for 4-6 hours.

18.4 Distribute into maximum-volume flasks or plates. For example, 30 x 15cm plates of 5 million cells and 12 mL media.

19 Add selection reagent at optimized concentration. To avoid disturbing cells, we add 3x concentrated stock, dissolved in medium, to the cells. For example, adding 6 mL of R10 + 18 ug/mL puromycin to 12 mL of medium yields 18mL media at a final concentration of 3 ug/mL. 1d

20 After 24 hours, start early time point pellet (ETP) collection, in vitro plating, and in vivo inoculation. 2w

20.1 Trypsinize and pool all cells together. Accurately count the number of cells via duplicate measurements using the Vicell.

20.2 Freeze two cell pellets of 1000x sgRNA representation for downstream determination of the “initial

representation" of each sgRNA.

20.3 Using standard tissue culture seeding densities, etc., passage duplicate "*in vitro* screens" at 1000x sgRNA representation, typically two T125 flasks.

Passage as needed and collect timepoints at days 7 and 14 from infection. For this *in vitro* arm, use plain R10 without antibiotics to mimic lack of selection markers *in vivo.*

20.4 Pellet 10 x desired cells/tumor from model development experiments. Should be in the range of 5-10 million cells. For example, 10 million cells per xenograft for slow-growing cell lines would require 100 million cells.

20.5 Resuspend in 500µL R10. Add 500µL matrigel. Keep 10 x 100µL aliquots on ice.

21 On the same day, inoculate mice with bilateral subcutaneous tumors. 4w

21.1 Check isoflurane levels and charcoal. Set up anaesthesia for tank and nose cone in vivarium procedure room.

21.2 Anaesthetize mice. One at a time, transfer mice to the nose cone in the hood and check depth of consciousness with a toe pinch.

21.3 Moving quickly, vortex cell/matrigel suspension and draw 100µL into syringe. Be careful to avoid bubbles and keep the cell/matrigel suspensions cold.

21.4 Inject on mouse flank, keeping bevel of the needle up and lifting skin with forceps to create a pocket. Repeat on opposite flank.

21.5 After injection, transfer mouse back to cage. Ensure mouse returns to alert and active consciousness.

21.6 Repeat for each of the 5 mice.

22 Complete health checks and tumor measurements every 3 days. 4w

22.1 Visually check health of the mice.

22.2 Weigh mice with scale, record on 'tumor-tracking' spreadsheet.

22.3 Measure width and length of tumors consistently using calipers.

23 To capture maximum guide dropout, collect *in vitro* pellets (2 x 7 million cells) on days 7 and 14. Time points should be adjusted for dependencies that confer viability effects at a different rate.

24 On days 14, 21 and 28, harvest tumors from randomly selected mouse and collect in vitro pellets:

24.1 Transfer all mice except the mouse to be sacked into a new cage.

24.2 Sack mouse in home cage using CO_2, verify with cervical dislocation.

24.3 Harvest both tumours, removing as much skin as possible. Dispose of carcass appropriately.

24.4 Weigh and wash tumors in PBS.

24.5 Flash freeze tissue sample in Liquid N2 until ready to isolate genomic DNA for sequencing.

25 Homogenize all tumor tissue samples by mincing then lysing overnight in ATL buffer

26 Repeat steps **11-13** to extract gDNA from each tissue and pellet sample using QIAGEN DNEasy Blood and Tissue kit, amplify barcode, and submit samples fro sequencing.

27 Repeat steps **14-15** to submit samples then use MAGeCK to extract read counts from Fastq files.

28 Suggested normalization steps to determine maximum efficacy/acceptable leakiness include:

28.1 Normalize read counts to total “mapped” counts.

28.2 Assess leakiness by normalizing “no dox” to early time point counts.

28.3 Assess efficacy by normalizing “dox” to “dox” negative control counts at each time point.

29