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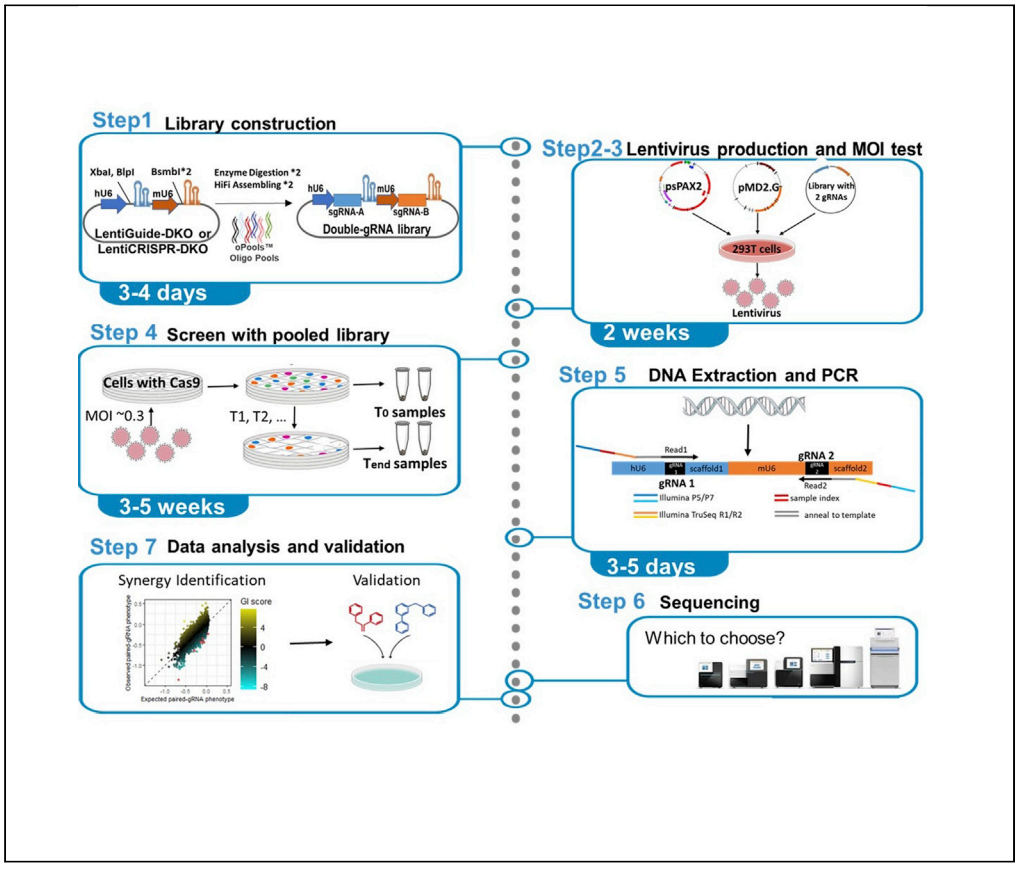
Protocol

Generation of dual-gRNA library for combinatorial CRISPR screening of synthetic lethal gene pairs



Combinatorial CRISPR screening is useful for investigating synthetic lethality (SL) gene pairs. Here, we detail the steps for dual-gRNA library construction, with the introduction of two backbones, LentiGuide_DKO and LentiCRISPR_DKO. We describe steps for *in vitro* screening with 22Rv1-Cas9 and SaOS2-Cas9 cells followed by sequencing and data analysis. By introducing two backbones, we optimized the library construction process, facilitated standard pair-end sequencing, and provided options of screening on cells with or without modification of Cas9 expression.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Shan Tang, Xue Wu, Jinghui Liu, ..., Fuhai Li, Lijun Cheng, Lang Li

shan.tang@osumc.edu (S.T.)
lang.li@osumc.edu (L.L.)

Highlights

Optimized library construction process by introducing two backbones

In vitro screening gene-gene interaction w/wo Cas9

Full screening process from gene selection to data analysis

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Generation of dual-gRNA library for combinatorial CRISPR screening of synthetic lethal gene pairs

Shan Tang,[1,5,*] Xue Wu,[2] Jinghui Liu,[3] Qiongsi Zhang,[3] Xinyi Wang,[3] Shuai Shao,[1] Birkan Gokbag,[2] Kunjie Fan,[2] Xiaoqi Liu,[3] Fuhai Li,[4] Lijun Cheng,[2] and Lang Li[2,6,*]

[1]College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

[2]Department of Biomedical Informatics, College of Medicine, The Ohio State University, Columbus, OH 43210, USA

[3]Department of Toxicology and Cancer Biology, University of Kentucky, Lexington, KY 40536, USA

[4]Institute for Informatics and Department of Pediatrics, Washington University in St. Louis, St. Louis, MO 63110, USA

[5]Technical contact

[6]Lead contact

*Correspondence: shan.tang@osumc.edu (S.T.), lang.li@osumc.edu (L.L.)
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SUMMARY

Combinatorial CRISPR screening is useful for investigating synthetic lethality (SL) gene pairs. Here, we detail the steps for dual-gRNA library construction, with the introduction of two backbones, LentiGuide_DKO and LentiCRISPR_DKO. We describe steps for *in vitro* screening with 22Rv1-Cas9 and SaOS2-Cas9 cells followed by sequencing and data analysis. By introducing two backbones, we optimized the library construction process, facilitated standard pair-end sequencing, and provided options of screening on cells with or without modification of Cas9 expression.

BEFORE YOU BEGIN

An overview of workflow chart can be found in Figure 1. Following this protocol, pooled library of 62,500 dual-gRNA combinations for targeting more than 1,000 gene pairs was successfully generated. This library was then applied to high-throughput combinatorial CRISPR screen on prostate cancer cell line 22Rv1-Cas9 and osteosarcoma cell line SaOS2-Cas9, to identify synergistic gene pairs that inhibited cancer cell growth when they were disrupted simultaneously.

In this study, we generated two backbone vectors, LentiGuide_DKO and LentiCRISPR_DKO (DKO refers to double knockout). The only difference between these two vectors is the additional Cas9-expressing sequence driven by EF-1-alpha promoter in LentiCRISPR_DKO. Cas9 is a bacterial protein and is not expressed by eukaryotic cells. Genetic screens in human cell lines with CRISPR technology often start with creation of cell line stably expressing Cas9, or using lentiviral backbone containing Cas9. With the built-in Cas9 expressing cassette, LentiCRISPR_DKO opens the option to perform the combinatorial CRISPR screen using cells that are not engineered to express Cas9.

In LentiGuide_DKO (Figure 2), two different small RNA promoters (hU6 and mU6) and two versions of gRNA scaffolds were built into one plasmid. After sequentially inserting two sets of sgRNA, the simultaneously functional knockout (KO) of two genes can be achieved with the presence of Cas9 protein. The introduction of two different sgRNA scaffolds lifted the constraint of using customized Illumina sequencing that is required by currently available combinatorial CRISPR screening method ((Han et al., 2017)). The base sequence difference between two scaffolds allows the specific binding of PCR-2R to the beginning of second scaffold, which makes the second gRNA insertion within the sequencing length (Figure 3). Both versions of scaffold have been used extensively in single sgRNA CRISPR screens (Luo et al., 2008; Hart et al., 2017; Gilbert et al., 2014).

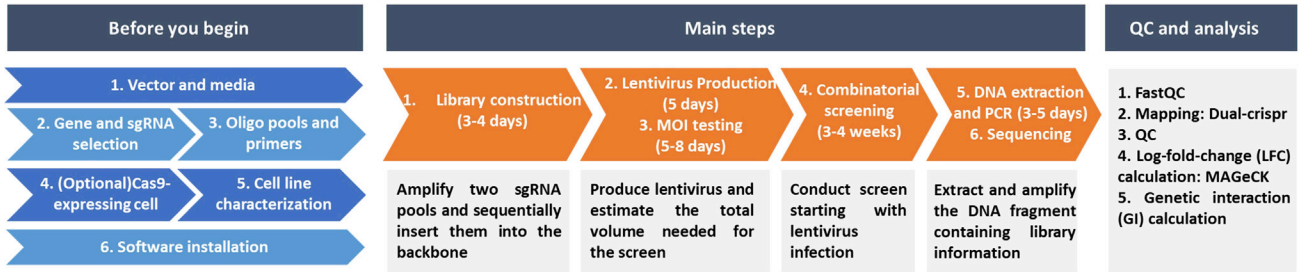

Figure 1. Protocol flow chart

This backbone design has been validated in two cell lines (C4-2-Cas9 and 22Rv1-Cas9) with dual-target of AR and CK1. The western blot result showed that both the expression of AR and CK1 were inhibited after functional KO of AR and CK1 using the LentiGuide_DKO plasmid. This result also validated the same performance of the two scaffolds in the newly-built backbone regardless of their differences in the sequence and structure.

Preparation one: Vector and media

Timing: 1–3 days

1. Purchase vector from Addgene: Addgene: LentiGuide_DKO (Plasmid #183193), or LentiCRISPR_DKO (Plasmid #183192). 2 μg of the backbone vector is needed in enzyme digestion to start the library construction (step 2).
2. Ensure all the media are prepared. A detailed list can be found in the sections of key resources table, materials and equipment.

Preparation two: Gene and sgRNA selection

A guideline for choosing gene and sgRNA to prepare for the library construction is provided here. Please refer to Table 1 for the summary of the experiment details of the previous combinational CRISPR screens from several groups.

3. Estimate library size and cell culture scale: The size of library is constrained by the cell culture limit; therefore, estimating the library size is critical before the experiment. For example, under the setting of library with 62,500 dual-gRNA constructions, ~0.3 MOI, triplicates for each time point and at least 200 cell coverages for each gRNA-gRNA construction during screening, the corresponding pooled dual-gRNA library size is 62,500 and at least 2.5 × 10^8 (62,500*200*6/0.3) cells will be needed at the time of infection.

   Note: There are basically two scenarios when "coverage" is used in this protocol. One is the coverage when constructing the dual-gRNA pool library (refer as library coverage); another is the coverage when conducting screening (refer as cell coverage). For a library with 62,500 constructions, a total number of 6.25 × 10^6 colonies are required to reach 100 library coverage. Later in the screen, at least 1.25 × 10^7 cells each sample are needed for 200 cell coverage.

4. Gene selection: The gene selection is mainly based on experiment objective and library size. Several commonly used selection criteria are listed below:
   a. Exclude the genes that its loss of function alone has detrimental effect on cell growth. The gene essentiality information can be obtained from previous screen or DepMap database (Broad, 2021). Note that gene essentiality is cancer type and cell-line specific.
   b. Prioritize the genes that have been validated as drug targets.
   c. Focus the genes from pathways of interest.

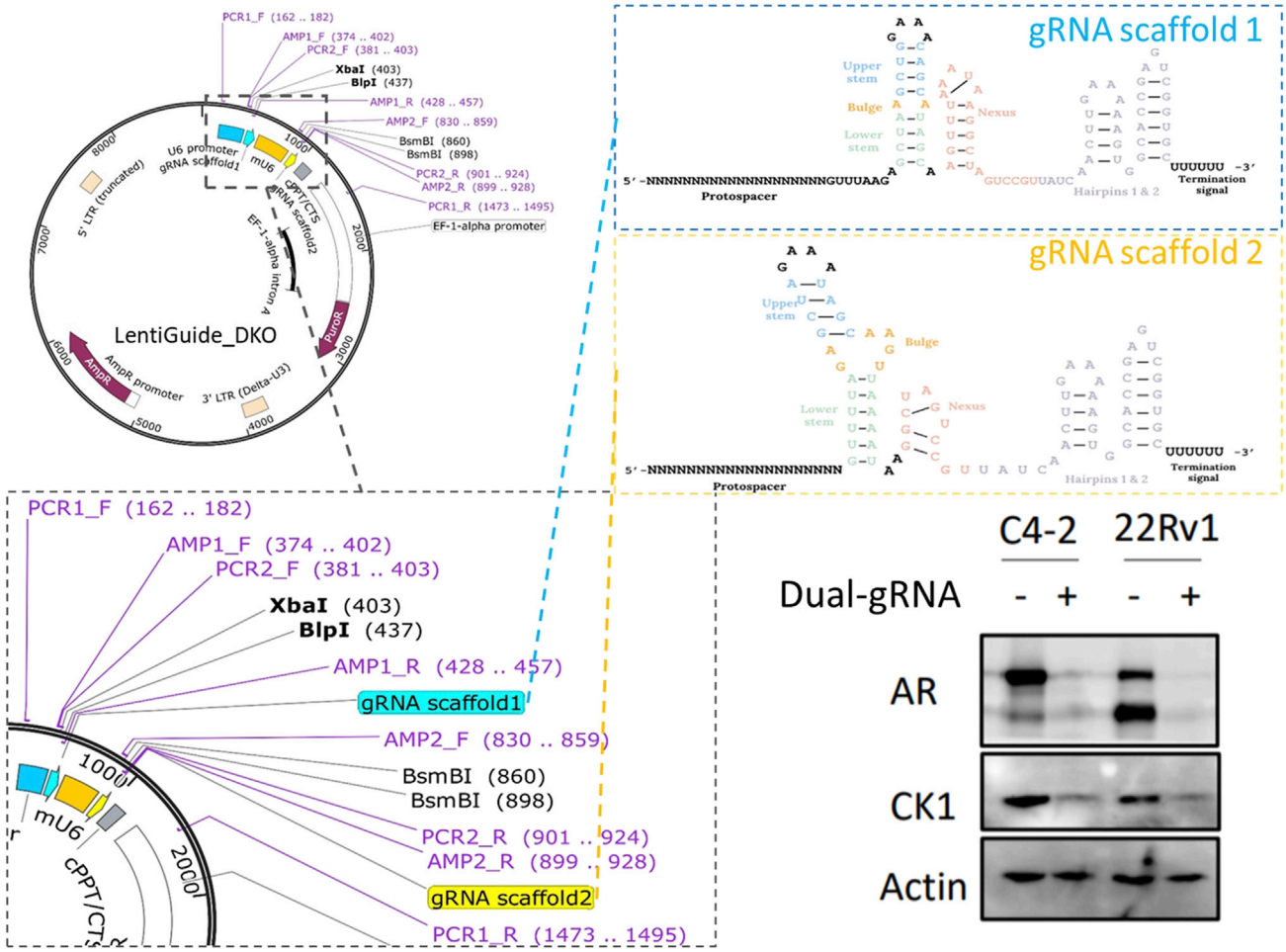

Figure 2. Backbone design and KO efficiency validation

d. Include 2–4 gene pairs with known synthetic lethality as positive controls.

In this study, we selected 56 genes that can be targeted by available drugs (Drug Bank (Wishart et al., 2018), DepMap) and have low essentiality scores (DepMap). Among them, HDAC1-WEE1, HDAC2-WEE1 and KDR-KDM6B serve as the positive controls (Yu et al., 2015; Wang et al., 2020).

5. sgRNA selection: typically 3 sgRNAs are required for each gene to ensure the quality of the screening. The tools for CRISPR/Cas9 sgRNA design were summarized by several groups (Cui et al., 2018; Doench et al., 2016; Liu et al., 2020).

The genes, sgRNAs and corresponding sources for generating our library are listed in the Table S1. In the project GitHub page, the sgRNA information and R code for information integration are provided under the sgRNA_info folder. In our library, 4 corresponding sgRNAs were selected for each gene with reference to available sgRNA sequence from current commonly-used CRISPR libraries, including TKOv3 (Mair et al., 2019), hGECKOv2 (Sanjana et al., 2014) and KinomeKO /Brunello(-Doench et al., 2016), as well as their VBC (Vienna Bioactivity CRISPR) scores (Michlits et al., 2020). We prioritize the gRNAs that have overlapping on the abovementioned libraries. Additionally, another 26 safe gRNAs that target non-functional parts of genome were added into the pooled library as negative controls.

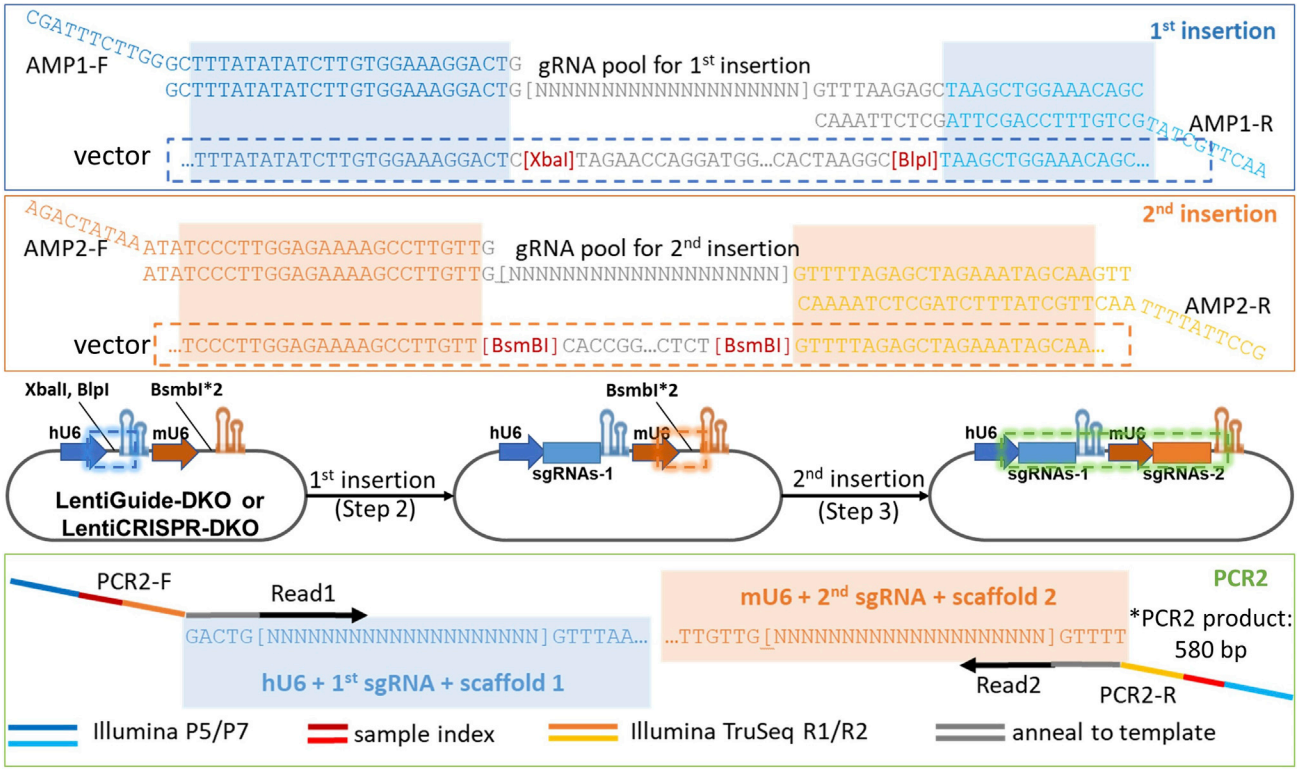

Figure 3. Overall library construction process and gRNA pools/primer design

⚠ CRITICAL: Make sure to EXCLUDE the gRNAs contain the three restriction sites (XbaI, BlpI, BsmBI) used in the library construction.

Preparation three: Design oligo pools and primers

During the library construction process, the DNA fragments of digested vector and amplified sgRNA pools with 20-bp overlap at both ends can be ligated by HiFi DNA assembly. Two sequential HiFi DNA assemblies generate the library containing two sgRNAs. The designing for oligo pools and primers can be found in Figure 3.

Note: An extra G is added to the 5′-end of gRNA, since human promoter (hU6) prefers a 'G' at the transcription start site to have high expression, though it is possible for the plasmid to still express without the G (Wang et al., 2019).

Note: We recommend using IDT Ultramer™ DNA oligonucleotides for PCR2 primer.

6. Order oligo pools from commercial site with added 5′-flank and 3′-flank sequence to the gRNA insertion. For reference, we bought the oligo pools from oPools Oligo Pools (10 pmol/oligo) from IDT.

	5′-flank	3′-flank
gRNA pool for 1st insertion	AAGTATCCCTTGGAGAACCACCTTG	GTTTAAGAGCTAAGCTGGAAACAGC
gRNA pool for 2nd insertion	TATCTTGTGGAAAGGACGAAACACC	GTTTTAGAGCTAGAAATAGCAAGTT

7. The primers are listed in the table below:

Primers	Sequence	Note
AMP1 _F	CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACTG	Amplification of 1st set of gRNA insertion (step 1)
AMP1 _R	AACTTGCTATGCTGTTTCCAGCTTAGCTCTTAAAC	
AMP2 _F	AGACTATAAATATCCCTTGGAGAAAAGCCTTGTT	Amplification of 2nd set of gRNA insertion (step 1)
AMP2 _R	GCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	
PCR1_F*	GAGGGCCTATTTCCCATGATT	1st round of the PCR after DNA extraction (step 20)
PCR1_R	GTTGCGAAAAAGAACGTTCACGG	

* PCR1_F can be used in Sanger sequencing in step2 – optional.

PCR2_F:

AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTCTTTCCCTACACGACGCTCTT CCGATCTTTATATATCTTGTGGAAAGGACTG.

PCR2_R:

CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCTTAACTTGCTATTTCTAGCTCTAAA.

Annotation: Illumina P5/P7, sample index (i5/i7 index), Illumina TruSeq R1/R2, anneal to template (22 bp). The i5/i7 indexes are listed in the table below:

i7 index name	i7 sequence	i7 index for sample sheet	i5 index name	i5 sequence	-i5 index for sample sheet
D701	CGAGTAAT	ATTACTCG	D501	TATAGCCT	TATAGCCT
D702	TCTCCGGA	TCCGGAGA	D502	ATAGAGGC	ATAGAGGC
D704	GGAATCTC	GAGATTCC	D503	CCTATCCT	CCTATCCT
D705	TTCTGAAT	ATTCAGAA	D504	GGCTCTGA	GGCTCTGA
D706	ACGAATTC	GAATTCGT	D505	AGGCGAAG	AGGCGAAG
D707	AGCTTCAG	CTGAAGCT	D506	TAATCTTA	TAATCTTA

Note: The number of PCR2 primer pairs should be equal to the total number of screen samples, and each sample should have a different i5/i7 index combination.

Preparation four: Cas9-expressing cell line generation

Timing: 1–2 weeks

Note: You may skip this step if you choose to use lentiCRISPR-DKO vector.

8. Please refer to Sanjana et al. (Sanjana et al., 2014) for generating the Cas9-expressing cell lines.

Preparation five: Cell line characterization

Timing: 1–2 weeks

9. Ensure cell line is mycoplasma free before starting screen.
10. Determine optimal cell plating density and passage number, typically in 15 cm plates. Measure and record the approximate cell doubling time.
11. Determine puromycin and polybrene (up to 8 μg/mL)/protamine sulfate (up to 5 μg/mL) sensitivity of cell lines. Concentration of puromycin to be used in screen should be 0.5–1 μg/mL higher than that required to kill all uninfected cells in at least 48 h. The antibiotic selection time in the main step 14 should be the same here.

Table 1. Current SL research using combinatorial CRISPR technology

Studies	Gene & sgRNA selection criteria	# of genes, control (ctrl)/safe gRNA and gRNA pairs	Coverages		Screen details (Cell line, MOI, etc.)	Harvest time[a]
			Library	Cell		
Wong et al., 2016	Epigenetics regulation	50 genes (3 gRNAs per gene), 3 ctrl gRNAs, 23,409 gRNA pairs	NA	300	OVCAR8-ADR-Cas9; MOI 0.3-0.5	Day 15, 20
(Han et al., 2017)	Non-essential and expressed druggable gene targets (previous screen and DrugBank)	207 genes (3 gRNAs per gene), 79 safe gRNAs, 21,321gRNA pairs	NA	1000	K562-Cas9; 2 replicates	Day 0, 14 post-selection
(Shen et al., 2017)	validated oncogenes, tumor-suppressor genes and cancer-relevant drug targets	73 genes (3 gRNAs per gene), 12 non-target gRNAs,141,912 gRNA pairs	100 (in LB medium)	200	HeLa-Cas9, 293T-Cas9, A549-Cas9; 2 replicates; MOI 0.1-0.4	Days 3, 14, 21, 28 post- infection
(Horlbeck et al., 2018)	Non-essential genes (CRISPRi v1 growth screen)	472 genes (1-3 gRNAs per gene), 18 ctrl gRNAs , 1,044,484 gRNA pairs	25	500 (initial 250)	K562-Cas9, Jurkat-Cas9; 2 replicates;	Day 0, ~10 doubling
(Zhao et al., 2018)	Genes represent glycolysis and the pentose phosphate pathway (PPP)	51 genes,11,475 gRNA pairs	50	80	HeLa-Cas9, A549-Cas9; 2 replicates; MOI 0.1-0.3	Days 3, 14, 21, 28 post-infection
(Diehl et al., 2021)	Autophagy pathway	160 genes, 247,032 gRNA pairs	NA	20, 200	RPE1-Cas9; triplicates; MOI 0.5	Days 2, 14 post-infection,
(Ito et al., 2021)	Paralog families	3,284 genes(6 gRNAs per gene), 110,874 gRNA pairs	NA	750-1000	11 cancer cell lines with Cas9; triplicates; MOI 0.3-0.5	NA
(Parrish et al., 2021)	Paralog and singleton essentiality	2,060 gene pairs, 33,170 gRNA pairs	1000	500	PC9-Cas9; triplicates; MOI 0.3	Plasmid pool, ~12 doubling
(Thompson et al., 2021)	Paralog families and SL partners from previous studies	1,191 gene pairs (3-5 gRNAs per gene), 41,838 gRNA pairs	NA	1000	RPE1-Cas9, A375-Cas9, MeWo-Cas9; triplicates; MOI 0.3	Days 7, 14, 28
This study	Genes from cell death pathways specified for prostate cancer; targetable genes for osteosarcoma	1,225 gene pairs (4 gRNAs per gene, 62,500 gRNA pairs)	10	200	22RV1-Cas9; SaOS2-Cas9; MOI 0.3	Day 0, 12 or 14 post-selection

[a]The sample harvest time is measured differently among different studies. For example, Han et al. harvested the samples at post-infection and puromycin selection day 0 and day 14. No info is presented if the time measurement is not specified in the original paper (such as the research done by Wong et al., 2016).

Preparation six: Software installation

Timing: 0.5 h

12. Mali-dual-crispr-pipeline: This pipeline is provided through conda and can be installed on any linux-64 or osx-64 platform (Windows installation is not available at this time). A detailed installation process can be found at https://github.com/ucsd-ccbb/mali-dual-crispr-pipeline.
13. MAGeCK: MAGeCK and associated software offer a range of functions and can be installed by conda/bioconda, Docker Image, or source code. We used MAGeCK RRA in the log-fold-change calculation. A detailed introduction and installation may be found at https://sourceforge.net/projects/mageck/.
14. R and R-Studio.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
psPAX2	Didier Trono	AddGene #12260
pMD2.G	Didier Trono	AddGene #12259
Endura Electro Competent Cells	Thermo Fisher Scientific/Lucigen	501047945

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Bovine Serum Albumin	Sigma-Aldrich	A9418
Polybrene Infection / Transfection Reagent	Sigma-Aldrich	TR-1003-G
Puromycin	Thermo Fisher Scientific	A1113803
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	11965118
GlutaMAX Supplement	Thermo Fisher Scientific	35050061
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific	11360070
Pen-Strep(5,000 U/mL)	Thermo Fisher Scientific	15070063
HEPES (1 M)	Thermo Fisher Scientific	15630080
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	31985070
Fetal Bovine Serum (FBS)	VWR/Avantor	97068-091
EZ-VISION Blue-Light DNA Dye	VWR	10791-798
XbaI	New England Biolabs	R0145S
BlpI	New England Biolabs	R0585S
BsmBI-v2	New England Biolabs	R0739S
X-tremeGENE™ 9 DNA Transfection Reagent	Roche/Sigma	6365787001
Critical commercial assays		
NEBNext Ultra II Q5 Master Mix	New England Biolabs	M0544L
NEBuilder HiFi DNA Assembly Cloning Kit	New England Biolabs	E5520S
QIAquick Gel Extraction Kit (50)	QIAGEN	28704
HiSpeed Plasmid Maxi Kit	QIAGEN	12663
QIAprep Spin Miniprep Kit	QIAGEN	27106X4
JetQuick Blood and Cell Culture DNA Maxiprep Kit	Thermo Fisher Scientific	A30706
Deposited data		
Sampler data and data analysis	Lang Li Lab (this paper)	https://github.com/Shantang3/Combinatorial-CRISPR-Screen-Anaylysis-Pipeline
Experimental models: Cell lines		
22RV1 with Cas9 expression	Xiaoqi Liu Lab	
SaOS2 with Cas9 expression	Lang Li Lab	
Oligonucleotides		
oPools Oligo Pools	This paper	IDT
PCR Primers	This paper	Thermo Fisher, A15612
Ultramer™ DNA oligonucleotides	This paper	IDT
Recombinant DNA		
LentiGuide_DKO	Lang Li Lab (this paper)	Addgene #183193
LentiCRISPR_DKO	Lang Li Lab (this paper)	Addgene #183192
LentiGuide_DKO based library	Lang Li Lab (this paper)	
Software and algorithms		
dual_crispr	Amanda Birmingham, CCBB, UCSD	https://github.com/ucsd-ccbb/mali-dual-crispr-pipeline
MAGeCK	Wei Li, et al.	https://sourceforge.net/projects/mageck/
R (version at least 4.0.3)		https://www.r-project.org/
R Studio		https://www.rstudio.com/
QC and Hit Identification	This paper	https://github.com/Shantang3/Combinatorial-CRISPR-Screen-Anaylysis-Pipeline
Other		
Gene Pulser/MicroPulser Electroporation Cuvettes, 0.2 cm gap	Bio-Rad	1652086
Petri Dishes with Clear Lid, 150 × 15 MM,100/CS	Thermo Fisher Scientific	FB0875714
Cell Culture Dish	VWR	10062-882
Plate Tissue Culture 6 Wells ST	VWR	10062-892

MATERIALS AND EQUIPMENT

Cell growth media		
Reagent	Final concentration	Amount
DMEM	N/A	500 mL
FBS	N/A	50 mL
GlutaMAX Supplement	N/A	5 mL
Sodium Pyruvate (100 mM)	~1 mM	5 mL
Pen-Strep(5,000 U/mL)	N/A	5 mL
HEPES (1 M)	~ 10 nM	5 mL
Total	**N/A**	**570 mL**
Store at 4°C up to two months.		

None-antibiotics media		
Reagent	Final concentration	Amount
DMEM	N/A	500 mL
FBS	10%	50 mL
GlutaMAX Supplement	N/A	5 mL
Sodium Pyruvate (100 mM)	~1 mM	5 mL
HEPES (1 M)	~ 10 nM	5 mL
Total	**N/A**	**565 mL**
Store at 4°C up to two months.		

Viral harvest media- Serum-Free, High-BSA 293T growth media		
Reagent	Final concentration	Amount
DMEM	N/A	500 mL
BSA	1.1 g/100 mL	5.5 g
GlutaMAX Supplement	N/A	5 mL
Sodium Pyruvate (100 mM)	~1 mM	5 mL
Pen-Strep (5,000 U/mL)	50 U/mL	5 mL
Total	**N/A**	**515 mL**
Store at 4°C up to two months.		

STEP-BY-STEP METHOD DETAILS

Part 1: Library construction & amplification

Timing: 3–4 days

This part generates the customized dual-gRNA screening library that contains 2 promoters (hU6 and mU6), 2 gRNAs, and 2 scaffolds of different versions within one vector. Briefly, the amplified gRNA pool inserts and digested vectors were ligated via their compatible ends.

Note: Refer to troubleshooting 1 for reducing recombination and troubleshooting 2 to improve efficiency.

1. (Day 1) Oligo pool amplification: assemble two PCR reactions for amplifying two gRNA pools separately on ice using corresponding primers, then follow the cycling conditions for each PCR reaction.

PCR reaction master mix: amplification of gRNA pool for 1st insertion	
Reagent	Amount
gRNA pool for 1st insertion (100 nM)	5 μL
Primer AMP1 _F (10 μM)	2.5 μL
Primer AMP1 _R (10 μM)	2.5 μL
2× NEBNext Mltra II Q5 Master Mix	25 μL
ddH_2O	15 μL

PCR reaction master mix: amplification of gRNA pool for 2nd insertion	
Reagent	Amount
gRNA pool for 2nd insertion (100 nM)	5 μL
Primer AMP2 _F (10 μM)	2.5 μL
Primer AMP2 _R (10 μM)	2.5 μL
2× NEBNext Mltra II Q5 Master Mix	25 μL
ddH_2O	15 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	15 cycles
Annealing	66°C	30 s	
Extension	72°C	10 min	
Final extension	72°C	4 min	1
Hold	4°C	Forever	

After PCR amplification, run out the reaction on an EZ-VISION Blue-Light DNA Dye-stained 1% agarose gel. Visualize and cut the DNA fragment at ~90 bp, then extract DNA using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions, elute in 20 μL ddH_2O.

⚠ CRITICAL: Elute the amplified gRNA and digested vector in ddH_2O to avoid introduction of the excess salt, which might influence the electroporation in later step.

Note: A guide line about the agarose gel concentration selection and optimization could be found at https://www.qiagen.com/us/knowledge-and-support/knowledge-hub/bench-guide/dna/analysing-dna/dna-analysis-using-analytical-gels.

2. (Day 1–2) Insert first set of gRNAs. Troubleshooting 1 and troubleshooting 2.
   a. Vector linearization (could be done in parallel to step 1): assemble the following reaction on ice and incubate at 37°C for 1 h.

Enzyme digestion reaction master mix	
Reagent	Amount
Vector: LentiGuide_DKO	2 μg
XbaI	1 μL
BlpI	1 μL
10× NEBuffer CutSmart	5 μL
ddH_2O	To 50 μL

Then run out the sample on an EZ-VISION Blue-Light DNA Dye -stained 1% agarose gel. Extract linearized vector using QIAGEN Gel Extraction Kit, elute with 30 μL ddH_2O.

   b. HiFi assembling: the molar ratio between the fragments of linearized vector: insertion (gRNA pools) is optimized to 1:100 with 100 ng of linearized vector. NEBioCacμlator helps to convert dsDNA mass to moles of dsDNA (or vice versa): https://nebiocalculator.neb.com/#!/dsdnaamt.

Assemble the reaction on ice, incubate at 50°C for 60 min (use thermocycler), then proceed to transformation (step 1c) directly.

HiFi reaction master mix				
Reagent	Length	Mass	Molar	Amount
Linearized LentiGuide_DKO	8750 bp	100 ng	18.5 fmol	(Based on concentration)
1st gRNA pool insertion	90 bp	103 ng	1850 fmol	(Based on concentration)
2× HiFi DNA Assembly Master Mix	–	–	–	10 μL
ddH_2O	–	–	–	To 20 μL

c. (Afternoon) Transformation by electroporation:
   i. Calculate the number of reactions needed based on the library size and coverage. For example, we usually get around 25,000–30, 000 colonies per reaction. For a gRNA pool with 250 gRNA, 1–2 electroporation reactions are required to reach at least 100 coverages (250*100 = 25,000) for each gRNA.
   ii. Prepare MicroPulser Cuvettes (2 mm) on ice for at least 10 min. Set up the electroporator with conditions of voltage 3,000 V, Capacitance 25 μF, resistance 200 Ω, cuvette 2 mm.
   iii. Thaw Endura Electrocompetent cells on ice for 15 min.
   iv. For each reaction: add 1 μL of the HiFi reaction product to Endura Electrocompetent cells, mix them gently. Then transfer the mixture into MicroPulser Cuvettes, electroporate at the setting listed in step ii, add 975 of Recovery Medium ASAP.

⚠ CRITICAL: Use fresh HiFi product for the transformation each time.

⚠ CRITICAL: Quickly adding the Recovery Medium into the cuvette ASAP after electroporation is one key step. The electroporation efficiency will dramatically decrease if this step is delayed.

Note: One trial run is suggested to test how many colonies can grow for each transformation reaction. Then calculate the coverage and corresponding number of reactions needed.

   v. Transfer cells mixture to a bacterial culture tube with additional 1 mL recovery medium (or SOC medium). Shake the tubes for 1 h at 37°C in a shaking incubator.

d. Titer the library: Set up dilutions for counting colonies: set up serial dilution (1:10, 1:100, 1:1000,…) of the culture and plate onto 10 cm ampicillin LB agar plate. Count the colonies next morning to calculate the library coverage. The diluted plate is used to calculate the total colony number to ensure library coverage.

e. Plate the library: Spread the original culture onto pre-warmed 15 cm ampicillin LB agar plate (0.5 mL/plate), incubate at 30°C for approximate 18 h.

⚠ CRITICAL: 30°C incubation is intended to minimize recombination of the lentiviral plasmid library.

f. Harvest colonies and maxi-prep: Transfer 6 mL of LB+ ampicillin medium on one 15 cm plate, scrape the colonies off with a cell spreader. Then use another 6 mL of LB+ ampicillin medium to wash the plate. Collect all colonies and proceed to max-prep according to QIAGEN Plasmid Kit procedures.

Optional: Sanger Sequencing. To ensure the success of gRNA pool's insertion, pick several colonies from the plate (2d or 2e) to 4 mL LB culture with ampicillin. Shake the tubes for at least 8 h at 37°C. Use QIAGEN Mini Prep Kit to extract the plasmid and send it to Sanger Sequencing with primer of PCR1_F.

3. (Day 2–3) Insert second set of gRNAs: The process is similar to step 2 of inserting first set of gRNA pools. The only difference is the enzyme digestion setting.
   a. Vector linearization: assemble the following reaction on ice and incubate at 55°C for 1 h.

Enzyme digestion reaction master mix	
Reagent	Amount
Vector: LentiGuide_DKO+1st gRNA	2 μg
BsmbI_v2	1 μL
10× N NEBuffer r3.1	5 μL
ddH_2O	To 50 μL

Then run out the sample on an EZ-VISION Blue-Light DNA Dye -stained 1% agarose gel. Extract linearized DNA using QIAGEN Gel Extraction Kit, elute with 30 μL ddH_2O. PCR purification kit could be used on the enzyme digestion product to save time.

   b. Repeat HiFi assembling as steps 2b to 2f. Harvest colonies and max prep are the same as step2.

Note: Step 3 usually requires much more reactions to reach appropriate library coverage, considering the combination of 2 sgRNAs. For example, for two sets of gRNA pools with 250 different gRNAs, there will be 62,500 combinations. To reach 10 coverages for each gRNA-gRNA construction, 20 electroporate reactions (62500*10/30,000) are needed.

4. Library QC: Directly proceed to steps 20–24 and data analysis part. Sequencing the whole library at least 50× coverage (refer to step 23 for how to choose sequencing service) for the QC purpose. Pay attention to mapping ratio, number/percentage of missed gRNA pairs, the distribution and evenness of the gRNA-gRNA constructions.
5. Library amplification: depending on the experiment designs, such as cell coverage and number of replicates, the amount of library generated from steps 1–3 might be insufficient. In this case, amplify the pooled library by electroporation as described in steps 2c–2e. Instead of using 1 μL of HiFi reaction product, dilute the pooled library to 50 ng/μL and then use 2 μL 50 ng/μL pooled library for each reaction.

Part 2: Large scale lentivirus production

Timing: 5 days

This part generates lentivirus that will be used to integrate dual-gRNA cassette into cellular genome during the screening steps. In this protocol we transfected 293T cells with psPAX2, pMD2.G and the dual-gRNA plasmid library for lentiviral production. psPAX2 and pMD2.G are the second generation lentiviral packaging plasmid and VSV-G envelope expressing plasmid, respectively.

⚠ CRITICAL: Biosafety precautions: Double gloves should be worn, and all virus waste requires incubation in 20% bleach for 30 min before disposal. No vacuum pumps can be used.

6. *Day 1.* Seed 8–9 × 10^6/15 cm plate 293T packaging cells (recommended: passage number < 15) in 20 mL none-antibiotic growth media. For 1 L virus production prepare ~60 plates. Incubate cells for 24 h (37°C, 5% CO_2), or until the following afternoon. The cells should be ~70% confluent at the moment of transfection.
7. *Day 2 afternoon.* Transfect 293T cells:
   a. Prepare the mixture of 3 transfection plasmid (~1:1:1 molar ratio) in OptiMEM:

Transfection plasmid mix (per 15 cm plate)			
Plasmid	Mass	Amount per plate	Total amount
psPAX2	6.5 μg per plate		
pMD2.G	3.6 μg per plate		
Library	5.4 μg per plate		
OptiMEM	–	To 200 μL	

b. For each plate, prepare one 1× OptiMEM and X-tremeGENE transfection reagent (i.e., 60 tubes OptiMEM and X-tremeGENE mix will be needed for 60 plates): directly add 48 μL X-tremeGENE into the tubes with 600 μL OptiMEM, and gently mix them by flicking the tubes.

⚠ CRITICAL: Do not mix by pipetting or vertex here.

c. After the incubation, add 200 μL OptiMEM with transfection plasmids drop-wise to each tube with OptiMEM and X-tremeGENE dilutions (1:3 ratio), then mix gently.
d. Carefully transfer the transfection mix to 293T packaging cells by drop-wise adding in circular motion.
e. Incubate cells for 18 h (37°C, 5% CO_2).

8. *Day 3 morning*. Replace the media containing transfection components with high-BSA growth media for viral production (~20 mL/15 cm plate).
9. *Day 4 morning*. Harvest media containing lentivirus at ~40 h post-transfection. Transfer media to 50 mL plastic tube. Spin the media containing lentivirus at 180 × *g* for 3 min, then collect and aliquot the supernatant into 15/50 mL sterile polypropylene storage tube. Lentivirus should be frozen at −80°C for long-term storage but can be stored temporarily at 4°C for hrs to days.

Note: Aliquot the lentivirus to smaller tubes according to the following steps' usage to avoid freeze/thaw cycles.

Optional: After harvesting the lentivirus at day 4 morning, add high-BSA growth media again for continuing viral production. Then harvest lentivirus at day 5 morning. Note that the lentivirus amount might drop a lot compared to day-4 batch.

Part 3: Multiplicity of infection (MOI) determine

Timing: 5–8 days

This part tests the titer of lentivirus produced from Part 2 and determine the volume of virus needed to reach the MOI ~0.3 in the lentivirus transduction of the pooled library in Part 4.

⚠ CRITICAL: The MOI of lentivirus must be determined for each batch and under the exact same cell culture conditions, such as media and cell density, used in the screening. Measurements from different conditions cannot be scaled to determine the MOI.

⚠ CRITICAL: Biosafety precautions for virus waste. In our institution, all virus waste requires incubation in 20% bleach for 30 min before disposal. No vacuum pumps can be used.

Note: To test MOI, two sets of samples with lentivirus infection are needed. The MOI can be calculated by measuring the ratio of living cells number in the lentivirus + antibiotics group over living cell number in the lentivirus only (no antibiotics selection, 100% survival control) group. Besides the samples with virus, another set of sample without virus but selected by antibiotics is needed for negative control purpose (0% survival control).

Note: The cells are transduced at a concentrated seeding density in 12-well plates while centrifuging ('spinfection'), to achieve higher transduction efficiency. For normal transduction without spinning, refer to Hart et al. (Hart et al., 2015). If the cells can reach MOI ~0.3 with sensible volume of lentivirus (<50% medium), normal transduction would be an easier option.

Note: The ideal spinfection conditions can be highly variable across cell type and should be optimized. A detailed protocol for the optimization of lentiviral transduction spinfection can be found at https://portals.broadinstitute.org/gpp/public/dir/download?dirpath=protocols/production&filename=Optimization_of_Lentiviral_Spinfection_Oct2018.pdf.

Note: The antibiotics selection time may vary from 2 to 5 days.

10. *Day 1.* Lentivirus spinfection.
    a. Thaw a fresh aliquot of pooled lentivirus on ice.
    b. Harvest cells for spinfection test and measure the number of cells/mL. Suspend the cells in small volume of median since spinfection requires concentrated seeding density.
    c. Design dilution series of virus for MOI determination between 0–1 mL (e.g., 0.1 mL, 0.2 mL, 0.5 mL). For each dilution series, prepare two sets (one for testing MOI, one for positive control). Add 1 mL of cell suspension and 1 mL of media/polybrene/lentivirus solution to the corresponding labeled wells (total 2 mL each well). Prepare another one set of samples without lentivirus for negative control purpose (i.e., one set of 6 × 10^6 SaOS2 in 8 mL media/polybrene solution ready for 4 wells).

Plating conditions	
Solution	Amount per well in 6-well plate
SaOS2-Cas9 cells	1.5 × 10^6 in 1 mL media
Polybrene	1.6 μL (final conc. 8 μg/mL)
Pooled lentivirus	
Median	To 2 mL

    d. Centrifuge the plate: spin the plates at 930 × *g* for 1.5 h at 30°C.
    e. After 1.5 h spinning, add 2 mL of media without polybrene dropwise on top of each well.
    f. Incubate cells for 24 h (37°C, 5% CO2).
11. *Day 2.* Transfer the cells into 15 cm plate and start antibiotics selection:
    a. Inspect cells visually, take note of the following things: cell conditions, the confluence of the wells, how adhered the cells are, approximate number of dead cells.
    b. Collect the cells from the 6-well plate.
        i. Remove the median, gently wash the cells with 1 mL pre-warmed PBS.
        ii. Add 250 μL of trypsin each well and let the cells detach from the plate.
        iii. Add 750 μL of media to each well and transfer the cell suspension to corresponding labeled tubes.
        iv. Spin down and then re-suspend the cells.
    c. Reseed the cells to 15 cm plate (4 corresponding wells for one 15 cm plate for SaOS2) with either media containing puromycin at required concentration (2.5 μL/mL for SaOS2) or media without puromycin. Add media containing puromycin to the negative control (cells + polybrene, no virus) set.

Note: If your incubator is not level, you can let plates sit level in hood for up to 1 h.

12. *Day 4.* MOI determination.
    a. After 48 h, all uninfected cells should be dead. There should be no living cells in the negative control group.

b. Remove media, gently wash the cells with pre-warmed PBS to remove all the remaining dead cells. Trypsinize and collect cells from each plate into 15 mL tubes.
c. Count the cells from all plates and two settings of with or without puromycin selection.
d. Determine the lentivirus volume that yields to ~30% survival with puromycin selection vs. without puromycin selection, which gives MOI ~0.3 and ensure each cell is infected by no more than one virus.

Part 4: Screening with customized library pool

Timing: 3–5 weeks

Part 4 performs pooled combinatorial CRISPR screen in human cells. The initial- and end- time point samples were harvested.

⚠ CRITICAL: Carefully consider the number of replicates and coverage you are going to use. Based on the previous MOI testing, cell coverage and replicate number, calculate the total number of cells, number of plates, and corresponding lentivirus volume (refer to step 13b).

⚠ CRITICAL: Biosafety precautions for virus waste (steps 12 and 13). In our institution, all virus waste requires incubation in 20% bleach for 30 min before disposal. No vacuum pumps can be used.

Note: Please refer to troubleshooting 3 to address the low infection efficiency issue.

Note: 1.25 × 10^7 cells give ~200-fold representation for each gRNA-gRNA construct in the 62,500 pool. If desired, you may use higher representation. In the previous combinatorial CRISPR screen, 80-fold is the minimum coverage ((Zhao et al., 2018)).

Note: Previous CRISPR/Cas9 screens have shown high genomic modification efficiencies after 6–12 days post-expression of Cas9 and gRNA. However, this genomic modification efficiency might be varied due to variations among gRNA performance and cell types. To confirm the ability of the CRISPR/Cas9 system to edit genes in specified cell lines, surveyor assay can be used (Wong et al., 2016). Nevertheless, the culture time for CRISPR/Cas9 screen post antibiotics selection typically ranges from 10 to 30 days (refer to Table1).

Note: A transient slowing of cell growth may be noticed. The cells should be able to return to grow normally after 5–15 days after infection, depending on the cell lines.

Note: Collect more cells if possible, considering the material loss during downstream processes such as DNA extraction.

13. *Day 1*. Spinfection. Troubleshooting 3.
    a. The following extra plates with same cell number each plate are needed for negative and positive control purpose: one negative control plate: no virus + puromycin (0% survival control); one positive control plate: virus + no puromycin (100% survival control).
    b. Calculate the plate number and total number of cells plated for infection with MOI ~0.3. As discussed in Preparation4, for the SaOS2-Cas9 with triplicate at each time point, at least 2.5 × 10^8 cells are needed at the time of infection, which requires ~30 6-wheel plates for spinfection.
    c. Thaw pooled lentivirus on ice.

d. Harvest cells for spinfection with corresponding virus volume to achieve ~0.3 MOI. Follow the spinfection process as step 9.

14. *Day 2*. Transfer the cells into 15 cm plate and start antibiotics selection: same as step 10.
15. *Day 4*. T0 sample collection and MOI checking.
    a. After 48 h, all uninfected cells should be dead. There should be no living cells in the negative control group.
    b. Remove media, gently wash the cells with pre-warmed PBS to remove all the remaining dead cells. Trypsinize and collect cells from each plate into 15 mL tubes.
    c. Count the cells from all plates and two settings of with or without puromycin selection. Ensure the MOI in the formal run is around ~0.3.
    d. Collect T0 sample: collect 3 replicates of 1.25 × 10^7 cells by centrifuge (500 × *g* for 5 min). Wash the T0 samples with PBS and centrifuge again. Label the tube and freeze the cell pellets at −80°C.
    e. Re-seed the rest of cells into three replicate groups. Ensure each replicate contains at least in total 1.25 × 10^7 cells to maintain the 200 cell coverage. Do NOT use puromycin in this or subsequent plating steps.
16. *Onward*. (T_1, T2, …, end point): The pool-infected cells should be cultured and passaged as the same way that you normally use. Each instance that you passage the cells out to the endpoint, ensure at least 1.5 × 10^7 cells in total for each replicate group.

    Note: Monitor the cell growth carefully, you may need to consider harvesting the T_{end} sample earlier if cells started dying fast.

17. *End time point*. Collect T_{end} samples as step 14d.

Part 5: Genomic DNA extraction and PCR

Timing: 3–5 days

Part 5 performs two rounds of PCR after genomic DNA (gDNA) extraction. PCR1 enriches dual-gRNA cassette regions from the whole genome. PCR2 adds Illumina adapters and sample indices to the amplified dual-gRNA to facilitate Illumina sequencing in part 6.

Note: IDT Ultramer™ DNA oligonucleotide is suggested for the long PCR2 primers (with adapter to flow cell, sample index, sequencing primer), which is more expensive than PCR1 primers. Thus two-step PCR is performed to reduce cost as well as PCR-induced error.

Note: Assuming a diploid genome is around 6.5 pg and one dual-gRNA construction per genome, 80 μg (62,500*200*6.5pg) of genomic DNA yields ~200-fold coverage of a library with size of 62,500. Note that the average 6.5 pg genome weight might not fit every cell line.

Note: Please refer to troubleshooting 4 for PCR failure.

18. gDNA Extraction: extract gDNA following the protocol of JetQuick Blood and Cell Culture DNA Maxiprep Kit.
19. Precipitate and purify gDNA, and then quantify gDNA using Qubit dsDNA BR Assay.
20. PCR1 (extract and enrich gRNA regions from the genome): add 2.5 μg of gDNA per 50 μL reaction. Set up identical 50 μL reactions to achieve the desired coverage.

    Note: Use the lowest amount of the gDNA among all samples to calculation the number of PCR1 reaction for each sample. For example, if 80 μg is the lowest yield from all gDNA samples, then set up 32 PCR1 reactions for all samples.

PCR1 reaction master mix	
Reagent	Amount
gDNA	2.5 μg
Primer PCR1_F (10 μM)	2.5 μL
Primer PCR1_R (10 μM)	2.5 μL
2× NEBNext Mltra II Q5 Master Mix	25 μL
ddH_2O	To 50 μL

PCR1 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25 cycles
Annealing	66°C	30 s	
Extension	72°C	15 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	
Pool all individual 50 μL PRC1 reaction products, mix by vertex. Then directly proceed to PCR2.			

Optional: Run 2 μL of PCR1 product on 1% gel. Visualize the PCR product on a gel imager. PCR1 yields a product of ~1,300 bp.

21. PCR2 (amplify gRNA with Illumina TruSeq adapters with i5/i7 indices and barcodes):

PCR2 reaction master mix	
Reagent	Amount
PCR1 product	5 μL
Primer PCR2_F (10 μM)	2.5 μL
Primer PCR2_R (10 μM)	2.5 μL
2× NEBNext Mltra II Q5 Master Mix	25 μL
ddH_2O	To 50 μL

PCR2 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	10 cycles
Annealing	66°C	30 s	
Extension	72°C	10 s	
Final extension	72°C	4 min	1
Hold	4°C	forever	

22. Gel extraction and QC:
    a. After PCR amplification, run out the reaction on an EZ-VISION Blue-Light DNA Dye-stained 1% agarose gel. Visualize and cut the DNA fragment at ~580 bp, then extract DNA using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions, elute in 20 μL ddH2O.
    b. Quantify the fragment concentration using Qubit dsDNA BR Assay.
    c. Run the sample in TapeStation DNA1000 or Bioanalyzer High Sensitivity DNA chip for QC check.

Part 6: Sequencing

Timing: depending on the sequencing core

Part 6 provides a guideline for selecting the sequencing service.

Note: Please refer to troubleshooting 5 for addressing the low diversity issue.

23. Choose appropriate sequencing service.
    a. Calculate the total reads: the total reads needed can be calculated as: coverages*library size*number of samples / mapping ratio. For example, to reach 200 coverage for a library with 62.5k different gRNA-gRNA constructions with triplicate in the screening (T_0 and T_{end} in triplicates, 6 samples in total), ~ 9.5 × 10^7 reads (200*62,500*6/0.8) will be needed. Ideally the mapping ratio should be greater than 0.8, this number can be reduced in read estimation if you were not confident about the library quality.
    b. Standard Pair-End (PE) sequencing with dual-index is required to sequence the two gRNAs on the both ends of the DNA fragment. At least 2 × 50 bp read cycle is suggested to cover the 20-bp gRNA sequence at both ends of the DNA fragment.
    c. Given the total reads and PE sequencing, you may choose the sequencing service. The library samples can be sequenced on NovaSeq, NextSeq and MiSeq, which we have all used previously.

EXPECTED OUTCOMES

During the library construction, ~30,000 colonies per electroporation are expected. In our study, less than 0.5% construction is missed in the final construction using 10 coverages in step 3. For the references, 300 μL lentivirus (LentiGuide_DKO backbone, spin-infection) is needed to reach ~0.3 MOI for 6 × 10^6 SaOS2 cells per 15 cm plate. In the gDNA extraction part, ~50 μg gDNA is the average yield for 20–25 × 10^6 cells. After two rounds of PCR and gel extraction (step 22), ~15 ng/μL DNA (for 20 μL elution, Qubit) is expected. It should be only one peak in the report of TapeStation DNA1000 Bioanalyzer. Note that the fragment size reported by TapeStation might be slightly larger than actual size.

Following this protocol, pooled library of 62,500 dual-gRNA combinations for targeting more than 1,000 gene pairs was generated. Then combinatorial CRISPR screen on prostate cancer cell line 22Rv1 and osteosarcoma cell line SaOS2 (Cas9-expressing) was conducted to identify gene pairs that inhibited cancer cell growth when they were disrupted simultaneously. The dual-gRNA construction in initial- and end-time point samples were successfully sequenced and quantified. The diversity of the large dual-gRNA library was maintained throughout library generation, delivery, and screening, as more than 99% of the expected dual-gRNA constructions were detected in plasmid, T_0 and T_{end} samples (Figure 4B). Additionally, the high mapping rate (above 90%, Figure 4A) and correlation between biological replicates (above 0.95, Figure 4C) further demonstrated the success of library construction and large-scale screening. Note that the difference in sequencing statistics between plasmid and screen samples is caused by the sequencing batch effect (MiSeq for single plasmid sample and NextSeq for multiple screen samples). Validation of growth-inhibiting effects of specific gene combinations that are identified by this study is in progress.

In general, a successful library construction should have a mapping rate greater than 80%, and the percentage of missed gRNA-gRNA construction should be less than 1%, or at most 5%. If any positive control is included, a substantial drop in the reads of the positive control constructs is expected.

QUANTIFICATION AND STATISTICAL ANALYSIS

Note: A small processed sampler dataset and all code for QC, hit identification etc. are available at GitHub: https://github.com/Shantang3/Combinatorial-CRISPR-Screen-Anaylysis-Pipeline.

1. FastQC: FastQC provides an overview of basic quality control metrics for raw next generation sequencing data. A detailed demonstration of FastQC can be found at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modμles/.

Sequencing Stat Summary

sample	reads	mapped	mapped%	zero_counts	zero_counts%	Gini_Index	above10	above10%
Library/Plasmid	16567397	14381187	86.80414	1642	2.6272	0.0555723	58165	93.0640
T0_1	6075441	5616461	92.44532	610	0.9760	0.0402558	61220	97.9520
T0_2	6892605	6345936	92.06876	605	0.9680	0.0398364	61407	98.2512
T0_3	6926165	6388158	92.23225	600	0.9600	0.0398576	61430	98.2880
Tend_1	8870135	8172896	92.13948	567	0.9072	0.0406178	61537	98.4592
Tend_2	9622123	8874311	92.22820	575	0.9200	0.0418790	61418	98.2688
Tend_3	11726836	10830207	92.35404	549	0.8784	0.0422329	61546	98.4736

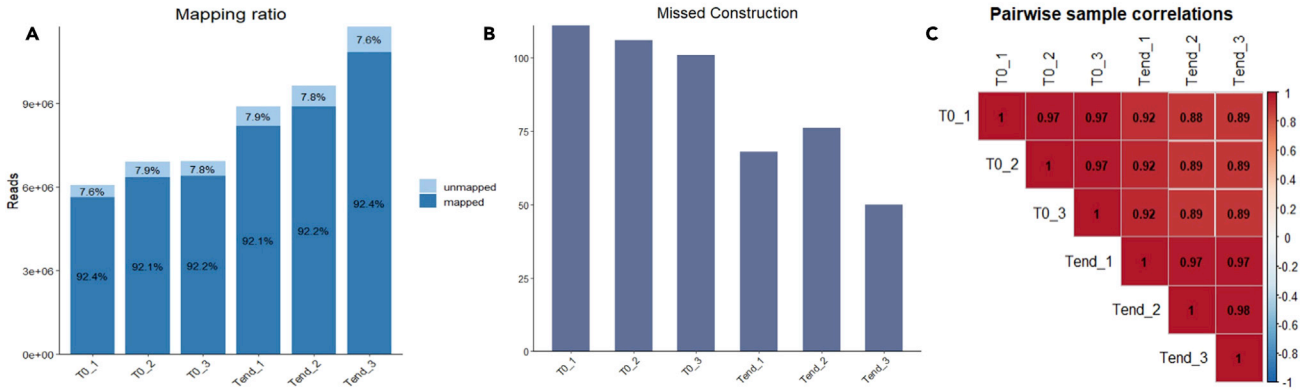

Figure 4. Expected sequencing outcomes

Expected sequencing outcomes: sequencing statistic summary with (A) mapping ratio and mapped reads; (B) number of missed constructions; (C) pairwise sample correlation.

2. Mapping and counting for gRNA-gRNA construction: Dual-crispr.
3. Quality control:
   a. Mapping rate: should be above 80%.
   b. Missed construct or zero count: should be less than 1%, or at most less than 5%.
   c. Count distribution.
   d. Sample correlation: should be greater than 0.8 between biological replicates.
   e. gRNA distribution evenness: In MAGeCK (Li et al., 2014), Gini index, a coefficient measuring the degree of income distribution inequality in economy, is used to measure the evenness of the gRNA distribution in different samples. Smaller Gini index represents more evenly distributed sgRNAs and thus is expected in the library and baseline samples.
4. Log fold change (LFC) for the abundance for each gene pair using MAGeCK RRA (Li et al., 2014).

MAGeCK RRA uses a modified robust ranking aggregation (RRA) algorithm and prioritizes genes whose sgRNA rankings are consistently higher than expected. In this step, the gene pairs with construct of A-B and B-A are considered to be the same. All dual-gRNA constructions targeting the same gene pair were pooled together when calculating the gene-level LFC.

5. Genetic Interaction (GI) calculation and hit identification on gene pairs:

The output file of "gene_summary.txt", specifically the LFC values, from MAGeCK RRA is used for the following analysis:

   a. Observed LFC for gene-gene pairs: by subtracting the LFC with median LFC for pairs in which both sgRNAs were safe controls. This step set the median LFC of safe-safe constructions to zero.
   b. Individual gene LFC: the median LFCs of all pairs compromising specified gene and other safe controls.

c. Expected LFC for gRNA-gRNA pairs: summing of LFCs of two individual genes from 5a.
d. GI calculation: the LFC deviation between observed and expected gene-gene pairs divided by the standard deviation of the individual genes' LFCs from 5a. The more negative GI score is, the higher possibility of synergistic lethality exists between the two genes.

LIMITATIONS

The capability of combinational CRISPR screen is mainly constrained by the library size and cell culture limit, especially for adherent cell lines. During the library construction, growing colony on plate from electroporation using HiFi assembly product has been optimized to reach the yield of 25,000–30,000 colonies per reaction in this protocol. This number yet needs to be improved for libraries with larger size or higher coverage. Instead of plating, some studies directly used LB median culture in the library construction process to reach higher library coverage. However, the problem of recombination using liquid median culture may compromise the library quality (Wang et al., 2016).

Moreover, the performance of CRISPR/Cas9 system relies on well-designed gRNAs. Despite that many models and computational tools have been developed to design gRNA, there is no golden-standard to quantify or compare the efficacy and specificity of gRNAs. Even though we selected multiple gRNAs targeting specified genes from validated merchandise or the commonly used CRISPR library, their performance might differ one from another.

Lastly, unlike single-gene CRISPR screen which has well-established analysis pipelines, limited analysis pipelines are available for the combinatorial CRISPR screen. The calculation of genetic interaction (GI) score and definition of SL gene pairs also vary study to study. With growing interest in combinatorial CRISPR screen, more bioinformatics tools are expected to facilitate the research community.

Nevertheless, this protocol, covering the whole combinatorial CRISPR screen process from gene selection to data analysis, provides the researchers with a framework for conducting experiment, analyzing data, and nominating potential combinational targets from scratch.

TROUBLESHOOTING

Problem 1

Plasmid library is recombined.

Potential solution

The gel electrophoresis can be used to assess the recombination issues. The use of Endura cells, growing bacteria on agar plate instead of in liquid culture, and incubation at 30°C are all intended to minimize recombination of the lentiviral plasmid library. Additionally, using the vector without Cas9 helps to reduce recombination during plasmid amplification.

Problem 2

Insufficient library coverage due to low transformation efficiency.

Potential solution

There are two common causes of this issue. Firstly, electrocompetent cells are no longer good to use. A test electroporation with control plasmid can be performed to check the condition of electrocompetent cells. Secondly, the salt in the HiFi product might be too much. To avoid introducing excess salt, we use ddH_2O to elute DNA in step1, step2a and step3a. Diluting the HiFi product with ddH_2O before transforming also helps.

Additionally, the transformation efficiency can be improved by adjusting the ratio of digested plasmid and insertion during HiFi assembly, the amount of HiFi product used, and electroporation

setting. Notably, we noticed that using more HiFi product in electroporation does NOT guarantee higher efficiency. Furthermore, unlike directly using plasmid DNA, the number of the colony each electroporation using HiFi product is restrained by the input of HiFi Assembly. For example, less than 20 fmol of the 8k linearized vector is suggested. Finally, adding recovery media to the competent cells right after electroporation is critical. For more guidelines for using HiFi DNA assembly, please refer to: https://www.neb.com/tools-and-resources/usage-guidelines/guidelines-for-using-nebuilder-hifi-dna-assembly.

Problem 3

Low MOI, or low transduction efficiency.

Potential solution

One factor that influences viral titers is the size of the vector. Higher viral titers (typically 20 to 100-fold) during viral packaging can be achieved using the vector without Cas9. Wang et al. suggested only who plant to conduct screens across multiple cell lines to clone libraries into the Cas9-containing vector (Wang et al., 2016).

The spin-infection is designed to achieve higher efficient transduction already. Additionally, using products like ViralBoost Reagent during packaging can increase viral titers.

Problem 4

PCR failure.

Potential solution

The most likely reason is the low quality of the gDNA. Check the DNA quality by A_{260}/A_{280}, or Bioanalyzer. More details about the troubleshooting of gDNA preparation can be found at: https://www.sigmaaldrich.com/US/en/technical-documents/technical-article/genomics/dna-and-rna-purification/problems-during-genomic-dna-preparation.

Problem 5

Low diversity libraries.

Potential solution

Low diversity libraries can suffer from Illumina focusing issues, a problem not found with random fragment libraries. Traditionally this problem can be solved in two ways: 1) 'Spiking in' a higher diversity sample such as PhiX, or RNAseq samples; 2) Use amplicon primers with a series of random 'N' (25%A, 25%T, 25%G, 25%C) bases upstream of their gene target. Note that Illuminia sequencer prefers shorter reads; therefore, if you want to sequence the libraries with RNA-seq samples, make sure the RNAseq samples' size are ~600 bp. Additionally, the mean insert size of the PhiX v3 library is approximately 400-bp, which is smaller than the 580-bp library. In one of our test runs using Illumina MiSeq with 40% PhiX spike-in, 75% reads went to PhiX in the end. Thus, consider lowering the PhiX spike-in ratio when loading together with the library samples.

Problem 6

False positive hits.

Potential solution

In our screen, the single genes that are already essential for cell growth tend to have a greater chance appearing in the SL gene pairs. Thus, excluding the single essential genes from the initial gene list (prep 4a) is critical for screen and hit identification. Alternatively, removing the gene combinations with single essential gene from the SL gene list is suggested after data analysis. In particular, following validation by means of transcriptional repression, protein inhibition or KO generation is needed to avoid false positive results.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lang Li (lang.li@osumc.edu).

Materials availability

Plasmids generated in this study have been deposited to Addgene: LentiGuide_DKO (Plasmid #183193), or LentiCRISPR_DKO (Plasmid #183192).

Cas9-expressing cell lines (22RV1-Cas9, SaOS2-Cas9) generated in relevant studies are available upon request.

Data and code availability

The original sequencing files (fastq) of the dual-gRNA library generated by this protocol are stored at Sequence Read Archive (SRA) under PRJNA835479 (BioSample accession SAMN28103246).

A sampler processed dataset and the code generated during this study is available at GitHub: https://github.com/Shantang3/Combinatorial-CRISPR-Screen-Anaylysis-Pipeline, with Zenodo https://doi.org/10.5281/zenodo.6592122.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101556.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.T., X.W., and L.L.; Gene selection: B.G., K.F., F.L., and L.C.; Library constriction: S.T., X.W., and S.S.; Screening *in vitro*: J.L., Q.Z., and X.W.; Data analysis: S.T.; Writing – original draft: S.T.; Writing – review and editing: X.W. and L.L.; Supervision: L.L. and X.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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