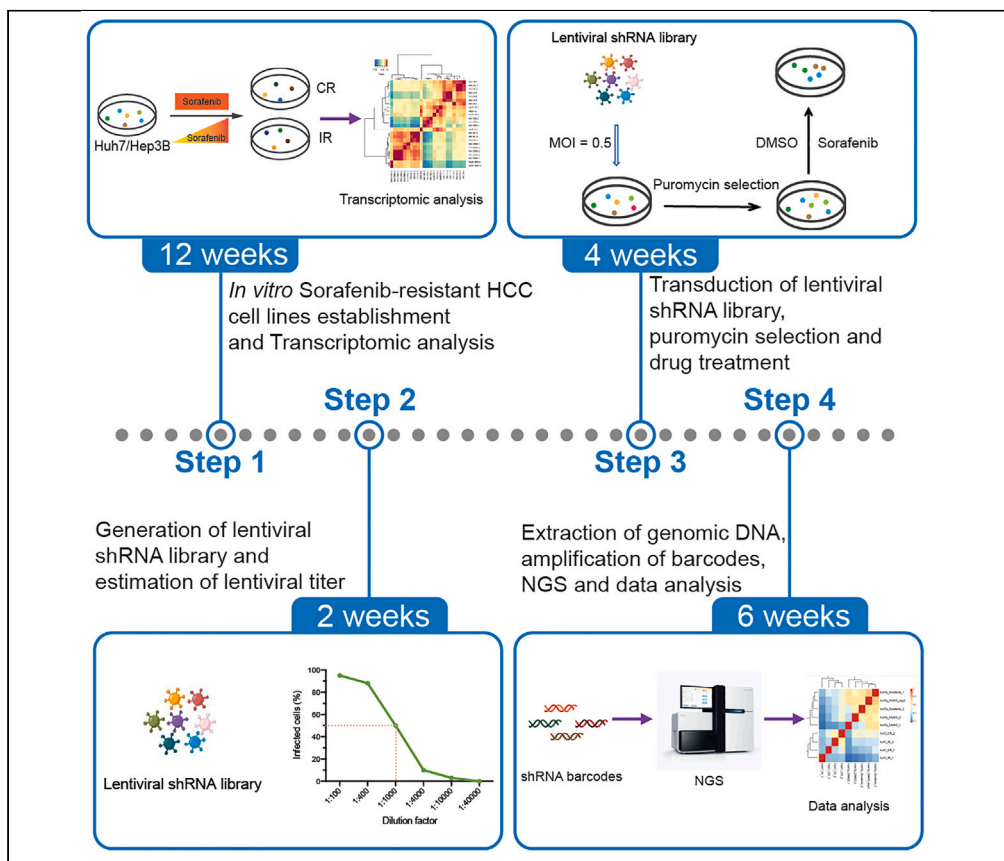


Protocol

Protocol to screen for Sorafenib resistance regulators using pooled lentiviral shRNA library and a Sorafenib-resistant hepatocellular carcinoma cell model



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Highlights

Establishment of *in vitro* Sorafenib-resistant HCC cell models

Establishment of intrinsic or acquired drug-resistant cancer cell lines

Global transcriptomic analysis by RNA sequencing

Synthetic lethal screen using genome-wide pooled lentiviral shRNA libraries

Approaches to study therapy resistance in HCC (hepatocellular carcinoma) are limited, especially when using HCC models *in vitro*. Here, we present a protocol to establish an *in vitro* Sorafenib-resistant human HCC cell model and conduct an shRNA-mediated synthetic lethal screen in established Sorafenib-resistant HCC cell lines to identify critical regulators of Sorafenib resistance. We describe steps for RNA sequencing and functional analysis to reveal the mode of action of potential candidates in conferring therapy resistance to HCC cells.

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

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Protocol

Protocol to screen for Sorafenib resistance regulators using pooled lentiviral shRNA library and a Sorafenib-resistant hepatocellular carcinoma cell model

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SUMMARY

Approaches to study therapy resistance in HCC (hepatocellular carcinoma) are limited, especially when using HCC models *in vitro*. Here, we present a protocol to establish an *in vitro* Sorafenib-resistant human HCC cell model and conduct an shRNA-mediated synthetic lethal screen in established Sorafenib-resistant HCC cell lines to identify critical regulators of Sorafenib resistance. We describe steps for RNA sequencing and functional analysis to reveal the mode of action of potential candidates in conferring therapy resistance to HCC cells.

For complete details on the use and execution of this protocol, please refer to Gao et al. (2021a)¹ and Gao et al. (2021b).²

BEFORE YOU BEGIN

Sorafenib is a small compound multikinase inhibitor, which targets c-RAF, PDGFR, EGFR, VEGFR, FLT-3 and c-KIT. It is a major first-line systemic treatment for advanced HCC patients, yet drug resistance develops very fast in most cases. Due to limited methods of studying Sorafenib resistance in HCC, establishment of *in vitro* models is a critical step for studying therapy resistance in HCC.

This protocol describes the details of the establishment of *in vitro* Sorafenib-resistant HCC cell models, their global transcriptomic analysis and a synthetic lethal screen of established Sorafenib-resistant HCC cell lines by using a human genome-wide pooled lentiviral shRNA library module 1 (50467 shRNAs; Vector: PRS116cb, Cellecta) which targets 6317 mRNAs involved in signaling pathways.

Institutional permissions

Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Establishment of Sorafenib-resistant human HCC cell lines

To delineate the molecular mechanisms underlying Sorafenib resistance in HCC, we established Sorafenib-resistant HCC cell models *in vitro*.

To find the most appropriate cell lines for the establishment of *in vitro* Sorafenib-resistant HCC cell models, we chose 14 different HCC cell lines and measured their IC50 values against the treatment with Sorafenib. The four cell lines most sensitive to Sorafenib were selected ones as candidates for



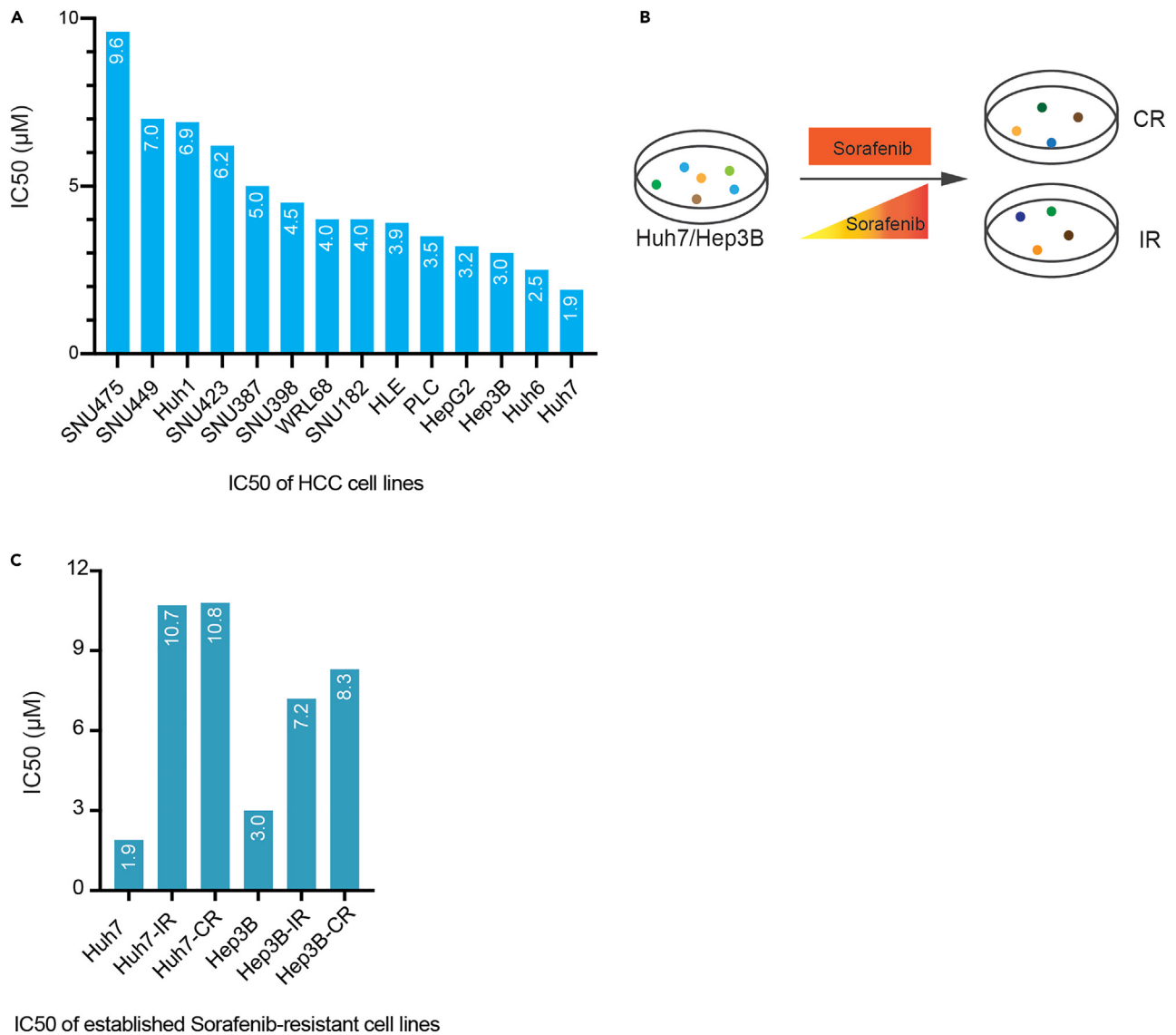


Figure 1. Establishment of Sorafenib-resistant HCC cell lines

(A) IC50 values of 14 HCC cell lines: 14 HCC cell lines were treated with increasing doses of Sorafenib, and the IC50 values for cell growth inhibition by Sorafenib were determined.

(B) Schematic representation of the establishment of Sorafenib-resistant derivatives of Huh7 and Hep3B HCC cell lines. Huh7 or Hep3B cells were treated with increasing (for Huh7 is from 0 to 10 μM, for Hep3B is from 0 to 5 μM) or with consistently high (7 μM for Huh7, 5 μM for Hep3B) concentration Sorafenib to establish acquired Sorafenib-resistant cell lines.

(C) IC50 values of Huh7-IR, Huh7-CR and Hep3B-IR and Hep3B-CR. These IC50 values are on the range of Sorafenib's clinically relevant concentration of 10 μM.

the establishment of Sorafenib-resistant cell lines *in vitro* (Figure 1A). To closely recapitulate the features of the establishment and progression of Sorafenib resistance in patients, we conducted two different treatment protocols on the selected four parental (Sorafenib-sensitive) HCC cell lines; They were either treated with a constant high concentration of Sorafenib or with gradually increasing concentrations of Sorafenib (Figure 1B). After approximately 8 weeks of treatment, when cells stably grow in the presence of the specific concentrations of Sorafenib, four different Sorafenib-resistant HCC cell lines were established. They were named Huh7-CR and Huh7-IR and Hep3B-CR and Hep3B-IR, originating from Sorafenib-sensitive Huh7 and Hep3B cell lines, respectively, and with

CR standing for a constant high concentration of Sorafenib and IR referring to the cell lines generated by gradually increasing the concentration of Sorafenib.

Below we provided the details of establishment of the Sorafenib-resistant HCC cell lines. This protocol could also be used as a reference for the establishment of drug resistance in other types of cancer cells.

Selection of Sorafenib-sensitive cell lines

⌚ Timing: ~ 2 weeks

1. Culture of HCC cell lines: all 14 HCC cell lines are cultured with complete high glucose DMEM at 37°C, 5% CO₂;
2. IC50 measurements:
 - a. Seed each cell line at 5 × 10⁴ cells into 24-well plates, 24 h later, add Sorafenib at final concentrations of 0 μM, 0.5 μM, 2 μM, 5 μM, 10 μM and 20 μM, determine cell growth in a Cell-IQ (Automated cell culture and analysis system, CM-Technologies) or a comparable device;
 - b. Refresh medium every 24 h for 3 times (at time points 48 h, 72 h and 96 h) with new medium containing the respective concentrations of Sorafenib as defined in step a.
 - c. Cell growth is recorded using a Cell-IQ, with the series of dose-response data to plot a x-y linear regression as $Y = a \cdot X + b$ (Y is the cell confluence/viability in %, X is the drug concentration), when Y = 0.5, X is termed the IC50. Thus IC50 values are calculated with the formulation:

$$IC\ 50 = (0.5 - b) / a$$

All IC50 values of 14 HCC cell lines are shown in [Figure 1A](#).

3. Huh7, HepG2, Hep3B and Huh6 present the 4 most sensitive HCC cell lines in response to Sorafenib treatment, with the IC50 values of 1.9 μM, 3.2 μM, 3 μM and 2.5 μM, these four cell lines are used for the next steps of establishing Sorafenib-resistant HCC cell lines.

Note: Alternative option: If Cell-IQ or a comparable device is not available, cell viability could be assessed using the CellTiter-Glo 2.0 viability Assay kit (Promega: Cat# G9241): Cells are seeded and cultured refer to step 2a–b. 96 h later, measure the cell viability with the CellTiter-Glo 2.0 viability Assay kit according to the [manufacturer's instructions](#), calculate the IC 50 values refer to step 2c.

Establishment of Sorafenib-resistant HCC cell lines

⌚ Timing: ~ 8 weeks

As HepG2 and Huh6 failed to survive upon long-term treatment with Sorafenib, below we provide the details of the establishment of Sorafenib-resistant cell lines derived from Huh7 and Hep3B cell lines, respectively.

4. Establishment of Huh7 Sorafenib-resistant cell lines:
 - a. Seed 1 × 10⁶ Huh7 cells into three T75 flask each. Treat the first flask with DMSO, treat the second flask with 7 μM Sorafenib;
 - b. Treat the last flask of cells with increasing Sorafenib concentration at the starting concentration of 1.5 μM. Increase the concentration by 0.2 μM after each two passages, terminate the concentration increase when the cells could not survive from higher concentrations of Sorafenib treatment.

- c. Keep the treatment until all cells remain stable, which needs approximately 8 weeks.
5. Establishment of Hep3B Sorafenib-resistant cell lines:
 - a. Seed 1×10^6 Hep3B cells into three T75 flask each. Treat the first flask with DMSO, treat the second flask with 5 μ M Sorafenib;
 - b. Treat the last flask of cells with increasing Sorafenib concentrations at the starting concentration of 2.5 μ M. Increase the concentration by 0.2 μ M after each two passages, terminate the concentration increase when the cells could not survive from higher concentrations of Sorafenib treatment.
 - c. Keep the treatment until all cells remain stable, which needs approximately 8 weeks, when cells could proliferate under the treatment of highest concentrations of Sorafenib.
6. We finally have succeeded in getting Huh7-IR/CR and Hep3B-IR/CR cell lines. Once the cells continually grow upon further culture in the presence of the highest doses of Sorafenib, freeze the cells for storage and further use (Figure 1B).
7. Measurement of the IC50 values:
 - a. To determine whether the established HCC cell lines are Sorafenib-resistant or not. Measure the IC50 values of the cells (Ref. Step 2) with a Cell-IQ or the CellTiter-Glo 2.0 viability Assay kit as described above. The respective IC50 values are shown in Figure 1C.
 - b. Compare the IC50 values with the clinically achievable Sorafenib drug level of 10 μ M (at which concentration has no clinical effect on non-responders). The IC50 values of established Sorafenib-resistant HCC cell lines (Figure 1C) are in the range of the concentrations obtained in patients in the clinics, meaning that the established cell lines are appropriately Sorafenib-resistant.³

Note: To define the concentrations of Sorafenib on the establishment of constant resistant cell lines, we tested gradient concentrations of 10 μ M, 9 μ M, 8 μ M, 7 μ M, 6 μ M, 5 μ M Sorafenib both on Huh7 and Hep3B and found that 7 μ M is the maximum tolerated concentration for Huh7 to maintain basal proliferation, and 5 μ M for Hep3B.

Global transcriptomic analysis of Sorafenib-resistant HCC cell lines

⌚ Timing: ~ 2 weeks

To delimit the molecular changes upon the establishment of Sorafenib-resistance in HCC cells, global transcriptomic analysis by RNA sequencing is performed on both Huh7/Hep3B-DMSO and Huh7-IR/CR and Hep3B-IR/CR Sorafenib-resistant cells.

8. Culture Huh7/Hep3B-DMSO cells and Huh7-IR/CR and Hep3B-IR/CR cells in T75 flasks:

(Huh7-IR/CR and Hep3B-IR/CR cells are always cultured at 7 μ M or 5 μ M Sorafenib, respectively). Once confluence reaches 80%, the cells are harvested, and RNA is extracted in biological triplicates by using miRNeasy Mini kit (Qiagen) according to the [manufacturer's instructions](#).
9. RNA quality control is performed using a fragment analyzer and standard or high-sensitivity RNA analysis kits (Labgene; DNF-471-0500 or DNF-472-0500).
10. Measure the RNA concentrations with a Quant-itTM RiboGreen RNA assay Kit (Thermo Fisher Scientific).
11. Take a total of 200 ng RNA per sample for the library preparation, with a TruSeq Stranded mRNA Library Prep Kit (Illumina) to generate mRNA and transcript the mRNA into cDNA.
12. Prepared cDNA library is sequenced using HiSeq SBS kit V4 (Illumina) on an Illumina HiSeq 2500 using [protocols](#) defined by the manufacturer.
13. Analysis of the RNA sequencing data:

- Map Single-end RNA-seq reads (81-mers) to the human genome assembly, version hg19 (GRCh37.75), with RNASTAR,⁴ with default parameters except for allowing only unique hits to genome (outFilterMultimapNmax = 1) and filtering reads without evidence in spliced junction table (outFilterType = "BySJout").
- Quantify expression levels per gene (counts over exons) for the RefSeq mRNA coordinates from UCSC using qCount function from QuasR package (version 1.12.0).
- Analyze the differentially expressed genes with the edgeR package (version 3.14.0). Genes with P-values smaller than 0.05 and minimum log₂-fold changes of ± 0.58 were considered as differentially regulated and were selected for functional and pathway enrichment analysis.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMSO	Sigma	Cat# D8418
Sorafenib	Selleckchem	Cat# S7397
Puromycin	Sigma	Cat# P8833
Crystal violet powder	Sigma	Cat# C0075
Polybrene	Sigma	Cat# 107689
DMEM high glucose	Sigma	Cat# D5671
L-Glutamine	Sigma	Cat# G7513
Penicillin-Streptomycin	Sigma	Cat# P4333
10 × Trypsin/EDTA	Sigma	Cat# T4174
FBS	Sigma	Cat# F7524
PBS	Sigma	Cat# D8537
Opti-MEM	Gibco	Cat# 51985034
FuGENE	Promega	Cat# E2311
RNase A	Qiagen	Cat# 19101
Buffer P1	Qiagen	Cat# 19051
Recombinant Proteinase K solution (20 mg/mL)	Thermo Fisher Scientific	Cat# AM2546
Critical commercial assays		
miRNeasy Mini kit	Qiagen	Cat# 217084
RNA analysis kit	Labgene	Cat# DNF4710500/4720500
Quant-it™ RiboGreen RNA assay Kit	Thermo Fisher Scientific	Cat# R11490
TruSeq stranded mRNA Library Prep Kit	Illumina	Cat# 20020594
HiSeq SBS kit V4	Illumina	Cat# FC-401-4002
HiSeq TruSeq SR Cluster Kit v3-cbot-HS	Illumina	Cat# GD-401-3001
QIAquick gel extraction kit	Qiagen	Cat# 28706
QIAquick PCR purification kit	Qiagen	Cat# 28104
NGS Prep Kit	CELLECTA	Cat# LNGS-120
Deposited data		
RNA-sequencing data on Sorafenib-resistant cell lines	This paper	GEO: GSE117116
Synthetic lethal barcode sequencing data	This paper	GEO: GSE158458
Experimental models: Cell lines		
Huh7	University Hospital Basel	N/A
HepG2	ATCC	Cat# HB-8065
Hep3B	ATCC	Cat# HB-8064
HLE	University Hospital Basel	N/A
SNU398	ATCC	Cat# CRL-2233
SNU449	ATCC	Cat# CRL-2234
SNU182	ATCC	Cat# CRL-2235
SNU475	ATCC	Cat# CRL-2236
SNU387	ATCC	Cat# CRL-2237
SNU423	ATCC	Cat# CRL-2238

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PLC/PRF/75	ATCC	Cat# CRL-8024
Huh1	University Hospital Basel	N/A
WRL68	ATCC	Cat# CL-48
Huh6	University Hospital Basel	N/A
HEK-293T	ATCC	Cat# CRL-3216
Recombinant DNA		
pMD2.G	Didier Trono Lab	Addgene Cat# 12259
pMDLg/pRRE	Didier Trono Lab	Addgene Cat# 12251
pRSV-Rev	Didier Trono Lab	Addgene Cat# 12253
Pooled lentiviral shRNA library	CELLECTA	Cat# HGW-M1-P2
Software and algorithms		
FastQC	N/A	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
R Development Core	N/A	https://www.r-project.org/
Adobe Illustrator	Adobe	N/A
Prism 9	GraphPad	N/A
Other		
12-well plate	Milian	Cat# 353043
24-well plate	Milian	Cat# 353047
T75 flask	Milian	Cat# 353136
T175 flask	Milian	Cat# 353112
0.45 µm syringe filter	Sigma	Cat# SLHV033R
15 mL tube	Sarstedt	Cat# 62.554.502
50 mL tube	Sarstedt	Cat# 62.547.254
Cryotube	Sarstedt	Cat# 72.380.007
Centrifuge	Eppendorf	5702
Cell-iQ	CM Technologies	N/A

MATERIALS AND EQUIPMENT

Complete high glucose DMEM medium

Reagent	Final concentration	Amount
DMEM high glucose	N/A	500 mL
L-Glutamine	2 mM	5 mL
Penicillin-Streptomycin	100 U/mL	5 mL
FBS	10%	50 mL
Total		560 mL

Note: Prepare in a sterile hood, store at 4°C for a maximum of 3 weeks.

0.5% Crystal Violet

Reagent	Final concentration	Amount
Crystal violet powder	0.5%	0.5 g
Distilled H ₂ O	N/A	80 mL
Methanol	N/A	20 mL
Total		100 mL

Note: Dissolve crystal violet powder in distilled H₂O and then add methanol. Store in the dark at around 23°C for a maximum of 2 months.

Lentiviral shRNA library

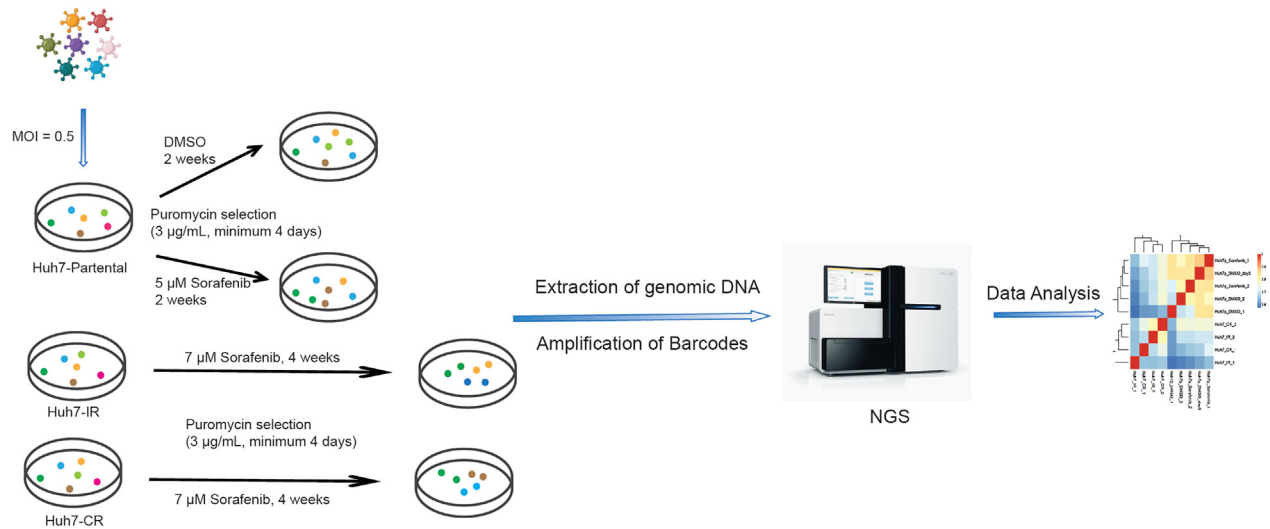


Figure 2. Schematic of the pooled lentiviral shRNA library screen (Created with BioRender.com)

Generated pooled lentiviral library was transduced into both Huh7-parental and Sorafenib-resistant cells. With 3 $\mu\text{g}/\text{mL}$ puromycin treatment for at least 4 days to confirm the library to be transduced into the cells successfully. Either DMSO or Sorafenib was added into DMEM, culture Huh7-parental for another 2 weeks, culture Huh7-IR and Huh7-CR cells for another 4 weeks; Following with the genomic DNA extraction, shRNA barcodes amplification and the NGS sequencing with Data analysis.

STEP-BY-STEP METHOD DETAILS

Generation of a pooled lentiviral shRNA library

⌚ Timing: \sim 1 week

This section details the step-by-step protocol for the generation of a pooled lentiviral shRNA library. The shRNA pools are purchased from Cellecta. A scheme of the synthetic lethal screen is shown in Figure 2.

⚠ CRITICAL: The library contains 50467 pooled lentiviral shRNAs targeting 6317 genes. To generate a sufficient virus titer, a batch of 100 μg plasmid is recommended for the production.

1. Seed 1×10^7 HEK-293T cells into seven T175 flasks each, culture cells with high glucose DMEM contains 10% FBS and 2 mM L-Glutamine at 37°C, 5% CO₂.

Note: Don't add antibiotics (Penicillin/streptomycin) to DMEM when producing lentivirus.

2. Transfection of pooled lentiviral shRNA library.
 - a. Once the cell confluence reaches approximately 50%, replace medium with 30 mL fresh medium.
 - b. Prepare two sterile 50 mL Falcon tubes and prepare the transfection system as follows: Tube A: 12 mL Opti-MEM, 37.5 μg pMD2.G, 75 μg pMDLg/pRRE, 25 μg pRSV-Rev, 100 μg library plasmid, mix well with pipette; Tube B: 12 mL Opti-MEM, 720 μL FuGENE, mix well with pipette.
 - c. Transfer tube B into tube A, mix well with pipette or vortex, then incubate for 15 min at around 23°C.
 - d. Transfer 3 mL mixture to each T175 flask and crossly rock the flasks to mix well with medium, then, put the cells to the incubator.

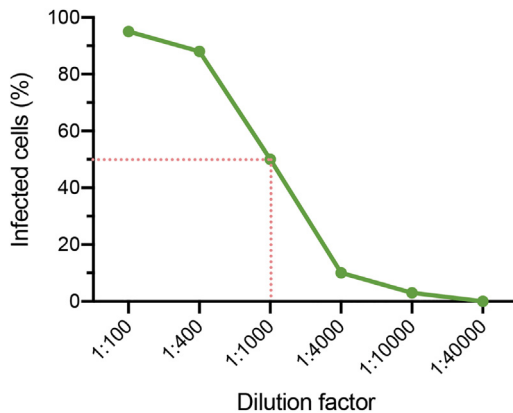


Figure 3. Estimation of the virus titer

A plot of the infected cells (%) versus dilution factors: when the virus is diluted with 1:1000, around 50% cells are infected, the estimated MOI is around 0.7. With the estimated MOI, the virus titer is with the formula: Virus titer = Cell numbers \times MO / Dilution factor.

- e. 18 h post transfection, change medium with 30 mL fresh high glucose DMEM.
3. Harvest the shRNA library virus.
 - a. 48 h post-transfection, collect all the culture medium in 50 mL falcon tubes.
 - b. Centrifuge the tubes at 500 g for 5 min, pass the supernatant through a 0.45 μ m syringe filter.
 - c. Freeze the viral supernatant in aliquots of 5 mL in 15 mL Falcon tubes at -80°C for further use.

Lentiviral virus titration

⌚ Timing: ~ 1 week

To determinate the virus titer, thaw one aliquot of the frozen viral supernatant and dilute it at different dilution factors and measure the transfection efficiency.

4. Seed cells and infect cells with diluted virus supernatant:
 - a. Seed 1×10^5 Huh7 cells per well in two 12-well plates.
 - b. Once the cell confluence reaches 50%, thaw one frozen viral supernatant on ice.
 - c. Dilute the virus supernatant with complete high glucose DMEM at a ratio of 1:100, 1:400, 1:1000, 1:4000, 1:10000, 1:40000 to a final volume of 10 mL virus solution (100 μ L, 25 μ L, 10 μ L, 2.5 μ L, 1 μ L, 0.25 μ L of virus respectively).
 - d. Take 3 mL diluted virus solution and add polybrene to 5 μ g/mL.
 - e. Transfer 1 mL diluted solution to each well (each ratio with duplicate wells).
 - f. Keep four wells without virus infection as control.

Note: Polybrene is used to enhance the infection efficiency.

5. 12 h later, change medium with 1 mL fresh complete high glucose DMEM.
6. 24 h post the infection, change with 1 mL fresh complete high glucose DMEM contains 3 μ g/mL puromycin (for the four control wells, two with puromycin, two without puromycin to confirm the efficacy of puromycin).
7. Culture cells for another 72 h, stain the cells with 0.5% Crystal Violet and calculate the transduction efficiency of different dilution factors.
8. Repeat the virus transduction two more times, plot the crystal violet staining ratio versus dilution factors into a chart, as shown in [Figure 3](#).
9. Calculate the viral concentration as the formula: Titer equals cell numbers \times estimated MOI/dilution factor, then calculate the virus volume required for the transduction to obtain a transduction efficiency of around 40%, which is considered a MOI of 0.5 for the next steps.

Transduction of library

⌚ Timing: ~ 4 weeks

The pooled lentiviral shRNA library will be transduced into Huh7-parental, Huh7-IR and Huh7-CR cell lines, a MOI of 0.5 will be used. After the transduction, cells will be treated with 3 µg/mL puromycin for at least 4 days, afterward, DMSO or Sorafenib will be added to the cells until harvest.

10. Set up four experimental groups as Huh7-parental + DMSO, Huh7-parental + 5 µM Sorafenib, Huh7-IR + 7 µM Sorafenib, Huh7-CR + 7 µM Sorafenib: take eight T175 flasks for each group, seed 1×10^7 cells per flask.
11. Infect cells and puromycin selection:
 - a. Take seven T175 flasks of cells per group when the cell confluence reaches 50%.
 - b. Thaw the frozen viral aliquots, dilute virus with complete high glucose DMEM at a MOI of 0.5.
 - c. Add polybrene to a final concentration of 5 µg/mL to the diluted virus and then infect the cells.
 - d. 24 h later, change medium with fresh complete high glucose DMEM containing 3 µg/mL puromycin.
 - e. Take the residual one T175 flask from each group (four flasks in total) as the control.
 - f. Treat the control cells with 3 µg/mL puromycin for at least 4 days to confirm the successful transduction of the pooled lentiviral shRNA library.
12. DMSO or Sorafenib treatment and cell harvest:
 - a. Treat the cells with either DMSO or Sorafenib as mentioned in step 9, culture Huh7-parental + DMSO / 5 µM Sorafenib for around 2 weeks.
 - b. Culture Huh7-IR/CR cells for over 10 passages to generate sufficient cell numbers.
 - c. Trypsinize the cells and combine all the cells from the same treatment group (6×10^7 cells are needed, which is 1000 times of the library size).
 - d. Centrifuge at 300 g for 5 min, remove the supernatant and collect the cell pellets for the next step.

Note: Upon treatment with Sorafenib, Huh7-parental cells will only survive for approximately 2 weeks. Hence, these cells will be harvested for analysis after 2 weeks, as the shRNAs which had dropped out in Huh7-parental cells upon acute treatment with 5 µM Sorafenib will be subtracted from the synthetic lethal gene list, to specifically enrich for the genes critical for the maintenance of Sorafenib resistance.

Extraction of genomic DNA for samples

⌚ Timing: ~ 1 week

To isolate whole genomic DNA from the cells of genetic screen, a conventional genomic DNA extraction protocol is used.

13. Resuspend cell pellets in 5 mL QIAGEN Buffer P1 (Cat# 19051) with 5 µL RNase A (final concentration is 100 µg/mL, Cat# 19101) with 15 mL Falcon tubes, add 250 µL 10% SDS, mix well and incubate at around 23°C for 5 min.
14. Sonicate to shear DNA into 10–100 kb sized fragments with an ultrasonic homogenizer at 4°C.
15. Add 10 µL Proteinase K (final concentration is 40 µg/mL), mix well and incubate at around 23°C for 15 min.
16. Add 5 mL Phenol: Chloroform: Isoamyl Alcohol solution (25:24:1), vortex and centrifuge with 6000 g for 60 min at 23°C.
17. Take 4 mL of upper phase, and transfer to new 15 mL Falcon tubes, add 500 µL 3 M Sodium Acetate and 4 mL Isopropanol, mix well and centrifuge with 6000 g for 30 min at 23°C.

18. Discard the supernatant and wash with 10 mL 70% Ethanol, centrifuge with 6000 g for 5 min at 23°C, discard the supernatant and dry the pellet.
19. Dissolve the pellet into ddH₂O, check the extraction quality by electrophoresis in 3.5% agarose gels and measure the concentrations of genomic DNA.

Amplification of Barcode Sequences from genomic DNA

⌚ **Timing:** ~ 1 week

The shRNA barcodes are amplified using NGS Prep Kit (CELLECTA, Cat# LNGS-120), the protocol is based on two rounds of PCR to amplify the shRNA barcode sequences. PCR products are purified using QIAquick PCR purification kit and separated by electrophoresis in 3.5% agarose gels, bands were excised and purified using QIAquick gel extraction kit.

20. First Round PCR.

The goal of the first round PCR is to amplify barcodes out of total genomic DNA. When amplifying the barcodes from samples which are generated by the positive selection screen, adequate amounts of genomic DNA are needed (up to 400 µg).

- a. Prepare the master mix according to the following table. A maximum of 50 µg genomic DNA is used in 100 µL volume per reaction. With 400 µg genomic DNA as template, eight reactions are set up at 100 µL each;

Component	Volume per reaction
Genomic DNA	x µL
Forward 1st Round PCR Primer	3 µL
Reverse 1st Round PCR Primer	3 µL
dNTP Mix	2 µL
Taq Polymerase Buffer 10×	10 µL
PCR-Grade H ₂ O	80 – x µL
Taq Polymerase	2 µL
Total volume	100 µL

- b. Mix well, centrifuge briefly, and start the PCR program with the following cycling conditions;

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 s	16–18
Annealing	65°C	30 s	
Extension	68°C	2 min	
Final extension	68°C	2 min	1
Hold	4°C	∞	

⚠ CRITICAL: The numbers of cycles depend on how many genomic DNA is used. If less than 50 µg are added in a 100 µL PCR reaction, one or two more cycles are recommended.

- c. Combine all the individual reactions into one tube for each group sample, the PCR products will be used for the second round PCR.

⏸ Pause point: The PCR product can be stored at –20°C for 6 months, if not perform the second round PCR immediately.

21. Second Round PCR.

Primers for the second round PCR contain the P5 and P7 sequences which are complementary to the immobilized primers in the NGS illumine flow cells. The NGS Prep Kits provide the index primer sets which contain different index sequences and can be used to deconvolute the sequencing results for each sample mixed together in the same flow cell or sequencing lane. Details are found in the User Manual of the NGS Prep kit.

- a. Use 5 μ L of PCR product from the first round PCR as the template, prepare the mix according to the following table (2 reactions with 100 μ L per reaction);

Component	Volume per reaction
First Round PCR Product	5 μ L
NFwd Primer	5 μ L
NRev(Index) Primer	5 μ L
dNTP Mix	2 μ L
Taq Polymerase Buffer 10x	10 μ L
PCR-Grade H ₂ O	71 μ L
Taq Polymerase	2 μ L
Total volume	100 μ L

△ CRITICAL: Each sample to be sequenced together in a single lane needs to use different NRev Index primers from the NGS Prep kit.

- b. Mix well, centrifuge briefly, and start the PCR program with the following cycling conditions;

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 s	12–14
Annealing	65°C	30 s	
Extension	68°C	2 min	
Final extension	68°C	2 min	1
Hold	4°C	∞	

22. PCR purification and extraction.

PCR products from the second round PCR are analyzed by gel electrophoresis to ensure equal amounts of amplified barcodes for all samples. Purify the PCR products by the following steps.

- a. Use the QIAquick PCR purification kit to purify the PCR products following the [manufacturer's protocol](#). For the last centrifugation step, take the maximum speed for 5 min to completely dry the membrane;
- b. Load the purified PCR products on a 3.5% agarose gel to separate the bands by electrophoresis;
- c. Excise the bands and extract DNA with QIAquick gel extraction kit;
- d. Quantitate the DNA concentration at A260nm using NanoDrop, and store for next generation sequencing with an Illumina HiSeq (Illumina).

▮▮ Pause point: The PCR product can be stored at -20°C for 6 months, if not perform the NGS sequencing immediately.

NGS (next generation sequencing) deep sequencing and data analysis

⌚ Timing: ~ 4 weeks

23. NGS deep sequencing.

Pooled amplified barcodes will be sequenced by NGS on an Illumina HiSeq (80–100 million reads per sample) using the GexSeq sequencing primer. Take 2 μL of 10 nM PCR product from the purified DNA band for the cluster generation step. The number of cycles for hGW library is 44, for the other libraries, the cycle numbers required depend on the length of barcode.

- a. Adjust the purified PCR samples to a final concentration of 10 nM.
- b. To each lane, add 2 μL sample and Phix174 control template based on the standard Illumina protocol for cluster generation using the Illumina single-read flow cell.
- c. Add GexSeq primer to the PhiX174 primer to get a final concentration of 0.5 μM for the NGS step.
- d. Run the NGS reaction for 44 cycles with a HiSeq TruSeq SR Cluster Kit v3-cbot-HS.

24. Data analysis.

The open-source processing pipeline in edgeR⁵ provides a complete analysis solution for the synthetic lethal screen data. It begins with the raw sequence reads and ends with a ranked list of candidate genes for downstream biological validation.

- a. First summarize the raw data contained in a fastq file into a matrix of counts (samples in the columns, genes in the rows) with options for allowing mismatches and small shifts in sequence position;
- b. Process Amplicons: process raw data from pooled genetic sequencing screens (<https://rdrr.io/bioc/edgeR/man/processAmplicons.html>);
- c. Multidimensional scaling plot of distances between digital gene expression profiles (<https://rdrr.io/bioc/edgeR/src/R/plotMDS.DGEList.R>);
- d. Calculate Normalization Factors to Align Columns of a Count Matrix (<https://rdrr.io/bioc/edgeR/man/calcNormFactors.html>);
- e. Compute GeneWise exact tests for differences in the means between two groups of negative-binomially distributed counts (exactTest: <https://rdrr.io/bioc/edgeR/src/R/exactTest.R>);
- f. Camera and Roast for GeneSet enrichment analysis.

EXPECTED OUTCOMES

The combination of transcriptomic analysis and pooled lentiviral shRNA library screen on Sorafenib-resistant HCC cell lines allows the identification of pivotal regulators of Sorafenib resistance in HCC. One recent example is the identification and functional validation of the YAP/TAZ-ATF4-SLC7A11 axis as a critical player in establishing and maintaining Sorafenib resistance by *in vitro/vivo* experiments and on HCC patients' samples, as reported in the original article by Gao et al. (2021b).² The raw RNA-sequencing data and pooled lentiviral shRNA library screen data has been deposited at Gene Expression Omnibus (GEO): GSE117116 (RNA-sequencing Sorafenib-resistant cell lines), GSE158458 (Synthetic lethal barcode sequencing).

LIMITATIONS

This protocol provides details of the *in vitro* establishment of Sorafenib-resistant HCC cell lines, a procedure which can also be used to establish other *in vitro* drug resistance models in different cancer cell types. Nevertheless, the establishment of drug-resistant cell lines may not be applicable for all types of cancer cell lines due to various response to different drugs, a batch of different cell lines needs to be tested to select the proper ones. The synthetic lethal pooled lentiviral shRNA library screen can also be modified for application with any other type of cell line. While such *in vitro* drug-resistant cell models may recapitulate the situation in drug-resistant HCC patients, they may be limited in fully mimicking the complex organismic interactions as observed in patients with drug-resistant HCC. Also, while the pooled lentiviral shRNA library screen has identified several pivotal regulators of drug-resistance, some critical players may be missed due to the complexity of a full genome-wide screening approach or due to the differences in the specific genetic and molecular make-up of the cell lines selected. Moreover, some of the newly identified players escape a functional validation in experimental animal models of HCC or in HCC patients.

TROUBLESHOOTING

Problem 1

Failed to establish Sorafenib-resistant cell lines on Huh6 and HepG2 cells.

Potential solution

High doses of Sorafenib will lead to severe cell death with Huh6 and HepG2 cells and only few cells did survive. One possible solution to generate the IR (Increasing resistant) cell lines is slowly increasing the concentration of Sorafenib, prolong the culture time with each concentration to generate sufficient cell numbers before the next stage of higher concentration treatment. For the constant high concentration of Sorafenib treatment, try to seed more cell numbers and culture for long enough time in the presence of the highest concentration of Sorafenib, which a few Huh6 and HepG2 cells may survive. These may take much longer times than was used with Huh7 and Hep3B cells.

Problem 2

Sorafenib-resistant cells are not growing well (Before you begin, step 6).

Potential solution

Sorafenib has to be added to the medium when the Sorafenib-resistant cells are in culture to maintain the resistance. For Huh7-IR/CR use 7 μ M Sorafenib, for Hep3B-IR/CR use 5 μ M Sorafenib. Culture medium has to be appropriately changed depending on the cells' growth conditions.

Problem 3

Low lentiviral titer (Generation of a pooled lentiviral shRNA library).

Potential solution

Low titer may be caused by: a. too high or too low cell density of HEK-293T, 50%–80% confluence is recommended; b. Low transfection efficiency: we tested 4 different transfection reagents: FuGENE, JetPEI, Lipo2000 and Lipo3000 to assess which one had the best efficiency of lentivirus production. CV staining (% to Control) of post-puromycin selected Huh7 or Hep3B was used as the criteria for the evaluation, FuGENE exhibited the highest efficiency of virus production in our system; c. Storage of aliquoted packaged library should be at -80°C . Don't freeze-thaw the virus more than one time.

Problem 4

Concentration of puromycin used for the selection (step 10).

Potential solution

The sensitivity to puromycin treatment varies between different cell lines. It is critical to generate a killing curve for determining the optimal concentration of puromycin.

Problem 5

No PCR product (Amplification of Barcode Sequences from Genomic DNA).

Potential solution

Ensure that the correct primers need to be used for each round of PCR, 10 ng plasmid library DNA maybe used as a positive control. Low numbers of barcodes in the genomic DNA could also block the amplification, the transduction efficiency of pooled lentiviral shRNA library has to be checked.

Problem 6

No barcodes present in the NGS sequencing (step 22).

Potential solution

Ensure that the correct sequencing primers are used when performing NGS. Use the Gexseq Sequencing primers from the NGS Prep kit.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the [lead contact](#), Ruize Gao (ruize.gao@lih.lu).

Materials availability

Plasmids and cell lines reported in this study will be available upon request.

Data availability

The sequencing files are deposited on GEO database under the accession numbers: GSE117116 (RNA-sequencing Sorafenib-resistant cell lines) and GSE158458 (Synthetic lethal barcode sequencing).

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AUTHOR CONTRIBUTIONS

Conceptualization, F.T., R.G., G.C.; Methodology, F.T., R.G.; Investigation, R.G.; Writing – Original Draft, R.G.; Writing – Review & Editing, G.C., F.T., R.G.; Supervision, G.C.; Project Administration, G.C.; Funding Acquisition, G.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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