Introduction to CRISPR Library Screening

Foreword

About Library Screening

CRISPR library screening is a biological tool based on the CRISPR/Cas9 system, used for high-throughput gene functional research. The background of this technology traces back to the birth of the CRISPR/Cas9 technology, a revolutionary gene-editing tool initially used for editing single genes. However, scientists soon realized that by integrating appropriate sgRNA sequences, it was possible to simultaneously intervene or edit multiple genes, leading to the development of CRISPR library screening technology.

CRISPR/Cas9 library screening enables high-throughput screening on a genome-wide scale. For each gene, 3-10 sgRNAs are designed, and tens of thousands of sgRNA probes covering the entire genome are synthesized on a chip at one time. These probes are cloned into a vector library, packaged into lentiviruses, and used to infect host cells at a low multiplicity of infection (MOI), theoretically integrating one sgRNA per cell to create a genome-wide gene knockout cell pool. After screening, genomic DNA is extracted, and the integrated sgRNA sequences are analyzed by Next-Generation Sequencing to assess the changes in sgRNA abundance before and after screening, identifying the corresponding candidate genes. Currently, comprehensive human and mouse genome libraries, as well as sub-libraries targeting various specific biological pathways, have been developed and refined.

The advent of CRISPR library screening technology allowed scientists to conduct precise functional research at the genomic level. By constructing CRISPR libraries, extensive gene screening and functional studies can be performed systematically. This technology has broad application prospects in disease treatment, agricultural improvement, and bioengineering. Through screening and identifying specific genes and pathways, scientists can better understand the mechanisms of biological organisms, reveal potential therapeutic targets, and optimize bioengineering processes. Given the powerful capabilities of CRISPR/Cas9 high-throughput library screening technology, it is foreseeable that it will undergo unprecedented development and application in the future.

This brochure will provide a detailed introduction to the CRISPR library screening process, enabling you to master your CRISPR library screening experiments.

TABLE OF CONTENTS

CRISPR Library Screening Procedure

I. Overall Steps:

- Packaging of Cas9 Lentivirus
- Determination of Screening Concentration
- Transduction of Cas9 Lentivirus
- Amplification and Quality Control of the sgRNA Library
- Packaging of sgRNA Lentivirus
- Determination of MOI for Transduction of Cas9+ Stable Cell Lines with sgRNA Lentivirus
- Transduction of Cas9+ Stable Cell Lines with sgRNA Lentivirus Library
- Expansion and Plating of Library Cells According to the Experimental Purpose
- Extraction of gDNA After Screening
- Construction of Sequencing Library
- NGS Sequencing and Analysis

II. Detailed Experimental Procedure:

1. Packaging of Cas9 Lentivirus

1) Approximately 24 hours before transfection, seed 4-5 × 10^6 Lenti-X 293T cells in a 10 cm dish with 8 mL of complete medium. Incubate overnight at 37 °C with 5 % CO_2. Cells should reach 80-90 % confluency by the time of transfection.

2) Mix the transfection reagents at room temperature and incubate for 10 minutes to form the complexes. After 10 minutes, centrifuge for 2 seconds to bring the liquid to the bottom of the tube.

3) Add the transfection complex solution dropwise to the 8 mL of culture medium prepared in step 1. Gently swirl the plate to mix.

Note: A slight change in the color of the medium is normal after adding the transfection complex solution.

4) Incubate the cells at 37 °C with 5 % CO_2.

Note: You can change the medium 4 hours after transfection. Alternatively, incubate overnight, though this usually doesn't improve transfection efficiency or titer.

5) After overnight incubation, add 6 mL of fresh complete medium. Incubate for additional 24-48 hours at 37 °C with 5 % CO_2. The virus titer typically peaks 48 hours post-transfection; however, you can collect supernatants at 48 and 72 hours for a combined yield.

6) If necessary, combine lentiviral supernatants from multiple dishes (store 48-hour samples at 4 °C until 72 hour samples are collected).

7) Centrifuge briefly (500g, 10 minutes) and filter the supernatants through a 0.2 μm filter to remove cell fragments. Aliquot and store at -80 °C.

Note: Use filters made of cellulose acetate or polysulfone (low protein binding) instead of nitrocellulose, as nitrocellulose binds proteins in the viral envelope, disrupting the virus.

8) Measure the viral titer of the stock solution and use the virus to transfect target cells.

Note: The titer may drop 2-4 fold with each freeze and thaw cycle.

2. Determination of Drug Selection Concentration

Before establishing stable and double stable cell lines using G418, Hygromycin, or Puromycin, it is essential to determine the optimal selection concentration of each batch of resistance reagents to the host cell lines. This is crucial as there is quite a efficiency difference between different batches of drugs. Therefore, each new batch of antibiotic reagents requires titration experiments to determine the optimal concentration. We recommend performing two experiments for each drug:

- Generate a cell kill curve to determine the optimal drug concentration.
- Conduct preliminary experiments to determine the optimal plating density. Even if a pre-established Cas9 cell line is used, it is advisable to perform this step.

Cas9 stable cell lines need to be established before the construction of double plasmid system CRISPR sgRNA library (eg. pLCKO2::mTKO). Prior to screening, ensure that the cell lines are free of mycoplasma. Determine the optimal cell seeding density in your chosen container to reach approximately 80% confluency after 3-4 days of culture. Record the approximate proliferation time of the cells.

2.1. Antibiotic Titration Cells

1) Plate 2×10^5 cells, seed each type of cells into six 10 cm dishes, each containing 10 mL of appropriate complete medium with varying concentrations of Hygromycin or G418 (0, 50, 100, 200, 400, and 800 μg/mL). For Puromycin, the concentrations should be 0, 1, 2.5, 5, 7.5, and 10 μg/mL.

2) Culture the cells for 10-14 days, replacing the selective medium every 2 days (or more frequently if necessary).

3) Check the dishes every two days for the live cells.

Note: To select stable transformants, use the lowest concentration that begins to cause significant cell death within approximately 5 days and leads to complete cell death within 7-10 days.

2.2. sgRNA Virus Transfection Resistance Curve

1) Determine the sensitivity of the cell line to Puromycin.

2) This can be performed in a 12-well plate and quantified using cell counting or Trypan Blue staining.

3) The range for Puromycin gradient dilution can be from 0 μg/mL to 10 μg/mL, with increments of 0.5 μg/mL.

4) Determine the minimal concentration that kills 100 % of uninfected cells within 48 hours.

5) Use the obtained resistance concentration to select infected cells.

6) The sensitivity and response time to Puromycin may vary depending on different cell line. Therefore, it is crucial to use the optimal resistance concentration that allows for selection of cells within 48-72 hours before starting phenotypic selection, thereby minimizing the loss of essential genes.

7) For cell lines with longer proliferation time, the duration of Puromycin selection can be extended appropriately. In these cases, the kill curve should be based on the time required for < 3 generations of proliferation.

2.3. Determination of Optimal Plating Density

After establishing the optimal drug selection concentration, use different cell densities to determine the optimal plating density. If cells are plated at too high a density, they will reach approximately 80 % confluency before the resistance selection takes effect. The optimal plating density depends on the cell proliferation time and cell size.

1) Plate cells at various densities in six 10 cm dishes containing 10 mL of selective medium. Recommended densities (cells/10 cm dish): 5×10^6, 1×10^6, 5×10^5, 2×10^5, 1×10^5, and 5×10^4.

2) Culture the cells for 5-14 days, change the selective medium every four days.

3) Check the dishes every two days for live cells.

4) To select stable transformants, utilize a plating density that allows the cells to reach approximately 80 % confluency before significant cell death begins (around day 5).

Note: This step need to be repeated when transfecting the sgRNA viral library into Cas9 stable transformants.

3. Amplification and Purification of the sgRNA Library

Materials:

- 50 µL competent cells (Lucigen, Endura™ Competent Cells 60240-1)
- 50 ng/µL plasmid DNA library
- 2 electroporation cuvettes (0.1 cm gap, Bio-Rad, 165-2089)
- 10 mL SOC (1X SOC, New England BioLabs, B9020S)
- 15 cm² plates (low salt LB agar + carbenicillin antibiotic)
- 2 Plasmid Maxi Kits (Qiagen Plasmid Maxi Kit 12163)
- Glass beads
- Electroporator (Bio-Rad GenePulser II)

Procedure:

1) Dilute the library plasmid to 50 ng/µL using TE buffer.

2) Use Endura™ Competent Cells (Lucigen, 60242) to transfect the plasmid library. Set up a total of 4 transfection groups:

a. Thaw the competent cells on ice and add 2 µL of the 50 ng/µL plasmid library, mix gently.

b. Transfer the mixture to pre-chilled electroporation cuvettes (1.0 mm) and perform electroporation according to the manufacturer's recommended conditions and protocols.

c. Within 10 seconds of post-electroporation, add 975 µL of SOC recovery medium to the cuvette.

d. Transfer the E. coli to a culture tube and add an additional 1 mL of recovery medium.

e. Place the culture tube in a shaking incubator at 37 °C with agitation at 250 rpm for 1 hour.

3) Set up dilution plates to titrate the library and estimate the transformation efficiency:

a. Mix the recovery liquid from all four groups (total of 8 mL).

b. Add 10 µL of the cell suspension into 990 µL of recovery medium for an 800 x dilution. Plate 20 µL of this diluted sample onto preheated 10 cm LB + carbenicillin (100 mg/mL) plates. This represents a 40,000 x dilution and can be used to calculate the transfection efficiency.

4) Spread the remaining cell suspension evenly across 20 preheated 15 cm LB + carbenicillin (100 mg/mL) plates, using 400 µL of the suspension per plate.

5) Incubate at 30°C for 14-16 hours. Cultivating at a lower temperature can reduce recombination at repetitive sequences in the genome.

6) Calculate Transformation Efficiency:

a. Count the number of colonies on the 40,000-fold dilution plate.

b. Multiply the number of colonies obtained in (a) by 40,000 to determine the total colony number.

c. Proceed to the next step once the total colony count is more than 200 x, ideally 500-1000 x. A sufficient number of colonies ensures high library coverage.

d. If the colony count is insufficient, increase the number of electroporations accordingly to achieve appropriate coverage.

7) Collect Colonies:

a. Add 7 mL of LB + carbenicillin liquid medium to a 15 cm culture dish.

b. Scrape the colonies off using a cell scraper.

c. Transfer the scraped cells into a 1 L culture flask.

d. Rinse the scraped plate with 5 mL of LB + carbenicillin medium and transfer the medium to the flask.

e. Repeat steps a-d for all plates, combining all scraped colonies into one flask.

f. Stir the combined culture at room temperature for 1 hour to disperse the bacterial clumps.

g. Transfer the cells to pre-weighed centrifuge tubes.

h. Centrifuge at 7,000 g and collect the bacterial pellet, discard the supernatant.

i. Weigh the bacterial pellet and record its weight.

8) **Purify the Plasmid Library:**

a. Use a plasmid DNA maxi or mega extraction kit.

b. Follow the extraction protocol according to the capacity of the purification column; typically, a maxi purification column can extract plasmids from 1 g of wet cell weight, while a mega purification column can extract from 2.5 g of wet cell weight.

4. Construction of Cas9 Stable Cell Lines

1) Prior to transfection, plate cells in a 6-well plate 12-18 hours in advance, ensuring that the cells reach 50-60% confluency before transfection.

2) Thaw one vial of Cas9 lentivirus.

3) Add polybrene to the cells to determine the optimal concentration.

4) Transfect the target cells with different amount/MOI of viruses; for example, the initial MOI can be set at 50-60, and then gradient dilute the viruses. If feasible, the efficiency of transfection can be improved by centrifugation.

Note: Centrifugation enhances transfection efficiency; we recommend centrifuging at 32°C, 1,200 g for 60-90 minutes.

5) After 8-24 hours of transfection, remove the medium containing the virus and replace it with fresh complete medium.

6) Continue to culture the cells for an additional 24 hours to allow them to recover and express the Cas9 protein.

7) Passage all the cells at different MOIs and screen with puromycin until untransfected cells are dead.

8) When the cells are enriched to a countable level, calculate the total number of cells when they reach 40-50% confluency. Continue to culture, passage, and count to obtain a growth curve.

9) Once the growth curve is established, select the MOI that yields the highest level of transduction without adversely affecting cell growth and viability. The optimal MOI may differ among various cell lines and will also depend on the cell line's tolerance to the Cas9 protein.

10) After selecting the most suitable Cas9 stable cell line, expand and cryopreserve the cells for future use.

11) Expand the required amount of Cas9 cells according to the size of the library.

Note: After constructing the Cas9 stable cell line, it is recommended to use previously validated sgRNAs for editing efficiency testing, as the editing efficiency directly impacts the effectiveness of subsequent selection processes.

5. Packaging of sgRNA Lentivirus Library

Materials:

- 293T packaging cells (recommended generation < 15)
- Transfection plasmids: CRISPR library plasmid, psPAX (Addgene plasmid #12260), pMD2.G (Addgene plasmid #12259)
- X-tremeGene 9 (Roche, 06 365 787 001)

- OPTI-MEM serum-free media (Invitrogen, #31985-070)
- Cell plating Medium - Low antibiotic growth medium (DMEM + 10 % FBS + optional: 0.1 x Pen/Strep)
- Virus collection medium - serum free, High-BSA 293T growth media (DMEM + 1.1 g/100 mL BSA + 1x Pen/Strep)

Procedures:

1) Using complete medium without bispecific antibody to plate the 293T cells, plate 8×10^6 cells in a 15 cm dish with 20 mL medium. To prepare 500 mL lentivirus, approximately 30 plates are needed.

2) Culture the cells for 24 hours at 37 ℃ with 5 % CO2, perform the transfection when the cells reach 70-80 % confluency.

3) Transfection of 293T cells.

a. Mix the 3 types of plasmids with Opti-MEM in a molar ratio of 1:1:1, and prepare the plasmids according to the number of dishes (+1) to be transfected, the specific volumes are as follow:

reagent	volume/15 cm dish	
	single vector system	Double vector system
Opti-MEM	100 µL	100 µL
psPAX2	4.8 µg	7.0 µg
pMD2.G	3.8 µg	4.0 µg
Library plasmid	8.0 µg	5.0 µg

b. Prepare X-tremeGENE 9 separately (volume as below). Transfer OpTi-MEM to 1.5 mL centrifuge tubes according to the amount of dishes to be transfected. Add X-tremeGENE 9 to the transferred Opti-MEM, mix gently and incubate at room temperature for 5 minutes.

Reagent	Volume/15 cm dish
pti-MEM	800 µL
XtremeGENE	948 µL

c. After 5 minutes of incubation, mix the plasmid mixture and XtremeGene 9 complex in a ratio of 1:3, mix gently and incubate for 30 minutes at room temperature.

d. Repeat the above step, until all mixtures are prepared.

Note: First, mix no more than 5 groups of XtremeGENE 9. After 3 minutes, mix the next group. Add DNA to the first group after 5 minutes, and do the same for the remaining groups. This helps avoid over-incubation.

e. After 30 minutes of incubation, carefully transfer the transfection mixture to each dish. Add the mixture with 1 mL pipette dropwise in a circular, Z motion without destroying the cell monolayer. Ensure that the incubator is level when incubating the cells.

4) Incubate cells for 18 hours at 37 ℃ with 5 % CO2.

5) After 18 hours, remove the transfection medium and gently add 18-20 mL virus-collection medium (serum-free with high BSA growth medium) in 15cm dishes.

6) Incubate cells for 24 hours at 37 ℃ with 5 % CO2.

7) After 24 hours, collect the medium containing virus and transfer into the polypropylene cryogenic vials.

8) Centrifuge the virus medium at 1000 rpm for 5-10 minutes to remove impurities such as cells, and then aliquot the virus into storage tubes.

9) Virus can be stored at 4 ℃ for short term (for hours or days), and need to be stored at -80 ℃ for long term. To reduce freezing and thawing, the virus can be aliquoted into small volumes for long-term storage.

6. Transduction of Cas9+ Stable Cell Lines With sgRNA Lentivirus Library

To construct a Cas9+/sgRNA+ cell library that can be used for screening, it's essential to control the depth of sgRNA. Meanwhile, ensure that each cell express only one single sgRNA. It's important to use the amount of virus which is needed to achieve approximately 30 % transfection efficiency in Cas9+ cell lines to transfect Cas9 stable cell lines. 30 % transfection efficiency (about 0.4-0.6 MOI) can produce the maximum number of cells expressing a single sgRNA, while maintaining a reasonable number of cells in culture before Hygromycin selection.

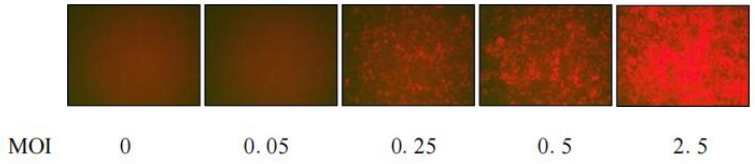

Fig1. Relationship between sgRNA library transfection efficiency and MOI in A375 - Cas9 stable cell lines)

6.1 Titration of Virus Volume on Target Cells

Determination of MOI must be performed under the same culture conditions as the formal screening experiments, including the same volume of culture vessel, composition and volume of medium, cell density, and using the same batch of virus. Test conditions performed on 6-well plate should not be used in formal experiments.

Day 1

1) Thaw a vial of virus and store it on ice.

2) Count the cells.

3) Design a 0-2 mL gradient to dilute the virus.

4) Add cells, medium, polybrene (typically at a final concentration of 8 μg/mL), and then virus into the culture vessel. Mix thoroughly then transfer to the incubator. Minimize the culture volume, e.g., 20 mL for a 15 cm dish.

5) Add the same volume of virus to each culture vessel with different concentration of virus.

6) Mix thoroughly for 2 minutes.

7) Transfer to the incubator.

Day 2

1) 24 hours after adding the virus, the cells appear to be infected and are firmly attached to the dish.

2) Remove the medium with a pipette.

3) Add fresh medium containing the desired concentration of puromycin to one bottle (20 mL to a 15 cm dish), and add medium without puromycin to the other bottle.

4) Transfer to the incubator and continue incubation.

Day 4

1) After 48 hours, all uninfected cells should be dead.

2) Digest and collect the cells, make sure that the cell clumps are gently dispersed.

3) Count the cells in all dishes, and calculate the selection results of groups with and without puromycin separately.

4) By comparing the virus amount of 30-40 % survival rate under resistance and non-resistance selection conditions, this is exactly the viral load of MOI 0.3-0.4.

6.2 Determine the Amount of Target Cells

To ensure the coverage of sgRNAs, the representative depth of sgRNAs should be more than 200. It's commonly recommended to be between 400-1000.

In addition, since the MOI is around 0.4, we recommend plating with 2-3 times as many cells to ensure that each cell carries only one single sgRNA.

Check the table below:

Item	Library screening
sgRNA amount	76612
Gene amount	19114
Cell amount needed for 400 x sgRNA representitive depth	3.1×10^7
Cell amount needed to be transfected	1×10^8
MOI	0.4
sgRNA representitive depth (recommended 400-1000 cells/sgRNA)	400

Additionally, good condition of cells is crucial to maintain the depth of each sgRNA, especially for negative screening or screening that requires higher sensitivity. Therefore, when passaging the cells, we recommend that the number of remaining cells should be more than 1000 times the number of sgRNAs during or after screening.

6.3 sgRNA Library Virus Transfect Target Cells

After confirming the amount of cells required, amplify the cas9 stable cell line and transfect sgRNA library virus. The transfection conditions must be the same as in the previous titration experiment. In addition, it is preferable that the conditions are the same as in the pre-experiment, e.g., antibiotic dosage, cell density, and screening duration.

(The following steps are based on a 15 cm dish for example)

Amplify the cells to 8-9 x 10^7 for Day 1 experiment.

Day1: Transfection

1) T0 generation cells should have approximately 1.5 x 10^8 cells.

2) The amount of cells required to be plated on 15 cm dish:

sgRNA library size * sgRNA depth (at least 200 x) ÷ 0.3 MOI = initiate cell amount (round numbers)

3) Determine the number of dishes needed, and consider setting up additional dishes for MOI swings and controls:

	Treatment	Dish amount
Treatment group	+library virus +puromycin	(sgRNA library size * 200 x) ÷ 0.3 MOI ÷ cell density of transfection = dish amount
Control group 1	No library virus +puromycin (0 % survival control)	1
Control group 2	No library virus + No puromycin (100 % survival control)	1

4) Collect cells into a bottle, seed desired amount of cells on each dish.

5) Add virus and polybrene 8 μg/mL.

6) Mix thoroughly for 2 minutes.

7) Transfer to incubator.

Day 2: Puromycin Resistance Screening

1) 24 hours after adding the virus, the cells appear to be infected and firmly attached to the dish.

2) Remove the medium with a pipette.

3) Add fresh medium containing the desired concentration of puromycin to treatment group and control group 1 dishes, and medium without puromycin to control group 2 dish.

Day 4: T0

1) 48 hours after administration, all uninfected cells were dead (Control group 1).

2) Remove the medium, rinse the dishes with pre-warmed PBS to remove any remaining dead cells.

3) Digest and collect the surviving cells into another container. Make sure that the cell clumps are gently dispersed.

4) Count the cells and calculate cell density (cells/mL) for the treatment group, control group 1, and control group 2, respectively.

5) Centrifuge and collect the cells. The sgRNA depth of collected cells should be more than 200 x, 400 x is preferable.

6) Centrifuge at 1,200 rpm for 5 minutes.

7) Rinse with PBS

8) Remove PBS, cryopreserve the cells at -80 °C, label as T0 sample.

9) Plate the remaining cells in three parallel groups, do not screen with puromycin from now on. Each group needs to have 1.9 x 10^7 cells (200 x depth). The amount of cells in each dish, as well as the cells needed for each parallel group need to be consistent. For example, if Group A has 4 dishes with 4.74 x 10^6 cells/dish for a total of 1.9 x 10^7 cells, Group B and Group C should have the same amount of cells as well.

Day 5 Subsequent experiments (T1, T2, ...T18), cells passaging

1) Most adherent cells need to be passaged every 2-4 days. During the expansion of infected cells, the density of the passaged cells should be the same as that of the normal passaged cells. There may be a brief period of slow growth after infection, but the cells will return to normal growth rate in 5-15 days after infection.

2) About 2 weeks after passaging:

a. Digest and collect the cells from all of the groups together, which helps minimize random growth effects in individual dish within the parallel group.

Note: Increasing the amount of cells in the plate (e.g., increasing to 2.5 x 107) may reduce the wasting of genome DNA extracted from downstream.

7. Screening

7.1 Positive Screening

Positive screening can screen genes that are sensitive to the screening conditions, when these genes are knocked out, the cells survive the screening conditions. During positive

screening, most cells are dead and only cells containing sgRNAs for genes sensitive to the screening conditions survive. The expected result is that the remaining cells will be enriched for these sgRNAs. In positive screening, it is important to culture the cells long enough to allow for the death of genes that are not relevant to the screening conditions before sequencing. According to our experience, 10 days to 2 weeks are enough.Positive screening is relatively robust, an NGS sequencing depth of approximately 1 x 10^7 reads is recommended.

7.2 Negative Screening

Negative screening can screen genes that are essential for survival under the stress of the screening conditions, where essential genes are knocked out or loss of function in the presence of sgRNAs, while cells carrying sgRNAs for other genes competitively have no affect on proliferation. Negative screening is relatively difficult, because most cells will survive the screening and require strict control of parameters, to ensure that statistically significant changes can be detected. To detect tiny changes in sgRNA in these negative screenings, an NGS sequencing depth of up to approximately 1 x 10^8 reads is required.

8. gDNA Extraction

High-quality genomic DNA extraction is important to maintain sgRNA coverage and to amplify the sgRNAs carried by the cells, it is recommended to use Qiagen or Omega Cellular Genome Extraction Kit to extract the genomic DNA. DNA purification columns should not be overloaded, as this will reduce the diversity of sgRNAs in the samples. The cell population should contain approximately 400-1000 cells per sgRNA, and the number of cells needs to be maintained throughout the screening process, including gDNA extraction.

9. PCR Amplification

To accurately identify sgRNAs present in the gDNA of cells isolated from screening, it's important to construct a high-quality NGS library.

EDITGENE provides library amplification primers, as well as corresponding adaptor and primer sets for sequencing.

Note: To avoid the experimental errors caused by PCR, we recommend not over-amplified when the number of PCR cycles used for library amplification is sufficient for the yield. Please refer to the next point.

10. gDNA PCR

Attention:

- It is recommended to extract gDNA with experimental and control groups in parallel;
- Refer to the figure below to optimize the PCR process.

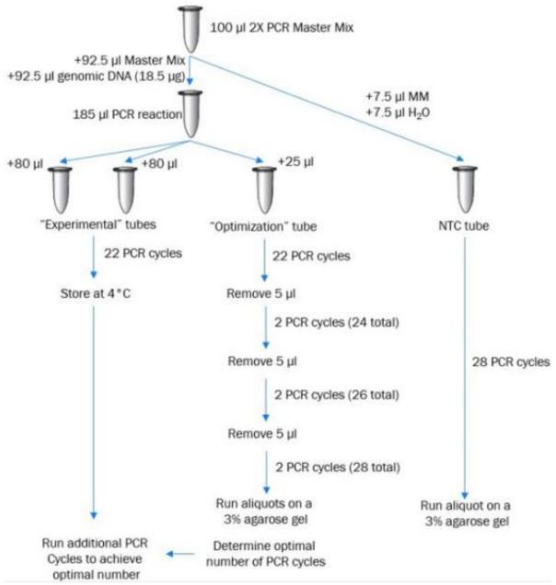

Fig2. PCR optimized procedure

1) Determine the band size of the PCR product: approximately 310 bp, NTC group should be blank.

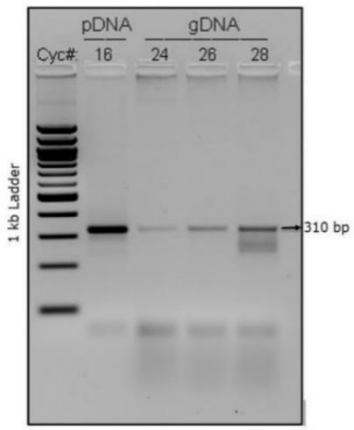

Fig3. sgRNA amplification electropherogram after optimization of PCR cycle number

2) Determine the optimal PCR cycle number, e.g., in the figure above, 24 cycles is sufficient to generate enough PCR products without generating other non-specific bands.

Note: The optimal PCR cycle number may be different in the gDNA samples of experimental and control groups.

3) After determination of the optimal PCR cycle number for different samples, transfer the products after amplification for 22 cycles into a tube, and store at 4 °C. Then, run the corresponding optimal cycle number.

Note: If the optimal cycle number is different for the experimental group and the control group, pause the PCR reaction and remove the tubes that have reached the optimal cycle number.

4) Ensure the PCR results of the experimental samples are consistent with that of the optimized samples, and are suitable for the next step of experiment and analysis.

11. Purification and Quantification of PCR Products

Run all PCR products in 2 % agarose gel, and cut the bands of target size (~ 310 bp) for purification.

11.1 DNA Purification and Quantification

1) Purify DNA fragments using gel purification kit (e.g. Takara NucleoSpin Gel or PCR Clean-Up kit).

2) Elute DNA with 20 µl NE Buffer.

3) Add 2 µl of purified DNA to 98 µl nuclease-free water for a 1:50 dilution.

4) Determine DNA concentration using Qubit dsDNA HS Assay Kit.

5) Dilute a small portion of the sample to 0.5 ng/µl. Analyze 1 µl (0.5 ng) of sample on Agilent High Sensitivity DNA Chip.

6) Expected result: a major peak at around 310 bp (as below).

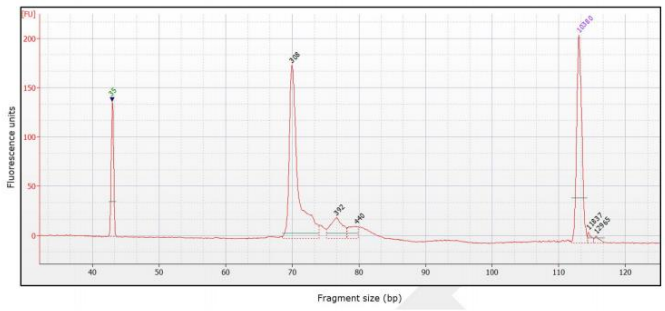

Fig4. Results of DNA purification and quantification

12. Library Preparation and Sequencing

Commonly use Illumina NextSeq500 or MiSeq sequencers for NGS. You can construct the NGS library according to the protocol for the sequencing instrument you are using.

13. Analyze:

After sequencing completed, use MAGeCKFlute to quality-control and analyze the result of sequencing.

According to our screening data and others, reads more than 1 x 10^7 should be sufficient to analyze complicate libraries, which is consisting of approximately 1 x 10^5 sgRNAs at the plasmid or infected cell population level. For positive screening under strong selection stress, a few million reads can yield sufficient results. However, for negative screening, where most cells survive, the may be only tiny significant changes. This may require deeper sequencing with more than 1 x 10^8 reads.

For standard screening of sgRNA activity leading to cytotoxicity, it is suggested to use transfected and unscreened cells as a control. The control cell population will be used for original plasmid of virus construction and plasmid obtained after transfection. When collect gDNA shortly after transfection, the frequency distribution of sgRNAs encoded in plasmid library is nearly identical to that observed in the efficiently transfected cell lines.

III. CRISPR Library Screening Frequently Asked Questions and Solutions

Question	Possible causes	Solution
Virus packing		
Low efficiency of transfection	Overcrowded when plating the cells	Plate the cells with the density of 4-5 x 10^6 cells/10 cm plate, if cell division is too fast, reduce the number of cells appropriately; perform the experiments at 50-80 % confluency.
	Determination of transfection efficiency is too short after transfection	The target gene can reach maximum expression only when 48 hours after transfection, at which time the transfection efficiency should be tested.
Low titer(< 105 cfu/mL)	Collect the virus too early	Collect the virus 48-72 hours after transfection.
	Have not used Polybrene, or not used the optimal concentration	Add 4 μg/mL polybrene during transfection or re-determine the optimal concentration (2-12 μg/mL).
	Freeze and thaw the virus for too many times	The titer decreases by 2-4 times per freeze-and-thaw, so reduce the times of freezing and thawing.
	Have not used the optimal screening concentration during titering	The resistance kill curve of the cells should be determined before titering, to determine the optimal screening concentration.
Transfection of target cells		
Low efficiency of transfection	Low titer	Concentrates viruses up to 100 x without ultracentrifugation.
	Low cell activity during	Virus packing medium may affect cell growth; dilute viral supernatant or reduce cell exposure time to virus.
		Overuse of polybrene: use titration to determine optimal dosage or shorten

	transfection	treatment time.
		Adjust the cells condition before transfection.
Toxic to cells during transfection	MOI is too high	Determine the optimal dosage of virus by titration, and dilute the virus.
	Toxicity of Polybrene	Reduce or optimize the concentration of polybrene; reduce transfection time.
	Cell supernatants of packing virus or medium are toxic to cells	Use medium to dilute and collect the virus.
Cells are dead or grow slowly after transfection with Cas9 virus	Cells are sensitive to Cas9 expression	Titrate the Cas9 virus using the target cell to ensure that one virus enters into one cell, or use another type of cell that is not sensitive to Cas9.
Extraction of genome DNA/PCR		
Low gDNA purification yield	Overloading of purification columns	Do not exceed the AXG500 maximum cell volume (~1 x 10^8 cells). Use multiple purification columns for large cell volumes.
Non-specific band observed in PCR		Optimize the cycle number.
Low/no PCR products		Optimize the cycle number.
Library		
Low editing efficiency	Low expression of Cas9	Use titration to determine optimal Cas9 virus concentration; use sgRNA with known editing efficiency to test.
	Not optimize transfection method yet	Use small amount of library viruses to optimize the transfection efficiency.
Representitive depth of gRNA is low after screening	Not optimize transfection method yet	Use small amount of library viruses to optimize the transfection efficiency.
	Over-passaging	Over-culturing can bias the pool. Screening should be performed when reaching the optimal number of cells.

IV. Q&A

1. How to transfect the cells with a high MOI?

If the target cells have a high MOI, you can try to transfect them with a lower viral load to avoid the adverse effects of over-expression of Cas9 to the cells. To address that, MOI optimization is required to determine the optimal virus load. This can be accomplished by gradually reducing the virus load and determining the transfection efficiency.

2. What if the plated bacteria always grow too little during CRISPR library construction?

The causes and suggestion are as followed:

1) Incorrect coating method: Make sure using fresh agar and the correct density of coated cells. In addition, try using different coating methods to improve the efficiency, such as rolling-coating or spraying-coating.

2) Low DNA yield or library quality: Repeat transformation with higher quality DNA or library samples. If these methods still do not solve your problem, you may need to re-evaluate your strain and culture conditions.

3. How is CRISPR KO plasmid library transformed and amplified? Does it need to be electrotransformed? Can it be done with a nucleotransformer?

The CRISPR KO library plasmids are usually transformed and amplified by electroporation or chemical transformation. Using a nucleotransformer to amplify is not recommended, while electroporation is currently the most efficient method. When performing electroporation, plasmid DNA is mixed with electroporated cells, and an electrical pulse is applied to facilitate the plasmids to entry into the cells. After electroporation, cells are seeded on selective medium to isolate colonies of the target plasmids.

4. How to perform CRISPR Cas9 library amplification? Why there is no result of sequencing?

CRISPR library usually applies PCR to amplify sgRNA sequences. If the sequences are not amplified, you may need to re-evaluate your PCR reaction conditions, such as primer concentration, PCR cycles, and reaction system. In addition, if you are using the Illumina sequencing platform, you may need to use the appropriate primers and sequencing method to ensure that the sgRNA is sequenced correctly. If you are still unable to obtain the expected sequence, it is recommended to optimize the method and quality of your library construction, ensuring that the sgRNA sequence has been correctly inserted into the library.

5. How to ensure the validity of library sgRNAs?

Methods to solve this problem:

1) Design high-quality sgRNAs to ensure high specificity and efficiency of gene targeting.

2) Conduct a pre-screening in certain test sites before the large-scale of screening, ensuring the validity of library sgRNAs.

6. How to ensure the integrity of library sgRNAs?

Ensure sufficient coverage of the library, the cellular coverages of each target gene depend on the type of screening. For positive screening, a target gene with a coverage of 100-200 × can be broken down to 4 gRNAs, with a coverage of 25-50 ×. For negative screening, a coverage of 500-1000 × per target gene is necessary to be sensitive enough to detect the essential genes. In addition, titer the virus with Cas9+ cell lines, it is important to determine the amount of sgRNA library virus required to achieve a transfection efficiency of approximately 30-40 %.

7. How to control the amount of viruses entering into the cells?

To control the amount of virus that enters the cell during CRISPR screening, the MOI (Multiplicity of Infection) and the density of the cells during infection should be determined. MOI is the ratio of the viral particles amount to the target cells amount, which can be adjusted by the virus amount added into the cells. The optimal MOI may vary depending on the cell type and virus species, so the appropriate MOI needs to be determined for specific experiments to ensure efficient transfection without causing cytotoxicity. In addition, cell density during infection can also affect transfection efficiency, thus, cell density needs to be optimized to ensure that the appropriate amount of cells are infected with the desired amount of virus. By controlling MOI and cell density, consistent and reproducible results can be obtained in CRISPR screening experiments.

8. How to detect sgRNAs in surviving cells?

To detect sgRNAs of surviving cells in CRISPR screening, collect the cells and extract their genome DNA (gDNA), then perform NGS analysis to detect sgRNAs. During NGS analysis, amplify and sequence the sgRNAs, then use software tools to analyze the sequencing results, to determine sgRNAs with increasing or decreasing frequency during screening. Specifically, the sequencing data can be trimmed using Cutadapt software, and then compare the sgRNA sequences (20nt) to a reference library or control group, determine fold changes in sgRNA frequency using CLC Genomics Workbench. Finally, use Excel to analyze the sequencing result, determine which sgRNAs have impact on cell survival.

9. **To make one virus transfect one single cell, why the library screening MOI required to be relatively low (approximately 30 %)?**

To ensure that only one virus transfects one single cell in CRISPR screening, the library screening MOI should be relatively low (approximately 30 %), only then the CRISPR gene editing of single cell can be performed. Too high MOI will result in multiple viruses entering one cell, leading to multiple gene editing sites and complicate the screening results. Therefore, to ensure that only one virus enters into one cell, the MOI value required to be controlled at around 30 %. This can be achieved by optimizing the amount of viral droplets in small-scale

experiments, to ensure that as many cells are infected as possible when only one virus enters in a cell.

10. What are the quality control criteria for CRISPR screening?

To ensure the accuracy of CRISPR screening results, the quality control criteria for CRISPR screening are as follow:

1) Quality of sgRNA plasmid library: Ensure the quality of sgRNA library, as well as the sgRNA sequence without mismatch or error. It is important to verify the accuracy and consistency of the sgRNA sequence.

2) sgRNA coverage: Check the coverage of the sgRNA library to ensure that each target gene has a sufficient number and diversity of sgRNAs.

3) Standardize the cell lines: Use standardized cell lines to ensure comparable screening results. It is also important to confirm the quality and certification of cell lines.

4) Control experiments: Perform appropriate control experiments to verify the validity and accuracy of the screening, including positive and negative controls, .

5) The efficiency of sgRNA introduction: Check the introduction efficiency of sgRNA, and ensure a sufficient amount of cells successfully receive sgRNAs.

6) Data analysis: Use appropriate statistical methods and data analysis tools to process and interpret screening data, and identify significant target genes.

11. What is the lead time for genome-wide CRISPR library screening?

The lead time for a genome-wide CRISPR screening varies depending on the specific experimental design and purpose, typically including the following steps:

1) sgRNA library preparation: This phase usually takes a few weeks to a month, depending on the design, synthesis and validation of sgRNAs.

2) Cell lines culture and standardization: During sgRNA library preparation, cell lines for screening need to be cultured and standardized. This phase can take several weeks.

3) sgRNA introduction and screening: This phase usually takes a few days to a few weeks, depending on the growth rate of the cells and the duration of the screening. Introducing sgRNA and screening may take some time to ensure that enough cells are infected.

4) Data collection and analysis: Once the screening is completed, the data collection and analysis phase may take a few weeks or more. In general, the lead time for genome-wide CRISPR screening typically ranges from a few months to half a year, depending on the scale of the experiments and the experimental objectives. The actual lead time may vary depending on the experimental conditions and complexity. Therefore, when performing a genome-wide CRISPR screening, it is important to make a detailed experimental plan in advance and arrange enough time for each step.