Protocol

Single Guide RNA Library Design and Construction

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This protocol describes how to generate a single guide RNA (sgRNA) library for use in genetic screens. There are many online tools available for predicting sgRNA sequences with high target specificity and/or cleavage activity. Here, we refer the user to genome-wide sgRNA sequence predictions that we have developed for both the human and mouse and that are available from the Broad Institute website. Once a set of target genes and corresponding sgRNA sequences has been identified, customized oligonucleotide pools can be rapidly synthesized by a number of commercial vendors. Thereafter, as described here, the oligonucleotides can be efficiently cloned into an appropriate lentiviral expression vector backbone. The resulting plasmid pool can then be packaged into lentiviral particles and used to generate knockouts in any cell line of choice.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agarose gels (1.0% and 2.0%)
BsmBI (New England Biolabs R0580S)
Endura Electrocompetent Cells (Lucigen 60242-0)
Ethidium bromide
Gel Extraction Kit (QIAGEN 28704)
Gibson Assembly Master Mix (New England Biolabs E2611S)
LB-ampicillin agar plates <R>
LB (Luria–Bertani) liquid medium <R>
Lentiviral single guide RNA (sgRNA) expression plasmid

Two expression plasmids are suitable—lentiCRISPR v2 (sgRNA expression plasmid with Cas9 [Addgene 52961]) or lentiGuide-Puro (sgRNA expression plasmid without Cas9 [Addgene 52963]). See Discussion for further details.

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Library polymerase chain reaction (PCR) primers
Forward: GGCTTTATATATCTTGTGGAAAGGACGAAACACCG
Reverse: CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

NEBuffer 3.1 (New England Biolabs B7203S, supplied by manufacturer as a 10× stock together with BsmBI)
Oligonucleotides, custom-made/ordered (see Step 4)
Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs M0531S)
Plasmid Plus Maxi Kit (QIAGEN 12963)
Recovery medium (Lucigen 80026-1)
Water (H_2O), PCR-grade

Equipment

Bacterial shaker(s) (at 30°C and 37°C)
E. coli Pulser Transformation Apparatus (Bio-Rad 165-2101)
Eppendorf tubes (1.5-mL)
Erlenmeyer flask (500-mL)
Gel electrophoresis apparatus
Gel imager
Heat blocks (at 50°C and 55°C)
Ice
Incubator(s) (at 30°C and 37°C)
MicroPulser Cuvettes (Bio-Rad 165-2089)
NanoDrop spectrophotometer (NanoDrop)
Online sgRNA sequence analysis tools (see Step 1)
Pipette
Thermocycler
Water bath
x-tracta gel extractor (USA Scientific 5454-0100)

METHOD

This protocol is intended primarily for users who wish to construct an sgRNA library targeting a customized set of genes. Many large-scale sgRNA libraries suitable for Cas9-based screening can be found on Addgene (http://www.addgene.org/CRISPR/libraries/). If a preexisting library is used, the investigator should skip to the Library Transformation section of this protocol (Steps 24–30).

sgRNA Sequence Design

1. Obtain a list of sgRNA sequences targeting the genes of interest.

   Investigators have a wide choice of online tools for determining sgRNA sequences that possess high target specificity and/or cleavage activity (Heigwer et al. 2014; Xie et al. 2014). For human and mouse genes, we have generated a set of sgRNA sequences that can be accessed at http://www.broadinstitute.org/~timw/CRISPR/. These sets of sgRNA predictions have been experimentally validated to show high on-target cleavage activity (T. Wang, unpubl.), and we recommend their use here.

2. Prepend the 5′ universal flanking sequence: TATCTTGTGGAAAGGACGAAACACC.

   An additional "G" must be prepended to sgRNA sequences starting with any other nucleotide to allow efficient transcription from the U6 promoter.

3. Append the 3′ universal flanking sequence: GTTTTAGAGCTAGAAATAGCAAGTTAAAAT.

4. Order custom oligonucleotide pools.

   Microarray-based oligonucleotide synthesis is a highly competitive and rapidly evolving industry, and, as such, many commercial vendors can provide similar product offerings. Many of the sgRNA libraries created to date have been synthesized by CustomArray Inc. (Bothell, WA), although we do recommend that the user identify a suitable vendor depending on the desired scale, accuracy, and speed of synthesis.

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Vector Preparation

5. Streak out a bacterial stab culture of sgRNA lentiviral expression vector obtained from Addgene on LB-amp plates and incubate overnight at 30°C.
6. Pick a single colony and seed into a 500-mL Erlenmeyer flask containing 100 mL of LB liquid medium containing 100 µg/mL ampicillin.
7. Incubate culture overnight at 30°C in a rotating bacterial shaker.
8. Prepare plasmid DNA from the bacterial culture using the QIAGEN Plasmid Plus Maxi Kit according to the manufacturer's instructions.
9. Assemble the following digestion reaction on ice.

Lentiviral sgRNA expression plasmid	3 µg
NEBuffer 3.1	3 µL
BsmBI	3 µL
H_2O	to 30 µL

10. Incubate overnight at 55°C in a water bath.
11. Run out the reaction on an ethidium-bromide-stained 1% agarose gel. Visualize the digested bands using a standard gel imager.
12. Cut the digested vector backbone using an x-tracta gel extractor tool.
13. Extract DNA using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions, eluting in 10 µL of water.

Library Amplification and Cloning

14. Assemble four replicates of the following PCR on ice, as follows.

Synthesized oligonucleotides	1 µL
Forward library PCR primer (10 µM)	2 µL
Reverse library PCR primer (10 µM)	2 µL
Phusion High-Fidelity PCR Master Mix with HF Buffer	25 µL
H_2O	20 µL

15. Amplify reactions in a thermocycler using the following program, varying the total number of cycles for each replicate.

1 cycle	98°C	2 min
8, 10, 12 or 16 cycles	98°C	10 sec
	60°C	15 sec
	72°C	45 sec
1 cycle	72°C	5 min
1 cycle	4°C	Hold

16. Run out the reactions on an ethidium-bromide-stained 2% agarose gel. Visualize the PCR bands using a standard gel imager.
17. For all reactions yielding a visible product at 92 base pairs, cut out the band using an x-tracta gel extractor tool.
18. Extract DNA using the QIAGEN Gel Extraction Kit according to the manufacturer's instructions, eluting in 10 µL of water.
19. Determine the PCR product concentrations using a NanoDrop spectrophotometer. Proceed to Gibson Assembly cloning using the sample amplified for the fewest cycles, with a product concentration >10 ng/µL.

20. Assemble two replicates of the following Gibson Assembly reaction on ice.

Digested vector from Step 13	100 ng
Gibson Assembly Master Mix	10 µL
H_2O	to 19 µL

21. Add 1 µL of the library PCR product to one reaction and add 1 µL of water to the other.
22. Incubate for 1 h at 50°C.
23. Place reactions on ice after completion.

Library Transformation

24. Warm Recovery Medium for 30 min in a 37°C water bath.
25. Warm an LB-ampicillin agar plate for 30 min in a 37°C incubator.
26. Thaw one vial of Endura Electrocompetent Cells and aliquot cells into two tubes on ice for 15 min.
27. Place two MicroPulser Cuvettes on ice.
28. For each reaction (control- and insert-containing) proceed as follows:
    i. Add 1 µL of the Gibson Assembly reaction product to bacterial cells.
    ii. Transfer 25 µL of the bacterial cell and Gibson Assembly reaction product mixture into MicroPulser Cuvettes.
    iii. Place cuvette into an *Escherichia coli* Pulser Transformation Apparatus and electroporate at 1.8 kV.
    iv. Quickly add 975 µL of the Recovery Medium into the cuvette and pipette up and down three times to resuspend the cells.
    v. Transfer mixture to a 1.5-mL microcentrifuge tube.
    vi. Place the tube in a shaking incubator for 1 h at 37°C.
    vii. Serially dilute 10 µL of the transformation mixture in Recovery Medium four times, using a dilution factor of 1/10 at each step.
    viii. Spot 10 µL of each dilution onto an LB-ampicillin plate.
    ix. Incubate plate overnight at 30°C.

    The number of colonies on these spots can be multiplied by 10^3, 10^4, 10^5, and 10^6, respectively, to estimate the total number of colony-forming units.

    See Troubleshooting.
29. For insert-containing reaction only, proceed as follows:
    i. Seed the remainder of the transformation mixture into a 500-mL Erlenmeyer flask containing 100 mL of LB liquid medium containing 100 µg/mL ampicillin.
    ii. Incubate culture overnight at 30°C.
    iii. If the transformation efficiency, as assessed by the serial plating, exceeds 20-fold of the library size and the transformation efficiency of the control reaction is <1% of the insert-containing reaction, then prepare plasmid DNA from the bacterial culture using the QIAGEN Plasmid Plus Maxi Kit according to the manufacturer's instructions.
30. To assess recombination, run out the amplified plasmid on an ethidium-bromide-stained 1% agarose gel. Visualize the plasmid DNA using a standard gel imager.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 28): The transformation efficiency is too low.
Solution: There are two common causes of this problem.

- There are bad electrocompetent cells. To check, perform a test electroporation with an intact control plasmid and compare with the advertised efficiency.
- The level of salt in the transformation is too high. Dilute the Gibson Assembly reactions 1:3 in water before transforming.

Finally, monitoring the time constant after electroporating cells can often serve as a useful indicator of transformation efficiency. A time constant between 3.5 and 4.5 msec is ideal.

Problem (Step 30): The plasmid library is recombined.
Solution: The use of Endura cells and incubation of bacteria at 30°C are both intended to minimize recombination of the lentiviral plasmid library. However, if a substantial fraction of the amplified plasmid library is recombined, as assessed by gel electrophoresis, it might be advisable to grow the transformation products on agar plates rather than in liquid culture.

DISCUSSION

The decision to use a vector with or without Cas9 for screening depends on several factors. Using a Cas9-containing backbone readily allows screening in any cell line without prior modification of the cell line. However, much less recombination during plasmid amplification and higher viral titers (typically 20- to 100-fold higher) during viral packaging can be achieved by using smaller vectors lacking Cas9. For this reason, we recommend that only users who plan to conduct screens across multiple cell lines should clone sgRNA libraries into a Cas9-containing vector. In contrast, those seeking to perform screens across multiple conditions in a single cell line should first derive a Cas9-expressing clone.

RECIPES

Ampicillin Stock Solution (100 mg/mL)

Ampicillin (sodium salt [sodium ampicillin], m.w. = 371.40)

Dissolve 1 g of sodium ampicillin in sufficient H_2O to make a final volume of 10 mL. If sterilization is required, prewash a 0.45- or 0.22-µm sterile filter by drawing through 50–100 mL of H_2O. Then pass the ampicillin solution through the washed filter. Store the ampicillin in aliquots at −20°C for 1 yr (or at 4°C for 3 mo).

LB Agar

Agar (20 g/L)
NaCl (10 g/L; Sigma-Aldrich S9625)
Tryptone (10 g/L; BD 211705)
Yeast extract (5 g/L; BD 212750)

Add H_2O to a final volume of 1 L. Adjust the pH to 7.0 with 5 N NaOH. Autoclave. Pour into Petri dishes (~ 25 mL per 100-mm plate).

LB-Ampicillin Agar Plates

Ampicillin, filter-sterilized (10 mg/mL stock)
LB agar

Autoclave 1 L of LB agar. Cool to 55°C. Add 10 mL of ampicillin stock. Pour into Petri dishes (~ 25 mL per 100-mm plate).

LB (Luria-Bertani) Liquid Medium

Reagent	Amount to add
H_2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Combine the reagents and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL). Adjust the final volume of the solution to 1 L with H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

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