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Generation of Knockout Cell Lines Using CRISPR–Cas9 Technology

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Ali Ghoochani[1,2,3,4], Monther Abu-Remaileh[1,2,3,4]

[1]Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA;
[2]Department of Genetics, Stanford University, Stanford, CA 94305, USA;
[3]The Institute for Chemistry, Engineering and Medicine for Human Health (Sarafan ChEM-H), Stanford University, Stanford, CA 94305, USA;
[4]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815, USA

asap

Ali Ghoochani
Stanford University

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We use this protocol and it's working

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Abstract

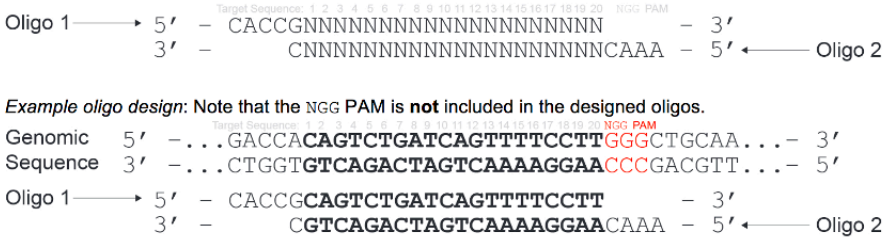
This protocol describes the generation of gene knockout cell lines using CRISPR–Cas9 genome editing. The procedure involves sgRNA design, cloning into a Cas9-GFP expression vector pX458, transfection, single-cell sorting, colony expansion, and validation by Sanger sequencing and ICE analysis.

Materials

- pX458 vector (expressing Cas9 and GFP) pSpCas9(BB)-2A-GFP (PX458) (Addgene #48138)
- Targeting sgRNA oligos
- XtremeGene 9 transfection reagent
- DMEM with 30% FBS
- Penicillin/Streptomycin
- 96-well plates
- Competent cells (recombination-deficient)
- T4 DNA ligase buffer (10X)
- BbsI enzyme
- PCR reagents and primers
- Sanger sequencing services
- ICE indel analysis tool (https://synthego.com)

Design sgRNAs

1



Annealing sgRNAs

2

- 1 µl Oligo 1 (100 µM)
- 1 µl Oligo 2 (100 µM)
- 1 µl 10X T4 Ligation Buffer (NEB)
- 7 µl ddH_2O
- Total: 10 µl

2.1 Thermocycler conditions for annealing:

- 37°C for 30 min
- 95°C for 5 min
- Ramp down to 25°C at 5°C/min

Golden Gate Ligation Reaction

3

- 1 µl of 1:100 diluted annealed oligos
- X µl backbone vector (20 ng)
- Add water to 16 µl total
- 2 µl 10× T4 ligase buffer
- 1 µl BbsI

3.1 Thermocycler conditions for ligation:

- 42°C for 5 min
- 42°C for 5 min

- 16°C for 5 min
- 55°C for 10 min

For 30 cycles

Transformation and Maxiprep

4 Transform 2 µL of the ligation reaction into 50 µL competent cells. Also include a negative control ligation. Continue transformation.

5 Purify the ligated sgRNA-pX458 construct using a Maxiprep kit

Transfection and Single-Cell Cloning

6 Transfect cells with the pX458-sgRNA plasmid using XtremeGene 9 at a 1:3 DNA-to-reagent ratio.

7 After 48 hours, sort GFP-positive cells using a flow cytometer into 96-well plates (1 cell/well) containing 200 µL of DMEM with 30% FBS and penicillin/streptomycin.

8 Incubate at 37°C and 5% CO_2 to allow colony formation

9 Once colonies are visible, harvest cells for genomic DNA extraction

Validation of Knockout

10 PCR amplify the sgRNA target region from each clone.

11 Submit PCR products for Sanger sequencing.

12 Analyze sequences using the Synthego ICE indel deconvolution tool.