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Generation of Knockout Cell Lines Using CRISPR–Cas9 Technology

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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

Oligo 1 → 5' — CACCGNNNNNNNNNNNNNNNNNNNN — 3'
3' — CNNNNNNNNNNNNNNNNNNNNCAA — 5' ← Oligo 2

Target Sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 NGG PAM

Example oligo design: Note that the NGG PAM is not included in the designed oligos.

Genomic 5' — ...GACCACAGTCTGATCAGTTTTCCTTGGGCTGCAA... — 3'
Sequence 3' — ...CTGGTGTCTAGTCAAAAGGAACTTCTGACGTT... — 5'

Oligo 1 → 5' — CACCGCAGTCTGATCAGTTTTCCTT — 3'
3' — CGTCAGACTAGTCAAAAGGAACAAA — 5' ← Oligo 2

Target Sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 NGG PAM

Annealing sgRNAs

2

- 1 µl Oligo 1 (100 µM)
- 1 µl Oligo 2 (100 µM)
- 1 µl 10X T4 Ligation Buffer (NEB)
- 7 µl ddH₂O
- 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.