pEcCas/pEcgRNA-Mediated Genome Editing in *E. coli* BL21(DE3)

Materials

Strains and DNA

E. coli BL21(DE3)
pEcCas plasmid
Target-specific pEcgRNA plasmid
Linear donor DNA
Wild-type BL21(DE3) as the control
E. coli DH5α for constructing target-specific pEcgRNA
E. coli DB3.1 for maintaining the ccdB-containing pEcgRNA backbone, if required

Reagents and Media

LB broth and LB agar
Kanamycin, 50 mg/mL stock solution
Spectinomycin, 50 mg/mL stock solution
Sterile 0.5 M L-arabinose stock
Sterile ice-cold 10% glycerol
L-rhamnose
Glucose
Sucrose
BsaI and corresponding restriction buffer
T4 DNA ligase and ligation buffer
Target-specific oligonucleotides
Colony-PCR reagents
Agarose gel-electrophoresis reagents
Genotype-verification primers

Equipment

37 °C shaking incubator
Refrigerated centrifuge
Spectrophotometer
Ice-water bath
Electroporator
Precooled 2-mm electroporation cuvettes
PCR thermocycler
Gel-electrophoresis and imaging system

Procedure

Construction of Target-Specific pEcgRNA

1. Digest pEcgRNA with BsaI to generate the overhangs 5′-TAGT-3′ and 5′-AAAC-3′.
2. Purify the linearized pEcgRNA backbone. Use it immediately or store it at −80 °C.
3. Design two 24-nt oligonucleotides containing the 4-nt overhang and the 20-nt target-specific sequence.
4. Anneal the two oligonucleotides to form double-stranded DNA.
5. Ligate the annealed oligonucleotides into the BsaI-linearized pEcgRNA backbone.
6. Transform the ligation product into *E. coli* DH5α and select transformants on LB agar containing spectinomycin.
7. Verify the target-specific pEcgRNA construct by colony PCR and DNA sequencing.

Introduction of pEcCas into BL21(DE3)

1. Transform pEcCas into competent *E. coli* BL21(DE3).
2. Recover the cells in antibiotic-free LB medium and plate them on LB agar containing kanamycin.
3. Incubate overnight at 37 °C.
4. Pick a single colony and culture it overnight in LB medium containing kanamycin.

Preparation of Electrocompetent Cells

1. Inoculate 15 mL of LB medium containing kanamycin with 1% overnight BL21(DE3)/pEcCas culture.
2. Add L-arabinose to a final concentration of 10 mM to induce the λ-Red system.
3. Incubate at 37 °C and 220 rpm until the OD_{600} reaches approximately 0.6.
4. Chill the culture in an ice-water bath for 30 min.
5. Centrifuge at 4,000 × g for 10 min at 4 °C and discard the supernatant.
6. Gently resuspend the pellet in 15 mL of ice-cold 10% glycerol.
7. Centrifuge again under the same conditions and discard the supernatant.
8. Repeat the glycerol washing step once.
9. Resuspend the final pellet in approximately 300 μL of ice-cold 10% glycerol.
10. Divide the cell suspension into 100 μL aliquots and keep them on ice.

Electroporation and Selection

1. Mix 100 μL of electrocompetent BL21(DE3)/pEcCas cells with approximately 100 ng of target-specific pEcgRNA and 400 ng of donor DNA.
2. Transfer the mixture into a precooled 2-mm electroporation cuvette.
3. Electroporate at 2.5 kV, 200 Ω, and 25 μF.
4. Immediately add 1 mL of antibiotic-free LB medium

5. Recover the cells at 37 °C and 220 rpm for 1 h.
6. Plate the recovered cells on LB agar containing kanamycin and spectinomycin.
7. Incubate overnight at 37 °C.

Verification of Edited Colonies

1. Pick individual colonies from the selection plates.
2. Perform colony PCR using primers located outside the donor homology arms.
3. Analyze the PCR products by agarose gel electrophoresis, using the wild-type strain as a control.
4. Purify PCR products of the expected size and verify the edited sequence by Sanger sequencing.

Plasmid Curing (Stepwise Plasmid Curing)

1. Inoculate a verified edited colony into 2 mL of LB medium containing 10 mM rhamnose and 50 μg/mL kanamycin.
2. Incubate overnight at 37 °C and 220 rpm.
3. Dilute the culture and plate it on LB agar containing 50 μg/mL kanamycin.
4. Screen individual colonies on separate LB agar plates containing 50 μg/mL kanamycin or 50 μg/mL spectinomycin.
5. Select colonies that remain kanamycin-resistant but become spectinomycin-sensitive. These colonies have lost pEcgRNA.
6. Inoculate a pEcgRNA-cured colony into LB medium containing 5 g/L glucose and incubate overnight at 37 °C and 220 rpm.
7. Plate the culture on LB agar containing 5 g/L glucose and 10 g/L sucrose.
8. Screen individual colonies on LB plates with and without kanamycin.
9. Select colonies that grow on antibiotic-free LB but not on LB containing kanamycin. These colonies have lost pEcCas.