Knock-Out of LysA Gene in E.coli BW25113 by λRed Recombination System

A. The Preparation of Targeting Vector

<table>
<tr><td>1</td><td>The Design of the Primers</td><td>
a. The knockout primers:
agcacttatctggagtttgttatgccacattcactgtGTGTAGGCTGGAGCTGCTTC
gtcatcatgcaaccagcgactaaccgcagttaaagcaATGGGAATTAGCCATGGTCC
(The part of lowercase letters is the kan resistance gene and the capital one is the LysA homology arms)
b. The detection primers:
TAGTAGTCCGACGCTGGTACGTCG
TTGCATAGACTCGACATAAATCGA
</td></tr>
<tr><td>2</td><td>PCR</td><td>
Amplify the targeting vector, using the plasmid pKD4 as template.
The PCR system:
PCR MIX 12.5μl
plasmid pKD4 1μl
upstream primer(10umol/L) 1μl
downstream primer(10umol/L) 1μl
sterile water 9.5μl
paraffin oil 10μl
The PCR processes:
<table>
<tr><td>Denaturation</td><td>94℃</td><td>20s</td><td rowspan="3">33 circulations</td></tr>
<tr><td>Annealing</td><td>52℃</td><td>20s</td></tr>
<tr><td>Elongation</td><td>72℃</td><td>5min</td></tr>
<tr><td>Final elongation</td><td>72℃</td><td>5min</td><td>1 circulations</td></tr>
</table>
</td></tr>
<tr><td>3</td><td>AGE</td><td>
To analyze whether the PCR product is correct.
a. The preparation of the agarose gel
1*TAE 30mL
agarose 300mg
genecolour I™ 3μl
heating 2min
clotting in the gel container
b. Electrophoresis
110V 35min
c. Analysis
Gel-Imaging System
d. Gel extraction
Gel Extraction Kit
</td></tr>
<tr><td>4</td><td>Purification</td><td>
To eliminate the false positives of pKD4
a. Enzyme digestion
System: 17μl extraction product, 1μl Dpn1, 2μl 10*buffer, 37℃, 1h
b. AGE
</td></tr>
</table>

B. The Transformation of Plasmid pKD46

1	The Preparation of Competent Cells($CaCl_2$)	a. Shake the E.coli BW25113 overnight at 30℃, then transfer it into a new liquid no resistant LB medium. b. Detect OD600 each hour. c. When OD600 is between 0.3 and 0.4, take 1.5ml bacterium solution into a 1.5ml sterile centrifuge tube, putting it into ice for 15-30min. d. Centrifuge the bacterium solution in the conditions of 4000rpm, 2min, 4℃, abandoning the supernatant. e. Add 800μl pre-cooling 0.1mol/L sterilized $CaCl_2$ solution to suspend the sediment. f. 30min ice bath. g. Centrifuge the bacterium solution in the conditions of 4000rpm, 2min, 4℃, abandoning the supernatant. h. Add 200μl pre-cooling 0.1mol/L sterilized $CaCl_2$ solution to suspend the sediment, take 50μl to transform.
2	The Transformation of Plasmid pKD46	a. Add 50μl competent cells and 5μl plasmid pKD46 to 1.5ml sterile centrifuge tube, taking 30min ice bath. b. After a 45s heating at 42℃ water, put the tube into ice rapidly for 2min, then adding 500μl liquid no resistant LB medium. c. Shake the tube for 1.5h at 30℃. d. Centrifuge the bacterium solution in the conditions of 5000rpm for 1min, abandoning the supernatant. e. Coat the ampicillin resistant solid LB medium with the bacterium solution, than cultivated overnight at 30℃. f. Picking a single bacterial colony into the ampicillin resistant liquid LB medium, cultivated overnight at 30℃.
3	The Verification	a. Bacteria keeping: add 700μl bacterium solution and 700μl 50% sterile glycerin into a 1.5ml sterile centrifuge tube, storing at -20℃. b. Extract plasmids: TIANprep Mini Plasmid Kit. c. AGE

C. Electroporation

1	The Preparation of Competent Cells(glycerin)	a. Add 100μl pKD46-transformed E.coli BW25113 to 7ml liquid LB medium, along with 7μl ampicillin solution, shaking overnight at 30℃. b. Take 5% of bacterium solution into 30ml no resistant liquid LB medium within 30μl ampicillin solution, cultivating at 30℃ shaker. c. Detect OD600 each hour until it is between 0.3 and 0.4. d. Add L-Arabinose until its concentration is 30mmol/L. Then shake it at 30℃ for

		1h. e. Take all of the bacterium solution into 1.5ml centrifuge tubes, ice bath for 10min. f. Centrifuge the bacterium solution in the conditions of 5000rpm and 4℃ for 10min, abandoning the supernatant. g. Suspend the sediment with pre-cooling 50% sterile glycerin till 1ml, centrifuging in the conditions of 5000rpm and 4℃ for 2min, repeat for 3 times. h. Suspend the sediment with 300μl glycerin.
2	Electroporation	a. Take 100μl bacterium solution and 10μl kan targeting vector into 1.5ml sterile centrifuge tube, mix, then adding into a pre-cooling electroporation cup with no bubbles. b. The size of electroporation cup:1mm or 2mm. c. Electroporation parameters: 2000kV, 25μF, controller 200Ω
3	Follow- up	a. Add 1 ml no resistant liquid LB medium immediately after electroporation, mix. b. Suck all the liquid out of the electroporation cup into a new sterile 1.5ml centrifuge tube, shaking it at 30℃, 180rpm for 1-1.5h. c. Coat the ampicillin resistant solid LB medium with the bacterium solution, cultivated overnight at 37℃.

D. The Verification of Strain

1	Bacteria Shaking Cultivating	Picking a single bacterial colony into the ampicillin resistant liquid LB medium, cultivated overnight at 30℃.
2	Bacteria Keeping	add 700μl bacterium solution and 700μl 50% sterile glycerin into a 1.5ml sterile centrifuge tube, storing at -20℃.
3	Plasmid Extraction	TIANprep Mini Plasmid Kit.
4	PCR	The PCR system(using the detection primers): PCR MIX 12.5μl plasmid pKD4 1μl upstream primer(10umol/L) 1μl downstream primer(10umol/L) 1μl sterile water 9.5μl paraffin oil 10μl
5	AGE	To analyze if the strain is correct.