

CRISPR/Cas9-mediated ssDNA Recombineering in *Corynebacterium glutamicum*

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[Abstract] *Corynebacterium glutamicum* is a versatile workhorse for industrial bioproduction of many kinds of chemicals and fuels, notably amino acids. Development of advanced genetic engineering tools is urgently demanded for systems metabolic engineering of *C. glutamicum*. Recently unveiled clustered regularly interspaced short palindromic repeats (CRISPR) and their CRISPR-associated proteins (Cas) are now revolutionizing genome editing. The CRISPR/Cas9 system from *Streptococcus pyogenes* that utilizes NGG as protospacer adjacent motif (PAM) and has good targeting specificity can be developed into a powerful tool for efficient and precise genome editing of *C. glutamicum*. In this protocol, we described the general procedure for CRISPR/Cas9-mediated ssDNA recombineering in *C. glutamicum*. Small modifications can be introduced into the *C. glutamicum* chromosome with a high editing efficiency up to 90%.

Keywords: CRISPR/Cas9, Genome editing, ssDNA recombineering, *Corynebacterium glutamicum*, Nucleotide editing, Lagging strand

[Background] The Gram-positive soil bacterium *Corynebacterium glutamicum* is a versatile workhorse for industrial bioproduction of amino acids, biofuels, and polymer building blocks (Becker *et al.*, 2016). At the early stage of engineering of *C. glutamicum*, random mutagenesis combined with positive selection by phenotypic resistance to amino acid analogs was the most commonly used strategy (Vertes *et al.*, 2005). Genetic manipulations in *C. glutamicum* were initiated in 1984 and became a key enabling strategy for strain improvement (Ozaki *et al.*, 1984). A routinely used method for gene disruption and insertion in *C. glutamicum* is based on integration of a suicide vector into its chromosome, followed by a second recombination event to remove the plasmid backbone and a counter-selection step using a conditionally lethal marker. Nevertheless, due to the frequent spontaneous inactivation of the counter-selectable marker *sacB*, up to 45% of colonies obtained in the screening process were false-positive, making this multi-step procedure time-consuming and inefficient (Schafer *et al.*, 1994). To engineer *C. glutamicum* more efficiently, simple but versatile genome editing tools are still in urgent demand.

Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and their CRISPR-associated proteins (Cas) have been explored as a leading-edge tool for bacterial genome editing (Choi *et al.*, 2016). The CRISPR/Cas9 system accepts NGG protospacer adjacent motif (PAM) and has good targeting specificity (Jiang *et al.*, 2013). Therefore, the CRISPR/Cas9 system is expected to possess

abundant editing targets in GC-rich *C. glutamicum*. We successfully developed a CRISPR/Cas9 toolbox for efficient and comprehensive engineering of *C. glutamicum* strains (Liu *et al.*, 2017; Wang *et al.*, 2018). By using the tailor-made CRISPR/Cas9 system, efficient deletion and insertion of large DNA fragments using plasmid-borne editing templates were achieved. By combining CRISPR/Cas9 and ssDNA recombineering, small modifications were introduced into the genome with efficiencies up to 90% (Liu *et al.*, 2017). Besides, targeted based editing without donor DNA was also realized using Cas9 and cytidine deaminase fusions (Wang *et al.*, 2018). The toolbox developed is simple and versatile, which is expected to overcome the major limitations of existing genome editing tools of *C. glutamicum* and advance the genetic manipulation of this industrial workhorse. Herein, we described the detailed protocol for CRISPR/Cas9-mediated ssDNA recombineering in *C. glutamicum*.

Materials and Reagents

1. 0.2 ml PCR tubes (Biosharp, catalog number: BS-02-P)
2. 1.5 ml Eppendorf tubes (Biosharp, catalog number: BS-15-M)
3. Pipette tips (Biosharp, catalog numbers: BS-10-T, BS-200-T, BS-1000-T)
4. Petri dishes (NEST Biotechnology, catalog numbers: 752001)
5. Competent *Escherichia coli* DH5 α cells (CWBIO, catalog number: CW0808)
6. pgRNA5 plasmid (Liu *et al.*, 2017)
7. pCas9 plasmid (Liu *et al.*, 2017)
8. Agarose (Biowest, catalog number: 111860)
9. GoldView I Nuclear Staining Dyes (10,000x) (Solarbio, catalog number: G8140)
10. AxyPrepTM DNA Gel Extraction Kit (Corning, Axygen[®], catalog number: AP-GX-250)
11. Tryptone (Oxoid, catalog number: LP0042)
12. Yeast extract (Oxoid, catalog number: LP0021)
13. Kanamycin (Solarbio, catalog number: K8020)
14. Chloramphenicol (Fisher Scientific, catalog number: BP904-100)
15. Q5[®] High-Fidelity DNA polymerase (New England Biolabs, catalog number: M0491L)
16. AxyPrepTM Plasmid Miniprep Kit (Corning, Axygen[®], catalog number: AP-MN-P-250)
17. Vazyme ClonExpress[®] II One Step Cloning Kit (Vazyme Biotech, catalog number: C112-01/02)
18. 2x EasyTaq PCR SuperMix (+dye) (Beijing TransGen Biotech, catalog number: AS111-11)
19. Tris base (Solarbio, catalog number: T8060)
20. Glacial acetic acid (Sinopharm Chemical Reagent, catalog number: 10000218)
21. EDTA (Solarbio, catalog number: E8030)
22. NaCl (Sinopharm Chemical Reagent, catalog number: 10019328)
23. Agar (Solarbio, catalog number: A8190)
24. (NH₄)₂SO₄ (Solarbio, catalog number: A8821)
25. K₂HPO₄ (Solarbio, catalog number: D9880)
26. NaH₂PO₄ (Solarbio, catalog number: S5830)

27. MgSO₄·7H₂O (Sinopharm Chemical Reagent, catalog number: 10013018)
28. Brain Heart Infusion (BHI) (BD, catalog number: 237500)
29. Tween 80 (Solarbio, catalog number: T8360)
30. DL-threonine (DL-Thr) (Tokyo Chemical Industry, catalog number: T3105)
31. Glycine (Gly) (Solarbio, catalog number: G8200)
32. Glucose (Beijing Dingguo Changsheng Biotechnology, catalog number: DS063)
33. Trisodium citrate (Solarbio, catalog number: S8220)
34. Sorbitol (Solarbio, catalog number: S8090)
35. Isonicotinic acid hydrazide (INH) (Sigma-Aldrich, catalog number: I3377)
36. IPTG (Solarbio, catalog number: I8070)
37. Sodium propionate (Sigma-Aldrich, catalog number: P1880)
38. Oligo for ssDNA recombineering:
AGGTAAGGCGCACACGAGCGACCTTACGAAGAGCAGAGTTAGGCTTGCGAGGGGTGGT
GGTGACACACGGGTGCATACGCCACGACGCT (synthesized and page-purified in Genewiz, Suzhou, China)
39. Primers for gRNA plasmid construction (synthesized and page-purified in Genewiz, Suzhou, China):
gRNA-1: AGAGCAGAGTTAGGCTTCTTGTTTTAGAGCTAGAAATAGCAAG
gRNA-2: TGCTCTTTCATTGATGGCTGG
gRNA-3: CAGCCATCAATGAAAGAGCAAC
gRNA-4: AAGAAGCCTAACTCTGCTCTTGAATTACACTGTACCTGTTGCGTC
40. 50x TAE electrophoresis buffer (see Recipes)
41. 1x TAE electrophoresis buffer (see Recipes)
42. LB medium (see Recipes)
43. LB solid medium (see Recipes)
44. BHI medium (see Recipes)
45. NCM medium (see Recipes)
46. BHIS medium (see Recipes)
47. LBHIS solid medium (see Recipes)
48. 25 mg/ml kanamycin stock (see Recipes)
49. 5 mg/ml chloramphenicol stock (see Recipes)
50. 0.1 M IPTG stock (see Recipes)
51. 500 g/L sodium propionate stock (see Recipes)

Equipment

1. Pipettes (Eppendorf, catalog number: 022575442)
2. Thermal cycler (Thermo Fisher Scientific, Thermo Scientific™, model: Arktik™ Thermal Cycler)
3. Water bath (Beijing Ever Bright Medical Treatment Instrument, model: DZKW-S-4)

4. NanoDrop (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 2000)
5. Milli-Q H₂O dispenser (Thermo Fisher Scientific, Thermo Scientific™, model: Barnstead™ GenPure™ Pro, catalog number: 50131950)
6. Centrifuge (Eppendorf, model: 5084 R, catalog number: 5805000696)
7. Electroporation cuvette (Bio-Rad Laboratories, catalog number: 1652082)
8. -20 °C freezer (Panasonic, catalog number: MDF-U548D-C)
9. pH meter (Fisher Scientific, model: Fisher Scientific™ accumet™ AB150, catalog number: 13-636-AB150)
10. DNA electrophoresis apparatus (Beijing LIUYI Biotechnology, catalog numbers: DYY-6C and DYCP-32B)
11. Electroporator (Eppendorf, model: Electroporator 2510)
12. Autoclave (SANYO, catalog number: MLS-3750)

Software

1. Primer Premier 5 (Premier Biosoft International)
2. sgRNAs9 (Xie *et al.*, 2014)

Procedure

A. Design mutation strategy and prepare ssDNA editing templates

1. Select the target position where the mutation will be introduced. Upload the sequence of the target region (23 mer upstream and downstream the target position, respectively, totally 47 mer) and the genome sequence of *C. glutamicum* to sgRNAs9 software (Xie *et al.*, 2014) to design gRNAs and evaluate the potential off-target sites.
2. Choose a proper gRNA with no potential off-target sites and make sure that the target mutation position locates in its seed region (green letters in Figure 1) or the “GG” region of the PAM sequence (Figure 1).

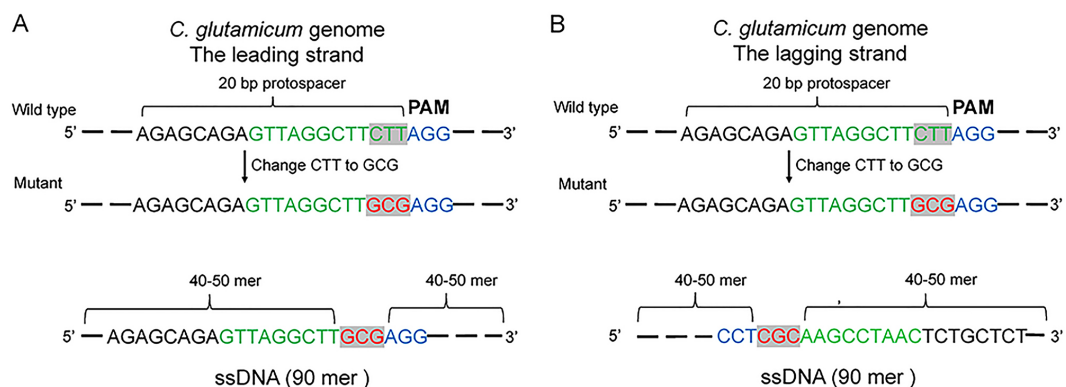


Figure 1. Design of protospacer, PAM site and ssDNA. A. Introducing mutations (red letters)

with a protospacer and a PAM sequence (5'-NGG-3', blue letters) in the leading strand of *C. glutamicum* genome. B. Introducing mutations (red letters) with a protospacer and a PAM sequence (5'-NGG-3', blue letters) in the lagging strand of *C. glutamicum* genome. The target mutation position should locate in the seed region (green letters) or the "GG" region of the PAM sequence. The ssDNA (90 mer) editing templates should be complementary to the lagging strand.

3. Design a 90 mer ssDNA using the 40-50 mer upstream and downstream the target position, respectively. Make sure that the 90 mer ssDNA is complementary to the lagging strand (Figure 1).

Note: A previous study demonstrated that ssDNA that is complementary to the lagging strand results in a higher recombination efficiency (Binder et al., 2013). According to the genome sequence of C. glutamicum ATCC 13032 published by Kalinowski et al. (2003), the bi-directional replication of the genome starts at the proposed oriC sequence near the dnaA gene (cg0001) and ends near to the calculated replication terminus at around 1.6 Mb. Therefore, regarding to genes located in 0-1.6 Mb, just copy the genome sequence to get the lagging strand sequence. For genes located in 1.6-3.3 Mb, the reverse complementary sequence of the genome sequence is the lagging strand sequence.

4. Synthesize PAGE-purified ssDNA (10 µg is used for each transformation).

B. Construct plasmid pgRNA-target

To construct plasmid pgRNA-target that is required for expression of specific gRNA, replace the original 20 bp spacer sequence of pgRNA5 with the target spacer sequence designed previously (Figure 2). Use Vazyme ClonExpress® II One Step Cloning Kit to construct the plasmid (this cloning technology is based on homologous recombination and overlapped sequences of 15-20 bp on both 5'- and 3'-end of PCR fragments are needed). Add the 20 bp spacer sequence in the primer and use the 20 bp spacer sequence as the overlap. Detailed procedure is as follows:

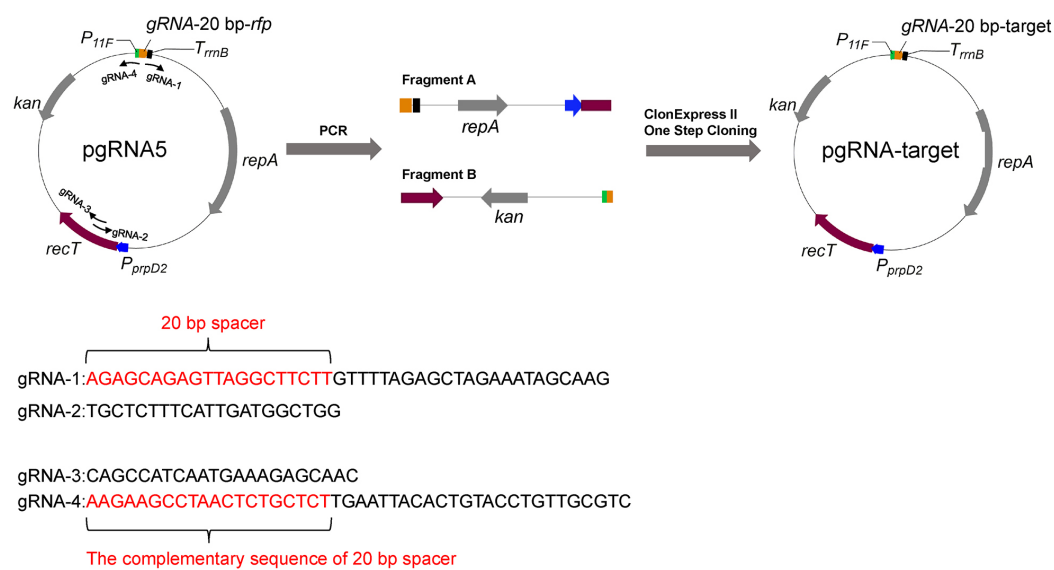


Figure 2. Construction of plasmid pgRNA-target

- Design primers for construction of plasmid pgRNA-target.
Primer gRNA-1: 20 bp spacer sequence + GTTTTAGAGCTAGAAATAGCAAG
Primer gRNA-2: TGCTCTTTCATTGATGGCTGG (permanent sequence)
Primer gRNA-3: CAGCCATCAATGAAAGAGCAAC (permanent sequence)
Primer gRNA-4: reverse complement of 20 bp spacer sequence + TGAATTACACTGTACCTGTTGCGTC
- Amplify Fragment A (3421 bp) and Fragment B (2812 bp) via PCR using pgRNA5 as a template and primer pairs gRNA-1/gRNA-2 and gRNA-3 and gRNA-4, respectively.
 - PCR program using Q5® High-Fidelity DNA polymerase:

98 °C	3 min	
98 °C	20 sec	
60 °C	30 sec	30 cycles
72 °C	2 min	
72 °C	5 min	
Hold at 4 °C		

- Purify PCR products by 1% agarose gel electrophoresis in 1x TAE electrophoresis buffer followed by gel extraction using AxyPrep™ DNA Gel Extraction Kit (Corning, Axygen®). Store the purified PCR product at -20 °C until used.

Note: GoldView I Nuclear Staining Dyes (10,000x) are used for DNA staining. According to the manufacturer's instructions, GoldView I should be protected from light and stored at room temperature. For 100 ml agarose gel solution, 10 µl GoldView I Nuclear Staining Dyes (10,000x) is used.

- Construct pgRNA-target plasmid

- a. Use Vazyme ClonExpress® II One Step Cloning Kit to construct pgRNA-target plasmid. The recombination reaction mixture (20 µl) contains 60-120 ng Fragment A, 60-120 ng Fragment B, 4 µl 5x CE II Buffer, 2 µl Exnase II. Incubate the reaction mixture at 37 °C in a thermocycler for 30 min and immediately place it on ice.
- b. Transform 100 µl chemically competent *E. coli* strain DH5α with 10 µl of the recombination products (make sure the transformation efficiency of competent cells is $> 10^7$ cfu/µg). Plate the transformation mix on LB solid medium containing kanamycin (25 µg/ml). Incubate the plate at 37 °C overnight.
- c. Verify the transformants via PCR using an upstream primer specific to the 20 bp spacer sequences and a downstream primer gRNA-5 (AGTCTTTTCGACTGAGCCTTTTCG) that locates on the plasmid backbone. The size of the expected fragment generated by PCR is approximately 150 bp.
- d. Cultivate the correct colonies and extract plasmids using AxyPrep™ Plasmid Miniprep Kit. Store the purified pgRNA-target plasmid at -20 °C until used.

C. CRISPR/Cas9-mediated ssDNA recombineering in *C. glutamicum*

The procedure for CRISPR/Cas9-mediated ssDNA recombineering is shown in Figure 3.

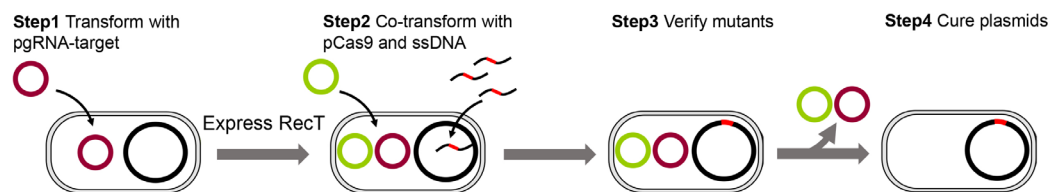


Figure 3. The procedure for CRISPR/Cas9-mediated ssDNA recombineering

1. Transform *C. glutamicum* with pgRNA-target plasmid

Prepare competent cells for *C. glutamicum* and transform plasmids according to the protocols described previously (Ruan *et al.*, 2015).

- a. Activate *C. glutamicum* strain by cultivation on LBHIS solid medium at 30 °C for 24 h.
- b. Inoculate a pre-culture of 20 ml BHI medium (in 100 ml shake flask) from a single colony and incubate at 30 °C with vigorous shaking (220 rpm) overnight.
- c. Dilute the overnight culture to an OD_{600nm} of 0.3 in NCM medium (100 ml) containing 0.46% INH, 0.11% Tween 80, 1.11% DL-Thr and 3.59 % Gly, and incubate at 30 °C with vigorous shaking (220 rpm) until the OD_{600nm} value reaches 0.9-1.0 (ca. 5-6 h).
- d. Chill the culture on ice for 15 min and harvest cells by centrifugation at 4 °C, 4,000 x g for 10 min.
- e. Discard the supernatant and resuspend the pellet in 50 ml ice-cold, sterile 10% glycerol. Centrifuge the resuspended cells at 4 °C, 4,000 x g for 10 min. Repeat this wash step four times.
- f. Discard as much supernatant as possible. Immediately resuspend the cell pellet in 0.4 ml

- ice-cold, sterile 10% glycerol, and divide the resuspended cells into 100 μ l aliquots.
- g. Add 0.5-1 μ g pgRNA-target plasmid (up to 5 μ l) to the competent cells and transfer the mixture to a 2 mm electroporation cuvette (Bio-Rad Laboratories). After electroporation at 2.5 kV, 5 msec, add 1 ml BHIS medium to the cuvette immediately, transfer the mixture into a 1.5 ml tube, and incubate the tube at 46 °C for 6 min. Then incubate the tube at 30 °C with vigorous shaking (220 rpm) for 2 h.
- h. Spread 50-100 μ l of the mixture on LBHIS solid medium containing 25 μ g/ml kanamycin. Incubate the plates at 30 °C for 24-36 h. The transformants are *C. glutamicum* (pgRNA-target) strains.
2. Transform *C. glutamicum* (pgRNA-target) with pCas9 plasmid and ssDNA
 - a. Prepare competent cells for *C. glutamicum* (pgRNA-target). Follow the procedures described above except that add 1 g/L sodium propionate into NCM medium to induce RecT recombinases expression.
 - b. Transform *C. glutamicum* (pgRNA-target) competent cells with 1-2 μ g pCas9 and 6-15 μ g synthetic ssDNA (total volume up to 10 μ l). After electroporation at 2.5 kV for 5 msec, add 1 ml BHIS medium to the cuvette immediately, transfer the mixture into a 1.5 ml tube, and incubate the tube at 46 °C for 6 min. Then incubate the tube at 30 °C with vigorous shaking (220 rpm) for 5-6 h.
 - c. Collect the cells by centrifugation at 5,000 \times g for 1 min. Remove the supernatant and resuspend the cells using 100 μ l BHIS medium. Spread all the cells on LBHIS solid medium containing 25 μ g/ml kanamycin, 5 μ g/ml chloramphenicol and 0.01 mM IPTG. Incubate the plates at 30 °C for 48-72 h until colonies appear.
3. Screen edited mutants

Design a primer pair that locates at approximately 200 bp upstream and downstream of the mutation site, respectively (Figure 4). Amplify the target fragment by using colony PCR and the above-mentioned primer pair. Sequence the PCR product by Sanger sequencing to verify the mutation was introduced and no other unexpected mutations were introduced.

Note: According to our experience, the editing efficiency is over 80%. Therefore, screening 5 colonies can ensure the acquisition of edited mutants.

For colony PCR, pick a speck of colony using a 10 μ l pipette tip and mix it with the PCR reaction mixture to introduce DNA template.

Colony PCR program using 2x EasyTaq PCR SuperMix (+dye):

94 °C	5 min	
94 °C	20 sec	30 cycles
50-60 °C	30 sec	
72 °C	1 min	
72 °C	5 min	
Hold at 4 °C		

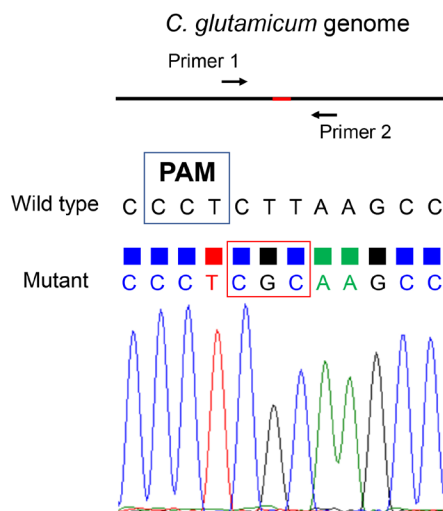


Figure 4. Verification of the mutants by Sanger sequencing

D. Plasmid curing

To remove pCas9 and pgRNA-target plasmids from the edited cells, inoculate the edited colony into 100 ml of BHI medium without antibiotics and cultivate it at 30 °C, 220 rpm for 12 h. Transfer 100 µl of the culture into 100 ml fresh BHI medium without antibiotics and cultivate it at 30 °C, 220 rpm for 12 h. Repeat this step for another time. Dilute the culture and spread 100 µl on LBHIS solid medium without antibiotics. Pick 10 to 20 isolates and inoculate LBHIS solid media with and without 25 µg/ml kanamycin (or 5 µg/ml chloramphenicol). Cultivate the plates at 30 °C overnight. The isolates that do not grow on LBHIS solid medium with 25 µg/ml kanamycin and LBHIS solid medium with 5 µg/ml chloramphenicol have been cured of the editing plasmid.

Note: According to our experience, the efficiency observed for curing strains of the plasmids is approximately 25%.

Data analysis

To verify the genome editing events, amplify the target region via colony PCR and subsequently sequence the PCR product. Align the reference sequence (wild-type) with the sequenced results to analyze whether the target mutations were introduced via CRISPR/Cas9-mediated ssDNA recombineering. The editing efficiency = No. of correct mutants/No. of colonies tested.

Note: After the CRISPR/Cas9-mediated ssDNA recombineering, some abnormally large colonies may appear on the plates (Figure 5). According to our experience, these abnormally large colonies are very likely false positives, which lose the cas9 expression plasmid during the editing process.

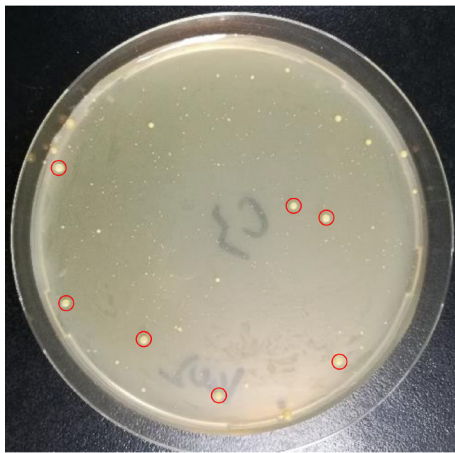


Figure 5. Transformants obtained by CRISPR/Cas9-mediated ssDNA recombineering. Colonies marked in red cycles were the so-called abnormally large colonies.

To assay the loss of editing plasmids pCas9 and pgRNA-target, pick 10 to 20 isolates from LBHIS solid medium without antibiotics and inoculate LBHIS solid media with and without 25 µg/ml kanamycin (or 5 µg/ml chloramphenicol). The isolates that are sensitive to both kanamycin and chloramphenicol have been cured of the editing plasmid. The efficiency of plasmid curing = No. of colonies without plasmid/No. of colonies tested.

Recipes

1. 50x TAE electrophoresis buffer
242 g Tris base
57.1 ml glacial acetic acid
100 ml EDTA, pH 8.0
Add Milli-Q H₂O to a total volume of 1 L
Store at room temperature
2. 1x TAE electrophoresis buffer
20 ml 50x TAE
980 ml Milli-Q H₂O
Store at room temperature
3. LB medium
10 g NaCl
10 g tryptone
5 g yeast extract
Add Milli-Q H₂O to a total volume of 1 L
Autoclave to sterilize
4. LB solid medium
1 L LB medium

18 g agar

Autoclave to sterilize

Allow cooling to about 60 °C. If antibiotics are required, they are added at this point

5. BHI medium

37 g Brain Heart Infusion (BHI)

10 g (NH₄)₂SO₄

0.2 g K₂HPO₄

0.3 g NaH₂PO₄

0.5 g MgSO₄·7H₂O

Adjust pH to 7.2

Add H₂O to a total volume of 1 L

Autoclave to sterilize

6. NCM medium

Containing 0.46% INH, 0.11% Tween 80, 1.11% DL-Thr and 3.59% Gly

a. NCM medium

17.4 g K₂HPO₄

11.6 g NaCl

5 g glucose

5 g tryptone

1 g yeast extract

0.3 g trisodium citrate

0.05 g MgSO₄·7H₂O

91.1 g sorbitol

Adjust pH to 7.2

Add H₂O to a total volume of 1 L

Autoclave to sterilize

b. Use sterilized NCM medium to prepare INH, DL-Thr and Gly solution

23 g isonicotinic acid hydrazide (INH)

5.5 g Tween 80

55.5 g DL-threonine (DL-Thr)

179.5 g glycine (Gly)

Add sterilized NCM medium to a total volume of 0.2 L

Filtration sterilization

c. Mix 0.8 L NCM medium with 0.2 L INH, DL-Thr and Gly solution

7. BHIS medium

18.5 g BHI

91 g sorbitol

Adjust pH to 7.2

Add H₂O to a total volume of 1 L

- Autoclave to sterilize
8. LBHIS solid medium
 - 5 g Tryptone
 - 5 g NaCl
 - 2.5 g yeast extract
 - 18.5 g BHI
 - 91 g sorbitol
 - 18 g agar
 - Adjust pH to 7.2
 - Add H₂O to a total volume of 1 L
 - Autoclave to sterilize
 - Allow cooling to about 60 °C. If antibiotics are required, they are added at this point
 9. 25 mg/ml kanamycin stock
 - 250 mg kanamycin
 - Add Milli-Q water to 10 ml
 - Filtration sterilization
 - Store at -20 °C
 10. 5 mg/ml chloramphenicol stock
 - 50 mg chloramphenicol
 - Add ethanol to 10 ml
 - Filtration sterilization
 - Store at -20 °C
 11. 0.1 M IPTG stock
 - 23.8 mg IPTG
 - Add Milli-Q water to 10 ml
 - Filtration sterilization
 - Store at -20 °C
 12. 500 g/L sodium propionate stock
 - 5 g sodium propionate
 - Add Milli-Q water to 10 ml
 - Filtration sterilization
 - Store at -20 °C

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Competing interests

The authors declare that they have no competing interests.

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