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Recombineering

Methods and Protocols

Edited by

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Preface

The development of recombineering techniques has facilitated genome editing across many organisms over the last three decades. The term “recombineering” was first coined as a portmanteau for “recombinogenic engineering,” describing techniques where DNA engineering occurs in vivo. In its original usage, recombineering referred to a method where the expression of genes from the defective Rac prophage or Lambda phage enhanced recombination efficiencies to unprecedented levels in *Escherichia coli*. The usefulness of this system was boosted by the demonstrations that regions of homology totaling only 40 bp were sufficient to enable site-specific integrations. Thus, primers could be synthesized with sufficient homology for target specificity, removing the need to clone regions of homology into a vector before the transformation. Single-stranded DNA can also be used as the editing template for recombineering with very high efficiency, negating the need to use antibiotic selection in some cases. In addition to *E. coli* genome editing, these technologies could be applied to bacterial artificial chromosomes and vectors used for mutagenesis in other organisms. The Lambda Red system, and homologous genes with similar functions, have also been ported to other bacteria, greatly improving the ability to perform genome editing in some model and non-model bacteria. More recently, techniques have been developed that use CRISPR/Cas programmable nucleases to facilitate genome editing. In some cases, the CRISPR/Cas nuclease acts as a counterselection tool by targeting the wild-type genome of unedited cells to remove them from the population. In other cases, double-strand break or nicking of the genomic DNA increases the recombination rates at the cut site. Some of these techniques require phage-derived proteins to increase efficiency, while others do not.

In this edition, a collection of methods are presented that enable genome editing across a variety of bacteria, phages, and plants. A broad interpretation of recombineering was employed, and protocols that do not specifically include phage-derived proteins for facilitating integration are included. Though these techniques do not fall into the traditional definition of recombineering, the methodology is similar, resulting in improved ability to perform genome editing.

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Chapter 1

Scarless Recombineering of Phage in Lysogenic State

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Abstract

We present a scarless recombineering-based method for introducing multiple point mutations into the genome of a temperate phage. The method uses the λ Red recombineering system to promote exogenous ssDNA oligos to anneal on the prophage lagging strand during host genome replication. DNA repair is suppressed by inducing the expression of a dominant-negative mutant protein of the methyl-directed mismatch repair system. Screening for recombinant cells without a selection marker is feasible due to its high recombination frequency, estimated as more than 40% after six cycles. The method enables scarless editing of the genome of a bacteriophage in 4–5 days.

Key words Scarless, Recombineering, Bacteriophage, Temperate, Lysogenic, Electroporation, λ Red, ssDNA

1 Introduction

With the emergence of multidrug-resistant bacteria (MDR) and the shrinkage of antibiotic pipelines, research towards alternative methods for treating bacterial infections began [1]. One such method is bacteriophage (phage) therapy [2]. Although phages were recognized as antimicrobial agents from the early twentieth century, phage therapy is yet to reach its full potential. Researchers therefore began modifying the phages to advance our understanding and broaden their role in therapeutic treatments [3]. Currently, native phages are engineered to meet MDA requirements [4] and to tackle the phage–bacterium specificity limitation by extending [5], expanding [6], or shrinking the host range [7] before dosage. Phages are also modified to serve as pathogen control agents to enhance antibacterial activity [8], for diagnostic purposes [9, 10], for anticancer or antigen delivery [11, 12], and for developing vaccines [13, 14].

Methods for genome and phage engineering are already established, with their own advantages and disadvantages [15]. Here we reappropriated the in vivo recombineering method, MAGE
(Multiplex-Automated Genome Engineering) [16], to modify the P2 bacteriophage in a lysogenic state without leaving a genetic scar and without incorporating off-target mutations. ssDNA is used here as a recombineering substrate. The pORTMAGE system is used as a more efficient MAGE recombineering system, as described before [17]. Although the method below was tailored to the P2 bacteriophage, the system can be used to edit any temperate bacteriophage in a λ-compatible host.

MAGE was reported originally for rapidly creating *E. coli* combinatorial genomic libraries using the λ red recombination system in the host [16]. Since induction of the phage λ in the presence of P2 prophage causes inhibition of protein synthesis leading to cell death [18], recombineering in P2 lysogens was overlooked until recently, with most of the work being performed in the lytic state [19]. Although there are standard methods for engineering bacteriophages in a lytic state, they take a longer time at best to generate and screen for recombinants [15]. Tackling the P2-λ interference mechanism, while at the same time maintaining the λ recombineering attributes, was therefore crucial for engineering mutants of bacteriophage P2. We recently found that a combination of recA host, old del1 P2 prophage and the pORTMAGE plasmid, can successfully delete genetic material and insert instead a selection marker to facilitate screening for the successful recombinant mutants [20]. We therefore used the same combinatorial attributes to also tackle the P2-λ interference in the MAGE workflow.

pORTMAGE encodes polycistronically the λ Red genes (gam, bet, and exo) and the negative mutant allele of MutL, MutL E32K, responsible for the inactivation of the methyl-directed mismatch repair system (MMR) [17]. The operon is under the P1 promoter and is being controlled by the cI857 temperature-sensitive λ repressor, allowing for timely induction of the recombineering pathway and inhibition of DNA repair [17]. Following six iterative cycles of λ Red enzymes and MutL E32K induction, ssDNA transformation (by electroporation) into the cells and recovery, we were able to introduce three point mutations independently in the gpH, gpG, gpP, and gpM gene. After the fourth cycle, nine colonies were picked, PCR amplified, and PCR products were sent for sequencing to find at best one positive with perfect sequencing trace and approximately three products with mixed WT/recombinant trace. When no pure positives were found, the respective mixed trace colonies were then used for another two cycles, resulting in more than 40% pure recombinants.

Compared to other techniques for scarless engineering of bacteriophages (e.g., CRISPR-Cas [21], Rebuilding/Refactoring Phage genomes in vitro [22], Whole-genome assembly from synthetic oligos [23] and the Yeast-based method [24, 25]), the altered MAGE is one of the most reliable and quickest methods for introducing point mutations without affecting the rest of the
sequence. This technique is especially relevant for further producing recombinant phage particles when the inflicted mutations are not inhibitory to phage particle assembly during phage awakening. It is however not suited for inserting, deleting, or substituting large fragments into the phage genome.

2 Materials

2.1 Reagents

- *Escherichia coli* C-5545 (*E. coli* C-1a containing old del1 P2 prophage), gift from Gianni Deho.
- pORTMAGE plasmid [17] (PBBR1 ORI, Ampicillin resistance), gift from Csaba Pál (Addgene plasmids #72680 or #72677).
- Luria Bertani broth (LB), prepared to manufacturer instructions and autoclaved.
- Ampicillin 100 mg/mL.
- Terrific broth (TB), prepared to manufacturer instructions and autoclaved.
- PCR purification kit.
- Ice-cold dH2O.
- 50% (vol/vol) glycerol.
- Dried ssDNA stocks for introducing point mutations.
- Phusion® High-Fidelity PCR Master Mix (or similar high-fidelity DNA polymerase).
- Flanking primers for diagnostic PCR.
- Dry ice.

2.2 Equipment

- 30 °C shaking incubator.
- 42 °C shaking water bath.
- Ice-cold sterile microcentrifuge tubes.
- PCR tubes.
- 10 mL serological pipettes.
- 5 mL serological pipettes.
- Pipette-aid.
- Spectrophotometer.
- Electroporator.
- 0.2 cm Electroporation cuvettes.
- Cuvettes for measuring OD.
- Insulating ice bucket.
- Nanodrop.
2.3 Oligo Design

The transformed synthetic oligonucleotides are thought to anneal on the prophage lagging strand like Okazaki fragments during genome replication [16]. As replication in *E. coli* occurs bidirectionally, the target strand changes according to the prophage coordinates [26]. In the case of P2 prophage, the target strand was shown to be edited successfully using an oligonucleotide sequence equivalent to the 5'-3' reverse complement sequence of the target loci. See Table 1 for optimal design parameters.

2.4 Reagents Setup

Transform competent *E. coli* strain C-5545 with the pORTMAGE plasmid. Recover the cells for 1 h at 30 °C to maintain tight repression of the λ Red genes encoded by the pORTMAGE plasmid. Plate on 100 μg/mL ampicillin plates to select for successful transformants (see Note 2).

Prepare 50 μM oligo stocks by resuspending the dried synthetic oligos in dH₂O (see Note 3).

### Table 1

| Parameter               | Optimum      | Reason                                           |
|-------------------------|--------------|-------------------------------------------------|
| Length                  | 90 nts       | >90 nts increases likelihood of secondary structures |
| Concentration           | 0.5–10 μM    |                                                 |
| Phosphorothioate bonds  | 2 at 5' end  | Prevents exonuclease-mediated degradation        |
| Folding energy          | > –9 kcal/mol at 42 °C | Unstructured ssDNA can hybridize easier on the target loci |
| Homology region         | >10 nts at both 5' and 3' ends | Mismatches close to the 5',3' ends could decrease the replication efficiency |
| Mismatch identities     | T-T; A-G; C-C | Mismatches are weakly recognized for repair by MutS (e.g., G-G is well recognized) |

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3.1 Inducing the $\lambda$ Red System

1. Grow overnight C-5545 + pORTMAGE cells in 5 mL LB + 100 $\mu$g/mL ampicillin (see Note 4), shaking (200 rpm) at 30°C.

2. Refresh the overnight culture (1 in 100 dilution) into 4 mL TB + 100 $\mu$g/mL ampicillin, in a culture tube.

3. Grow cells shaking (250 rpm) at 30°C until $\text{OD}_{600} = 0.3–0.6$ (~3 h) (see Note 5).

4. Transfer the culture tube to a 42°C shaking water bath for 15 min to induce $\lambda$ Red expression and shake vigorously (250 rpm) (see Note 6).

5. Transfer the culture tube to ice on a slow rocker for a minimum of 10 min to delay further cell growth (see Note 7).

3.2 Preparing Induced Cells for Electroporation

1. Thaw the 50 $\mu$M oligo stock on ice. Prepare 1 $\mu$L oligo solution by mixing 1 $\mu$L 50 $\mu$M oligo stock with 49 $\mu$L chilled dH$_2$O. Keep on ice until needed.

2. Transfer 1 mL of induced culture into a prechilled microcentrifuge tube.
3. Pellet/wash cells twice in ice-cold dH₂O. Keep all centrifugations at maximum speed ~14k rpm for 30 s, at 4 °C (see Note 8).

4. Resuspend the final cell pellet in the 1 μM oligo solution prepared in step 1. Continue directly to electroporation and recovery (see Note 9).

3.3 Electroporation and Recovery

1. Transfer the oligo/cell mixtures to 0.2 cm ice-cold electroporator cuvettes. Gently flick the cuvettes to ensure full coverage of the bottom of the cuvette and no bubbles in the mixture. Leave standing for one minute.

2. Electroporate the mixtures one pulse at a voltage of 2.5 kV.

3. Immediately add 1 mL of room temperature TB.

4. Incubate the cultures at 30 °C shaking (250 rpm) for 2 h or overnight.

Repeat the recombineering cycle three more times before screening. To begin a new cycle of recombineering, start from inducing the λ Red system step 3, or step 2 if the cells were left growing overnight. After four cycles, dilute the culture to 10⁻⁴, 10⁻⁵ in LB and plate on LBA + 100 μg/mL ampicillin. Incubate the plate at 30 °C overnight and screen for positives the next day (see Note 10).

3.4 Screening

1. Resuspend nine potentially recombinant colonies into 10 μL LB each (see Note 11).

2. Perform a diagnostic PCR on 1 μL of each of the colony resuspensions, using the Phusion® High-Fidelity PCR Master Mix with HF Buffer. Run PCR as recommended in the Phusion MM protocol.

3. Add 10 μL 50% glycerol to the remaining resuspended colonies (final concentration 25%) and snap-freeze on dry ice. Store at −80 °C.

4. Purify the PCR products using a PCR purification kit and send for Sanger sequencing to confirm genotype (see Note 12).

When the sequencing trace consists of only mixed WT/recombinant peaks, run another two recombineering cycles. Begin a new recombineering cycle from Inducing the λ Red system step 1. Use as inoculation material the respective WT/recombinant colony resuspended and frozen during screening.
4 Notes

1. For consistency, the folding energy was calculated using the IDT Oligoanalyzer tool with the following parameters: 0.5 μM oligo, 50 mM Na\(^+\), 1.5 mM Mg\(^{2+}\), 0.2 mM dNTPs.

2. Perform here diagnostic PCR and run on a 1% agarose gel to confirm successful transformation with the pORTMAGE plasmid. Pick the respective positive colony and grow overnight at 30 °C. Make glycerol stocks the next day by mixing 300 μL 50% glycerol with 800 μL culture. Snap-freeze on dry ice before storing at −80 °C.

3. For preparing the 50 μM oligo stock, resuspend the dried oligos with \((X \times 20)\) μL dH\(_2\)O, where \(X\) is the specific nm value written on each tube for the respective oligo. Mix well, without vortexing the tube.

4. Inoculate the fresh media preferably from a single colony by gently touching the top of the colony with an autoclaved tip and dispensing the tip into the culture tube with fresh media containing appropriate antibiotics. You can also use a glycerol stock of an overnight culture grown from a single colony.

5. Grow the culture preferably towards the lower end of the \(\text{OD}_{600} = 0.3–0.6\) range, to allow for repeated recombineering cycles in one day without needing to refresh the culture. Aim for \(\text{OD}_{600} = 0.3\).

6. Submerge the tube enough for all the culture to be in indirect contact with the water. This is to ensure the cells are subjected to a uniform rise in temperature. While vigorously shaking, make sure no water gets into the tube by examining the water bath until the shaking speed reaches 250 rpm.

7. You can keep the culture longer than 10 min on ice, but ideally not longer than 3 h.

8. Preferably perform this step in a cold room to maintain the cells cold at all times. Pipette the supernatant out instead of pouring, to avoid losing the pellet in between the washes. Mix gently by flicking the tube instead of vortexing.

9. Do not freeze at this point the now \(\lambda\) Red-induced electro-competent cells and continue directly to electroporation and recovery. You need to induce the \(\lambda\) Red system before electroporation every time, for the ssDNA to immediately begin recombining once inside the cells.

10. It is important here you have single colonies. If no detectable single colonies are found, streak a new plate with appropriate antibiotics and incubate the plate overnight. Check for individual colonies the next day.
11. Gently touch the top of a potentially recombinant lysogen colony with an autoclaved tip and pipette up and down into 10 μL LB to inoculate/resuspend the cells in fresh media.

12. Elute the sample with 25 μL Elution Buffer. Wait one minute before spinning to allow the EB to soak freely into the column. Quantify the sample via Nanodrop. Expect pure sample ~50 ng/μL. Prepare the purified sample for Sanger sequencing according to the company’s sample submission instructions. After four recombineering cycles expect at best one recombinant with perfect trace, and approximately three with WT/recombinant mixed trace.

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Lambda Red Recombineering of Bacteriophage in the Lysogenic State

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Abstract

We present a recombineering-based method for editing the genome of a temperate phage. The method uses the lambda Red recombination system to edit the genome of a lysogenized host with a prophage compatible with bacteriophage lambda. Linear DNA is used as the recombination substrate and antibiotic resistance is used as the basis for selection of recombinants. The method enables the genetic manipulation of a prophage in 3–5 days.

Key words Recombineering, Bacteriophage, Temperate, Lysogenic, Electroporation, Lambda red

1 Introduction

As it becomes increasingly apparent that the way bacterial infections are treated cannot continue in its current form, it becomes increasingly urgent to develop alternative treatment options [1]. One such option is bacteriophage (phage) therapy, the treatment of bacterial infections by the application of bacteria-specific viruses [2]. For this option to become viable, however, it may be necessary to manipulate the genomes of bacteriophages, either to study them or to render them suitable for pharmaceutical applications. We focus here on bacteriophage P2 as an example of temperate phage, the genome of which we routinely manipulate by homologous recombination. However, as the interaction between the old exonuclease of P2 bacteriophage and the gam protein of the lambda Red recombination system is lethal to the host cells—the reason for exclusion of the growth of phage lambda in P2 lysogens—it is nontrivial to manipulate the genome of P2 prophage [3–5]. The method here overcomes this limitation by using the strain *Escherichia coli* C-5545 (A P2 lysogen containing the del1 mutation to the P2 old gene [6]), which is compatible with the lambda Red
recombination system. The method we present here is tailored for bacteriophage P2 but could be adapted to any temperate phage that is compatible with the phage lambda.

We have used the method to delete P2 genes and to insert exogenous genes, using antibiotic resistance as the selective marker. However, in addition, the method could be adapted to insert/delete/replace very short sequences by using the MAGE (Multiplex Automated Genomic Engineering) method, which does not rely on selective markers but has been reported to work with such a high frequency that screening a small number of plated colonies yields positive results with over 30% success [7–10].

Typically, when the genome of a bacteriophage is edited, it is done so in the lytic state [11]. For this, the genetic manipulations can be made using recombineering in the host bacterium [12], by molecular cloning methods, e.g., Gibson assembly [13] or by using recombineering in nonhost cells e.g. *Saccharomyces cerevisiae* [14]. While these techniques have been used with success, the major advantage of editing temperate—rather than lytic—bacteriophage genomes is the availability of selective markers. To select for recombinant lytic bacteriophage there are two reported methods: (1) The identification of a host gene that is nonessential to the host but essential to the bacteriophage and is not involved in processes prior to phage DNA injection. In a host strain deficient in the gene, therefore, only the bacteriophage that carries the bacteriophage-essential gene and expresses it in the deficient host, i.e., recombinant bacteriophage, will propagate [15]; (2) The CRISPR-Cas (clustered regularly interspaced short palindromic repeats—CRISPR-associated protein) system can be used to select against the area of the wild type sequence that is to be modified, thus allowing only recombinant bacteriophage to propagate [16]. The disadvantage of the first approach is that it requires a priori knowledge about the interaction of the bacteriophage with its host: there must be at least one known host gene that is essential to the bacteriophage but nonessential to the host to enable the use of a selection marker. The disadvantage of the CRISPR-Cas approach is the lack of stringency of selection compared to the first method: In the case of bacteriophage T7, deletion of trxA from the host (bacteriophage-essential but host-nonessential gene) leads to a 10^{10}-fold loss of viral titer, whereas use of CRISPR-Cas yields approximately a 100–1000-fold loss of viral titer [17]. The CRISPR-Cas basis for selection of recombinant bacteriophage is thus much less stringent than the first approach, although there is more flexibility in terms of selection of a marker, as no knowledge about the host–bacteriophage interaction is required. As prophages are incorporated into the host genome, however, selection for recombinant bacteriophage in the lysogenic state can be done on the basis of antibiotic resistance. This is advantageous over manipulating lytic phage genomes as this type of selection marker is not
specific to the phage being manipulated (c.f. identification of selection marker genes essential to the phage but not to its host). As such, any antibiotic resistance the host strain does not already possess may be used to select for recombinant bacteriophage when in the lysogenic state. The stringency of antibiotic selection markers is dependent on the type of antibiotic used and can be tuned by altering its concentration in the growth medium. Overall, as there is a vast array of antibiotic selection markers in routine use in microbiology research and as their selection stringency can be tuned for the given bacterial host, they present a convenient option for selection for recombinant bacteriophages in the lysogenic state.

In the presented approach, electrocompetent cells with the lambda Red proteins induced are prepared and then transformed with linear DNA that encodes an antibiotic resistance cassette flanked by regions homologous to the target sequence in the prophage. The transformed cells are then recovered for much longer than is typical in transformation procedures and then plated on selective agar. Finally, colonies are screened for the desired insertion by diagnostic PCR.

Other than the specific lysogen strain, the method presented requires standard laboratory equipment and reagents and can yield recombinant P2 prophage in 3–5 days. Recombination occurs at a low frequency (in our experience, fewer than 50 colonies per experiment is typical) but false positives occur infrequently, thus only five to ten colonies are recommended for screening by diagnostic PCR.

### 2 Materials

#### 2.1 Generation of Linear DNA Fragment by PCR

1. Recombination substrate: Linear, double-stranded oligonucleotide (see Subheading 3.1 for design protocol; see Note 1).
2. Phusion (or polymerase of comparable high fidelity) High-Fidelity PCR Master Mix, store at −20 °C (see Note 2).
3. Forward/reverse primers to amplify linear fragment.
4. Nuclease-free water.
5. Dimethyl sulfoxide (DMSO).
6. PCR purification kit.
7. Agarose/electrophoresis tank/power pack/TAE buffer/DNA stain.
8. Nanodrop spectrophotometer.

#### 2.2 Preparation of Electrocompetent Cells

1. Escherichia coli C-5545 (see Note 3).
2. pPORTMAGE plasmid [PBBR1 ORI (copy number approximately 40); Variety of antibiotic selection markers available (see Note 4); Heat-inducible lambda Red system [18]).
3. 5 mL Luria Bertani broth prepared to manufacturer instructions and autoclaved.
4. Antibiotic to which the chosen pORTMAGE plasmid provides resistance (see Note 4).
5. 10 mL Terrific broth prepared to manufacturer instructions and autoclaved.
6. 80 mL ice-cold dH₂O.
7. 80 mL sterile 50% glycerol.
8. Sterile 150 mL conical flask.
9. 30 °C shaking incubator.
10. 42 °C shaking water bath (see Note 5).
11. Ice-cold 50 mL conical centrifuge tube ×2.
12. Ice-cold sterile microcentrifuge tubes.
13. 10 mL serological pipettes.
14. Pipette-aid.
15. Centrifuge (large enough for 50 mL conical tubes) prechilled to 4 °C.
16. Spectrophotometer.
17. Cuvettes.

2.3 Recombineering

1. 20 mL Terrific broth prepared to manufacturer instructions and autoclaved.
2. Antibiotic to which the recombined DNA provides resistance.
3. Linear DNA from PCR protocol.
4. LB agar plate supplemented with selection antibiotic ×6.
5. Sterile 150 mL conical flask ×3.
6. Ice-cold electroporator cuvettes ×3.
7. Electroporator.

3 Methods

3.1 Design of Recombination Substrate

1. The desired insert sequence (selection marker) should be flanked by regions homologous to the desired position of insertion in the prophage genome. Eighty base pairs of homology on each side is optimal for recombination.
2. Order the required recombination substrate as a linear, double-stranded oligonucleotide (see Note 1).

3.2 Generation of Recombination Substrate by PCR

1. Resuspend linear oligonucleotide to 50 ng/μL in nuclease-free water. Resuspend primers to 100 μM in nuclease-free water. Store all at −20 °C.
2. Set up a PCR reaction containing the following components: 25 μL Phusion High-Fidelity PCR Master Mix, 2.5 μL forward primer, 2.5 μL reverse primer, 1.5 μL DMSO, 1 μL linear oligonucleotide, 7.5 μL nuclease-free water.

3. Run the PCR reaction in a thermocycler with the following parameters:
   - 98 °C for 30 s.
   - 98 °C for 10 s; primer melting temperature +3 °C for 30 s; 72 °C for the time specified by polymerase manufacturer—For 35 cycles.
   - 72 °C for 10 min.
   - 4 °C for ∞.

4. Purify the PCR product using a PCR purification kit. Elute DNA in 50 μL of nuclease-free water.

5. Visualize the purified PCR product on a 1% agarose gel. Only the desired product should be visible (see Note 6).

6. Measure the concentration of DNA in the PCR product using a Nanodrop spectrophotometer or by gel densitometry if a Nanodrop spectrophotometer is not available (≥70 ng/μL is ideal).

7. Store the PCR product at −20 °C until required.

3.3 Preparation of Recombineering Strain

1. Transform pORTMAGE DNA into C-5545 electrocompetent cells (electroporate at 2.5 kV in a 0.2 cm cuvette). All incubations following electroporation must be at 30 °C to maintain tight repression of the lambda Red genes encoded by the pORTMAGE plasmid. Spread transformed cultures on LB agar supplemented with the antibiotic to which the chosen variation of pORTMAGE provides resistance.

2. Grow an overnight culture of the recombineering strain (30 °C with shaking). Mix an aliquot of the overnight culture with an equal volume of 50% sterile glycerol (25% final concentration of glycerol) and store at −80 °C.

3.4 Preparation of Electrocompetent Cells

1. Grow an overnight culture of the recombineering strain in 5 mL LB supplemented with the appropriate antibiotic to maintain the chosen pORTMAGE plasmid, shaking at 30 °C.

2. Dilute the overnight culture 1 in 100 into 10 mL TB supplemented with the appropriate antibiotic to maintain the chosen pORTMAGE plasmid in a sterile 150 mL conical flask (see Note 7).

3. Incubate the culture shaking at 30 °C until OD_{600} = 0.4–0.6 [19].
4. Transfer the flask to a 42 °C shaking water bath for 15 min to induce lambda Red expression.

5. Transfer the flask to ice on a slow rocker for a minimum of 10 min to halt further cell growth.

6. Transfer the culture into a prechilled conical tube. Keep on ice. Prepare another conical tube of equal mass as a counterbalance for centrifugation.

7. Pellet/wash cells twice in ice-cold dH2O (all centrifugations at 2490 RCF for 8 min). Transport the cultures on ice and do the resuspensions in a 4 °C room (see Note 8).

8. Resuspend the final cell pellet in 160 μL of ice-cold dH2O. Divide into 40 μL aliquots in ice-cold microcentrifuge tubes [18]. Keep on ice and use immediately in recombinering protocol below (see Note 9).

### 3.5 Recombineering

1. On ice, combine three 40 μL aliquots of electrocompetent cells with 200, 300, and 0 ng (negative control) of PCR product (see Note 10).

2. Transfer the three DNA/cell mixtures to ice-cold electroporation cuvettes. Gently flick the cuvettes to ensure full coverage of the bottom of the cuvette and no bubbles in the mixture.

3. Electroporate the mixtures (2.5 kV, 0.2 cm cuvette).

4. Immediately after electroporation, add 1 mL of room temperature TB.

5. Transfer the cultures into 4 mL of room temperature TB in sterile 150 mL conical flasks.

6. Incubate the cultures on a rocker at room temperature for 3 h.

7. Add selection antibiotic to the cultures.

8. Incubate overnight on the rocker at room temperature [20].

9. Pellet the cultures (2490 RCF for 2 min).

10. Pour off and discard all but around 200 μL of the supernatant. Resuspend the pellets in the remaining supernatant.

11. Spread each resuspension across two selection agar plates.

12. Incubate overnight at 30 °C.

13. Check plates for colonies (see Note 11).

14. Confirm recombination by growing five to ten colonies overnight in LB with selection antibiotic, and then performing a diagnostic PCR on the cultures the following day using the same primers used to amplify the linear DNA fragment (see Note 12).
4 Notes

1. The recombination substrate could be amplified from a plasmid using primers with 80-base pair homology tails and the template then removed by DpnI restriction enzyme digestion. However, as the template plasmid here would contain the selection marker, any undigested plasmid could be transformed into the *E. coli* C-5545 cells, yielding false positives. Thus, as linear DNA will not be maintained in the cells without incorporation into replicable DNA, the use of linear DNA as the PCR template is critical here.

2. High-fidelity polymerase must be used to generate the linear fragment for recombineering. Mutations in the homology regions of the linear fragment lead to decreased efficiency of recombination.

3. *Escherichia coli* C-5545 is *E. coli* C-1a containing a P2 prophage with a mutant *old* gene. This mutation permits the use of the lambda Red recombineering system. Otherwise, the combination of P2 and lambda Red is lethal to the cells.

4. pPORTMAGE-3 encodes kanamycin/neomycin resistance. pPORTMAGE-4 encodes chloramphenicol resistance [18]. Any lambda Red-encoding plasmid would be suitable but would be induced differently to the plasmids listed here. We thus recommend pPORTMAGE, as the protocol has been optimized for this.

5. A shaking water bath must be used rather than an air incubator. This is to enable sufficient heat-transfer in the induction time.

6. If other bands are present, alter the PCR conditions to rectify appropriately. If there are additional bands to the desired one, increase the annealing temperature to reduce nonspecific priming.

7. A conical flask is critical here to enable rapid heat transfer during the 15-min induction step.

8. Linear DNA is transformed $10^4$-fold less efficiently than circular DNA, so care when making competent cells is critical [21]. Performing handling steps in a 4 °C room is a way to limit the exposure of the competent cells to heat and is recommended to maximize competency.

9. The induced electrocompetent cells are not stored at −80 °C. They are made fresh every time.

10. A range of DNA amounts are used to improve the chance of success. It has been observed in our research that 300 ng of DNA in 40 μL of electrocompetent cells is optimal.
Occasionally however, 200 ng of DNA yields more colonies than when 300 ng of DNA is used, hence these two amounts are recommended here.

11. Check plates at the beginning of the day. If there are no colonies, continue incubation and check again at the end of the day (they often require a long time until they are visible). Expect zero colonies on the negative control. This protocol has yielded ~200 colonies on the 300 ng DNA plate and ~10 colonies on the 200 ng DNA plate. If no colonies are visible after 24 h at 30 °C, it has been found that leaving the plates at room temperature for a further 1–2 days can yield colonies. But be sure that no colonies grow on the negative control during this additional time.

12. If the diagnostic PCR yields bands indicating mixed cultures of wild type and recombined bacteriophage, dilute the overnight culture $10^{-5}$ in LB and spread 100 μL on LB agar supplemented with selection antibiotic. Grow overnight at 30 °C and repeat the diagnostic PCR.

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Recombineering-Mediated Genome Editing in Burkholderiales Strains

Xue Wang, Jiaqi Liu, Wentao Zheng, Youming Zhang, and Xiaoying Bian

Abstract

Red/ET recombineering is primarily mediated by the E. coli recombinase pair Redα/Redβ from λ phage or RecE/RecT from Rac prophage, which is applied in E. coli and also closely related Gram-negative bacteria for efficient genome editing. However, some distant bacterial species like Burkholderiales strains require host-specific Redα/Redβ recombinase pair for highly efficient genome editing. A pair of recombinases Redβ7029 from the Burkholderiales strain DSM 7029, recently identified as Schlegelella brevitalea, were identified for efficient genetic manipulation in the native strain and several other Burkholderiales strains. In this chapter, we describe a detailed protocol for genome engineering in Burkholderiales strains via the Redγ-Redβ7029 recombineering and Cre/loxP site-specific recombination.

Key words Burkholderiales strains, Recombineering, Genome editing, Site-specific recombination, SacB counterselection

1 Introduction

Burkholderiales belong to the β-proteobacteria of Gram-negative bacteria and exists in virtually all niches [1]. The Burkholderiales include a genus Burkholderia containing pathogenic organisms, such as Burkholderia mallei (glanders), B. pseudomallei (melioidosis), and B. cepacia complex, and a genus Paraburkholderia harboring environmental species, for example, plant growth-promoting endophyte Paraburkholderia phytofirmans [2] and P. rhizoxinica, an endosymbiont of Rhizopus microspores [3]. Burkholderiales strains have become an emerging source of bioactive natural products, and many biosynthetic gene clusters (BGCs) are still cryptic or silent [4–7]. How to activate these biosynthetic gene clusters to obtain compounds with novel structure and/or remarkable activity becomes a study hotspot. But most of the Burkholderiales strains are nonmodel species, and lack of streamlined genome engineering

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techniques thus limits rapid activation and mining of silent gene clusters.

Red/ET recombineering is a homologous recombination genetic manipulation technique firstly used in *Escherichia coli* by using short homology arms (~50 bp) based on the expression of recombinase proteins Redα and Redβ derived from *E. coli* λ phage or RecE and RecT derived from Rac prophage [8–10], which could generate desired mutations in any part of the DNA, even genome. Thus, it is an ideal tool for efficient bacterial genome engineering and mining for novel natural products. The *E. coli* Redαβ system was only directly used for genetic modification in native and some closely related Gram-negative bacteria such as γ-proteobacteria [11–17], while recombineering in some more distant bacterial species require host-specific Redαβ recombinase systems for efficient genome manipulation [18–23]. Hoang group utilized mutated *E. coli* Redαβγ recombinases to perform knockout and subcloning of genomic sequences in two naturally transformable species *B. thailandensis* and *B. pseudomallei*, but they were not workable in unnaturally transformable species [24]. Therefore, developing a straightforward genetic manipulation tool is essential for the genome editing and natural product mining in Burkholderiales strains. Recently, we discovered a pair of bacteriophage recombinases Redαβ7029 from the Burkholderiales strain DSM 7029, recently reclassified as *Schlegelella brevitalea* [25], which can be used for efficient genetic editing in the native strain and also several other Burkholderiales strains that lack effective genetic tools, such as *P. phytofirmans*, *P. rhizoxinica*, and *Paraburkholderia megapolitana* [26, 27]. Using specialized recombinase-assisted insertion of functional promoters in the genomes, we successfully mined or activated six cryptic nonribosomal peptide synthetase/polyketide synthase BGCs and four classes of novel lipopeptides, glidopeptins, rhizomides, holrhizins, and haereomegapolitanin, were structurally elucidated [26–28]. In addition, deletions of large genome sequences (100–200 kb) were also conducted using this recombineering, and the biomass and growth rate of genome reduction mutants were improved compared with the wild type strain [26]. Therefore, we staged a technical platform for precise genome editing of Burkholderiales strains to promote cryptic BGC mining and genome reduction.

In this chapter, we describe a detailed protocol for application of this Redαβ7029 recombinase-assisted genetic editing in Burkholderiales strains, which consists of genome modification in Burkholderiales strain DSM 7029, and the seamless deletion in genome using recombineering together with Cre/loxP site-specific recombination (Fig. 1).
2 Materials

2.1 Reagents

1. Luria Broth (LB): Dissolve 5 g yeast extract, 10 g tryptone, and 1 g NaCl in 1 L ddH₂O, adjust the pH to 8.0, and transfer 100 or 200 mL aliquots into a glass stock bottle and autoclave at 121 °C for 20 min. Add appropriate antibiotics before use.

2. LB agar plates: Add 1.2% agar (w/v) into the liquid LB and autoclave at 121 °C for 20 min. Add appropriate antibiotics when the medium has cooled to 50 °C if it is required. Pour 20–25 mL medium into each petri dish and allow the agar to solidify in the sterile hood.

3. CYMG Broth: Dissolve 8 g Casein peptone, 4 g Yeast extract, 4.06 g MgCl₂·2H₂O, 10 mL glycerol in 1 L ddH₂O, and transfer 100 or 200 mL aliquots into a glass stock bottle and autoclave. Add appropriate antibiotics before use.

4. CYMG agar plates: Add 1.2% agar (w/v) into the liquid CYMG and autoclave. Add appropriate antibiotics when the medium has cooled to 50 °C if it is required. Pour 20–25 mL medium into each petri dish and allow the agar to solidify in the sterile hood.
5. 10% glycerol: 50 mL pure glycerol is mixed with 450 mL ddH$_2$O. The solution can be kept at room temperature (RT) for several months after autoclaving. The medium supplemented with sucrose should be sterilized at 115 °C for 30 min.

6. Antibiotic stock solutions: The antibiotics are stored in stock solution; dissolve apramycin (50 mg/mL), kanamycin (30 mg/mL), and gentamicin (50 mg/mL) in autoclaved ddH$_2$O, respectively, for the preparation of different antibiotic stock solutions. Pass the solutions through 0.2-μm syringe filters to sterilize in a clean bench and divide 1 mL aliquots into sterile 1.5-mL tubes. The stock solution can be stored at −20 °C.

7. 10% (w/v) L-(+)-arabinose: Dissolve 10 g L-(+)-arabinose powder in 80 mL ddH$_2$O and bring to a final volume of 100 mL. Pass the solutions through 0.2-μm syringe filters to sterilize in a clean bench and divide 1 mL aliquots into sterile 1.5 mL tubes. The stock solution can be stored at −20 °C.

8. 10% (w/v) L-(+)-rhamnose: Dissolve 10 g L-(+)-rhamnose powder in 80 mL ddH$_2$O and bring to a final volume of 100 mL. Pass the solutions through 0.2-μm syringe filters to sterilize in a clean bench and divide 1 mL aliquots into sterile 1.5-mL tubes. The stock solution can be stored at −20 °C.

9. 0.8% (w/v) agarose gels: Add a certain amount of agarose to appropriate volume 1× TAE buffer (w/v = 0.8%) fully melt the agarose power in a microwave and then cool the gel to 60 °C. Add 5% (v/v) ethidium bromide solution (10 mg/mL), mix thoroughly, pour the liquid gel into a gel mold with an appropriate hole card and waiting for solidification.

10. 1× TAE electrophoresis buffer: Dilute 50× TAE buffer with ddH$_2$O to a 1× solution and store it at room temperature for several months.

11. Oligonucleotides.
12. DNA Marker.
13. Restriction enzymes.
14. PrimeSTAR Max DNA Polymerase (or equivalent).
15. 10× Tris/Boric Acid/EDTA (TBE) buffer.
16. Ethidium bromide solution (10 mg/mL).
17. Agarose.
18. Universal DNA Purification kit.
19. Ethanol absolute.
20. RNase A (10 mg/mL; DNase and protease free).
21. L-(-)-Arabinose.
22. L-(-)-Rhamnose.
23. Sucrose.

2.2 Equipment
1. High-speed microcentrifuge.
2. Mini centrifuge.
3. Benchtop refrigerated microtube centrifuge kept at 2 °C.
4. Thermo Shaker Incubator.
5. Thermomixer.
6. Electroporation cuvettes with 1-mm gap.
7. Electroporator.
8. Mini Vortex meter.
9. Digital gel imaging system.
10. UV spectrophotometer.
11. UV-Vis spectrophotometer.
12. Gel electrophoresis apparatus.
13. Multipette.
14. 10 mL Combitips
15. Incubator.
16. Sterile 10 μL inoculation loop.
17. Petri dishes, 94 mm × 16 mm.
18. Syringe needle, 25G 5/8, 0.5 mm × 16 mm.
19. 0.2-μm syringe filter.

2.3 Strains and Plasmids
1. Burkholderiales strain DSM 7029 = Schlegelella brevitalea [29].
2. E. coli GB05-dir [30].
3. pBBR1-Rha-gba-Km [26].
4. pBBR1-Rha-Redγ-Redβ7029-Km [26].
5. pR6K-lox71-Genta-lox66-fleQ [16].
6. pRK2-Apra-Km [24].
7. pRK2-BAD-Cre-Apra-SacB (Fig. 3a, this study).

2.4 Primers
1. Primers for Subheading 3.1 [26].
2. Primers for Subheading 3.2 (Table 1).
3 Method

3.1 Redγ-Redαβ7029 Recombineering in Burkholderiales Strains

3.1.1 General Scheme for Genome Engineering of Burkholderiales Strains

Figure 2 illustrates the genome engineering of Burkholderiales strains DSM 7029. It is based on Redαβ7029 recombinases from DSM 7029. Redγ is an exonuclease inhibitor derived from E. coli; the addition of Redγ notably improves the recombination efficiency in Burkholderiales strains [26]. The recombinase proteins Redγ-Redαβ7029 are introduced into the middle copy pBBR1 plasmid (×20 per cell) and under the control of an L-(+)-rhamnose-induced Rha promoter for transient expression, which ensures their high-level expression for efficient recombination, and diminishes their toxicity and possible undesirable genome rearrangements. The elimination of selection marker is performed by Cre/loxP site-specific recombination to reach the seamless deletion in genome.

3.1.2 Construction of the Redγ-Redαβ7029 Transient Expression Plasmid

1. The recombinase expression plasmid pBBR1-Rha-Redγ-Redαβ7029-Km is based on the pBBR1 origin and under the control of an L-(+)-rhamnose inducible promoter (Rha).
2. Digest pBBR1-Rha-Redgba-Km with HindIII and DraI, and purify the 6892 bp fragment.
3. Amplify the Redβα7029 fragment from DSM 7029 genomic DNA by PCR.

Table 1
Primers used in Subheading 3.2

| Primers          | Primer sequences (5’-3’)                                      | Application                      |
|------------------|---------------------------------------------------------------|----------------------------------|
| 01 RK2-Apra-1    | TTGGGCTATCTTTAAATACTG                                       | pRK2-BAD-Cre-Apra-SacB construction |
|                  | TAGAAAGAGGAAGAAATAATAATG                                     |                                  |
|                  | GCAATACGAATTCGCAAAGAAG                                       |                                  |
| 02 sacB-Apra-2   | AGAAATATCATATAATATCATTTCACATAAATAA                          |                                  |
|                  | TAGTGAACCTACGCAAATCGACTGGGACG                                |                                  |
| 03 sacB-1        | GTTCACTTAATTATTAGGAAATGAG                                    |                                  |
| 04 RK2-sacB-2    | TGCGGCTCCTGCATTGCACATCG                                      |                                  |
|                  | TTAGGTTGTTTTG                                                 |                                  |
| 05 glbB-check-1  | ATTTGCTGTCCGAGTTTTCCAC                                       | DSM 7029ΔglbB selection marker elimination check |
| 06 glbD-check-2  | TGCCGAGCGTGGCTGGGACG                                         |                                  |
| 07 genta-3out-seq| CATTTCGACATGTTAGGCTCG                                        |                                  |
| 08 genta-5out-seq| GGATCGTCACCGGTAATCGC                                         |                                  |
| 09 sacB-ins-1    | ATACAACGGGTGAGGAGGTTAT                                       | pRK2-BAD-Cre-Apra-SacB cure check |
| 10 sacB-ins-2    | TGTGCCCTTATTCTGATTGTG                                        |                                  |

*a* The underlined bases represent homologous arms (HAs)
4. Co-transform the above fragments into *E. coli* GB05-dir to build pBBR1-Rha-Redγ-Redαβ7029-Km.

5. The recombinants are selected on LB plates containing kanamycin (15 μg/mL) and incubated at 37 °C. Correct colonies are verified by restriction analysis and sequencing.
6. The correct recombinase expression plasmids should be transformed into the Burkholderiales strains by electroporation and checked by colony PCR.

3.1.3 Transformation of Recombinase Expression Plasmid into Burkholderiales Strains

1. Inoculate single colony of wild type Burkholderiales strains in fresh liquid CYMG medium without any antibiotics at 30 °C for 16–18 h until the OD600 is around 1.5.
2. Spin it at 10,303 × g for 30 s in room-temperature centrifuge. Discard the supernatant as much as possible.
3. Resuspend the pellet in 1 mL of room-temperature ddH2O.
4. Repeat steps 2–4 twice and finally resuspend the electrocompetent cells in 35 μL ddH2O.
5. Add 200 ng plasmid to the electrocompetent cells and pipette the mixture into the room-temperature 1-mm electroporation cuvette.
6. Set the electroporator to 1300 V, 10 μF, 600 Ω (see Note 2).
7. Gently tap the cuvette on the bench to remove air bubbles and dry the metallic sides of the cuvette with a tissue.
8. Place the cuvette into the holder of the electroporator, insert, and push the ‘start’ button once.
9. Add 1 mL fresh CYMG medium with no antibiotics to the cuvette. Mix the cells gently by pipetting up and down and transfer into the reaction tube.
10. Incubate the cultures on a shaking incubator at 950 rpm for 180 min.
11. Take out 100 μL cultures and spread the cells by three-zone streak on kanamycin (10 μg/mL) CYMG plates.
12. After incubation at 30 °C for 72 h, check a single colony for whether the recombinase expression plasmid pBBR1-RhaRedγ-Redαβ7029-Km is correctly transformed into Burkholderiales strains by colony PCR.

3.1.4 Preparation of “Knock-In” or “Knock-Out” Cassette

1. Attaching homology arms to the cassette for chromosomal fragment deletion or promoter insertion by PCR amplification. The 70-mer oligonucleotides include ~50-nt homology arms flanking the targeting region and ~20-nt PCR primers at their 3’ ends.
2. Digest pR6K-lox71-Genta-lox66-fleQ with NcoI and pRK2-Aprar-Km with BstZ17I to avoid the background from the intact plasmids (see Note 1).
3. Once digestion completed, the mixture is precipitated by sodium acetate and ethanol using one-tenth volume of 3 M sodium acetate trihydrate and threefold volumes of ethanol. The DNA pellet is then rinsed with 70% ethanol. After air
drying the DNA pellet, the digested plasmid should then be diluted to 2.5 ng/μL with ddH₂O before use.

4. Use the digested plasmids (pR6K-lox71-Genta-lox66-fleQ or pRK2-Apra-Km) as the PCR templates, and the reaction conditions are listed in Tables 2 and 3.

3.1.5 PCR Purification

1. 1 μL of the PCR products are checked by electrophoresis on 0.8% agarose gel.

2. Rest of the yield should be purified using Universal DNA Purification Kit and eluted with 40 μL ddH₂O (see Note 3).

3.1.6 Procedure of Recombineering in Burkholderiales Strains

1. Inoculate single colony of DSM 7029 harboring pBBR1-Rha-Redγ-Redβ7029-Km in fresh CYMG with kanamycin at 30 °C for 24 h.

| Table 2 | Regular PCR system |
|---------|-------------------|
| **Template** | **pR6K-lox71-Genta-lox66-fleQ** | **pRK2-Apra-Km** |
| 2 x PrimeSTAR Max premix | 25 μL | 25 μL |
| Oligo 1 (50 pmol/μL) | 0.8 μL | 0.8 μL |
| Oligo 2 (50 pmol/μL) | 0.8 μL | 0.8 μL |
| Template | 1 μL of 2.5 ng/μL | 1 μL of 2.5 ng/μL |
| H₂O to total | 50 μL | 50 μL |

*For the lox71-Genta-lox66 PCR, digest about 10 μg plasmid with NcoI, precipitate and dissolve in ddH₂O to the final concentration of 2.5 ng/μL as PCR template.

b For the P_apra-Apra PCR, digest about 10 μg plasmid with BstZ17I, precipitate and dissolve in ddH₂O to the final concentration of 2.5 ng/μL as PCR template.

| Table 3 | Cycle setting for regular PCRs |
|---------|-----------------------------|
| **Temperature** | **Time** | **Cycles** |
| 98 °C | 4 min | 1 |
| 94 °C | 15 s | 30 cycles |
| 52 °C | 15 s | 30 cycles |
| 72 °C | 40 s | 30 cycles |
| 72 °C | 10 min | 1 |
| 4 °C | End | 1 |

*a The PCR products of lox71-Genta-lox66 is used in the chromosomal fragment deletion of Burkholderiales strain DSM 7029.

b The PCR products of P_apra-Apra is used in the promoter insertion of Burkholderiales strain DSM 7029.
2. Dilute 100 μL of the cultures (starting OD<sub>600</sub> around 0.15) into 1.3 mL fresh CYMG medium with kanamycin and grown at 30 °C, 950 rpm for 16–18 h (see Notes 4 and 5).

3. Add l-(-)-rhamnose to a final concentration of 2.5 mg/mL to induce the expression of the recombinases, the culture continued to be cultivated at 30 °C, 950 rpm for 90 min.

4. Spin it at 9600 rpm for 30 s in room temperature centrifuge.

5. Discard the supernatant as much as possible and resuspend the pellet in 1 mL of room temperature ddH<sub>2</sub>O.

6. Repeat steps 2–4 twice and finally resuspend the electrocompetent cells in 35 μL ddH<sub>2</sub>O.

7. Add 1 μg PCR products into the electrocompetent cells of Burkholderiales strains harboring the pBBR1-Rha-Redγ-Redαβ7029-Km expression plasmid and blending slightly.

8. Set the electroporator to 1300 V, 10 μF, 600 X.

9. Gently tap the cuvette on the bench to remove air bubbles and dry the metallic sides of the cuvette with a tissue, place the cuvette into the holder of the electroporator, insert, and push the “start” button once.

10. Add 1 mL fresh CYMG medium with no antibiotics after electroporation.

11. Incubate the cells at 30 °C, 950 rpm, 240 min for recovery and then spread on kanamycin (10 μg/mL) and gentamicin (20 μg/mL) CYMG plates.

12. After incubation at 30 °C for 72 h, check whether the recombinants are correct by colony PCR (see Note 6).

3.1.7 Identify the Transformants

1. After incubation at 30 °C for 72 h on kanamycin (10 μg/mL) and gentamicin (20 μg/mL) CYMG plates, pick 24 single colonies checking with at least two pairs of check primers (Table 1, primer 05, 06 and primer 07, 08).

2. Inoculate the single colony in fresh liquid CYMG medium with kanamycin (10 μg/mL) and gentamicin (20 μg/mL) at 30 °C for 16 h.

3. Take out 100 μL cultures to a new 1.5-mL EP tube and spin the cells at 12,000 rpm for 1 min and prepare backup tubes before starting.

4. Discard the supernatant as much as possible. Add 1 mL of sterilized ddH<sub>2</sub>O.

5. Resuspend the pellet by a Vortex.

6. Place in boiling water for 10 min.

7. Colony PCR using boiled cultures as DNA template (see Note 7).
8. PCR products are checked by electrophoresis on 0.8% agarose gel (see Note 8).

9. Pick correct single colony and preserve strain with 25% glycerol in −80 °C refrigerator.

3.2 Selection Marker Elimination by Site-Specific Recombination

The selection marker elimination is critical for consecutive genome fragment deletions in one strain. In this section, we present a detailed procedure for the elimination of the selection marker by Cre/loxP site-specific recombination and curing of the Cre expression plasmid using SacB counterselection.

3.2.1 General Scheme for the Workflow

The site-specific recombinase Cre was constructed on a replicated RK2 origin plasmid, and the l-(-)-arabinose inducible BAD promoter was employed to control the transient expression of the Cre recombinase (Fig. 3a). The SacB counterselection marker is also included in this plasmid to cure the Cre expression plasmid after selection marker cassette is eliminated (Fig. 3a) [31]. Hence, the markerless mutant can be generated by Cre site-specific recombination under multiple induction by adding l-(-)-arabinose (Fig. 3b). Figure 3c illustrates the method to cure Cre site-specific recombinase expression plasmid with continuous culturing in CYMG liquid media with different sucrose concentration. The curing of Cre expression plasmid also need to be verified by double-streak and colony PCR.

3.2.2 Elimination of Selection Marker on the Genome

1. To remove the gentamicin selection marker on the chromosome of Burkholderiales strain DSM 7029 by site-specific recombination, the plasmid pRK2-BAD-Cre-Apra-SacB should be introduced into Burkholderiales strain DSM 7029 by electroporation (Table 1, primers 01, 02, 03, 04, 05 to construct this plasmid).

2. Inoculate correct single colony in 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL) and apramycin (30 μg/mL) at 30 °C, 950 rpm for 14 h, 10 μL l-(-)-arabinose (100 mg/mL) should be introduced to induce the expression of Cre.

3. Transfer 30 μL of the 14 h cultures into 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL), apramycin (30 μg/mL), and 10 μL l-(-)-arabinose (100 mg/mL) at 30 °C, 950 rpm for approximate 14 h.

4. Repeat step 3 for 3–5 times.

5. Take out 100 μL cultures and spread the cells by three-zone streak on kanamycin (10 μg/mL) /apramycin (30 μg/mL) CYMG plates.

6. After incubation at 30 °C for 72 h, check single colonies and accomplish the removal of the loxP-Genta cassette. Perform
colony PCR (Table 1, primer 05, 06 and primer 07, 08) to confirm whether the gentamicin selection marker was thoroughly excised from the genome of Burkholderiales strain DSM 7029 (see Note 9).

7. Pick the correct single colony and preserve strain with 25% glycerol in −80 °C refrigerator.

3.2.3 Curing of Cre Expression Plasmid

To cure the Cre expression plasmid, we transferred the culture multiple times in a liquid CYMG medium with increasing concentrations of sucrose. The curing of site-specific recombinase Cre expression plasmid needs to be verified by colony PCR and double-streak (see Note 10).

Fig. 3 Schematic diagram of selection marker elimination. (a) Construct of the Cre-inducible expression and SacB expression plasmid pRK2-BAD-Cre-Apra-SacB. (b) Elimination of gentamicin selection marker by Cre site-specific recombination using multiple rounds of L-(+)-arabinose induction. (c) Curing of Cre expression plasmid pRK2-BAD-Cre-Apra-sacB. Plasmid carrying SacB counterselection marker is cured by adding increasing concentrations of sucrose in medium
1. Inoculate a single colony in 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL) and 5% sucrose at 30°C, 950 rpm for 16 h.

2. Transfer 30 μL cultures into 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL) and 7% sucrose at 30°C, 950 rpm for 16 h.

3. Transfer 30 μL cultures into 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL) and 10% sucrose at 30°C, 950 rpm for approximately 16 h.

4. Transfer 30 μL cultures into 1.3 mL fresh liquid CYMG medium with kanamycin (10 μg/mL) and 15% sucrose at 30°C, 950 rpm for approximately 16 h.

5. Perform step 4 twice.

6. Take out 100 μL cultures and spread the cells by three-zone streak on kanamycin (10 μg/mL) CYMG plates.

7. After incubation at 30°C for 72 h, check single colony whether the Cre expression plasmid (pRK2-BAD-Cre-Aprα-SacB) is thoroughly cured in Burkholderiales strain DSM 7029 by colony PCR (use primer 09, 10 in Table 1).

8. Double-streak correct single colony on kanamycin (10 μg/mL) CYMG plates and kanamycin (10 μg/mL)/gentamicin (20 μg/mL) CYMG plates.

9. Pick the correct single colony growing only on kanamycin (10 μg/mL) CYMG plates and preserve strain with 25% glycerol in −80°C in a refrigerator.

With seamless mutagenesis of Burkholderiales strain DSM 7029, several large nonessential chromosomal DNA fragments have been continuously deleted to form appropriate genome-reduced microbial chassis cell. Hence, the method we described here combining recombineering with site-specific recombination can be employed circularly and efficiently to achieve genome-reduced mutants.

### Notes

1. To make markerless mutants of Burkholderiales, the Cre-loxP system with the variant lox66 and lox71 sites are used to prevent the instability caused by multiple loxP sites in the genome.

2. Setting the electroporator to 1300 V is based on an Eppendorf® Electroporator 2510 using a 1 mm gap cuvette.

3. The purification is essential to get rid of inappropriate oligonucleotides and salts. The yield should be more than 5 μg at
150–300 ng/μL. For recombineering, 1–1.5 μg PCR products are needed for one electroporation.

4. The optimal OD\textsubscript{600} is around 1.8 before preparing electro-competent cells. The cultivation time can be extended if the OD\textsubscript{600} does not reach this value.

5. For electrocompetent cells for recombineering, grow fresh culture at 30 °C, 950 rpm for 16–18 h until OD\textsubscript{600} of 1.8. Then add L-(+)-rhamnose to a final concentration of 2.5 mg/mL, the cells are grown at 30 °C, 950 rpm for 90 min until the OD\textsubscript{600} to 2.0.

6. When identifying the transformants, pick the large colonies. The small colonies generally cannot grow in the liquid medium with kanamycin (10 μg/mL) and gentamicin (20 μg/mL).

7. The regular PCR and colony PCR reaction conditions are listed in Tables 2, 3, 4, and 5.

8. For large chromosomal DNA fragment deletion, the recombinants should be checked at both junctions by colony PCR and

### Table 4

| Template                      | Boiled cultures\textsuperscript{a} |
|-------------------------------|-----------------------------------|
| 2× PrimeSTAR Max premix       | 10 μL                             |
| Oligo 1 (50 pmol/μL)          | 0.3 μL                            |
| Oligo 2 (50 pmol/μL)          | 0.3 μL                            |
| Template                     | 1 μL of 20 ng/μL                  |
| H\textsubscript{2}O to total  | 20 μL                             |

\textsuperscript{a} For the boiled cultures, the concentration of nucleotide should be approximately 20–50 ng/μL.

### Table 5

| Temperature | Time | Cycles |
|-------------|------|--------|
| 98 °C       | 4 min| 1      |
| 94 °C       | 15 s | 30 cycles |
| 52 °C       | 15 s |         |
| 72 °C       | 40 s |         |
| 72 °C       | 10 min| 1      |
| 4 °C        | End  | 1      |

\textsuperscript{a} For the boiled cultures, the concentration of nucleotide should be approximately 20–50 ng/μL.
the PCR products should be sequenced when the insertion sequence is large.

9. When inducing the expression of Cre recombinase under the arabinose inducible BAD promoter, the cells grow at 30 °C after the addition of L-(+)-arabinose to a final concentration of 2.5 mg/mL, 950 rpm for approximate 24 h. Then take out 30 μL cultures into fresh liquid CYMG medium with kanamycin (10 μg/mL) and apramycin (30 μg/mL) with the addition of L-(+)-arabinose to a final concentration of 2.5 mg/mL.

10. All the operations here should be strictly sterilized to avoid contamination. More importantly, the operation time for electroporation should be as short as possible.

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**High-Efficiency Multi-site Genomic Editing (HEMSE) Made Easy**

Tomás Aparicio, Víctor de Lorenzo, and Esteban Martínez-García

**Abstract**

The ability to engineer bacterial genomes in an efficient way is crucial for many bio-related technologies. Single-stranded (ss) DNA recombineering technology allows to introduce mutations within bacterial genomes in a very simple and straightforward way. This technology was initially developed for *E. coli* but was later extended to other organisms of interest, including the environmentally and metabolically versatile *Pseudomonas putida*. The technology is based on three pillars: (1) adoption of a phage recombinase that works effectively in the target strain, (2) ease of introduction of short ssDNA oligonucleotide that carries the mutation into the bacterial cells at stake and (3) momentary suppression of the endogenous mismatch repair (MMR) through transient expression of a dominant negative *mutL* allele. In this way, the recombinase protects the ssDNA and stimulates recombination, while MutL	extsubscript{E36K} temporarily inhibits the endogenous MMR system, thereby allowing the introduction of virtually any possible type of genomic edits. In this chapter, a protocol is detailed for easily performing recombineering experiments aimed at entering single and multiple changes in the chromosome of *P. putida*. This was made by implementing the workflow named High-Efficiency Multi-site genomic Editing (HEMSE), which delivers simultaneous mutations with a simple and effective protocol.

Key words HEMSE, Cycled recombineering, ssDNA, Multiplex genome editing, *Pseudomonas putida*, Synthetic biology

**1 Introduction**

Different Bio-related technologies pursue production of high-added value chemicals from renewable resources, new (bio)-materials for the industry as well as living catalysts to alleviate environmental pollution. These technologies promise to facilitate the transition from petrochemical-based processes to a more sustainable and circular economy. To carry out this challenge, biologists rely on the combination of multiple disciplines such systems biology, metabolic engineering, and synthetic biology. All these disciplines merge to divert the nature of cells into adapted chemical factories and that implies not only to target a couple of genes but to tackle complex metabolic routes. For multiple reasons, this process...
is quite challenging and requires many attempts and iterations of the typical Design-Build-Test (DBT) cycle, borrowed from engineering disciplines, to finally obtain an optimal performer [1]. One of the limiting steps of this circular process is our ability to efficiently perform genome-scale engineering, tasks that require at least two different mutations within the chromosome [2] of a given bacterial species to develop the desired chassis. Even though there are a wide number of genetic tools based on homologous recombination to mutate the extant chromosome, they are tedious and time-consuming [3, 4]. Using those classic techniques, the process of obtaining deep engineered bacterial chassis is a daunting and challenging task. However, the appearance of recombineering, in particular single-stranded (ssDNA) recombineering, has pushed these technologies forward first in *E. coli* [5] and later in other biotechnologically interesting bacteria [6–8], thus helping to reduce the building time of the corresponding DBT cycle (Fig. 1). This approach takes advantage of the heterologous expression of phage recombination proteins to introduce any type of substitution at any possible chromosomal position while it also eliminates unnecessary cloning steps and just requires the design of a short ssDNA molecule (~90 bp) as template to introduce the desired mutation [5]. In *E. coli*, multiple variants of this technique such as MAGE (multiplex automated genome engineering) [9] and DIvERGE (directed evolution with random genomic mutations) [10] have been instrumental to bring about several genome-scale engineering projects [11–14]. Although these approaches work well in *E. coli*, their implementation in other bacteria is not obvious. So, its implantation to other biotechnologically interesting microorganisms is crucial. Among those bacteria, the environmental and safe-to-use *Pseudomonas putida* [15] offers a high potential for metabolic engineering [16–20] due to its inherent metabolism
that favors the production of reduction power through the EDEMP cycle [21]. Several works have been necessary to finally develop a prime version of ssDNA recombineering suited for this organism. It all starts with the identification of efficient recombinases [22, 23]. Then followed with the use of a proper heterologous DNA expression system that allows a temporary inhibition of the endogenous MMR system by expressing a negative dominant \textit{mutL} allele [24]. Once these setting were identified, optimal configuration required the tandem expression of the Rec2 phage recombinase and \textit{mutL_{E36K}PP} allele by the heat-inducible, tightly controlled expression system \textit{cI857/P_{L}} [25]. And finally, all of these steps merged to allow cycling the procedure to obtain a High-Efficiency Multi-site genomic Editing (HEMSE) protocol that increased the frequency of substitutions up to 10% per individual mutation in \textit{P. putida} EM42 [26].

The protocol described in this chapter explains an easy-to-do methodology to perform ssDNA recombineering in a multiplexed way in \textit{P. putida}. The protocol can be applied to either introduce multiple different mutations simultaneously or to diversify specific regions of the genome to randomly explore the DNA sequence space to identify the best combination for the desired application.

## Materials

### 2.1 Bacterial Strains and Plasmids

The protocol for cycled recombineering experiments described in this chapter has been tested on \textit{P. putida} KT2440 derivative EM42 [27]. \textit{E. coli} CC118 [\Delta(ara-leu) araD \DeltalacX74 galE galK phoA20 thi-1 rpsE rpoB argE.- (Am) recA1] [28] was used for cloning and plasmid maintenance. When plasmid transfer to \textit{P. putida} (or any \textit{Pseudomonas}) was required, \textit{E. coli} HB101 [F\`{\textprime} \lambda \hspace{0.1cm} hsdS20(r_{B} \textprime} \ m_{B} \textprime}) recA13 lenB6(\textprime) araC14 \Delta (gpt-proA)62 lacT1 galK2 (Oc) xyl-5 mtl-1 thiE1 rpsL2(\textprime) SmR]) was used as conjugation helper in mating experiments. The plasmid \textit{pSEVA2314-rec2-mutL_{E36K}PP} \hspace{0.1cm} [oriV(pBBR1), \hspace{0.1cm} cI857-P_{L} \rightarrow \text{rec2-mutL_{E36K}PP}, \hspace{0.1cm} Km^{R}; \hspace{0.1cm} [26]] (see \textbf{Note 1}) is required in order to perform ssDNA recombineering in \textit{P. putida} (Fig. 2) (see \textbf{Note 2}).

### 2.2 Culture Media and Chemicals

1. LB liquid medium. The components of LB per liter are: 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl. The components are solved in H_{2}O and autoclaved.
2. Terrific Broth (TB) without glycerol. The components of this medium per liter are: 12 g of tryptone, 24 g of yeast extract, 2 g KH_{2}PO_{4}, and 9.4 g of K_{2}HPO_{4}.
3. Rich solid media was obtained complementing the liquid preparation with 1.5% (w/v) agar.
4. M9 minimal medium supplemented with 0.2% (w/v) citrate as the sole carbon source (see Note 3). Importantly, the components of this minimal medium have to be prepared, autoclaved, and stored individually. The individual components of this medium are arranged as follows: (1) first a 10× stock of M9 salts is prepared by dissolving 42.5 g of Na₂HPO₄·2H₂O, 15 g of KH₂PO₄, 2.5 g of NaCl and 5 g of NH₄Cl in H₂O up to a final volume of 500 mL; (2) 100 mL of 1 M MgSO₄ solution is prepared by dissolving 12 g of MgSO₄ in H₂O; (3) finally, the citrate carbon source is prepared as a 20% (w/v) stock solution (see Note 4). When needed, all components are mixed to have a final concentration of 1× M9 salts, 0.2% (w/v) of citrate, and a 2 mM MgSO₄ solution.

For M9 medium plates, prepare a separate 1.6% (w/v) agar solution in H₂O and sterilize by autoclaving. Keep agar solutions stored at room temperature (solidified) and melt it in the microwave before use. In that way, mix the melted agar solution with the rest of the components to render a final 1.4% (w/v) agar concentration.

Add 20–25 mL of molten medium to prepare 90-mm Petri dish plates while 60–70 mL for the 145-mm ones. Then, let the media solidify at room temperature.

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**Fig. 2** Genetic organization of the business part of the pSEVA2314-rec2-\textit{mutL}_{E36K}^{PP} plasmid. The plasmid region between \textit{PacI} and \textit{SpeI} spans two functional domains. The first one, within \textit{PacI} and \textit{AvrII}, corresponds to the SEVA expression system \textit{d857-\textit{P}_{L}} (cargo # 14) [24]. The second part is the recombineering module, integrated by the \textit{rec2} recombinase from \textit{P. putida CSV86} that belongs to the ERF superfamily of ssDNA binding proteins [23] and the \textit{mutL}_{E36K}^{PP} allele. The asterisk within the \textit{mutL} gene represents the GAA → AAA mutation that changes the natural glutamic acid amino acid at position 36 for lysine. The \textit{mutL}_{E36K}^{PP} variant acts as a negative dominant allele inhibiting the MMR system of \textit{P. putida} [25]. The orange circles correspond to the ribosome binding sequence used to translate the \textit{rec2} and \textit{mutL}_{E36K}^{PP} pair. The SEVA \textit{T0} terminator (in red) blocks any transcriptional readthrough to the plasmid backbone [31]. The picture is not drawn to scale.
5. Antibiotics are prepared as 1000× concentrated stocks, kanamycin at 50 mg/mL and chloramphenicol at 30 mg/mL. Kanamycin is dissolved in H2O while EtOH is used for chloramphenicol. Both antibiotic stocks are sterilized by filtration (0.45 μm) and stored at −20 °C.

6. Prepare a 300 mM sucrose solution by dissolving 25.6 g of sucrose in 250 mL of H2O, sterilize by filtration, and store it at room temperature (see Note 5).

7. SYBR™ Safe DNA gel stain (Thermo Fisher Scientific; MA; USA) or equivalent.

8. Prepare a concentrated stock of TAE buffer (50×) by dissolving 242 g of Tris-base in 600 mL of H2O, add 100 mL 0.5 M EDTA and 57.1 mL of glacial acetic acid. Complete with H2O to 1 L. Store at room temperature. For DNA electrophoresis running buffer dilute the 50× TAE buffer with H2O to 1×.

2.3 Molecular Biology Techniques

1. Plasmid DNA purification kit.

2. Agarose gel extraction and PCR purification kit.

3. Colony PCR for amplification of DNA. Bacterial cells are streaked out from a fresh plate with a sterile toothpick and transferred to the PCR tube containing H2O (see Note 6). Independently, prepare a PCR reaction mixture with primers, buffers, dimethyl sulfoxide (DMSO; if required), dNTPs, and Taq DNA Polymerase following the specific manufacturer indications. Then, aliquot the exact amount to each tube and set up the PCR reaction with the proper Tm and amplification time.

2.4 Laboratory Equipment and Supplies

1. 10 mL plastic tubes.

2. 1.5 mL microcentrifuge tubes.

3. Sterile toothpicks.

4. Pipettes and tips.

5. Erlenmeyer flasks of different capacities (50 and 100 mL).

6. 50 mL conical tubes (Sterile).

7. Electroporation system.

8. 2-mm gap width electroporation cuvettes.

9. PCR machine.

10. Water bath (42 °C).

11. Stove incubator (30 °C).

12. Shaking incubator (170 rpm) at 30 °C.

13. 90 mm and 145 mm round sterile plastic Petri dishes.

14. 3 mm-diameter Glass beads for bacterial plating. Used glass beads can be recycled by washing them with 70% (v/v) ethanol, dried and autoclaved in glass bottles. Then, distribute sterile beads in 50 mL conical tubes for daily use.
15. Spectrophotometer to quantify DNA concentration and to read OD\textsubscript{600} of bacterial cultures.
16. DNA horizontal electrophoresis system.
17. Benchtop centrifuge for 1.5 mL tubes.
18. Centrifuge for 50 mL conical tubes.
19. Blue-Light Transilluminator.

2.5 Oligonucleotides

Acquire oligonucleotides from your favorite supplier as desalted DNA and, when received, add H\textsubscript{2}O to obtain oligo stocks of either 5 \(\mu\)M for PCR and sequencing or 100 \(\mu\)M for recombineering. Then, keep primers stock at \(-20\) °C.

2.5.1 Recombineering Oligonucleotides

Oligonucleotides to produce the desired genomic changes (point mutations, insertions and/or DNA variability) should be around 60–90 bp long (see Note 7, Fig. 3 and examples below). It is also important to design the oligo against the DNA lagging strand [5]. For that, it is necessary to locate within the chromosome the origin of replication (\textit{oriC}) and the termination (\textit{dif}) regions. The works of [32, 33] provide info related to the \textit{oriC} in \textit{Pseudomonas} while [34] offer an extended list with the \textit{dif} coordinates for a

![Fig. 3 Outline of the HEMSE procedure. The main steps of the HEMSE protocol are depicted. 1—Mutagenic oligonucleotides are designed with the genomic edition (single base changes, insertions, or sequence randomizations) located in the middle part of the sequence. Oligo sizes range between 60 and 90 nt, displaying two arms of homology of 30–45 nt with the genome target (yellow line). 2—Plasmid pSEVA2314-\textit{rec2-mutLE\textsubscript{E36K}}\textsuperscript{PP} is transformed by mating into \textit{P. putida} (strain of choice). 3—HEMSE protocol is applied by growing a culture of \textit{P. putida} harboring the pSEVA2314-\textit{rec2-mutLE\textsubscript{E36K}}\textsuperscript{PP} plasmid, inducing the expression of \textit{rec2} and \textit{mutLE\textsubscript{E36K}}\textsuperscript{PP} by heat shock (42 °C/15 min), preparing competent cells, electroporating the oligos, and recovering the culture until OD\textsubscript{600} \approx 1.0. Once the first cycle is accomplished, the culture enters a second round of mutagenesis, applying again the thermal induction and following the protocol in an iterative fashion. When a single cycle is performed, recovery can be extended overnight. 4—Once the desired HEMSE cycles are completed, the culture is plated after the last recovery step. The resulting colonies are screened by PCR and sequencing to find a correctly edited clone.](image-url)
broad number of microorganisms (see Note 8). The last factor to consider in the oligo design process is the optimization (when possible) of its folding energy to $\Delta G > -16$ kcal/mol [22]. Use DNA folding form from the mfold web server (http://www.unafold.org/mfold/applications/dna-folding-form.php; [35]) to estimate the $\Delta G$ of the designed oligonucleotides and choose the ones with the higher $\Delta G$ ($-13$ kcal/mol is better than $-16$ kcal/mol, for instance).

Examples of recombineering oligos for HEMSE are the following described in [26]:

Oligonucleotide to introduce DNA variability (sequence randomization):

**RBS-Deg9:**

**5’-TCTAGAGTCGACCTGCAGGCATGCAAGCTTTARRRRRRRTAAAAACATATGAGTAAAGGAGAA**

**GAACTTTT-3’.**

This oligonucleotide aims to generate variability at the RBS level of a heterologous RBS-less GFP construct. It is designed to introduce 9 nucleotides (bold) of which 6 positions are degenerated (R) to introduce either A or G. While the short homology arms of 30 nt surrounding the insertion are enough to promote recombineering, the low performance observed with these oligos [26] suggests that longer arms might increase the recombineering efficiency in the specific case of insertions.

Oligonucleotides to introduce single point mutations:

**SR:**

**5’-G*T*C*A*GACGCACACGGCATACTTTACGCAAGG**

CCGAGTTAGGGTTTTTGTCCGGCGTGGTGTTGT

ACACACGGGTGCACAC. **GCCACGACGCTGC-3’.**

This oligo (90 bp) introduces a point mutation (in bold) in the rpsL gene that renders cells SmR. The * symbol denotes phosphorothioate bonds (see Note 9).

**RR:**

**5’-TCCGAGAGGCTTGTCTGGTCCATGAACAGGGG**

ACAGCTGGCTGGAACCAGAAACTCT-3’. **ACACACGGGTGCACAC.**

This oligo (60 bp) introduces a point mutation (in bold) in the rpoB gene that renders cells RifR.

**PR:**

**5’-AGGTCCAAGAACAATCTCGAAGCCCTTTGTCACA**

CAGGGTCTTAGACAATGCCCCGAAGCGCTGCTG-3’. **ACACACGGGTGCACAC.**

This oligo (63 bp) introduces a point mutation (in bold) in the pyrF gene that renders cells auxotroph for uracil while resistant to 5-fluorotic acid (5-FOA).

Oligonucleotide to introduce a double point mutation:

**NR:**

**5’-AACGAGAACGGCTGGGCCCATAACGCACAGGATGG TAT**

TGTTAGAACCAGTGTCCGCGTGGTGGTGA-T-3’. **ACACACGGGTGCACAC.**
This oligo (65 bp) introduces two mutations (in bold) in the gyrA gene that renders cells Nal $^R$.

Oligonucleotide to introduce extra nucleotides within a specific ORF.

CR: 5'-GCAGCACGCGCAGAATGATCTGCTTGAAGCGCGG GTTTCCCCTATTATCATTCGGCATGGTCCAGGCAAGCAGCTACCCGGT TGAAGAAGGCT-3'.

This oligo (89 bp) introduces 9 nucleotides (in bold) encoding three stop codons in the catA-1 gene. catA-1$^-$ cells develop a brownish pigmentation in the presence of benzoate.

2.5.2 Diagnostic and Sequencing Primers to Confirm Mutations

To verify that bacteria have acquired the desired mutation, design oligonucleotides to amplify the region of interest for each of the target genes. Ideally, design primers that have similar melting temperatures to obtain a DNA amplicon of around 500 bp.

3 Methods

The protocol described here shows the necessary steps to do single ssDNA recombineering experiments and how to perform a cycled and multiplexed version (HEMSE) in P. putida EM42. A schematic picture of the process is represented in Fig. 3. The first step of the procedure is to design the oligonucleotides to introduce the desired mutations or DNA variability (see Subheading 2.5). This is followed by the introduction of the recombineering plasmid into your bacterial host. Next, the proper recombineering experiment is performed in a single or cycled manner. After a number of cycles, the mutagenized culture is plated and the proper mutant/s selected and purified. Finally, the last step is to eliminate the recombineering plasmid from the selected strain/s.

3.1 Delivery of the Recombineering Plasmid into Target P. putida Strain

The first step of the protocol is to have transformed the P. putida strain of interest with the recombineering plasmid. For plasmid delivery we recommend the drop-mating procedure described below.

1. Grow overnight the following bacterial strains (described in detail in Subheading 2.1):

   (a) E. coli (pSEVA2314-rec2-mutL$_{e36K}^{PP}$) in LB with Km at 37 °C (donor).
   (b) E. coli (pRK600) in LB with Cm at 37 °C (mating helper).
   (c) P. putida EM42 in LB at 30 °C (recipient).
2. Take 1 mL of the overnight grown cells and centrifuge (7200 × g, 2 min, room temperature). Remove the supernatant and add 1 mL of 10 mM MgSO$_4$ and gently resuspend the bacterial pellets.

3. Mix 100 μL of each of the three bacterial strains and centrifuge (7200 × g, 2 min, room temperature). Eliminate the 300 μL of the supernatant and add 20 μL of 10 mM MgSO$_4$. Gently resuspend the pellet and spot the cells on the surface of an LB agar plate.

4. Incubate the mating plate at 30 °C for 4–6 h.

5. Using a bent pipet tip, scrape cells from the agar plate and transfer them to a microcentrifuge tube containing 1 mL of 10 mM MgSO$_4$. Resuspend the mating pellet by vortexing.

6. Plate 10 μL and 100 μL of the resuspended mating mix on M9 + 0.2%(w/v) citrate + Km plate to select *P. putida* cells that acquired the pSEVA2314-rec2-mutLE36K$_{PP}$ plasmid. Keep the rest of the mating mix solution at 4 °C until checking that the dilutions plated rendered bacterial colonies.

7. Select a few colonies and purify them on a fresh M9 + 0.2%(w/v) citrate + Km plate.

8. Check the presence of the pSEVA2314-rec2-mutLE36K$_{PP}$ in *P. putida* by isolating plasmid DNA. Then, confirm plasmid integrity by DNA restriction with either AvrII/SpeI (expected DNA band sizes: 4.4 + 2.8 Kb) or EcoRI/HindIII (4.5 + 1.3 + 0.9 + 0.5 Kb).

9. Keep a −80 °C frozen stock of a correct recombinant *P. putida* (pSEVA2314-rec2-mutLE36K$_{PP}$) strain in 20% (v/v) glycerol.

### 3.2 HEMSE Protocol

The protocol will be outlined with the two examples described in [26]. The first one illustrates the use of recombineering to introduce DNA variability at a defined locus and the second describes how to cycle and multiplex the process to introduce several mutations simultaneously.

#### 3.2.1 Introducing DNA Variability at a Defined Genomic Locus with a Single Cycle

In this example, a strain of *P. putida* EM42 bearing a genomic construct $P_{EM/7-gfp}$ without a functional ribosome binding site (RBS) is subjected to one cycle of recombineering with the oligo RBS-Deg9 (Subheading 2.5.1). This soft-randomized oligo inserts 64 different combinations of six A/Gs and the restoration of functional (yet diverse in activity) RBSs is easily screened by visual inspection of fluorescent colonies. To perform a single recombineering experiment (cycle = 1) one need to: (1) grow the bacterial culture; (2) induce the expression of the recombinase together with the negative dominant *mutL* allele to temporarily inhibit the bacterial MMR System; (3) electroporate the recombineering
oligonucleotide to introduce random DNA variability; (4) allow the cells to recover and plate the culture. Then, the whole library can be stored or directly screened to select an optimal clone.

Culture Growth and Transient Induction of the Recombinase and MutLE36KPP Allele

1. Directly from the −80 °C frozen stock, inoculate a test tube with the P. putida strain harboring the recombineering plasmid in LB + Km.
2. Incubate overnight at 30 °C with shaking.
3. Measure OD<sub>600</sub> and dilute to 0.1 in 20 ml LB.
4. Incubate at 30 °C with shaking until OD<sub>600</sub> reaches 1 (~4 h).
5. Transfer the Erlenmeyer flask to a shaking water bath set at 42 °C for 15 min with vigorous shaking (250 rpm). This incubation at 42 °C inactivates the cI857 repressor, liberating the P<sub>L</sub> promoter and activating the expression of the rec2-mutL<sub>E36K</sub><sup>PP</sup> genes.
6. Quickly place the Erlenmeyer flask in ice and incubate for 5 min to cool down the culture. This step allows the cells to again produce the cI857 repressor to block back the P<sub>L</sub> promoter. This procedure allows for a short and transient (but sufficient) expression of the recombinase and the inhibitor of the MMR System.

Preparation of P. putida Electrocompetent Cells

1. Transfer 10 mL culture into a 50 mL conical tube.
2. Centrifuge at 3220 × g for 5 min at room temperature.
3. Remove the supernatant and add 10 mL of 300 mM sucrose. Gently resuspend the bacterial pellet.
4. Centrifuge at 3220 × g for 5 min at room temperature.
5. Remove the supernatant and add 5 mL of 300 mM sucrose. Gently resuspend the bacterial pellet.
6. Centrifuge at 3220 × g for 5 min at room temperature.
7. Remove the supernatant and add 1 mL of 300 mM sucrose. Gently resuspend the bacterial pellet and transfer it to a 1.5 mL microcentrifuge tube.
8. Use now a benchtop centrifuge at 10,600 × g for 1 min at room temperature.
9. Remove the supernatant and add 200 μL of 300 mM sucrose. Gently resuspend the bacterial pellet.
10. Pipet 100 μL of the electrocompetent mix to a fresh 1.5 mL tube (see Note 10).

Oligonucleotide Electroporation for a Single Recombineering Experiment

1. Add 1 μL of the 100 μM recombineering oligo (e.g: RBS-Deg9) to the 100 μL of fresh electrocompetent cells. This produces a final oligo concentration of ~1 μM.
2. Pipet the ssDNA + cells mixture into a 2-mm gap width electroporation cuvette avoiding the formation of air bubbles.

3. Clean the electroporation cuvette.

4. Set the appropriate parameters in the electroporator apparatus (2.5 kV) and proceed with the electroshock.

5. Quickly add 900 μL of TB to the electroporation cuvette.

6. Ideally, the electroshock timing should be ~5 ms or higher (see Note 11).

**Cell Recovery and Plating**

1. Gently pipet up and down the transformed cells and pass the 1 mL mix to a 100 mL Erlenmeyer flask containing 4 mL of TB (see Note 12).

2. Incubate overnight (~16 h) at 30 °C with 170 rpm shaking (see Note 13). Normally, the OD_{600} of the culture in that condition reaches 8–10.

3. Plate a 10^{-6} dilution on a 145 mm Petri dish to get around 500 colonies per plate. With the expected conditions for single changes (~1 x 10^{-2}) one would expect around 5 mutated colonies per plate. However, in some cases due to different circumstances (short homology regions within the oligo or the extended length of the introduced sequence) this frequency could drop down to 5.9 x 10^{-4} to 9.9 x 10^{-4} [26]. For that reason, we recommend spreading the maximum possible number of plates (see Note 14).

4. Incubate plates overnight at 30 °C (see Note 15).

5. Count total CFUs and perform the screening (visual) to pinpoint clones with different levels of fluorescence. This would translate into selecting mutated clones that have incorporated different variants of the RBS.

6. Re-streak the identified clones on a fresh plate.

7. PCR amplify the mutated region with the oligonucleotides designed in Subheading 2.5.2.

8. Purify the resulting DNA fragment and sequence using the same primers used for the PCR.

9. To estimate the recombineering frequency divide the confirmed mutated clones by the total CFUs and normalize that number to 10^{9} cells.

10. Prepare a –80 °C frozen stock of the different and nonredundant identified mutated clones.

**3.2.2 Cycled and Multiplexed Recombineering Experiment (HEMSE)**

In case one is interested in introducing more than one mutation simultaneously within the same *P. putida* bacterial strain, the ssDNA recombineering protocol should be cycled to increase the frequency of multiple changes. As an example of the HEMSE
procedure, we are going to protocolize the experiment detailed in [26], in which five independent mutations were introduced into the genome of *P. putida* EM42 by applying 10 recombineering cycles.

The procedure to perform HEMSE is similar to the one described above for single cycle ssDNA recombineering, with some modifications. One need to: (1) grow the bacterial culture; (2) transiently induce the co-expression of the rec2-mutL<sub>E36K</sub><sup>Pp</sup> genes; (3) electroporate with the recombineering oligonucleotide mix (n = 5) and (4) recover the culture and repeat ten times the whole process from step 1. After cycling the protocol, cell culture must be plated, screened for multi-edited cells, and finally the recombineering plasmid is curated from the correct clone.

This protocol is basically identical to the one described in Subheading 3.2.1 except for the oligonucleotide transformation and cell recovery steps. Only those stages are described below.

---

**Oligonucleotide Electroporation for a HEMSE Experiment**

1. Prepare a mixture of all recombineering oligos (n = 5) by adding 10 μL of the 100 μM stock of each (oligo names: SR, NR, RR, PR and CR; see Subheading 2.5.1).

2. Add 3 μL from the oligo mix to the 100 μL of the freshly prepared electrocompetent cells. This renders a final concentration of ~0.6 μM for each oligo.

3. Pipet the ssDNAs + cells mixture into a 2-mm gap width electroporation cuvette avoiding the formation of air bubbles.

4. Clean the electroporation cuvette.

5. Set the appropriate parameters in the electroporator apparatus (2.5 kV) and proceed with the electroshock.

6. Quickly add 900 μL of TB + Km to the electroporation cuvette.

7. Ideally, the timing of the electroshock current should be between ~5 ms or higher (see Note 11).

---

**Cell Recovery and Preparation for Another Recombineering Cycle**

1. Smoothly transfer the 1 mL of electroporated cells to a 100 mL Erlenmeyer flask containing 4 mL of TB + Km (see Note 16).

2. Incubate transformed cells at 30 °C with 170 rpm shaking until OD<sub>600</sub> ~1 (~4 h). Then, proceed with a new induction cycle.

3. If needed, this recovery step is performed overnight without shaking to limit cell growth.

4. Also, when the OD<sub>600</sub> reaches the expected value late in the day, the bacterial culture can be kept at 4 °C overnight. In this case, cells are reactivated the next morning for 30 min at 30 °C with shaking before starting the induction stage.

5. After repeating the whole protocol 10 times, plate different aliquots in the appropriate medium.
6. Purify selected clones by re-streaking them again in the right selective medium.

7. Check the correct incorporation of the mutations by PCR amplification and sequencing of target genes.

8. Prepare a frozen stock with a strain that bears all mutations.

3.3 Curing of Recombi neering Plasmid

Once having the final mutated strain ready, after 1 or 10 cycles, and stocked at −80 °C, proceed to eliminate the recombineering plasmid before doing the desired experiments. To do that, simply follow the next steps:

1. Use the −80 °C frozen stock to inoculate a 10 mL test tube containing 3 mL of LB.

2. Incubate overnight at 30 °C with shaking.

3. Prepare a 10⁻³ dilution in 1 mL and inoculate 10 μL from that solution in 3 mL fresh LB.

4. Incubate for at least 6 h at 30 °C with shaking.

5. Transfer 3 μL to 3 mL fresh LB.

6. Incubate overnight at 30 °C with shaking.

7. Repeat steps 3–6 for 10 times.

8. Then, plate several dilutions on LB to obtain single colonies.

9. Re-streak a number of colonies on LB + Km and LB agar plates.

10. Select a clone that is sensitive to Km.

11. Check the absence of plasmid either by miniprep or by PCR using a proper combination of the SEVA backbone primers \[31\].

12. Maintain a frozen stock of the mutated \(P.\ putida\) plasmid-less strain.

4 Notes

1. There are also other plasmids available to perform ssDNA recombineering or HEMSE: pSEVA2214--rec2-mutL\(_{E36K}\)\(^{PP}\) \((\text{oriV(RK2)}, cI857-P\_L \rightarrow \text{rec2-mutL}\_E36K^{PP}, \text{Km}\_R; [26])\) or pSEVA2514-\(\text{rec2-mutL}_E^{PP}\) \(\text{oriV(RSF1010)}, cI857-P\_L \rightarrow \text{rec2-mutL}\_E36K^{PP}, \text{Km}\_R; [25]\).

2. Even though this protocol is optimized for \(P.\ putida\) this could also work in other \textit{Pseudomonas} or other bacterial species given that the plasmids replicate in that organism. We recommend first testing whether the recombinase and \textit{mutL} allele works in the target bacterial species by performing a recombineering experiment with a reporter gene (\textit{rpsL, gyrA, rpoB, pyrF}, etc.).
3. Other media to counterselect for *Pseudomonas* can also be used, such as cetrimide agar or Pseudomonas Isolation Agar (PIA).

4. If experimentally required, other C-source (glucose, succinate, fructose, etc.) could be added to the M9 minimal media.

5. To prevent contamination, it is recommended to aliquot the 300 mM sucrose solution stock in different 50 mL conical tubes.

6. We recommend to add 5 μL extra of H₂O to account for what the toothpick absorbs.

7. For single nucleotide changes, short oligos of 60 bp length work fine. For DNA insertions and deletions, longer oligos (80 nt or longer) are preferred: an insertion of 9 nt, for instance, should be included in the middle of an 89-nt oligonucleotide (homology arms of 40 nt).

8. When the coordinates of the oriC and dif are not known, design a test oligo against both strands to introduce a point mutation in a reporter gene and test the efficiency of both oligos and select the most efficient one.

9. As detailed in [36], the design of oligos with phosphorothioate bonds at the 5'-end is not needed.

10. In case something goes wrong with the electroporation, there is an extra 100 μL of competent cells to repeat the electroporation.

11. Lower (<5 ms) electroshock time values might produce poor transformation efficiencies, which translates to low recombineering performance.

12. In one-cycle experiments, maintenance of the recombineering plasmid inside the cells is not needed and Km is not necessary in the culture recovery step.

13. Depending on the particularities of the experiment, different incubation times could be used.

14. It is recommended to keep a −80 °C frozen stock of a part of the transformed culture.

15. Depending on the specific screening, the incubation time and the components of the selective medium may vary.

16. When cycling the recombineering experiments, it is important to keep the recombineering plasmid in bacterial cells throughout the cycles. Add the proper antibiotic (Km in this specific case) to avoid plasmid loss during the process.
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Chapter 5

Creation of Markerless Genome Modifications in a Nonmodel Bacterium by Fluorescence-Aided Recombineering

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Abstract

Metabolic engineering of nonmodel bacteria is often challenging because of the paucity of genetic tools for iterative genome modification necessary to equip bacteria with pathways to produce high-value products. Here, we outline a homologous recombination-based method developed to delete or add genes to the genome of a nonmodel bacterium, \textit{Zymomonas mobilis}, at the desired locus using a suicide plasmid that contains \textit{gfp} as a fluorescence marker to track its presence in cells. The suicide plasmid is engineered to contain two 500 bp regions homologous to the DNA sequence immediately flanking the target locus. A single crossover event at one of the two homologous regions facilitates insertion of the plasmid into the genome and subsequent homologous recombination events excise the plasmid from the genome, leaving either the original genotype or the desired modified genotype. A key feature of this plasmid is that Green Fluorescent Protein (GFP) expressed from the suicide plasmid allows easy identification and sorting of cells that have lost the plasmid by use of a fluorescence activated cell sorter. Subsequent PCR amplification of genomic DNA from strains lacking GFP allows rapid identification of the desired genotype, which is confirmed by DNA sequencing. This method provides an efficient and flexible platform for improved genetic engineering of \textit{Z. mobilis}, which can be easily adapted to other nonmodel bacteria.

Key words  Markerless method, Fluorescence activated cell sorter (FACS), Green fluorescent protein (GFP), \textit{Zymomonas mobilis} ZM4, Nonmodel bacteria, Genetic tools, Suicide plasmid, Homologous recombination

1 Introduction

Homologous recombination has enabled development of a variety of genetic tools for bacterial systems [1]. In the last half-century, several plasmids have been engineered for deletion or insertional inactivation of genes capitalizing on pathways for homologous recombination [1–4]. However, the development of genetic tools for nonmodel bacteria has generally trailed behind that of more genetically tractable systems such as \textit{Escherichia coli}. \textit{Zymomonas mobilis} ZM4 is a natural ethanologen with several features that
make it attractive as an industrial microbial biocatalyst and for strain engineering [5–7]. However, this bacterium is limited in genetic tools to delete or add genes into the genome. Host homologous recombination strategies that replace a gene typically do so with an antibiotic resistance marker cassette [8, 9]. *Z. mobilis* is naturally resistant to several antibiotics [10], thus limiting the iterative use of this deletion strategy in *Z. mobilis*. A few strategies have been developed to avoid the permanent incorporation of an antibiotic resistance cassette. For example, the use of FLP recombinase [11] eliminates the antibiotic resistance gene but leaves behind FRT sites in the modified genome [1], and the use of *sacB* [12] provides a growth counter-selection, instead of an antibiotic resistance selection, but cannot be easily used in strains with a native *sacB* gene. Recently, we developed a recombination-based method [13] to delete or add genes iteratively that does not require permanent addition of antibiotic resistance genes, and is greatly accelerated by use of a fluorescence activated cell sorter (FACS). The method leverages the simple and efficient process of broad-host-range conjugation and provides more flexibility for strain engineering than previously described approaches [1, 8, 9, 11, 12].

The method involves two homologous recombination events of a recombineering suicide plasmid (Fig. 1). The suicide plasmid pPK15534 [13] contains the pACYC184 origin of replication (*ori*) that allows replication in *E. coli* but not in *Z. mobilis*. The broad host range mobilization element in pPK15534 is from pSUP202 [14], which allows conjugation of plasmids from an *E. coli* donor strain to *Z. mobilis* strains. The plasmid also contains an antibiotic resistance marker cassette, chloramphenicol acetyltransferase (*cat*), for plasmid selection and the gene encoding the green fluorescence protein (*gfp*) for plasmid screening. Expression of *gfp* was also optimized to maximize the fluorescence signal produced from a single genomic copy. This facilitates sorting of strains that have lost the plasmid by tracking the loss of fluorescence using a blue light transilluminator or a FACS.

Deletion of genes requires cloning 500 bp upstream (UP) and downstream (DN) of the target gene, which will define the deletion boundaries, into the SpeI restriction site of the plasmid pPK15534 (Fig. 2). These homologous sequences in the plasmid direct recombination of the plasmid into the recipient genome at either the region upstream or downstream of the target locus. For insertion of heterologous DNA into the genome, the heterologous gene cassette is cloned between two *Z. mobilis* regions in the suicide plasmid, which will direct the location of the cassette in the genome. The engineered plasmid is transformed into *E. coli* WM6026 [15] and mobilized from this donor strain into the *Z. mobilis* recipient strain via conjugation. The plasmid recombinant strains are selected based on their chloramphenicol resistance. In the next step, recombinants are grown without selection to allow the growth of cells that have undergone a second recombination
event that results in plasmid loss and either a modified genome or the original strain genotype (Fig. 1). As recombination events occur at a low frequency (~3 out of $10^3$ cells), FACS facilitates enrichment of strains that have lost the plasmid (Fig. 3) because they are no longer fluorescent. Manual enrichment by the screening of colonies for loss of fluorescence using a blue-light transilluminator is also feasible (Fig. 3). In either case, the nonfluorescent colonies that have resolved the plasmid are screened by PCR amplification for the correct genotype and then sequence-verified.
Fig. 2 Gibson assembly of a recombineering suicide plasmid. (a) Position of primers to amplify the upstream (UP) and the downstream (DN) regions flanking the target gene. (b) Scheme for Gibson assembly of UP and DN fragments at the SpeI restriction site in pPK15534. The PCR amplified fragments contain overhangs that match with the end of the joining DNA fragments. The gray-colored fragment is the recombineering plasmid pPK15534 that was digested at the SpeI restriction site for adding UP and DN sequences. Positions of the P1 and P2 primers are shown, which are used to verify cloning of the UP and DN fragments into the pPK15534 plasmid. (ori—origin of replication from pACYC184; cat—chloramphenicol resistance gene cassette; gfp—gene encoding green fluorescent protein; mob—mobilization element carrying oriT region; UP—500 bp region homologous to upstream of the target gene; and DN—500 bp region homologous to downstream of the target gene)
2 Materials

2.1 Bacterial Strains, Plasmids, and Growth Media

1. *Z. mobilis* ZM4 (ATCC # 31281).
2. *E. coli* DH5α [16] for plasmid cloning and propagation.
3. A *m*-diaminopimelate (DAP) auxotroph *E. coli* WM6026 for conjugating plasmids into *Z. mobilis* strains.
4. Suicide plasmid pPK15534 [13].
5. ZRMG: 1% yeast extract, 0.2% KH₂PO₄, 2% glucose. Add 10 g yeast extract and 2 g KH₂PO₄ to 900 ml water, and autoclave. Autoclave 100 ml of 10% glucose solution separately. Combine both solutions for use. Ultrapure water was used for preparing all solutions in this protocol.
6. Solid media for conjugation, ZRMGc: 1% yeast extract, 0.2% KH₂PO₄, 2% glucose, 10 mg/ml tryptone, 0.1 mM DAP. Add 10 gm yeast extract, 10 g tryptone and 2 g KH₂PO₄ and 15 g Bacto agar to 900 ml water. Prepare 100 ml of 10% glucose solution. Autoclave these solutions separately. Combine both solutions and add 10 ml of filter sterilized 10 mM DAP

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Fig. 3 Workflow for enrichment of nonfluorescent cells and screening to identify the desired genotype
solution once the two solutions are cooled to below 50 °C. Pour medium into disposable sterile petri plates. Solidify overnight and store at 4 °C.

7. Luria-Bertani (LB) liquid and solid medium.

8. Stock solution of 20 mg/ml chloramphenicol (in ethanol). Chloramphenicol is used at a final concentration of 20 μg/ml for *E. coli* strains and at a final concentration of 120 μg/ml for *Z. mobilis* strains.

### 2.2 Screening Cells or Colonies for the Presence or Absence of GFP Fluorescence

1. Fluorescence activated cell sorter (FACS), Sony MA900 or equivalent. This protocol was developed using Sony MA900 equipped with a 70 μm sorting chip, 488 nm excitation laser, and 525/50 nm emission detector. It requires sheath fluid and calibration beads for the calibration of the machine.

2. Bleach and 100% ethanol for FACS.

3. 5 ml capped sterile tubes.

4. Blue light transilluminator (Thermofisher) or equivalent.

5. Phosphate buffer saline (PBS): 1.37 mM NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 18 mM KH₂PO₄.

### 2.3 Assembly, Amplification, and Purification of the Recombineering Plasmid

1. Q5 Polymerase or other high-fidelity polymerase for amplification of DNA fragments for cloning or sequence verification.

2. GOTaq® Flexi DNA polymerase colorless mastermix (Promega) or equivalent for screening or verification of genome modification.

3. 10 mM dNTPs mixture.

4. GeneJET PCR Purification Kit (Thermofisher) or equivalent.

5. GeneJET Plasmid Miniprep Kit (Thermofisher) or equivalent.

6. PureLink Quick Gel Extraction Kit (Thermofisher) or equivalent.

7. NEBuilder® HiFi DNA assembly cloning kit for Gibson assembly of DNA fragments into suicide vector, or equivalent enzymes.

8. SpeI restriction enzyme and Cutsmart buffer (New England Biolabs).

9. 1 Kb plus DNA ladder.

10. Agarose gel and apparatus.

11. NanoDrop™ microvolume UV-Vis spectrophotometer (Thermo Scientific) or equivalent.

### 2.4 Conjugation

1. 30 °C and 37 °C incubators and water baths.

2. 2 ml microcentrifuge tubes.

3. DAP.
### Method

#### 3.1 Cloning of 500 bp Z. mobilis Regions into the Suicide Plasmid (Fig. 2)

**3.1.1 Primer Design**

Design primers using NEBuilder assembly tool (https://nebuilder.neb.com/) to amplify 500 bp genomic regions (see Note 1). NEBuilder assembly tool is programmed to design primers to amplify DNA sequence with matching overhangs for Gibson assembly.

1. Input the sequence of the plasmid (pPK15534) as new fragment in NEBuilder assembly tool (https://nebuilder.neb.com/).
2. Specify the fragment generated by restriction enzyme digestion using SpeI.
3. Input the 500 bp sequence upstream of the target gene and then the 500 bp sequence downstream of the target gene as a new fragment. This will generate a set of primers to amplify all fragments for Gibson assembly.

**3.1.2 PCR Amplification and Purification of the Genomic DNA Fragments**

The 500 bp regions upstream and downstream of the target gene are amplified by colony PCR using high-fidelity Q5 polymerase.

1. Pick a single colony of Z. mobilis and resuspend in 50 μl of water.
2. Label two PCR tubes for amplification of UP and DN regions.
3. Prepare a reaction mixture in each tube containing 5 μl Q5 buffer, 1 μl 10 mM dNTPs, 2.5 μl 10 μM forward primer and 2.5 μl of 10 μM reverse primer, 2 μl of cell suspension, 1 unit of Q5 polymerase, and water to adjust final volume to 50 μl. Use UP and DN region amplification primers that were designed in Subheading 3.1.1, in their respective tubes.
4. Incubate the reaction mixtures in a thermocycler with the following settings: 1 cycle for initial denaturation and cell lysis (at 98 °C for 30 s), 30 cycles for denaturation (at 98 °C for 30 s), annealing (at 5 °C less than Tm of primers for 20 s) and elongation (at 72 °C for 30 s per Kb), 1 cycle for final elongation (at 72 °C for 2 min), and final hold at 10 °C.
5. Electrophorese PCR reaction mixtures on 0.8% agarose gel.
6. Excise band of interest and elute DNA using PureLink Quick Gel Extraction Kit.
7. Measure the concentration of DNA using a NanoDrop™ microvolume spectrophotometer or similar device.
3.1.3 Digestion and Purification of the Plasmid pPK15534

1. Prepare the following reaction mixture in a 1.5 ml tube: 5 μl Cutsmart buffer, 1 μg pPK15534 plasmid, 1 unit SpeI enzyme, and water to adjust total volume to 50 μl.

2. Incubate at 37 °C overnight or as recommended by the enzyme manufacturer.

3. Inactivate the enzyme by heating the reaction mixture at 85 °C for 20 min or as recommended by the enzyme manufacturer.

4. Electrophorese plasmid digestion reaction mixtures on 0.8% agarose gel.

5. Excise band of interest and elute DNA using PureLink Quick Gel Extraction Kit.

6. Measure the concentration of DNA using a NanoDrop™ microvolume spectrophotometer or similar device.

3.1.4 Gibson Assembly of Fragments into Recombineering Plasmid

1. Calculate the concentration of DNA fragments in pmol/μl using the following formula:

   \[ \text{pmol/μl} = \frac{\text{Concentration in ng/μl} \times 1000}{\text{Fragment size in bp} \times 650} \]

2. Set up following reaction mixtures:

|              | Tube A | Tube B (negative control) |
|--------------|--------|---------------------------|
| HiFi master mix | 10 μl  | 10 μl                     |
| UP fragment   | 0.05 pmol | x                     |
| DN fragment   | 0.05 pmol | x                     |
| Linearized plasmid | 0.1 pmol | 0.1 pmol              |
| Water         | To 20 μl | To 20 μl                |

3. Incubate mixture at 50 °C for 30 min in a thermocycler or as recommended by the NEBuilder assembly tool (https://nebuilder.neb.com/).

3.2 Transformation of Gibson Assembly Mix into DH5α

1. Transform the 20 μl reaction mixture into 45 μl chemical competent DH5α [16] or other similar strain with settings as directed in [17].

2. Add 1 ml LB to transformed cells, incubate for 1 h at 37 °C, centrifuge at 17,300 \( \times g \) for 30 s at room temperature, decant supernatant, suspend pellet in 50 μl LB, plate on LB agar plate containing 20 μg/ml chloramphenicol and incubate overnight at 37 °C aerobically. Streak-purify multiple transformants onto a LB agar plate with 20 μg/ml chloramphenicol and incubate overnight at 37 °C aerobically.
3. Identify fluorescent colonies with blue light transilluminator. Inoculate 5 ml LB containing 20 μg/ml chloramphenicol with a single fluorescent colony of each isolate and incubate at 37 °C aerobically overnight. Extract plasmid from the cell suspension using a DNA miniprep kit.

4. Verify plasmid construct by PCR using primers P1 (5’ GTAG CACCTGAAGTCAGCCC) and P2 (5’ GCTGGCGAAGATCGAAGAG) that flank the SpeI site of pPK15534 (Fig. 2). Prepare a reaction mixture containing 10 μl Flexi GoTaq colorless master mix, 2 μl forward primer (final concentration—1 μM) and 2 μl reverse primer (final concentration—1 μM), 1 μl of plasmid (10–15 ng) and water to adjust volume to 20 μl. Incubate the reaction mixture in a thermocycler with the following setting: 1 cycle for initial denaturation and cell lysis (at 95 °C for 2 min), 30 cycles for denaturation (at 95 °C for 30 s), annealing (at 5 °C less than Tm of primers for 30 s) and elongation (at 72 °C for 1 min per Kb), 1 cycle for final elongation (at 72 °C for 5 min), and final hold at 10 °C.

5. Analyze fragment size of PCR amplification product on a 0.8% agarose gel. It should be 1.27 Kb if plasmid was assembled correctly.

6. Confirm sequence of “UP” and “DN” fragments and the genome junction regions in the recombineering plasmid by Sanger sequencing using primers P1 and P2, described in step 4.

### 3.3 Transformation of the Recombineering Plasmid into Donor Strain WM6026

1. Grow *E. coli* strain WM6026 in LB with 0.1 mM DAP at 37 °C aerobically to early exponential phase and prepare chemically competent cells using method described in [17].

2. Transform recombineering plasmid into WM6026 strain using method described in [17].

3. Add 1 ml LB containing 0.1 mM DAP to transformed cells, incubate for 1 h at 37 °C, plate cells on LB agar plate containing 20 μg/ml chloramphenicol and 0.1 mM DAP and incubate at 37 °C aerobically.

4. Streak-purify at least three colonies on LB agar plate supplemented with 0.1 mM DAP and 20 μg/ml chloramphenicol to obtain isolated colonies. Grow each isolate in LB supplemented with 0.1 mM DAP and 20 μg/ml chloramphenicol at 37 °C aerobically, flash freeze 1 ml with glycerol (30% final concentration) in dry ice and store at −80 °C.

### 3.4 Conjugation for Targeted Integration of the Recombineering Plasmid into the Genome

1. Streak the donor strain WM6026 that contains the recombineering plasmid on LB solid media containing 20 μg/ml chloramphenicol and 0.1 mM DAP, and the recipient *Z. mobilis* strain on ZRMG solid media. Incubate the donor strain at 37 °C aerobically and the recipient *Z. mobilis* strain at 30 °C anaerobically.
2. Inoculate a colony of the recipient *Z. mobilis* strain into 5 ml ZRMG and grow ~24 h at 30 °C without shaking to late exponential phase until OD$_{600}$ reaches 0.5–1.0. (Inoculate in the morning of day 1). The doubling time for *Z. mobilis* is ~70 min.

3. Inoculate a colony of the donor *E. coli* strain (WM6026 that contains the recombineering plasmid) in 5 ml LB containing 0.1 mM DAP and 20 μg/ml chloramphenicol and grow overnight at 30 °C aerobically. (Inoculate in the evening of day 1).

4. In the morning of day 2, subculture donor *E. coli* strain into 5 ml LB containing 0.1 mM DAP to an OD$_{600}$ of 0.15. Incubate at 30 °C with shaking until OD$_{600}$ reaches 0.5.

5. In the afternoon of day 2, add 1 ml of the donor and 1 ml of the recipient cells (adjust to OD$_{600}$ of 0.5 with ZRMG if necessary) together in a 2 ml microfuge tube for mating. Also aliquot 1 ml of donor and recipient into separate tubes as negative controls. Centrifuge at 17,300 × $g$ for 30 s at room temperature.

6. Decant supernatant. Let tubes sit for 2–3 min to loosen the pellet. Gently resuspend pellet in remaining media in tube.

7. Transfer the cell suspension to the center of the prewarmed ZRMGc conjugation plate as one drop.

8. Incubate plates with conjugation mixture and controls overnight (12–15 h) at 30 °C with agar side down.

9. In the morning of day 3, add 1 ml ZRMG medium to the center of the plate, pipetting up and down a few times to resuspend and collect all cells. Tilting plate slightly may facilitate collecting liquid from the edge of the plate. Alternatively, scrape cells using sterile spatula or scraper and resuspend cells in 1 ml ZRMG medium in a tube. Transfer cells to a 2 ml microfuge tube.

10. Shake tubes vigorously for 10 s to disrupt mating pairs. Centrifuge cells at 17,300 × $g$ for 30 s at room temperature, and then resuspend pellet in 1 ml ZRMG. Incubate at 30 °C for 3–5 h.

11. Plate 100 μl of the cell suspension on a ZRMG agar plate containing 120 μg/ml chloramphenicol. Incubate at 30 °C anaerobically for 2–4 days; expect no colonies on the negative control plates and between 20 to 100 colonies on the plate containing conjugation mixture. These colonies result from conjugation and recombination of the recombineering plasmid into the genome.

12. Colonies with the plasmid should be fluorescent because of expression of GFP from the recombineering plasmid. Fluorescence can be viewed on a blue light transilluminator after incubating plates in the presence of oxygen for 4 h, which allows for maturation of GFP (see Note 2).
13. Pick at least three fluorescent colonies and streak-purify twice on fresh ZRMG agar plates containing 120 μg/ml chloramphenicol, incubated at 30 °C anaerobically.

14. From a streak-purified colony, inoculate 5 ml ZRMG containing 120 μg/ml chloramphenicol and grow at 30 °C anaerobically with or without agitation to late exponential phase. Transfer 1 ml to a freezer stock vial, flash freeze with glycerol (30% final concentration) in dry ice, and store at −80 °C.

3.5 Verification of Integration Locus of the Recombineering Plasmid

Verify insertion of the plasmid into the correct location of the genome by colony PCR using primers P3 and P4. Primer P3 is designed to anneal upstream of the 500 bp UP region and P4 is designed to anneal downstream of the 500 bp DN region in the genome as shown in Fig. 5.

1. Resuspend a single colony of the recombinant strain in 50 μl of water.

2. Prepare a reaction mixture containing 10 μl Flexi GoTaq® colorless master mix, 2 μl forward primer (final concentration—1 μM) and 2 μl reverse primer (final concentration—1 μM), 2 μl of cell suspension and water to adjust volume to 20 μl.

3. Incubate the reaction mixture in a thermocycler as described in Subheading 3.2, step 4.

4. Analyze the size of PCR amplified fragment by 0.8% agarose gel electrophoresis. The fragment size should be the sum of the size of the recombineering plasmid (5.7 kb) + the size of the target gene for deletion + the size of the DNA fragment from P3 primer annealing position to the start position of the target gene + the size of the DNA fragment from the end of the target gene to the primer P4 annealing position. See Note 3.

3.6 Resolving the Plasmid and Enrichment of the Desired Strains Using FACS

The final step of this protocol requires growth of the recombinant strain without selection pressure (i.e. no antibiotic) to allow growth of recombinants that have lost the plasmid. Recombination events that occur either between the two homologous “UP” regions or the two “DN” regions yield either the wild-type genotype or depending on the plasmid design, deletion of a target gene or insertion of a heterologous sequence. Recombination events in Z. mobilis occur at a frequency of ~0.3%, indicating that the desired genotype is a minority in the total population. While methods such as replica plating can be used to identify colonies that have lost the antibiotic resistance of the plasmid, we developed a fluorescence-based method to first enrich for cells that had lost the plasmid to limit the number of colonies needed to be screened. There is sufficient GFP and fluorescence signal produced from an integrated copy of the suicide plasmid pPK15534 in Z. mobilis that single cells
FACS can be analyzed by FACS. Utilizing FACS, it is possible to sort fluorescent cells that have the plasmid from nonfluorescent cells that have lost the plasmid (Fig. 3). Fluorescence-activated cell sorting is a technique to sort populations of cells based on their physical and chemical characteristics such as size and fluorescence. In this technique, a cell suspension is injected into a stream of sheath fluid that passes through a vibrating nozzle that creates droplets, with each drop containing ideally a single cell. The cytometer is calibrated to flow one droplet at a time through a laser beam. Cells expressing a fluorescence marker, such as GFP, absorb light of a specific wavelength and emit light of a different wavelength that is measured by specific detectors. To separate droplets that exhibit fluorescence from those that do not, an electric charging ring adds opposite charge to only droplets with a fluorescent signal and subsequently, an electromagnetic plate deflects droplets into collection tubes based on their charge [18]. Millions of cells can be quickly passed through the FACS and sorted based on fluorescence. We took advantage of this high-throughput method to enrich for nonfluorescent cells and simultaneously remove the fluorescent cell population based on the parameters described below. This approach (see Note 4 for an outline of the method) greatly reduced the number of colonies that are subsequently screened for their genotype.

3.6.1 Prepare Cell Sample to Flow in FACS

Inoculate the wild-type Z. mobilis strain in 2 ml ZRMG, and the recombinant Z. mobilis strain that has the recombineering plasmid integrated into the genome in 2 ml ZRMG with and without antibiotic. The recombinant strain grown with antibiotic (GFP strain) and the wild-type strain (GFP/C0 strain) are used as reference controls for identifying the fluorescence maxima of GFP + and GFP/C0 strains in the fluorescence-intensity plot generated by FACS (Fig. 4a, b).

1. Grow at 30 °C anaerobically until early exponential phase (5–7 generations).
2. Transfer all culture tubes to an aerobic environment for 2–5 h to mature GFP and allow gfp+ cells to become fluorescent.
3. Label 1.5 ml microfuge tubes for the recipient strain, the recombinant strain grown with antibiotic, and the recombinant strain grown without antibiotic. Add 980 μl sterile 1/C2 PBS buffer and 20 μl of cell suspension to each tube to prepare a diluted suspension of each culture for loading into the FACS instrument. Keep all tubes on ice.

3.6.2 Determine the Sorting-Gate for Nonfluorescent Cells

1. Calibrate the FACS for 488 nm. These directions will be instrument-dependent.
Fig. 4 Creating a selection gate for the nonfluorescent strains in plots generated by FACS. (a) GFP+ Z. mobilis strains were flowed into the instrument and the appropriate events were selected by creating a selection gate in a backward scatter—area (BSC-A) versus forward scatter—area (FSC-A) plot (left panel) and then in a forward scatter—height (FSC-H) versus forward scatter—width (FSC-W) plot (middle panel). The selected events were plotted with the number of events against the fluorescence intensity (right panel). (b) GFP− Z. mobilis strains were flowed into the instrument and the appropriate events were selected (left and middle panels, row b) by using selection gates from the left and middle panels, row a that were made for the GFP+ strains. A selection gate “C” was generated in the fluorescence intensity-region for selection of the nonfluorescent strains (right panel). (c) The recombinant Z. mobilis strains grown without selection were flowed into the instrument and appropriate events were selected (left and middle panels, row c) using the same procedure done for the previous two reference strains (GFP+ and GFP− strains). Sorting of the nonfluorescent strains were then achieved by selecting gate “C” generated from the right panel of row b (right panel, row c) At the top left within each plot are the gate name (A, B or C) and the percentage of events that are selected by that gate. The data were generated using a Sony MA900 instrument for deletion of the ldh (ZMO0256) gene in Z. mobilis ZM4.
2. Begin with flowing of the diluted GFP+ cell suspension prepared in Subheading 3.6.1 that contains recombinant cells grown with antibiotic.

3. Identify the droplet events of interest by creating the appropriate selection gate in output plots with different axis-parameters; Make a first selection based on size of the cells by creating a selection gate for cellular events in a forward scattering–area (FSC-A) versus backward scattering–area (BSC-A) plot as shown in the left panel of Fig. 4a (see Note 5). Plot those events in a forward scatter–height (FSC-H) versus forward scatter–width (FSC-W) plot and make a selection for droplet events with single cell by creating a gate to select events in the linear range as shown in the middle panel of Fig. 4a (see Note 6). Plot those events as the number of events versus fluorescence intensity as shown in the right plot of Fig. 4a.

4. Flow the diluted GFP wild type cell suspension. Make a selection gate for the nonfluorescence droplet events (in a number of events versus fluorescence intensity plot) as shown in the right panel of Fig. 4b. This gate will be used in the next step to sort nonfluorescent strains from a mixture of fluorescent and nonfluorescent strains (see Note 7).

### 3.6.3 Sort gfp Nonfluorescent Cells from a Population of gfp+ Recombinant Strains that Was Grown Without Selection

1. Flow the diluted strain suspension that contains the recombineering plasmid and grown without selection, and identify strains of interest as described in the previous two steps using the same settings and plots.

2. Sort the nonfluorescent strains in 1 ml of ZRMG medium using a selection gate that was made for selecting nonfluorescent strains in the previous step (right panel of Fig. 4b, c).

3. Plate 100 μl of the sorted cell suspension on a ZRMG agar plate. Incubate anaerobically at 30 °C for 2–3 days for colonies to form.

4. Transfer plates to an aerobic environment for 2–5 h and identify nonfluorescent colonies using a blue light transilluminator (Fig. 5a).

### 3.7 An Alternate Method to Sort Nonfluorescent Cells Using a Blue Light Transilluminator Without FACS

In this alternative method, the strain containing the recombineering plasmid recombinated onto the genome is grown without selection and plated on ZRMG agar plates directly to screen ~10,000 colonies with a blue light transilluminator for colonies that have lost fluorescence (Fig. 3).

1. From a fluorescent colony of the recombinant strain, inoculate 5 ml ZRMG and grow at 30 °C anaerobically to an OD_600 of 0.5. Prepare serial dilutions and plate the appropriate dilution on 100 ZRMG plates to achieve approximately 100 colonies on each plate.
2. Incubate at 30 °C anaerobically until colonies form. Transfer plates to an aerobic environment for 5 h for GFP maturation. Visually screen plates with a blue light transilluminator and identify nonfluorescent colonies.

3. Streak-purify nonfluorescent colonies onto ZRMG agar plates and incubate 2–3 days at 30 °C anaerobically.

3.8 Determining the Genotype of Strains by Colony PCR Assay

There are two possible genotypes of the nonfluorescent strains that result from a second recombination event: a wild type genotype or a deletion mutant genotype (Fig. 1). Ideally, the probability of one of the two genotypes is 50%. To identify the desired strain, a PCR assay is done using primers that anneal upstream of the “UP” sequence and downstream of the “DN” sequence in the genome.

Fig. 5 (a) Phenotype of colonies following enrichment of nonfluorescent cells by FACS plated on a nonselective media. Plate was imaged in Azure C600 imager for GFP fluorescence (left figure) and in visible light (right figure). The blue dots in the left figure indicate those few colonies that are still fluorescent. This figure is reprinted from [13]. (b) Genotyping strains by PCR with specific primers. Using primers P3 and P4 that anneal to sequences flanking the regions of homology cloned on the suicide plasmid allows for discrimination between the wild-type and deletion genotype after separation of PCR products by gel electrophoresis.
For example, the fragment size of the deletion strain should be the sum of the size of DNA fragment from the P3 primer annealing position to the start position of the target gene and the size of the DNA fragment from the end of the target gene to the primer P4 annealing position (Fig. 5b) (see Note 8). The fragment size of the wild type strain will have the additional number of base pairs of the gene (Fig. 5b). The nonfluorescent strains should not have the recombineering plasmid, which can be further verified by PCR assay; the size of the PCR amplified fragment by P3 and P4 primers will be the sum of the size of the DNA fragment from the P3 primer annealing position to the start position of the target gene, the size of the gene, size of the DNA fragment from the end of the target gene to the primer P4 annealing position and the size of the recombineering plasmid.

1. Resuspend a colony of nonfluorescent strains in 50 μl of water. Prepare the assay for colony PCR as described in Subheading 3.1.2 using primers P3 and P4. Incubate the reaction mixture in a thermocycler with the appropriate settings.

2. Electrophorese the PCR amplified fragment on 0.8% agarose gel, and identify strains with the correct band size for the deletion or insertion mutant. Verify sequence by Sanger sequencing of the DNA fragment.

4 Notes

1. Other software are also available to design primers.

2. GFP requires oxygen for maturation of the fluorophore. Therefore, plates are exposed to aerobic conditions for a couple of hours if the colonies were initially grown in anaerobic conditions.

3. There could be minor bands on the gel in addition to the major band for the integrant. This indicates that the plasmid is dynamically recombining in and out of genome, creating a mixed population.

4. The first step in this method is to determine a “sorting-gate” that identifies all nonfluorescent-droplet events and excludes all fluorescent-droplet events [18]. Events in this gate are selected to sort nonfluorescent cells from a mixed population of fluorescent and nonfluorescent cells. To determine a sorting-gate, fluorescent strains that contain the recombineering plasmid (gfp⁺) are passed through the FACS to identify a fluorescence-intensity peak in an output plot. Similarly, the nonfluorescent Z. mobilis strain that lacks the recombineering plasmid (gfp⁻) are passed through the FACS and its
fluorescence-intensity peak in the plot is identified. A sorting gate is then set by the fluorescence-intensity peak region for the nonfluorescent events, as discussed below and shown in Fig. 4a, b. These determined parameters are then used to sort the recombinant cell suspension grown without antibiotic by passing it through the FACS, such that nonfluorescent cells are sorted through the gate designated for the nonfluorescent events as shown in right plot of Fig. 4c.

5. Smaller particles or debris tend to have lower forward scatter levels, and are often found at the lower left corner of the FSC versus SSC density plot. Therefore, this strategy helps in excluding cell debris and other small particles in the cell suspension.

6. A droplet may have two or more cells if the cell suspension is dense. A forward scatter—height (FSC-H) versus forward scatter—width (FSC-W) helps identifying events representing single cells [18]. Events with one cell fall on a straight line. However, events with multiple cells fall towards the right side in the density-plot. To remove all events with more than one cell, a gate in straight line is selected (middle plot of Fig. 4a–c).

7. A single parameter histogram is generally used to further identify distinct cell types that express a particular marker (such as GFP) in a population of cells [18]. Droplet events with GFP− cells usually produce less fluorescence. Making a selection gate in this region helps to sort GFP− strains from a mixture of GFP− and GFP+ strains.

8. In the case of insertion of heterologous DNA into the genome, the fragment size will be the sum of the size of the DNA fragment from the P3 primer annealing position to the insertion locus in the genome, the size of heterologous sequence and the size of the DNA fragment from the insertion locus in the genome to the primer P4 annealing position.

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Chapter 6

Plant Gene Modification by BAC Recombineering

Zhoubo Hu, Ajit Ghosh, and Csaba Koncz

Abstract

Recombineering approaches exploiting the bacteriophage λ Red recombination functions are widely used for versatile modification of eukaryotic genes carried by bacterial artificial chromosomes (BACs) in E. coli. Whereas BAC transformation provides a simple means for integration of modified genes into the genomes of animal cells to generate knock-in and knockout lines, successful application of this strategy is hampered by low frequency of homologous recombination in higher plants. However, plant cells can be transformed at a high frequency using the transferred DNA (T-DNA) of Agrobacterium, which is stably and randomly integrated into the plant genome. The function of plant genes that are modified by recombineering and transferred by Agrobacterium T-DNA vectors into plant cells can thus be suitably studied using genetic complementation of knockout mutations induced by either T-DNA insertions or genome editing with T-DNA-based Crisp/Cas9 constructs. Here we describe two recombineering protocols for modification and transfer of plant genes from BACs into Agrobacterium T-DNA plant transformation vectors. The first protocol uses a conditional suicide ccdB gene cassette to assist the genetic complementation assays by generation of point mutations, deletions, and insertions at any gene position. The second “turbo”-recombineering protocol exploits various I-SceI insertion cassettes for fusing of fluorescent protein tags to the plant gene products to facilitate the characterization of their in vivo interacting partners by affinity purification, mass spectrometry, and cellular localization studies.

Key words  Recombineering with antibiotic resistance gene cassettes, Conditional suicide ccdB gene, I-SceI excision cassette, Gap-repair recombination, Agrobacterium plant transformation vectors, Fluorescent protein tags

1 Introduction

Since the recognition that phage-encoded enzymes can mediate recombination of linear DNA molecules flanked by 30–50 nt homology arms with corresponding sequences of chromosomal and plasmid DNAs, the technology of recombineering was continuously improved and simplified [1–3]. From the many available options, the bacteriophage λ Red (exo, bet, and gam) recombination system remained popular. In the commonly used E. coli host strain SW102 [4], the λ Red genes are carried by a chromosomally integrated prophage and expressed by the pL promoter, which is
tightly controlled by the thermosensitive cI857ts repressor. The integration of modified sequences into target genes is traditionally monitored using a marker exchange strategy: the target gene is first labeled with a selectable marker, such as the \textit{galK}^{+} gene in a \textit{galK}^{-} mutant background, which is then replaced by modified gene sequences using suitable counter-selection, e.g., by selecting for the lethality conferred by \textit{galK} in the presence of deoxy-galactose [4]. Due to considerable time requirement of selection and counter-selection on minimal medium at permissive (32 °C) temperature, the use of \textit{galK} and other metabolic markers was replaced by the application of antibiotic resistance and conditionally inducible suicide genes [5]. Inactivation of methyl-directed mismatch repair system [6] also allowed simple modification of target gene sequences by homologous recombination with single-stranded oligonucleotides [7]. Recombineering procedures designed for modification of eukaryotic genes carried by BACs (bacterial artificial chromosomes) in \textit{E. coli} became amenable to automation [8] and widely employed in genome-wide modification and knockout of gene functions using DNA transformation and homologous recombination e.g., in \textit{Caenorhabditis elegans} and mouse [9, 10]. In comparison, recombineering remained largely unexploited in plant research primarily because, except for the moss \textit{Physcomitrella patens} [11, 12], the frequency of gene targeting events by homologous recombination is low in higher plants [13]. Instead of DNA transformation, high-frequency gene transfer into plants is achieved by exploiting the natural gene transfer systems of Agrobacterium Ti and Ri (tumor- and root-inducing) plasmids. DNA sequences flanked by direct 25 bp repeats of transferred T-DNAs of Agrobacterium plasmids are processed to a single-stranded (ss) intermediate, and then transferred into plants by a type IV (T4SS) conjugation system [14]. A pilot protein (VirD2), which is covalently linked to the 5’-end of conjugated DNA strand aids both nuclear import and chromosomal integration of gene-shuttle T-DNAs. Whereas T-DNA insertions into the plant genomes result in random mutations, their combination with Crisp/Cas constructs facilitates the induction of gene-specific point and T-DNA insertion mutations [15–17]. However, gene editing by homologous recombination or gene conversion remains a challenging task in plants. Nonetheless, the availability of large collections of insertion mutations in several plant species, such as Arabidopsis and rice [18, 19], provides an excellent basis for studying the functions of genes that are precisely modified by recombineering and introduced into knockout mutants, using genetic complementation assays. To support such studies, plant genes were initially modified by recombineering either in Agrobacterium transformation-competent TAC vectors [20–22] or transferred from BACs into smaller Agrobacterium T-DNA vectors by gap-repair after recombineering [23]. However, plant
transformation frequencies obtained with TACs are relatively low due to the large size (100–120 kb) of their transferred DNAs. In addition, deletions in the TAC T-DNAs are common because plant DNA sequences resembling the T-DNA 25 bp border repeats are recognized by the Agrobacterium conjugation system. On the other hand, insertion of homology arms for transferring individual genes from BACs into Agrobacterium T-DNA vectors by gap-repair is a laborious step preventing high-throughput applications. Consequently, the initial plant recombineering technologies did not become widely exploited.

Here we describe two protocols improving the efficacy of plant BAC recombineering. For marker exchange, the first protocol employs a selectable antibiotic resistance gene (e.g., spectinomycin resistance, SpecR) linked to a gyrase-inhibitor killer gene as a counter-selectable marker, in which the expression of an arabinose-inducible promoter (pBad)-driven ccdB gene is controlled by the araC repressor (Fig. 1, left panel). By using the ccdB-cassette, point mutations, deletions, and insertions of suitable tags can be introduced into any position of a plant gene in a BAC. The second protocol is specifically designed for the replacement of translational start and stop codons of plant genes with coding sequences of Target gene

Fig. 1 Recombineering schemes using the ccdB and I-SceI exchange cassettes. The work-flow of recombineering is illustrated by replacement of the stop codon of a plant target gene by GFP coding sequences. The left panel shows cassette exchange with the inducible counterselection gene ccdB. The right panel shows the fast-track method where the I-SceI is used to remove the marker.
fluorescent protein (e.g., GFP and mCherry) tags that are linked to an antibiotic resistance gene flanked by recognition sites of the I-SceI homing endonuclease (Fig. 1, right panel). As the E. coli and Arabidopsis genomes lack I-SceI sites, the antibiotic selectable marker can be precisely excised from the recombineering constructs by I-SceI digestion after the gap repair step. To resolve the bottleneck of transferring of modified genes from BACs into Agrobacterium, new PCR amplifiable plant transformation vectors were designed (Table 1). In the new pGAPBR vectors, the plant selectable marker genes are placed in the vicinity of the 3'-end (i.e., so-called left border, [14]) of transferred DNA to ensure a selection for the integration of full-length T-DNAs (i.e., which are located between the 5' and 3' 25 bp repeats of transferred ssT-DNA) into plant chromosomes. The vectors are equipped with a cosmid-derived stable replicon, which can carry up to 40–50 kb plant DNA inserts. Upon linearization by a restriction endonuclease cleavage in their T-DNA regions, the vector sequences are PCR amplified with primers that carry 50 nucleotide arms for recombination with homologous sequences flanking the modified plant genes in the BACs (Figs. 1 and 2). Gap-repair with the PCR-amplified vectors thus removes the necessity of previous cloning steps and opens the way to high-throughput applications of PCR-based plant BAC recombineering. An application of described protocols is illustrated by labeling of the Arabidopsis TFIIH general transcription factor-associated CDKF;1 and CDKD kinases with

Table 1  
Vectors used in the BAC recombineering. (ABRC, Arabidopsis Biological Resource Center, The Ohio State University)

| Vectors             | Origin                  | Primary use                  | References | Available from |
|---------------------|-------------------------|------------------------------|------------|----------------|
| pGAPBRHg            | pPCV plasmids [24] and pHC79 cosmid | Agrobacterium binary vector  | [25]       | ABRC Ohio CD3-2800 |
| pGAPBRKm            | pPCV plasmids [24] and pHC79 cosmid | Agrobacterium binary vector  | [25]       | ABRC Ohio CD3-2801 |
| C-GFPStop-SpectR    | –                       | C-terminal I-SceI cassette with SpR | [25]       | ABRC Ohio CD3-2813 |
| C-mCherryStop-SpectR | –                       | C-terminal I-SceI cassette with SpR | [25]       | ABRC Ohio CD3-2814 |
| N-SpectR-GFP        | –                       | C-terminal I-SceI cassette with SpR | [25]       | ABRC Ohio CD3-2807 |
| N-KmR-mCherry-ccdB  | –                       | N-terminal I-SceI cassette with KmR | [25]       | ABRC Ohio CD3-2806 |
| SpectR-araC-ccdB    | –                       | SpR-ccdB exchange            | [25]       | ABRC Ohio CD3-2804 |

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GFP and mCherry tags for studying genetic complementation of knockout mutations, purification and mass spectrometry analysis of protein complexes, and cellular co-localization of labeled TFIIH kinase subunits [25].

2 Materials

All solutions were prepared using deionized distilled water unless indicated otherwise.

2.1 Reagents

1. Solution I (50 mM Tris.HCl [pH 8.0], 50 mM glucose, 20 mM EDTA, and 1 mg/mL lysozyme).
2. Solution II (0.2 N NaOH and 1% SDS), always prepare fresh.
3. Solution III (3 M Na-acetate [pH 4.8]), store at room temperature.
4. 3 M Na-acetate [pH 5.6].
5. TE buffer (50 mM Tris.HCl [pH 7.5], 1 mM EDTA).
6. Ethanol (EtOH).
7. Isopropanol.
8. Proteinase K.
9. RNase A (70 Kunitz Units/mg).
10. Phenol/chloroform-isoamyl alcohol (25:24:1).
11. Chloroform-isoamyl alcohol (24:1).
12. 3 M Na-acetate (pH 5.4).
13. Restriction endonucleases (\textit{BamHI}, \textit{KpnI} and \textit{I-SceI}).
14. Antarctic phosphatase.
15. T4 DNA ligase.
16. Liquid and solid LB medium.
17. Taq DNA Polymerase.
18. Q5 High-Fidelity DNA Polymerase (or equivalent high-fidelity polymerase).
19. 10 mM dNTPs (mix of 10 mM dATP, dTTP, dCTP, and dGTP in equal amount).
20. Oligonucleotides, designed in Fig. 2.
21. Agarose for gel electrophoresis.
22. Gel running buffer (1× TAE buffer, 40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA; 0.5 μg/mL ethidium bromide).
23. 6× DNA Loading dye: 60% (v/v) glycerol, 0.03% (w/v) bromophenol blue, 60 mM EDTA, 20 mM Tris.HCl pH 7.6.
24. 1 kb DNA ladder.
25. PCR/Gel cleanup kit.

### 2.2 Equipment

1. 32 °C, 37 °C, and 42 °C incubators.
2. Tabletop centrifuge with cooling system.
3. Electroporator.
4. Electroporation cuvette (2 mm).
5. Gel electrophoresis chambers and gel casting trays.
6. PCR thermocycler.
7. Microwave.
8. Vacuum desiccator.
9. Eppendorf tubes.
10. Pipettes.
3 Method

3.1 Preparatory Steps of Recombineering

3.1.1 Primer Design for BAC Recombineering (Fig. 2)

1. For insertion of ccdB and I-SceI exchange cassettes, the forward and reverse primers are planned with 50 nt homology arms flanking the designed positions of insertions (i.e., start or stop codons in the case of I-SceI cassettes).

2. For PCR monitoring of the insertion and cassette exchange events, the forward and reverse gene-specific PCR primers are planned such that the size of the amplified gene segment carrying the target site is between 0.3 and 0.6 kb. The primers used for PCR amplification of pGAPBR binary vectors are placed typically either upstream or downstream, or within the genes, which flank the modified target gene.

3. Attention should be paid for maintaining bidirectional promoter regions upstream and overlapping transcribed regions producing potential natural antisense (nat-si) RNAs downstream of the modified target gene. Examples for planning of primers for recombineering are provided in the Supplement of publication [25].

3.1.2 BAC Plasmid Verification and Isolation

Genomic or cDNA sequence of the targeted plant gene is subjected to a BLAST search using the TAIR10 whole genome (BAC clones) database (https://www.arabidopsis.org/Blast/index.jsp) to obtain the BAC number, and then the BAC clone is purchased from the Ohio Arabidopsis Stock Centre (https://abrc.osu.edu/).

1. E. coli strains with BAC clones carrying the target plant genes are maintained by selecting for the BAC-encoded antibiotic resistance marker (see Notes 2 and 3).

2. A single E. coli colony carrying the BAC is inoculated in 5 mL LB medium with the appropriate antibiotics (Table 2) and grown overnight (O/N) by shaking (250 rpm) at 37 °C.

3. Cells from 2 mL culture are pelleted by centrifugation in an Eppendorf centrifuge at 12,000 rpm for 3 min at room temperature.

### Table 2
Antibiotics stocks and their working concentrations

| Antibiotic          | Stock concentration | Working concentration for E. coli |
|---------------------|---------------------|----------------------------------|
| Ampicillin (Amp)    | 100 mg/mL           | 100 μg/mL                        |
| Chloramphenicol (Cm)| 25 mg/mL            | 25 μg/mL                         |
| Kanamycin (Km)      | 50 mg/mL            | 25 μg/mL                         |
| Spectinomycin (Spect)| 50 mg/mL           | 50 μg/mL                         |
4. The cell pellet is resuspended in 200 μL of Solution I, and incubated at room temperature (RT) for 30 min. Then, 200 μL freshly prepared Solution II is added and the samples are mixed gently at RT until the lysate becomes water clear (max. 5 min).

5. Then, the SDS-solubilized proteins and cell debris are precipitated by addition of 200 μL ice-cold Solution III and incubation of well mixed lysate on ice for at least 30 min.

6. The cell debris is removed by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant is transferred into a new tube, and then 0.6 volume of ice-cold isopropanol is added to precipitate the BAC DNA on ice for 30 min.

7. The DNA is pelleted by centrifugation at 12,000 rpm for 10 min at 4 °C. The pelleted BAC DNA is washed with 1 mL 75% ethanol (EtOH), dried in a vacuum desiccator for 2–3 min, and dissolved in 250 μL TE buffer.

8. 250 μg/mL heat-treated RNase A is added to the BAC DNA solution to digest the contaminating RNA for 2 h at 37 °C. Subsequently, 500 μg/mL proteinase K (predigested for 10 min at 37 °C) is added to remove protein contaminants by further incubation at either 37 °C for 2 h or RT for O/N.

9. The sample is then extracted twice with equal volume of phenol/chloroform-iso-amylalcohol (25:24:1) followed by centrifugation, and then the upper DNA-containing water phase is re-extracted twice with ice-cold chloroform-isoamyl alcohol (24:1).

10. The DNA is then precipitated by addition of 0.1 volume 3 M NaOAc (pH 5.6) and 0.7 volume ice-cold isopropanol on ice for at least 30 min.

11. The purified BAC DNA is pelleted by centrifugation at 13,000 rpm for 10 min at 4 °C, the DNA pellet is washed with 75% ethanol (−20 °C), dried briefly under vacuum, and dissolved in 100 μL H₂O.

12. The presence of the target gene in the BAC is verified by PCR amplification with two gene-specific primers flanking the position of the target site (either start or stop codon, when using the I-SceI cassettes) in the studied plant gene (see Note 2).

3.1.3 Preparation of Electrocompetent E. coli Cells

1. From a single colony, a starter culture of 2 mL is grown O/N at 37 °C.

2. Inoculate 20 mL fresh LB liquid medium with 200 μL starter and grow the cells to OD₆₀₀: 0.4–0.6.

3. Then, 2 mL culture is pelleted by centrifugation at 8000 × g at 4 °C, and the pellet is washed three times with sterile ice-cold ddH₂O.

4. Finally, bacterial cells are suspended in 100 μL ddH₂O for electro-transformation and stored on ice until use.
3.1.4 Preparation of Heat-Induced Electrocompetent E. coli SW102 Cells (Table 3)

1. A starter culture of 2 mL from a single colony is grown O/N at 32 °C using appropriate antibiotics to select for the BAC-encoded resistance marker.

2. 200 μL starter culture is inoculated into 20 mL fresh LB liquid medium and the culture is harvested at OD 600: 0.4–0.6.

3. 2 mL aliquots of cells are pelleted by centrifugation at 8000 × g at 4 °C and the cell pellets are resuspended in 1 mL LB-medium followed by incubation at 42 °C with shaking for 15 min to induce the expression of λ Red genes.

4. After centrifugation, the cells are resuspended in 1 mL ice-cold sterile ddH2O, and then resuspended and pelleted similarly in three recurrent cycles, and finally resuspended in 100 μL H2O.

3.1.5 Transformation of Purified PCR Products/Plasmids into Electrocompetent Cells

1. The BAC DNA, or PCR-amplified ccdB and I-SceI cassette fragments, or linearized pGAPBR vector DNAs are drop-dialyzed on Millipore membrane filters (MFTM 0.025 μm VSWP) floating on sterile H2O before transforming into E. coli by electroporation [27].

2. The desalted DNAs are electroporated in a prechilled electroporation cuvette (2 mm) using an electropulse at 2.5 kV [i.e., 12.5 kV/cm cuvette with], 25 μF, and 200 Ω) for 4–5 ms in an electroporator.

3.1.6 PCR Template Preparation

All the necessary PCR templates need to be purified and subjected to quality assessment before starting the recombineering experiments.

1. The ccdB exchange cassettes were PCR amplified with the primers depicted in Fig. 2, digested by DpnI to cleave the contaminating template plasmid, and isolated from agarose gels by electro-elution after size separation.

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Table 3

Bacterial strains used in the BAC recombineering

| Strains | Primary use | Relevant genotype | References | Available from |
|---------|-------------|-------------------|------------|---------------|
| DH10B   | E. coli host for plasmid preparation | mcrA Δ(mrr-hsdRMS-mcrBC)O80dlacZ ΔM15ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697galU pglΔ8 rpsL nupG | [26] | Invitrogen |
| SW102   | BAC recombinocering | DY380 | [4] | ABRC Ohio CD3-2815 |

(see Note 1)
2. The *I-See* insertion cassettes were excised from the corresponding pBSK vectors as *BamHI*-*KpnI* fragments and gel purified.

3. The pGAPBR binary vectors are linearized by *BamHI*, followed by alkaline phosphatase treatment, and gel purified by electro-elution.

4. Aliquots of purified *ccdB* and *I-See* cassettes and self-ligated pGAPBR vectors are electroporated into *E. coli* DH10B cells to test and exclude the presence of contaminating cassette-source vectors and circular pGAPBR plasmids (see Note 4).

3.2 Recombineering with *ccdB* Cassettes

1. After electroporation of the BAC DNA carrying the target plant gene into *E. coli* SW102, the cells are incubated in 1 mL LB medium at 32 °C without antibiotics for 1 h. Then, 100 μL culture is spread on a solid LB plate with appropriate antibiotics to select for the BAC-encoded resistance marker at 32 °C.

2. The linear SpectR-araC-ccdB cassette is PCR amplified using (GeneF)SpectR-araC-ccdB5F and (GeneR)SpectR-araC-ccdB3R primers (Fig. 2), and then subjected to gel purification.

3. The purified cassette fragment (~100 ng) is transformed by electroporation into heat-induced SW102 competent cell carrying the target BAC. Then, the transformed cells are supplemented with 1 mL LB medium, incubated at 32 °C for 2 h, and plated onto LB agar plate containing Spect and 0.5% glucose and incubated at 32 °C overnight to select for the integration of the *ccdB* cassette into the BAC.

4. Subsequently, three single SpecR colonies are inoculated into 2 mL LB medium with Spect and 0.5% glucose, and then used as template for colony PCR with the GeneseqF and GeneseqR primers (Fig. 2). Clones carrying the *ccdB* cassette are expected produce a PCR product larger than 2 kb. For further use, only those colonies are selected, which do not carry empty BACs without the *ccdB* cassette insertion.

5. The *ccdB* cassette is replaced either by in-frame coding sequences of fluorescent proteins or modified gene sequences. The GFP/mCherry cassettes are PCR amplified with the GFP/mCherry5F and GFP/mCherry3R primers (Fig. 2), and then isolated from agarose gels after size separation. For exchanging the *ccdB* cassette with modified gene sequences, two single-stranded oligonucleotides carrying 50 nt homology flanks and 25 nt overlap including the sequence modification are mixed, denatured at 100 °C for 5 min, and annealed by cooling down to room temperature. Subsequently, single-stranded ends of annealed oligonucleotides are filled-in with T4 DNA polymerase.
6. The PCR amplified GFP/mCherry DNA fragments (~100 ng), as well as the double-stranded oligonucleotides carrying sequence alterations, are electroporated into heat-induced competent SW102 cells that harbor only BACs carrying the confirmed ccdB cassette insertion.

7. Following electroporation, the cells are suspended in 1 mL LB medium and incubated at 32 °C for 2 h. The cells are then plated onto LB agar containing 0.2% arabinose and antibiotics to select for the BAC-encoded resistance marker at 32 °C for O/N. Subsequently, three colonies are regrown in 2 mL LB medium with 0.2% arabinose plus antibiotics and plated again to single colony on LB-antibiotics plate.

8. The replacement of ccdB cassette with the GFP/mCherry coding sequences or modified gene sequences is confirmed by colony PCR using the GeneseqF and GeneseqR primers. In the case of GFP/mCherry insertions, the size of PCR fragments is expected to be larger than 1 kb. Replacement of the ccdB cassette by modified sequences of double-stranded oligonucleotides is confirmed by sequencing of the PCR products.

3.3 Fast-Track Recombineering Using I-SceI Insertion Cassettes

1. Purified linear C-GFPStop-SpectR, C-mCherryStop-SpectR, or N-SpectR-GFP cassette DNAs (2 ng) are used as templates for PCR amplification with the primers depicted in Fig. 2, and then the PCR products are subjected to gel purification (see Note 5).

2. About 100 ng purified DNA fragment is transformed into heat-induced SW102 competent cells carrying the target BAC by electroporation. The transformed cells are incubated at 32 °C in 1 mL LB medium without antibiotics for 1 h, and then plated on LB-Spect agar plates to select for transformants at 32 °C.

3. Three single colonies are regrown in 2 mL LB-Spect medium, and then used as a template for colony PCR with GeneseqF and GeneseqR primers. Successful recombineering will yield a PCR product of about 2–3 kb.

3.4 GAP Repair

1. Two nanograms of the linearized pGAPBR vector is used as a template for PCR amplification with GeneFlank1 and GeneFlank2 primers (Fig. 2). The amplified linear vector DNA is then isolated from 0.8% agarose gel by electro-elution and purified (see Notes 6 and 7).

2. Around 100 ng purified pGAPBR vector DNA is transformed into heat-induced SW102 competent cells carrying a BAC with the inserted C-terminal C-GFPStop-SpectR/mCherryStop-SpectR or N-terminal N-SteptR-GFP/N-SpectR-mCherry cassettes by electroporation and plated on LB agar medium containing Amp.
3. Three colonies are picked from LB-Amp plate and regrown in 2 mL LB medium with Amp for plasmid DNA isolation. When transferring genes into pGAPBR vectors from BACs, which were modified by the help of ccdB cassettes, the isolated plasmid DNAs are verified using PCR with the GeneseqF and GeneseqR primers, and then sequenced with the same primers. When transferring genes modified by the I-SceI cassettes into the pGAPBR vectors, the isolated plasmid DNA is digested by I-SceI (see Note 8) to remove the antibiotic resistance gene (a fragment of about 1 kb is cleaved out from the vector). After gel isolation, the purified pGAPBR vector is self-ligated with T4 DNA ligase and transformed into *E. coli* DH10B cells by electroporation. Transformed cells are plated on LB agar containing Amp and grown at 37 °C for O/N.

4. The transformants are replica plated onto LB-Amp and LB agar plates containing the antibiotics corresponding to the resistance marker of the removed I-SceI cassette. Finally, two independent clones are subjected to colony PCR using the GeneseqF and GeneseqR primers, and then the PCR product is subjected to sequencing using the same primers. In addition, the transfer of desired plant DNA region from the BAC into the pGAPBR vector is tested by fingerprinting the final clone with diagnostic restriction enzyme digestions.

## 4 Notes

1. SW102 is sensitive to temperature. Never grow the SW102 strain at higher temperature than 32 °C.

2. The BAC clones are distributed by the Arabidopsis Biological Resource Center of the Ohio State University (ABRC, Ohio). The presence of the plant target gene in the ABRC delivered BAC should be verified by PCR amplification (e.g., by detecting a fragment flanking its start or stop codon with genespecific primers; black arrows in Fig. 1) before introduction into the *E. coli* recombineering host SW102. The same genespecific PCR primers are used for monitoring the insertion and replacement of ccdB and I-SceI cassettes.

3. The gap-repair binary vectors pGAPBRKm and pGAPBRHyg were designed such that they share no homology with the BAC vectors that were used for construction of Arabidopsis genomic libraries.

4. Sometimes, the digestion of pGAPBRs remains incomplete. Therefore, before PCR amplification of pGAPBRs for gap-repair, it is necessarily to verify the lack of undigested circular and self-ligation competent vector DNAs in the linearized PCR templates.
5. The annealing temperature for amplification of GFP/mCherry-SpectR I-SceI-cassettes is around 71 °C. We recommend a two-step PCR reaction, where the denaturation step (95 °C) is followed by 72 °C annealing and extension. When using a lower annealing temperature, a shorter PCR product of about 1 kb might also appear in addition to the correct products of about 2 kb.

6. The size of pGAPBR vectors is about 7 kb. For PCR amplification of linear pGAPBR DNAs, Q5® High-Fidelity DNA Polymerase (NEB) is used in combination with NEB enhancer buffer.

7. The length of the pGAPBR-specific primers might be extended from 25 to 35 bp for a better amplification of pGAPBR plasmids.

8. If the I-SceI digestion would not produce the excision of the antibiotic resistance gene fragment, plasmid DNA could be bulk isolated from a mixture of SW102 transformants, and then retransformed into E. coli DH10B followed by screening for the loss of resistance marker using replica plating.

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Chapter 7

Dup-In and DIRex: Techniques for Single-Step, Scar-Free Mutagenesis with Marker Recycling

Joakim Näsvall

Abstract

This chapter describes two related recombineering-based techniques: “Duplication Insertion” (Dup-In) and “Direct- and Inverted Repeat stimulated excision” (DIRex). Dup-In is used for transferring existing mutations between strains, and DIRex for generating almost any type of mutation. Both techniques use intermediate insertions with counter-selectable cassettes, flanked by directly repeated sequences that enable exact and spontaneous excision of the cassettes. These constructs can be transferred to other strains using generalized transductions, and the final intended mutation is obtained following selection for spontaneous loss of the counter-selectable cassette, which leaves only the intended mutation behind in the final strain. The techniques have been used in several strains of *Escherichia coli* and *Salmonella enterica*, and should be readily adaptable to other organisms where λ Red recombineering or similar methods are available.

Key words Scar-free mutagenesis, λ Red, Recombineering, Duplication, Direct repeat, Inverted repeat, Marker recycling

1 Introduction

1.1 Why Is Simple Marker Recycling Useful?

Techniques for transferring mutations from one bacterial strain to another or for generating designed mutations on bacterial chromosomes almost always require a method for selecting successful recombinants. With recombineering this can be done by inserting an antibiotic resistance (Ab^R) cassette so that it either directly disrupts a gene or so that it is genetically linked to a gene or mutation of interest. Once a specific Ab^R cassette insertion has been generated it is easily transferrable between related strains by generalized transduction without the need for repeating the more time-consuming recombineering step.

Since the number of usable Ab^R cassettes are limited, the number of mutations that are possible to introduce in a single strain is limited to a handful. Because of this limitation, marker recycling methods for removing a previously introduced Ab^R cassette before introducing the next cassette can be very useful for leaving more
options for the following steps during the construction of strains with multiple mutations. Methods for marker recycling often include a second transformation step after the insertion of a cassette: (1) a second recombineering step for the removal of a counter-selectable cassette [1–3] or (2) the introduction of a Flp recombinase expression plasmid for removing AbR cassettes flanked by Flp Recombinase Target (FRT) sites [4]. The latter method has the additional drawback that it leaves a single FRT “scar”, which may cause unexpected phenotypes or cause problems in later recombineering steps. Methods that completely avoid AbR cassettes usually require additional helper plasmids for expression of e.g. guide RNAs and CRISPR/Cas9 [5].

1.2 Single-Step, Scar-Free Mutagenesis with Marker Recycling

This chapter describes two related techniques that enable quick and easy marker recycling without leaving any unwanted “scar” sequence behind. In both techniques a temporary insertion containing a positively (AbR) and a negatively (sucrose or rhamnose sensitivity) selectable marker is generated in a single transformation step. By flanking the insertions with repeating DNA sequences on both sides, they are inherently unstable and spontaneously lost. The first technique, “Duplication-Insertion” (Dup-In; Figs. 1a, 2, and 3) is useful for inserting temporary selection markers, which allows for existing genes, mutations, or artificial constructs to be transferred between related strains by generalized transductions. The Dup-In technique entails insertion of a cassette flanked by a duplication of the target DNA. This is accomplished by recombination between a PCR product and two sister chromosomes in the same cell (Fig. 3c) [6]. As relatively large (hundreds of bps) repeated sequences are highly prone to homologous recombination [7], such Dup-Ins are lost at high enough frequencies to be easily isolated through negative selection. The Dup-In technique has also been used for generating transcriptional and translational fusions by “trapping” duplications with selectable cassettes with reporter genes at the junctions, making it possible to generate fusions even to essential genes in their native loci [8].

The second technique, “Direct and Inverted Repeat stimulated excision” (DIRex; Figs. 1b, 4, 5, and 6), is useful for making deletions, single nucleotide substitutions, small insertions, or replacements, as long as the mutation can be contained within long PCR primers. DIRex involves the generation of insertions flanked by very short duplicated sequences that we refer to as direct repeats (DRs). The DRs are short enough (25–30 bps) to be included as part of 5′-overhangs in PCR primers and can contain any designed mutation [9]. As the DRs are included in the PCR primers and can be derived from the target locus, any type of mutation that can be contained within the primers can be engineered. Because the DRs are too small to be efficient targets for homologous recombination, their loss is instead stimulated by including large (~800 bps)
Fig. 1 Principles of the Dup-In and DIRex techniques. (a) Dup-In. In order to transfer a mutation of interest from one strain to other strains, recombineering is used to generate a duplication (~500 bp to several kb) with a selectable and counter-selectable (S/C) cassette at the junction. (b) DIRex. In order to engineer a mutation of interest, recombineering is used to generate a complex insertion (DIRex intermediate) containing a S/C cassette with long (~800 bp) inverted repeats (IRs) flanked by short (≥25 bp) direct repeats (DRs). The mutation of interest is included either within or just outside the DRs (see Fig. 5 for more detailed descriptions). Letter combinations ab and cd indicate adjacent sequences in the target sequence, while ce, fb, and e'c indicate artificial junctions between the target sequence and the inserted cassette. Both Dup-Ins and DIRex intermediates are transferable by generalized transduction and are spontaneously lost at high frequencies, leaving no selection markers or scar sequences behind. Clones that have lost the intermediates are easily isolated by negative selection. These properties make Dup-In and DIRex ideal techniques for combining multiple mutations without running out of selectable markers or repeating the transformation steps.
Fig. 2 Outline of using a Dup-In for transferring a mutation from strain “A” to strain “B.” (Day 1) A culture expressing λ Red is transformed with a PCR product to generate a semi-stable duplication-insertion containing a selectable and counter-selectable cassette at the junction. (Day 2) Transformants are isolated and colony-purified on selective medium. (Day 3) Cultures of isolated Dup-In containing clones are started for preparation of transducing phage lysates. (Day 4) Phage lysates are prepared, and used to transduce the Dup-In and the linked mutation into a recipient strain. (Day 5) Transductant clones are isolated and colony-purified on selective medium. (Day 6) Pure, Dup-In containing clones are picked from selective medium and streaked on counter-selective medium. (Days 7 and 8) Spontaneous segregants lacking the Dup-In grow as colonies on counter-selective medium. Screening for the mutation can be made either before or after loss of the Dup-In
A - Primer design

B - Generate DNA for recombineering

C - λ Red recombineering

D - Dup-In

Fig. 3 Detailed outline for primer design for generating a Dup-In that will be used in order to transfer a mutation of interest (marked with an asterisk *) to other strains. PCR primers are drawn as thin lines with arrow heads in the 3’ ends, and are mapped above (forward direction) and below (reverse direction) the corresponding identical sequences in the target DNA. Regions of the primers that matches the target DNA are drawn as lines that are parallel to the target DNA. (a) A span close to the mutation of interest is chosen for duplication. Primers “H1-P1” and “H2-P2” are designed with the homology tails (H1 and H2) in an “ends-in” orientation in order to duplicate the sequence between H1 and H2 in the target DNA. (b) An S/C cassette is PCR-amplified with primers “H1-P1” and “H2-P2” in order to generate the substrate DNA for recombineering. (c) λ Red mediates recombination between the homology tails of the PCR product and the identical sequences in two sister chromosomes in the same cell. (d) The structure of the resulting Dup-In.
inverted repeats (IRs) in the inserted cassettes. Using DIRex, we have made single nucleotide substitutions, promoter insertions/replacements, and deletions spanning in size from single bps to tens of kbs.

The combination of Dup-In and DIRex allowed reconstruction of more than a hundred mutants found in whole genome sequences of evolved populations of *E. coli* and *S. enterica* adapted to four different laboratory media [10]. Although not demonstrated, additional uses include inserting short sequences such as protein binding sites in DNA, His-tags or FLAG-tags to detect or purify proteins expressed from their native loci, or signal peptides for degradation or secretion of proteins.

### 1.3 Genetic Requirements

The generation of Dup-Ins and DIRex intermediates requires expression of the λ Red recombination proteins Gamma, Beta, and Exo from a helper plasmid or λ prophage, whereas the loss of

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**Fig. 4** Outline of using DIRex for (a) generating a mutation or (b) transferring an already generated mutation into another strain. (Day 1) A culture expressing λ Red is transformed with two PCR products to generate a semi-stable DIRex intermediate containing a selectable and counter-selectable cassette. (Day 2) Transformants containing the DIRex intermediate will appear blue (due to expression of AmiICP from the cassette). Colonies are picked and colony-purified on selective medium. (Day 3) Colonies growing on selective medium are picked and streaked on counter-selective medium. (Day 4) Nearly 100% of colonies growing on counter-selective medium contain the designed mutation, and will appear white/uncolored due to the loss of *amiICP*. (b) Transferring a previously constructed mutation into another strain by generalized transduction. A phage lysate grown on a strain containing a DIRex intermediate is used as donor in the transduction. The steps involved are the same as in (a) except for a transduction instead of a recombineering step on day 1.
the intermediates is spontaneous and does not require expression of any extrinsic factor. Once the intermediate has been generated it can be transferred to many other strains using generalized transduction without the need to repeat the more laborious transformation step.

Loss of Dup-Ins through homologous recombination is expected to require RecA and e.g. the RecBCD or RecFOR pathways [11]. The genetic requirements for the loss of DIRex

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**Fig. 5** Detailed outline for primer design for generating four different kinds of mutations using DIRex. In each panel, the structure of the DIRex intermediate is shown below the target locus. PCR primers are drawn as thin lines with arrow heads in the 3' ends, and are mapped above (forward direction) and below (reverse direction) the corresponding identical sequences in the target DNA. Regions of the primers that matches the target DNA are drawn as lines that are parallel to the target DNA. (a) Deletions. Two “outer” primers are designed with 55 nt 5' tails matching 40 nt on the 5' side (h1-d and h2-r', respectively), and 15 nt on the 3' side (r and d', respectively), of the designed deletion. The sequences d-r and their complementary sequences d'-r' form direct repeats (DRs) surrounding the insertion, each DR containing one copy of the deletion junction. (b) Point mutations (single nucleotide substitutions). One of the “outer” primers (h1-*dr-P1) is designed with a ≥50 nt homology tail containing the point mutation in the middle (i.e. between h1 and dr), while the other (h2-dr'-P1) is designed with a ~ 40 nt homology tail that leads up just next to the position of the mutation. For maximum efficiency of generating the wanted mutation, the primers need to be designed so that the mutation ends up next to the DR that is nearer to the origin of replication. (c and d) Primers for replacements and insertions are designed similarly to (a), except the DRs are composed entirely or partially of artificial sequences (red). Homologies for recombineering are chosen with a gap between them in order to replace a sequence (green line in c), or without a gap in order to generate a precise insertion. Note that there is no size limitation for the sequence to replace (green), but the size of the new sequence (red) is limited by primer synthesis.
A - Generate DNAs for recombineering

B - λ Red recombineering

C - DIReX intermediate

D - Final mutant

Fig. 6 Generation of a DIReX intermediate by two-fragment recombineering. (a) Two separate PCR reactions are set up, with a combination of “outer” primers, internal primers, and compatible template cassettes in order to generate two overlapping “half-cassettes” with identical but inversely oriented sequences (IR) next to the designed DRs. (b) The two PCR products are co-electroporated into cells expressing λ Red. Recombination occurs between the partial antibiotic resistance genes in the half-cassettes as well as between the cassettes and the chromosome. (c) Structure of the resulting intermediate insertion. The inserted cassette contains an antibiotic resistance marker for positive selection and a counter-selectable marker for negative selection, flanked by identical but inversely oriented 840 bp IR sequences. Flanking the IR sequences are the designed DR sequences (in this example containing a deletion junction). (d) The large inverted repeats stimulate precise deletion of the DIReX intermediate, leaving one copy of the DR behind.
intermediates have not been examined, but according to the suggested mechanism for the spontaneous deletion of palindromic DNA between direct repeats [12] it may need only the normal components of the replication machinery. None of the counter-selectable cassettes described here (Fig. 7) require any specific strain background, but the *Atox*-cassettes require the regulators (RhaS and RhaR) of the rhamnose regulon, as well as intact rhamnose uptake and degradation pathways (RhaT and RhaBAD), which are present in wild-type *Escherichia coli* and *Salmonella enterica* strains.

Both of these techniques have been used successfully in *Escherichia coli* (K12, ST131, and O6) and *Salmonella enterica* (ssp. *enterica* serovar Typhimurium, strains LT2 and ATCC 14028), but should be readily adaptable to work in other bacteria where λ Red recombineering or similar methods are available.

## 2 Materials

Prepare all growth media and solutions using deionized or distilled H₂O. Liquid media can be stored at room temperature and, when needed, add antibiotics just prior to use. Agar plates containing antibiotics (especially chloramphenicol or tetracycline which are light-sensitive) should be stored in a cool, dark place.

### 2.1 Growth Media and Solutions

1. **Low-salt Lysogeny Broth (low-salt LB, LB [Luria [13]]):** Mix 8 g tryptone, 4 g yeast extract, 0.4 g NaCl, 800 mL H₂O, and 160 μL 5 M NaOH in a 1 L autoclavable glass bottle. Autoclave for 20 min at 121–125 °C.

2. **Low-salt LB + 0.2% glucose:** To 100 mL low-salt LB, add 1 mL sterile filtered 20% (w/v) glucose.

3. **SOB:** Mix 16 g Tryptone, 4 g yeast extract, 0.46 g NaCl, 0.15 g KCl, 800 mL H₂O, and 160 μL 5 M NaOH in a 1 L autoclavable glass bottle. Autoclave for 20 min at 121–125 °C.

4. **SOC:** To 100 mL SOB, add 2.2 mL sterile filtered 20% (w/v) glucose and 2 mL sterile filtered 1 M MgSO₄.

5. **LB Agar (LA) plates:** Mix 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g Bacto Agar, 500 mL H₂O, and 80 μL 5 M NaOH in a 1 L autoclavable glass bottle. Autoclave for 20 min at 121–125 °C. Cool to 55–60 °C, add antibiotics if needed and pour in Petri dishes.

6. **Sucrose selection plates (for counter-selection against *sacB*):** Mix 6 g tryptone, 3 g yeast extract, 9 g Bacto Agar, and 500 mL H₂O in a 1 L autoclavable glass bottle. Autoclave for 20 min at 121–125 °C. Cool to 55–60 °C and add 100 mL sterile filtered 30% (w/v) sucrose before pouring in Petri dishes.
**Fig. 7** Useful selectable and counter-selectable cassettes. All cassettes except Acatsac3 and Akansac3 can be used for generating Dup-Ins, using primer binding sites “P1” and “P2” for PCR amplification. For DiRex, a combination of two compatible cassettes are used as templates: Acatsac1 and Acatsac3, Akansac1 and Akansac3, or Atox1 and Atox2. P1, primer binding site 1; P2, primer binding site 2; F, internal primer (forward direction); R, internal primer (reverse direction); cat, chloramphenicol acetyl transferase, chloramphenicol resistance (Cam\(^\text{R}\)); sacB, levansucrase from *Bacillus subtilis*, sucrose sensitivity (Suc\(^\text{S}\)); kan, neomycin phosphotransferase II (nptII), kanamycin resistance (Kan\(^\text{R}\)); amilCP, *Acropora millepora* chromoprotein (Blue); dhfr, dihydrofolate reductase (trimethoprim insensitive), trimethoprim resistance (Tmp\(^\text{R}\)); PrhaB-orph11, CdiA\(_{\text{E}689}\), Zn\(^{2+}\)-dependent DNAse toxin controlled by a rhamnose inducible promoter, rhamnose sensitivity (Rha\(^\text{S}\)). GenBank accession # (when available) and cassette sizes are indicated below the cassette names, and the sizes of the DiRex “half-cassettes” and length of overlaps are indicated between each cassette pair.
7. 20× M9 salts (for M9 plates): Dissolve 96 g Na₂HPO₄ (x0 H₂O), 48 g KH₂PO₄, 8 g NaCl, and 16 g NH₄Cl in ~800 mL H₂O. Adjust the volume to 1000 mL and autoclave for 20 min at 121–125 °C.

8. M9 + rhamnose plates (for counter-selection against Prha-orph11 in Atox cassettes): Autoclave 7.5 g Bacto agar in 469 mL H₂O for 20 min at 121–125 °C. Cool to 55–60 °C and add 5 mL sterile filtered 20% (w/v) rhamnose and, in the following order: 500 μL 0.1 M sterile CaCl₂, 500 μL 1 M sterile filtered MgSO₄, and 25 mL 20× M9 salts. Mix after each addition to avoid precipitation. Pour into Petri dishes.

9. 10% glycerol. Sterile-filtered or autoclaved. Store at +4 °C and place in an ice-water bath before use.

10. Antibiotics: See Table 1 for stock solutions, concentrations in media, and storage conditions.

### 2.2 DNA Cassettes and Template DNA for PCR

The DNA cassettes used for Dup-In and DIRex are described in Fig. 7. Except for the Acatsac3 and Akansac3 cassettes, all cassettes can be PCR-amplified using primer binding sites “P1” and “P2” for use in Dup-Ins or “regular” recombineering, while a combination of “P1” and the “F” or “R” binding sites (Fig. 7) are used to amplify the overlapping “half-cassettes” for DIRex (see Fig. 6). SacB (levansucrase) from Bacillus subtilis converts sucrose to levan polymers. When expressed in the periplasm of Gram-negative bacteria, SacB causes toxic buildup of levan polymers [14]. The Acatsac- and Akansac-cassettes confer sucrose sensitivity, and resistance to chloramphenicol (Cam<sup>R</sup>) or kanamycin (Kan<sup>R</sup>), respectively. The Atox cassettes carry the dhfr gene, conferring resistance to trimethoprim (Tmp<sup>R</sup>), and a gene encoding the CdiA-CT<sub>o11</sub>EC869 DNase toxin [15] from *E. coli* EC869 expressed from the rhamnose-inducible Prha<sub>B</sub> promoter, conferring lethality on minimal medium with rhamnose as only carbon source. All template DNAs are available upon request (as chromosomal insertions in *E. coli* K12 MG1655).
2.3 Recombineering Helper Plasmid

pSIM5-Tet (pSC101Ts, λ [gam-bet-exo Δkil ΔrexAB::tetAR ci857] [16]) was constructed from pSIM5 [17] by exchanging the chloramphenicol-resistance gene with tetR and tetA (tetracycline resistance) genes from Tn10. The plasmid is maintained at 30 °C (with tetracycline) but is easily lost by growth at 37 °C or above (without tetracycline). The Red genes (gam, bet, and exo) are expressed from their native promoter (λ PL), which in turn is repressed by a temperature-sensitive mutant λ CI repressor (CI857). The plasmid is available upon request.

2.4 Bacteriophages for Generalized Transductions (Optional)

1. For *E. coli* K12 strains: P1 vir [18].
2. For *S. enterica* strains: P22 HT/105 int [19].

2.5 PCR Primers

The DNA sequences of the cassette-specific internal primers and the 3’-ends of recombineering primers are listed in Table 2. See Subheadings 3.1 and 3.3, Figs. 3 and 5 for primer design guidelines.

### Table 2

**PCR primers**

| Primer | Sequence |
|--------|----------|
| P1a    | (40–80 nt. 5’-tail) - GTGTAGGCTGGAGCTGCTTC |
| P2a    | (40–80 nt. 5’-tail) - CATATGAATATCCTCCTTACGTCC |
| cat-midF | CGACGATTTCCCGCAGTTTC |
| cat-midR | GCCGACATGGAAAGCAATCAC |
| kan-midF | GCCGGTCTTTCGATCAGGA |
| kan-midR | ACCGTAAGCACGAGGAAGC |
| tmp-midF | AACTACCGCGCCTGCGGCTG |
| tmp-midR | GGAAGACGGCGTCAACCCTCG |

a These primers are the same as the “P1” and “P2” primers from Datsenko and Wanner [4], except “P2” is extended by 4 nt in its 3’-end to increase its annealing temperature.

3 Methods

The two techniques follow the same general procedures, but differ in primer design and substrate DNAs for recombineering. For Dup-Ins, follow Subheadings 3.1 and 3.2 and for DIRex, follow Subheadings 3.3 and 3.4. The protocols for the PCR amplifications have been determined using Phusion HF DNA polymerase (Thermo Fisher) and may need to be adapted if another polymerase will be used.
### 3.1 Dup-In: Primer Design

Start by selecting a span close to the mutation of interest to duplicate (see Notes 1–4). Use the last 40 nt of the “upper” strand of this span (“H1” in Fig. 3) as 5'-end on one primer and add one of the 3'-primer ends (P1 or P2). Use the “lower” strand of the first 40 nt of the span (“H2” in Fig. 3) as 5'-end of the other primer, and add the other 3'-primer end (P2 or P1).

### 3.2 Dup-In: Preparation of DNA for Recombineering (PCR)

1. Prepare template DNA: resuspend a single colony, or scrape cells from a frozen (−80 °C) stock, in 100 μL H2O in a microcentrifuge tube.

2. Prepare a 40 μL PCR reaction:
   (a) 18 μL H2O
   (b) 8 μL Phusion HF buffer (5×)
   (c) 4 μL dNTP (2 mM each)
   (d) 4 μL DMSO
   (e) 2 μL oligo XXX1-P1 (5 μM)
   (f) 2 μL oligo XXX2-P2 (5 μM)
   (g) 2 μL template cell suspension
   (h) 0.2 μL Phusion DNA polymerase

3. Run the PCR according to the cycling parameters in Table 3.

4. Analyze the PCR products by agarose gel electrophoresis. The correct fragment sizes are indicated in Fig. 7.

5. Concentrate and de-salt the PCR products (see Note 5).

### 3.3 DIRex: Primer Design

The primer design for DIRex is described in Fig. 5, and the PCR, the recombineering step, the structure of the DIRex intermediate, and the final mutant are described in Fig. 6 (see Note 1). Briefly, for generating deletions, replacements, or small insertions, the long

| Temperature | Time     |
|-------------|----------|
| 98 °C       | 2 min    |
| 98 °C       | 20 s     |
| 72 °C<sup>a</sup> | 5 s<sup>4</sup> |
| 57 °C (0.5 °C/s ramp rate) | 20 s  |
| 72 °C (0.5 °C/s ramp rate) | 1 min/kb |
| 98 °C       | 20 s     |
| 72 °C       | 1 min/kb |
| 72 °C       | 10 min   |
| 4 °C        | Hold     |

<sup>a</sup> This step was included in order to cool to 72 °C at normal rate before changing to the slower ramp rate.

---

Table 3

| Temperature | Time     |
|-------------|----------|
| 98 °C       | 2 min    |
| 98 °C       | 20 s     |
| 72 °C<sup>a</sup> | 5 s<sup>4</sup> |
| 57 °C (0.5 °C/s ramp rate) | 20 s  |
| 72 °C (0.5 °C/s ramp rate) | 1 min/kb |
| 98 °C       | 20 s     |
| 72 °C       | 1 min/kb |
| 72 °C       | 10 min   |
| 4 °C        | Hold     |

<sup>a</sup> This step was included in order to cool to 72 °C at normal rate before changing to the slower ramp rate.
“outer” primers are designed so that the mutation will be present as part of the DRs on both sides of the intermediate insertion. Each of the primers for making deletions will have 40 nt identical to the 5’-side of the deleted sequence and 15 nt identical to the 3’-side. This results in 30 bp DRs containing the deletion junction in the middle. To make replacements or insertions, the DRs are instead composed partially or completely from artificial sequences.

When using DIRex for generating point mutations, less than 100% of the transformants inherit the mutation from the PCR product. We have found that the most optimal way to design primers for point mutations is to make one of the “outer” primers with a 51 nt 5’-tail with the point mutation in the middle, and the other “outer” primer with a 40 nt 5’-tail leading up to the nucleotide just next to the mutation (but not containing the mutation). In this way the mutation will be present just outside the DR on only one side of the intermediate insertion. Additionally, we have determined that to maximize the number of transformants that have the mutation, the mutation must be on the side of the cassette that is closer to the origin of replication (i.e. next to the left DR in Fig. 5b). In addition to the specific recombinogenic 5’-ends, each of the long “outer” primers should have the identical 20 nt 3’ primer ends (P1).

### 3.4 DIRex: Preparation of DNA for Recombineering (PCR)

As the DIRex intermediate will have identical but inversely oriented ~800 bp sequences in each end, the cassette can only be amplified as two overlapping “half-cassettes” that are assembled by recombination between identical sequences in the Ab^R^-genes during recombineering. The two compatible “half-cassettes” are amplified in separate PCR reactions, mixed, and used to co-transform the recipient cells in Subheading 3.5. The internal primers are designed to produce overlaps of 277 (Cam^R^), 288 (Kan^R^), and 184 (Tmp^R^) bp between the respective “half-cassette” pairs.

1. Prepare template DNAs: resuspend a single colony, or scrape cells from a frozen (−80 °C) stock, in 100 μL H₂O in a microcentrifuge tube.

2. Prepare two 40 μL PCR reactions for each transformation:

   **Tube I:**
   
   - (a) 18 μL H₂O
   - (b) 8 μL Phusion HF buffer (5×)
   - (c) 4 μL dNTP (2 mM each)
   - (d) 4 μL DMSO
   - (e) 2 μL oligo XXX1-P1 (5 μM)
   - (f) 2 μL oligo “R” (cat-midR, kan-midR or tmp-midR; 5 μM) *(see Note 6)*
Table 4

| Temperature                   | Time     |
|-------------------------------|----------|
| 98 °C                         | 2 min    |
| 98 °C                         | 20 s     |
| 72 °C (0.5 °C/s ramp rate)    | 5 s\(^a\) |
| 57 °C (0.5 °C/s ramp rate)    | 20 s     |
| 72 °C (0.5 °C/s ramp rate)    | 1 min/kb\(^b\) |
| 98 °C                         | 20 s     |
| 65 °C                         | 20 s     |
| 72 °C                         | 1 min/kb\(^b\) |
| 4 °C                          | Hold     |

\(^a\) This step was included in order to cool to 72 °C at normal rate before changing to the slower ramp rate

\(^b\) Use the size of the longer of the two “half-cassettes” to decide the proper extension time

(g) 2 μL template cell suspension (Acatsac1, Akansac1 or Atox1)

(h) 0.2 μL Phusion DNA polymerase

**Tube II:**

(a) 18 μL H₂O

(b) 8 μL Phusion HF buffer (5×)

(c) 4 μL dNTP (2 mM each)

(d) 4 μL DMSO

(e) 2 μL oligo XXX2-P1 (5 μM)

(f) 2 μL oligo “F” (cat-midF, kan-midF or tmp-midF; 5 μM) (see Note 6)

(g) 2 μL template cell suspension (Acatsac3, Akansac3 or Atox2)

(h) 0.2 μL Phusion DNA polymerase.

3. Run the PCR according to the cycling parameters listed in Table 4.

4. Analyze the PCR products by agarose gel electrophoresis. The correct fragment sizes are indicated in Fig. 7.

5. Concentrate and de-salt the PCR products (see Note 5).

### 3.5 Transformation

The following protocol is enough for ten transformations (see Note 7):

1. Grow the recipient strain containing pSIM5-Tet O/N at 30 °C in low-salt LB + glucose + tetracycline (see Notes 8 and 9).
2. Dilute 1:100 in 25 mL 30 °C prewarmed low-salt LB + glucose + tetracycline in a 125 mL Erlenmeyer flask (see Note 10).

3. Grow at 30 °C until OD600 ≈ 0.2 (~60–70 min for a wt strain; see Note 11).

4. To induce Red expression, move the flask to a 42 °C shaking water bath (see Note 12) and shake at 185 rpm for 15 min (OD600 will reach ~0.3; see Note 11).

5. Quickly transfer the flask to an ice-water bath and let it cool for at least 10 min and swirl the flask a few times to cool as quickly as possible.

6. Spin down the cells (~4000 × g, 7 min, 4 °C) in a prechilled 50 mL tube. Pour off all liquid and carefully remove any remaining drops with a pipette.

7. Wash once with 1 mL ice-cold 10% glycerol (pellet the cells at ~4000 × g, 3 min, 4 °C). Use a pipette to carefully aspirate all liquid.

8. Resuspend the cells in 200 μL ice-cold 10% glycerol.

9. Mix 20 μL cells and de-salted DNA (see Note 13) in an electroporation cuvette (1 mm gap width) on ice.

10. Electroporate at 2.5 kV, 25 μF and 200 Ω. Immediately but carefully resuspend the cells in 200 μL prewarmed (42 °C) SOC and transfer to tubes (10 or 50 mL tubes) in a 42 °C water bath (shaking is not necessary).

11. Let the cells recover at 42 °C for 15 min (for chloramphenicol or trimethoprim resistance, for other antibiotics up to 1 h recovery may be required). Shaking is not required during the recovery step.

12. Plate on prewarmed plates (see Note 14) with the appropriate selective medium, incubate the plates overnight at 37 °C.

13. Pick single colonies and purify by streaking for single colonies on appropriate selective medium. Colonies with amilCP-containing cassettes will appear blue–purple (see Note 15).

14. Continue with generalized transduction and/or segregation.

3.6 Generalized Transduction (Optional)

1. Please refer to another protocol for transduction with phages P1 vir or P22 HT/105 int, e.g. Thierauf et al. [20].

2. Select for transductants on the appropriate selective medium, incubate the plates overnight or until colonies appear. Colonies with amilCP-containing cassettes will appear blue–purple.

3. Pick single colonies and purify by streaking for single colonies on appropriate selective medium. Colonies with amilCP-containing cassettes will appear blue–purple.
4. Verify that the clones are phage-free and phage-sensitive (see e.g. Thierauf et al. [20]) before proceeding to the segregation/excision step (see Note 16).

3.7 Segregation/Excision of Dup-Ins and DIRex Intermediates by Counter-Selection

1. Pick a single colony of a clone containing a Dup-In or DIRex intermediate (see Note 15). Streak on appropriate negative selection medium (sucrose selection plates for the sacB cassettes, M9 + rhamnose for the Atox-cassettes).

2. Incubate at 37 °C overnight (sucrose selection with sacB cassettes) or 2 days (rhamnose selection with Atox-cassettes). Segregants that have lost an amilCP-containing cassette will become uncolored/white at this step.

4 Notes

1. For primer design, it can be helpful to see the structure of the intermediate that you want to accomplish, e.g. make a drawing of the intermediate and its surrounding sequence, or even better, put the sequence together in a sequence editor and copy the primer sequences from there.

2. When choosing which span to duplicate, a general rule is to place it close (1 to a few kb) to the mutation of interest and to keep the duplication relatively small (~0.5–1 kb). This ensures a high frequency of co-transduction of the Dup-In and the linked mutation.

3. The mutation should preferentially not be included in the duplication, as this could result in formation of “heterozygous” transductants that have the mutation in only one copy of the duplicated sequence. Upon loss of the Dup-In, such transductants will segregate into both parental genotypes with frequencies that depend on the distance between the cassette and each copy of the mutant locus. If the mutation needs to be inside the duplicated area it is especially important to keep the duplication small to reduce the frequency of “heterozygous” transductants and that the mutation is close to the middle of the duplication in order to avoid a bias in which allele is left after segregation of “heterozygotes.”

4. If the mutation of interest is in an essential gene or in an operon with essential genes, it may be needed to either make the duplication larger or to place it further away from the mutation to leave at least one intact copy of the gene or operon.

5. For high efficiency of transformation, it is critical that the DNA is concentrated and salt-free. We routinely use SureClean (Bio-line) for cleanup and concentration, but any similar product or ethanol precipitation should work. Spin column kits usually
result in lower DNA concentrations, which may cause lower transformation efficiencies.

6. As the two outer “XXX-P1” primers have identical 3’-ends, it does not matter for the final outcome which of the outer primers are used with which “half-cassette,” but as the orientation of the internal primer binding sites relative to the “P1” binding sites differ between the template cassettes, only one combination of template cassette and internal primer will produce a usable product. See Fig. 7 for which combination to use for the different cassettes.

7. To scale up, increase the volumes in steps 2, 7, and 8.

8. During growth of *E. coli* and *S. enterica* in LB (without added glucose) the rapid exponential growth phase ends at OD600 ≈ 0.3, followed by several phases of slower growth as the medium is depleted for utilizable carbon sources [21]. The addition of glucose (0.2%) to LB extends the exponential growth phase beyond OD600 = 0.3, resulting in more reproducible timing between inoculation and induction.

9. The use of low-salt LB (LB [Luria; 0.5 g/L NaCl [13]]) allows preparation of electrocompetent cells with only one wash. Other LB formulas (LB [Lennox; 5 g/L NaCl [22]] and LB [Miller; 10 g/L NaCl [23]]) require three washes.

10. To ensure good aeration of the culture, use an Erlenmeyer flask (E-flask) that is graded at least five times the culture volume, i.e. for a 50 mL culture use a 250 mL (or larger) E-flask.

11. In our experience it is critical that OD600 does not reach much higher than ~0.3 for *S. enterica*, while *E. coli* remains transformable at higher OD.

12. It is critical that a water bath is used in order to heat up from 30 °C to 42 °C quickly to ensure efficient induction during the 15 min induction period. Shaking is necessary to ensure that the growth of the cells does not slow down due to oxygen deprivation. If possible, keep the water bath shaking for the entire incubation.

13. We have noticed the highest transformation efficiencies with ~0.15 pmol of DNA in 1 μL (or less) H2O. The amount (in ng) of DNA to take can be approximated by dividing the length of the DNA (in bp) with 10, e.g. 280 ng of a 2800 bp DNA corresponds to approximately 0.15 pmol. If a single PCR reaction does not yield enough DNA, pool from several reactions.

14. Using too cold or too dry plates may result in lower recovery of transformants. Therefore, use freshly prepared plates and allow them to warm up to 37 °C during preparation of the cells for transformation.
15. Before negative selection, it is of critical importance that transformants and transductants are purified through at least one single cell bottleneck. Directly after transformation or transduction the colonies contain surviving recipient cells that will survive negative selection and form false negative (Suc$^R$ / Rha$^R$) clones.

16. Here it is possible to save time by integrating the post-transduction cleanup with the segregation/excision procedure, e.g. purify transductants on antibiotic-containing (Cam or Tmp) EBU-plates [20] before negative selection, and test clones for phage-sensitivity in parallel with the negative selection.

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Chapter 8

Genome Editing in *Klebsiella pneumoniae* Using CRISPR/Cas9 Technology

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**Abstract**

CRISPR/Cas9 systems have been widely adopted for genetic manipulation in diverse biological systems owing to the ease of use and high efficiency. We have recently developed a CRISPR/Cas9-based genome editing system (pCasKP-pSGKP) by coupling a CRISPR/Cas9 system with the lambda Red recombination system as well as a cytidine deaminase-mediated base editing system (pBECKP) in *Klebsiella pneumoniae*, enabling rapid, scarless, and efficient genetic manipulation in diverse *K. pneumoniae* strains. In this chapter, we introduce the detailed procedures of using these two tools for genome editing in *K. pneumoniae*.

**Key words** CRISPR/Cas9, *Klebsiella pneumoniae*, Genome editing, Base editing, pCasKP-pSGKP, pBECKP

1 Introduction

CRISPR/Cas9 systems, originally discovered in bacteria and archaea, have been harnessed for efficient genetic manipulation in diverse organisms, such as mammalian cells [1], plants [2], yeast [3], and some bacterial species [4–7]. The Cas9 nucleases from CRISPR/Cas9 systems are capable of cleaving genomic DNAs in a programmable manner with the guidance of a single guide RNA (sgRNA), generating a double-stranded DNA break (DSB) in a target locus. Because most bacterial species, including *Klebsiella pneumoniae*, lack the nonhomologous end joining (NHEJ) repair pathway, which is the major pathway for DSB repair in eukaryotes, DSBs in bacteria are lethal and thereby CRISPR/Cas9 systems have been used as an effective counter-selection system in bacteria. By coupling the lambda Red recombination system with the CRISPR/Cas9 system from *Streptococcus pyogenes*, we developed a two-plasmid system, pCasKP-pSGKP, for rapid, efficient, and scarless genetic manipulation in *K. pneumoniae*, including gene insertions, gene deletions, and point mutations (Fig. 1a) [6].
In addition, we developed a cytidine deaminase-mediated base editing system (pBECKP) for precise C to T conversion in *K. pneumoniae* by fusing the SpCas9 nickase (D10A) and rat APOBEC1 (rAPOBEC1) deaminase (Fig. 1b) [6]. In this base editing system, the target C is directly converted to T using a catalytic deamination reaction and a subsequent DNA replication pathway without generating a DSB break. Thereby, the system is more amenable for genetic manipulation of plasmids and transposable elements in *K. pneumoniae*. By catalyzing the conversions of CAA, CAG, CGA, or TGG codons to premature stop codons, the system can be used as an effective tool for gene inactivation.

## 2 Material

All solutions should be prepared using Milli-Q water (18.2 MΩ·cm) and analytical grade reagents.

### 2.1 Bacterial Culture

1. Luria-Bertani broth medium (LB medium, for both *E. coli* and *K. pneumoniae*).
2. Agar.
3. Apramycin (50 μg/ml for both *E. coli* and *K. pneumoniae*).
4. Kanamycin (50 μg/ml for both *E. coli* and *K. pneumoniae*).
5. Hygromycin B (100 μg/ml for both *E. coli* and *K. pneumoniae*).
6. Spectinomycin (50 μg/ml for *E. coli*, 50–100 μg/ml for *K. pneumoniae*).
2.2 Construction of the Editing Plasmid

1. pCasKP-apr, a temperature-sensitive plasmid vector that expresses the Cas9 nuclease and the lambda Red recombination proteins for genome editing in *K. pneumoniae*, in which the resistance marker is apramycin (Addgene # 117231).

2. pCasKP-hph, a temperature-sensitive plasmid vector that expresses the Cas9 nuclease and the lambda Red recombination proteins for genome editing in *K. pneumoniae*, in which the resistance marker is hygromycin B (Addgene # 117232).

3. pSGKP-km, a plasmid vector that expresses the sgRNA, of which the resistance marker is kanamycin (Addgene # 117233).

4. pSGKP-spe, a plasmid vector that expresses the sgRNA, in which the resistance marker is spectinomycin (Addgene # 117234).

5. pBECKP-km, a cytidine deaminase-mediated base-editing plasmid in *K. pneumoniae*, in which the resistance marker is kanamycin (Addgene # 117235).

6. pBECKP-spe, a cytidine deaminase-mediated base-editing plasmid in *K. pneumoniae*, in which the resistance marker is spectinomycin (Addgene # 117236).

7. Oligonucleotides.

8. T4 polynucleotide kinase.

9. 5 M NaCl.

10. *Bsa*I-HF.

11. T4 DNA ligase.

12. *Xba*I.

13. *E. coli* DH5α competent cells.

14. High-fidelity DNA polymerase.

15. PCR Cleanup Kit.

16. Gibson DNA Assembly Master Mix.

17. Plasmid mini-prep kit.

18. PCR thermocycler.

19. DNA gel electrophoresis.

2.3 Genome Editing in *K. pneumoniae*

1. 10% v/v glycerol.

2. 5% w/v sterile sucrose.

3. Bacterial genomic DNA extraction kit.

4. Electroporation cuvette (2 mm).

5. Electroporation System.
3 Methods

The experiments should be carried out in biosafety laboratories when handling harmful pathogens. The procedures below are using pCasKP-apr, pSGKP-km, and pBECKP-km plasmids for illustration (see Note 1). Schemes of gene editing procedures using pCasKP-pSGKP and pBECKP are shown in Figs. 2 and 3, respectively.

3.1 Insertion of a Spacer into the sgRNA Expression Plasmid (pSGKP or pBECKP)

1. Design spacer primers by picking a 20 bp-spacer ahead of the PAM sequence (5'-NGG-3') in the target locus (see Note 2). Synthesize two oligos in the following form (see Note 3).

   spacer-F: 5'- TAGTNNNNNNNNNNNNNNNNNNNCAAA -3'
   spacer-R: 3'- NNNNNNNNNNNNNNNNNNNNNNCAGA -5'

2. Linearize the sgRNA expression plasmids (pSGKP or pBECKP) by incubating the following mixture at 37 °C for 1–2 h.

| Reagent               | Volume |
|-----------------------|--------|
| BsaI-HF               | 1 µl   |
| 10× Cutsmart buffer   | 5 µl   |
| pSGKP or pBECKP       | X µl (1 µg) |
| Milli-Q H₂O           | 44-X   |
| Total                 | 50 µl  |

3. Purify the digested plasmid with a PCR Cleanup Kit.

4. Phosphorylate the oligos by incubating the mixture listed below at 37 °C for 0.5–1 h.

| Reagent               | Volume |
|-----------------------|--------|
| spacer-F (100 µM)     | 1 µl   |
| spacer-R (100 µM)     | 1 µl   |
| T4 polynucleotide kinase | 1 µl  |
| 10× T4 DNA ligase buffer | 5 µl |
| Milli-Q H₂O           | 42 µl  |
| Total                 | 50 µl  |

5. Anneal the oligos by adding 0.5 µl of 5 M NaCl to the phosphorylated oligo pairs. Incubate at 95 °C for 3 min and then gradually cool it down to room temperature using a
thermocycler, during the process of which the temperature decreases by 0.5 °C per 10 s. Dilute the annealed oligos 20-fold to 100 nM with Milli-Q H₂O.

6. Ligate the annealed oligo into the BsaI-linearized plasmids (pSGKP or pBECKP) by mixing the following reagents to insert the spacer into the linearized plasmid and incubate the mixture at room temperature for more than 1 h, or 16 °C overnight.
7. Transform the 10 μl of the ligated product into 100 μl *E. coli* DH5α competent cells and recover for 1 h at 37°C. Plate the cells onto an LB agar plate containing 50 μg/ml kanamycin, and then incubate the plate at 37°C overnight.

8. Verify the successful cloning of pSGKP-XX or pBECKP-XX by colony PCR with the M13 reverse sequencing primer (5'-CAGGAAACAGCTATGAC-3') and spacer-F primer (Fig. 4). Run an agarose gel to check for the size of the PCR product, for which the theoretical size is 220 bp. Select several colonies confirmed by colony PCR and separately inoculate them into 5 ml LB broth containing 50 μg/ml kanamycin and incubate them at 37°C overnight by shaking.

9. Extract the spacer-introduced pSGKP-XX or pBECKP-XX plasmid by plasmid mini-prep kit.

10. Verify the pSGKP-XX or pBECKP-XX plasmid by sequencing to confirm that the spacer has been successfully inserted into the plasmid. The primer used for the sequencing can be M13 reverse sequencing primer (5'-CAGGAAACAGCTATGAC-3').
3.2 Preparation of the Repair Template

3.2.1 Prepare the Single-Stranded DNA as the Repair Template (Fig. 5a)

1. Design two 45 nt DNA sequence flanking the cleavage site as the left and right repair templates, respectively.
2. Splice the two 45 nt DNA sequence, then synthesize them as a 90 nt oligonucleotide (ssDNA) repair template.

3.2.2 Prepare the Plasmid-Borne Double-Stranded DNA as the Repair Template (Fig. 5b)

1. Linearize the constructed pSGKP-XX plasmid with XbaI:

| Reagent                  | Volume     |
|--------------------------|------------|
| XbaI                     | 1 μl       |
| 10× CutSmart buffer      | 5 μl       |
| pSGKP-XX plasmid         | X μl (1 μg)|
| Milli-Q H₂O              | 44-X μl    |
| Total                    | 50 μl      |

2. Incubate the mixture at 37 °C for 1–2 h. Purify the digested pSGKP-XX plasmid with PCR Cleanup Kit.
3. Generate the repair templates with the repair arms (500 bp each) by PCR amplification (see Note 5). Purify the PCR product with PCR Cleanup Kit.
4. Insert the repair template into the XbaI-linearized pSGKP-XX plasmid via Gibson assembly and incubate the mixture at 50 °C for 1 h.

| Reagent                      | Volume       |
|------------------------------|--------------|
| Gibson DNA Assembly Master Mix | 10 μl       |
| Linearized plasmid           | X μl (~50 ng)|
| Repair arm (up)              | Y μl (~25 ng)|
| Repair arm (down)            | Y μl (~25 ng)|
| Milli-Q H₂O                  | 10-X-2Y μl   |
| Total                        | 20 μl        |

5. Transform 10 μl Gibson assembly product into 100 μl of DH5α competent cells. Plate the cells onto an LB agar plate containing 50 μg/ml kanamycin. Incubate the plate at 37 °C overnight with shaking.

6. Verify the correct transformants by colony PCR. Select colonies confirmed by colony PCR. Inoculate correct clones into 5 ml LB broth containing 50 μg/ml kanamycin and incubate them at 37 °C overnight.

7. Extract the constructed pSGKP-XX-HR plasmid using a plasmid mini-prep kit.

8. Send the pSGKP-XX-HR plasmid for sequencing to confirm that the repair arms have been successfully inserted into the plasmid.

### 3.3 Prepare K. pneumoniae Electrocompetent Cells

1. Pick a single fresh colony of the *K. pneumoniae* strain and culture it in 5 ml LB broth at 37 °C overnight.

2. Dilute 1 ml overnight culture into 100 ml of LB broth, shake the culture at 37 °C until the optical density at 600 nm (OD₆₀₀) reaches approximately 0.7.

3. Chill the culture on ice for 20 min.

4. Harvest the cells by centrifugation (7200 × *g*, 5 min, 4 °C). Discard the supernatant.

5. Resuspend the cells with 15 ml of sterile ice-cold 10% v/v glycerol.

6. Centrifuge the cells (7200 × *g*, 5 min, 4 °C). Discard the supernatant (see Note 6).

7. Repeat steps 5 and 6 once.

8. Resuspend the cells with 1 ml of ice-cold 10% v/v glycerol.

9. Dispense the cells into 50 μl aliquots in 1.5-ml sterile Eppendorf tubes. Freeze the tubes in liquid nitrogen and store them at −80 °C.
3.4 Electroporate the pCasKP Plasmid into K. pneumoniae

1. Take out a tube of *K. pneumoniae* electrocompetent cells from −80 °C and thaw it on ice for approximately 5 min.

2. Add no less than 200 ng of pCasKP plasmid to the thawed electrocompetent cells, and then transfer the mixture into a 2 mm electroporation cuvette.

3. Perform the electroporation at an Electroporation System with the following parameters: voltage, 2500 V; capacitance, 200 Ω; resistance, 25 μF.

4. Add 1 ml of LB broth into the cuvette to resuspend the cells immediately after the electroporation. Then transfer the cells into a 1.5-ml sterile Eppendorf tube.

5. Shake the tube for 1 h at 30 °C.

6. Plate the cells on an LB agar plate containing 50 μg/ml apramycin. Incubate the plate at 30 °C overnight (see Note 7).

3.5 Prepare pCasKP-Harboring K. pneumoniae Electrocompetent Cells

1. Pick a single colony of the pCasKP-harboring *K. pneumoniae* strain from the plate. Inoculate it into 5 ml of LB broth containing 50 μg/ml apramycin and incubate it at 30 °C overnight.

2. Dilute 1 ml overnight culture into 100 ml of LB broth, and shake the culture at 30 °C.

3. Add 1 ml of 20% L-arabinose to induce the expression of the lambda Red recombination system, when OD<sub>600</sub> of the cell culture reaches approximately 0.2.

4. Incubate the cells at 30 °C with shaking for another 2 h.

5. Chill the culture on ice for 20 min.

6. Harvest the cells by centrifugation (7200 × g, 5 min, 4 °C). Discard the supernatant.

7. Resuspend the cells with 15 ml of sterile ice-cold 10% v/v glycerol.

8. Centrifuge the cells (7200 × g, 5 min, 4 °C). Discard the supernatant (see Note 6).

9. Repeat steps 7 and 8 once.

10. Resuspend the cells with 1 ml of ice-cold 10% v/v glycerol.

11. Dispense the cells into 50 μl aliquots in 1.5-ml sterile Eppendorf tubes (see Note 8).

3.6 Electroporate Repair Template into K. pneumoniae

1. Take a tube of fresh pCasKP-harboring *K. pneumoniae* electrocompetent cells.

2. Add ~300 ng pSGKP-XX-HR plasmid, or ~200 ng pSGKP-XX plasmid with 3 μl 90 nt ssDNA (100 μM) repair template into the L-arabinose-induced pCasKP-harboring electrocompetent cells (see Note 4), and then transfer the mixture into a 2 mm electroporation cuvette.
3. Perform the electroporation at a Electroporation System with the following parameters: voltage, 2500 V; capacitance, 200 Ω; resistance, 25 μF.

4. Add 1 ml of LB broth into the cuvette to resuspend the cells immediately after the electroporation. Then transfer the cells into a 1.5-ml sterile Eppendorf tube.

5. Incubate the tube at 30 °C for ~1.5 h with shaking.

6. Plate the cells onto an LB agar plate containing 50 μg/ml apramycin and 50 μg/ml kanamycin. Incubate the plate at 30 °C overnight.

7. Amplify the target locus in the K. pneumoniae genome by colony PCR. The locations of the two PCR primers should be outside of the repair template in the genome. Send the desired PCR products for sequencing to confirm the successful genome editing.

3.7 Cure Only the pSGKP-XX Plasmid in the Desired Mutant

1. Pick a colony of the confirmed K. pneumoniae mutant and inoculate it into 5 ml of LB broth containing 50 μg/ml apramycin. Shake the culture at 30 °C overnight.

2. Plate the cells onto an LB agar plate containing 50 μg/ml apramycin and 5% w/v sucrose (see Note 9). Incubate the plate at 30 °C overnight.

3. Pick several colonies, and individually streak them onto apramycin-containing (50 μg/ml) LB agar plates in the presence or absence of 50 μg/ml kanamycin. Incubate the plates at 30 °C overnight.

If there are some colonies on the kanamycin-free plate, but no colony on the kanamycin-containing plate, it means that the pSGKP-XX plasmid has been cured.

3.8 Cure Both the pCasKP and pSGKP-XX Plasmids in the Desired Mutant

1. Pick a colony of the confirmed K. pneumoniae mutant. Inoculate it into 5 ml of antibiotic-free LB broth and incubate it at 37 °C overnight (see Note 7).

2. Plate the culture onto an LB agar plate containing 5% w/v sucrose (see Note 9). Incubate it at 37 °C overnight.

3. Pick several colonies and individually streak them onto antibiotic-free LB agar plates and LB agar plates containing 50 μg/ml apramycin or 50 μg/ml kanamycin. Incubate the plates at 30 °C overnight.

If there are some colonies on the antibiotic-free plate, but no colony on the apramycin or kanamycin-containing plate, it means that both the pCasKP and pSGKP-XX plasmids have been cured.
3.9 Base Editing in K. pneumoniae by pBECKP Plasmid

1. Insert the spacer into the pBECKP plasmid to construct the editing plasmid pBECKP-XX. The detailed procedures are described in Subheading 3.1.

2. Electroporate the editing plasmid pBECKP-XX into *K. pneumoniae* electrocompetent cells. The electrocompetent cell preparation methods and plasmid transformation followed the same procedures as that of the pCasKP plasmid. The only difference is that the temperature of culturing the *K. pneumoniae* harboring the pBECKP-XX plasmid can be adjusted to 37 °C.

3. Evaluate the base editing efficiency by amplifying the editable sites from the target locus of *K. pneumoniae* genome by colony PCR. Then, send the PCR product for sequencing to evaluate the base editing efficiency.

3.10 Cure the pBECKP-XX Plasmid in the Desired Mutant

1. Pick a colony of the confirmed *K. pneumoniae* mutant. Inoculate it into 5 ml of antibiotic-free LB broth and incubate it at 37 °C overnight.

2. Plate the cells onto an LB agar plate containing 5% w/v sucrose (see Note 9). Incubate the plate at 37 °C overnight.

3. Pick several colonies and individually streak them onto LB agar plates in the presence or absence of 50 μg/ml kanamycin. Incubate the plates at 37 °C overnight.

   If there are some colonies on the kanamycin-free plate but no colony on the kanamycin-containing plate, it means that the pBECKP-XX plasmid has been cured.

4 Notes

1. Given that some of clinically isolated *K. pneumoniae* strains are resistant to widely used apramycin and/or kanamycin, we constructed other editing plasmids pCasKP-hph, pSGKP-spe, and pBECKP-spe, by replacing the antibiotic marker of the plasmids (pCasKP-apr, pSGKP-km, or pBECKP-km) with hygromycin B (hph) or spectinomycin (spe) marker. The editing plasmids can be further modified with other antibiotic markers, depending on the antibiotic resistance of the target *K. pneumoniae* strains.

2. Find a 20 bp-spacer on the 5’ side of the PAM (5’-NGG-3’), in the form of 5’-spacer (20 nt)-NGG-3’. In addition, a suitable spacer can also be designed by the sgRNAcas9 software [8].

   The base editor has a high C-to-T conversion efficiency within the activity window. We find that the activity window of the pBECKP system in *K. pneumoniae* was from positions 3 to 8. Thereby, the target mutation site should be located in the
activity window (positions 3–8). In addition, a suitable spacer for producing a stop codon can also be designed by the CRISPR-CBEI webtool (https://taolab.nwsuaf.edu.cn/CrisprCBEI).

3. Given the cleavage sites of the restriction enzyme BsaI (Fig. 4), the 5' end of the 20 bp spacer and the reverse complementary sequence of the 20 bp spacer need to be added with the sequences 5'-TAGT-3' and 5'-AAAC-3', respectively.

4. The lambda Red recombination system can use linear homologous DNA fragments or plasmid-borne donor DNA as the repair template. Using the plasmid-borne donor DNA as the repair template has a similar gene editing (deletion, insertion, and point mutation) efficiency as ssDNA or linear dsDNA in K. pneumoniae.

5. Ensure that adjacent segments (linearized plasmid, upstream of the repair template, and downstream of repair template) have identical sequences on the ends when designing primers used to amplify the repair template from the K. pneumoniae genome (Fig. 5b). The length of repair arms can be 500 bp each. The Gibson Assembly webtool (http://nebuilder.neb.com/) can be used to design suitable primers for Gibson Assembly reactions in this study.

6. Sometimes the K. pneumoniae cultures cannot be fully pelleted after centrifugation. However, it does not influence the transformation of the editing plasmid in the subsequent procedures.

7. The temperature-sensitive replicon repA101(Ts) were introduced into the pCasKP plasmids for curing the pCasKP plasmid after editing. Thus, the K. pneumoniae cells harboring the pCasKP plasmid must be incubated at 30 °C.

8. To get a high-editing efficiency, we suggest using freshly prepared pCasKP-harboring K. pneumoniae electrocompetent cells to perform the editing experiment.

9. The sucrose-sensitive gene sacB was introduced into the pSGKP and pBECKP plasmids for curing the plasmids after editing. 5% w/v sucrose is lethal for the cells harboring the pSGKP or the pBECKP plasmid.

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Chapter 9

Generating Single Nucleotide Point Mutations in E. coli with the No-SCAR System

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Abstract

Genetic manipulation of microbial genomes is highly relevant for studying biological systems and the development of biotechnologies. In E. coli, λ-Red recombineering is one of the most widely used gene-editing methods, enabling site-specific insertions, deletions, and point mutations of any genomic locus. The no-SCAR system combines λ-Red recombineering with CRISPR/Cas9 for programmable selection of recombinant cells. Recombineering results in the transient production of heteroduplex DNA, as only one strand of DNA is initially altered, leaving the mismatched bases susceptible to repair by the host methyl-directed mismatch repair (MMR) system and reduces the efficiency of generating single nucleotide point mutations. Here we describe a method, where expression of cas9 and the MMR-inhibiting mutLE32K variant are independently controlled by anhydrotetracycline- and cumate-inducible promoters from the pCas9CyMutL plasmid. Thus, MMR is selectively inhibited until recombinant cells have undergone replication and the desired mutation is permanently incorporated. By transiently inhibiting MMR, the accumulation of off-target mutations typically associated with MMR-deficient cell types is minimized. Methods for designing the editing template and sgRNA, cloning of the sgRNA, induction of λ-Red and MutLE32K, the transformation of editing oligo, and induction of Cas9 for mutant selection are detailed within.

Key words Recombineering, Genome editing, No-SCAR, Cas9, Mismatch repair, mutL

1 Introduction

Recombineering has revolutionized the genome editing landscape, enabling rapid and efficient construction of desired mutants. In E. coli, λ-Red recombineering is among the most widely used methods for gene editing [1]. In this technique, linear DNA substrates encoding the desired edits and flanked by homology arms corresponding to the target locus are transformed into cells expressing exo, bet, and gam genes from the λ-bacteriophage [2]. The λ-Red proteins process the linear DNA substrate and promote annealing to the target region while protecting the template from degradation by host nucleases. Both double- and single-stranded DNA can be used as editing substrates for creating insertions,
deletions, and point mutations with fewer than 50 bp of homology to the target [1, 3].

Traditionally, incorporation of an antibiotic resistance cassette in addition to the intended mutation is required to select for recombinant cells followed by subsequent removal of the marker, leaving behind a scar site [1, 4]. The scarless Cas9-assisted recombineering (no-SCAR) system was developed as a single-step method for selecting recombinant cells without the introduction of scar sites [5, 6]. The no-SCAR system uses the programmable S. pyogenes Cas9 nuclease, targeted to a specific sequence by a single-guide RNA (sgRNA) to remove unedited cells from the population selectively. Expression of cas9 is controlled by the anhydrotetracycline (aTc)-inducible promoter, P_{Tet}, on the pCas9CR4 plasmid and was engineered for tight transcriptional control, enabling co-maintenance with the sgRNA-encoding pKDsgRNA plasmid. The pKDsgRNA plasmid encodes exo, beta, and gam and the programmable sgRNA, allowing for efficient recombineering and mutant selection with a two-plasmid system. Further, Cas9-cleavage can be easily retargeted to any locus containing a protospacer adjacent motif (PAM) by modifying the 20 bp spacer region of the sgRNA.

Recombineering occurs at the replication fork and exhibits a bias for the lagging strand template [3, 7–11]. Only one of the two DNA strands is initially modified during recombineering, creating heteroduplex DNA that can be repaired by the endogenous methyl-directed mismatch repair (MMR) system. Mutator strains with specific genetic backgrounds that disrupt mismatch repair (ΔmutS, ΔmutL, ΔmutH, or ΔuvrD) have been shown to improve the efficiency of recombineering [8]. However, permanent inhibition of MMR results in an over 100-fold increase in background mutation rate [12]. Careful design of the editing substrate has also been shown to improve efficiency by taking advantage of certain blind spots in the MMR system. For example, MMR can be successively avoided by designing the oligo such that it creates a C:C mismatch within 6 bp of the desired mutation, four or more adjacent mismatches, or mismatches at four or more consecutive wobble positions [10]. However, such design constraints are not always possible to accommodate depending on the intended application. Another strategy has been to inhibit MMR only briefly using temperature-sensitive or dominant-negative alleles of the MMR proteins MutS and MutL [13, 14].

Here we describe a technique that employs the dominant-negative MutL_{E32K} variant with the no-SCAR system, allowing efficient generation and selection of single nucleotide recombinants. We incorporated the MutL variant into the pCas9CR4 plasmid and placed expression under control of the cumate-inducible promoter/regulator pair, P_{cymRC}/CymR [15], creating pCas9Cy-MutL (Fig. 1a). When combined with pKDsgRNA (Fig. 1b), the
design allows for independent induction of all effectors starting with the expression of the λ-Red genes and the MutL\textsubscript{E32K} variant by addition of arabinose and cumate, respectively, allowing for successful incorporation and stability of single nucleotide point mutations and prevention of removal by MMR. After recovery and genome replication, Cas9-counterselection against unedited cells is induced by plating onto a medium with aTc (Fig. 2).

2 Materials

2.1 Media

1. Super optimal broth (SOB) is made by adding 20 g tryptone, 2.4 g MgSO\textsubscript{4}, 5 g yeast extract, 0.186 g KCl, and 0.5 g NaCl to 1 L dH\textsubscript{2}O.

2. SOB with catabolite repression (SOC) is made by adding 20 mM filter-sterilized glucose to the sterilized SOB.

3. 50 mg/mL spectinomycin (spec) stock is prepared by dissolving in dH\textsubscript{2}O and sterilizing through a 0.2 μm filter and added to media at a concentration of 50 μg/mL.

4. 40 mg/mL chloramphenicol (chlor) stock is prepared by dissolving in 70% EtOH and added to media at a concentration of 40 μg/mL.
5. 100 nM anhydrotetracycline (aTc) stock is prepared by dissolving in 70% EtOH. The aTc is light-sensitive in solution and in agar plates and thus should be stored in the dark.

6. 100 mM cumate stock is prepared in 70% EtOH and used in media at 0.1 mM.

7. 1.33 M L-arabinose (ara) stock is prepared in dH$_2$O and sterilized through a 0.2 μm filter.

8. Luria Bertani (LB) broth is made by adding 10 g tryptone, 5 g yeast extract, and 10 g NaCl to 1 L deionized water (dH$_2$O).

9. LB agar is made by adding 15 g/L agar to LB broth.

10. LB agar plates with chlor, chlor + spec, and chlor + spec + aTc + cumate.
2.2 Donor DNA and sgRNA Design

1. DNA sequence of target region.
2. DNA editing program (see Note 1).

2.3 sgRNA Cloning

1. SgRNA retargeting primers (designed in Subheading 3.2).
2. Primer gam-F—5’- ggaattcgagctctaaggaggtta-3’.
3. Primer gam-R—5’- tttaacctcttagctga-3’.
4. Plasmid miniprep purification kit of choice.
5. pKDsgRNA (SpecR; Addgene #62654 or #62656) for PCR template.
6. High Fidelity DNA polymerase.
7. DNA assembly mastermix, such as NEBuilder HiFi DNA Assembly.
8. Chemically competent _E. coli_ for cloning.
9. _DpnI_ Restriction Enzyme.
10. Agarose.
11. 1× Tris-Acetate-EDTA (TAE) Buffer: For 10× TAE stock, add 48.4 g Tris Base, 11.4 mL glacial acetic acid, and 20 mL 0.5 M EDTA pH 8.0 to 800 mL dH2O and stir to dissolve. Adjust volume to 1 L with dH2O.
12. 1 kb DNA ladder.
13. DNA stain compatible with blue light transilluminator.
14. 6× gel-loading dye.
15. Gel purification kit.
16. Taq DNA polymerase.
17. Primer sgRNAcheck-F—5’- cagtgaatggggtaaatgg -3’.
18. Primer sgRNAcheck-R—5’- gcctgcagtctagactcgag -3’.
19. Primer sgRNAseq—5’- agctttcgctaaggatgattt -3’.

2.4 Recombineering, Cas9 Counterselection, and Plasmid Curing

1. pCas9CyMutL (Addgene #166981).
2. _E. coli_ host strain.
3. Plasmid miniprep purification kit.
4. Retargeted pKDsgRNA-xxx (made in Subheading 3.3).
5. DNA editing oligo (designed in Subheading 3.1).
6. Sterile 20% Glycerol/1.5% Mannitol Solution.
7. Sterile dH2O.
8. 0.1 or 0.2 cm gap electroporation cuvettes.
9. Electroporator.
10. Primers for DNA sequencing or allele-specific PCR.
11. pKDsgRNA-p15 (Addgene #62656).
3 Methods

3.1 Donor DNA Design

1. Obtain the DNA sequence up- and downstream of the target locus and paste it into a DNA editing program (see Note 1; Fig. 3a).

2. Model the desired genotype in silico by modifying the DNA sequence to include the intended point mutations (Fig. 3b).

3. Select ~80 bp of sequence that spans the desired mutation, place the mutation near the middle of the selected region, and copy the sequence corresponding to the lagging strand in the chosen *E. coli* strain (see Note 2). This is the oligonucleotide sequence that will serve as the editing template (Fig. 3c).

4. Check the secondary structure of the oligo using a DNA secondary structure prediction program (see Note 1). If the ΔG is less than −12.5 kcal/mol, then the oligo should be shifted up- or downstream to decrease the secondary structure, while maintaining at least 15 bp between the mutation and the end of the oligo.

5. Add 2–4 phosphorothioate bonds between the nucleotides at the 5′ end of the oligo (see Note 3).

6. Design primers that bind the approximately 400–500 bp up- and downstream of the mutation (xxx-MutCheck-F and xxx-MutCheck-R; where “xxx” represents the target locus), as well as a third primer (xxx-MutSeq) that binds approximately 100 bp from the mutation. These primers will be used to confirm recombinant cells later.

3.2 Identifying and Designing sgRNA Target

1. Using the same DNA sequence for the target locus obtained above, identify all NGG PAM sites within 15 bp of the mutation on both strands (Fig. 3d).

2. Choose a PAM site that positions the mutation on the 5′ side of the NGG motif. This way the mutation will disrupt sgRNA binding either by altering the chosen PAM site or the 12 bp seed region (Fig. 3e).

3. Copy the 20 bp immediately 5′ of the chosen PAM site and add the sequence “gttttagagctagaaatagcaag” to the 3′ end, creating primer xxx_sgRNA-F.

4. Obtain the reverse complement of the 20 bp target sequence from the previous step and add the sequence “gtgctcagtatctc-tatcactgataga” to the 3′ end to make primer xxx_sgRNA-R.

5. Use “Cas-OFFinder” (http://www.rgenome.net/cas-offinder) [16] to identify potential off-target sites for the designed sgRNA. Select the PAM Type, SpCas9: 5′-NRG (see Note 4).
6. Under the target genome section, select “Others” for organism type, then select your *E. coli* strain. Enter the designed sgRNA sequence in the “Query Sequences” box and set the “Mismatch Number” to 3 (*see Note 5*). Click submit.

7. The results are listed by genomic location. Ensure that only the intended target matches the 12 bp seed region exactly. If more than one exact match is found then another sgRNA target site should be chosen.

Fig. 3 Example for target identification and design of editing template and sgRNA sequences. (a) DNA sequence for the target locus, *rpoB*, with the residue to be mutated highlighted. (b) CCT → CTT single point mutation generating the RpoB*P564L* variant. (c) 80 bp region containing the target mutation to be used for the editing template targeting the lagging strand (blue oligo). (d) Identified PAM sites located within 15 bp of the intended mutations. PAM sites 1–4 contain the mutation 5’ to the NGG motif and are suitable for sgRNA targeting. (e) 20 bp targeting region (orange oligo) for the sgRNA using PAM site 4.
8. If no off-target results are found, then proceed with ordering the designed primers (xxx_sgRNA-F and xxx_sgRNA-R) as well as the five other primers listed above in Subheading 2.4.

### 3.3 Retargeting the sgRNA by Gibson Assembly Cloning

The sgRNA sequence that provides target specificity is modified using a ligation-independent cloning with overlapping linear DNA fragments. We present the method using the NEB HiFi DNA Assembly Kit, though other techniques are expected to work (see Note 6). An overview of this method is shown in Fig. 4.

1. Grow the *E. coli* strain harboring the pKDsgRNA plasmid (Addgene #62654 or #62656) in LB broth + spec at 30 °C, 200 rpm in a shaking incubator overnight.
2. Purify the pKDsgRNA plasmid with the plasmid miniprep purification kit.
3. Obtain primers gam-F, gam-R, and the sgRNA retargeting primers (xxx_sgRNA-F and xxx_sgRNA-R) designed above.
4. PCR amplify the pKDsgRNA-xxx plasmid in two separate 25 μL reactions using a high-fidelity DNA polymerase, such as Q5 High Fidelity 2× Master Mix, with primers gam-F and xxx_sgRNA-R for PCR 1 and xxx_sgRNA-F and gam-R for PCR 2. For Q5 polymerase, program the thermal cycler with the following conditions:

![Schematic of sgRNA retargeting by Gibson assembly cloning. The pKDsgRNA plasmid is PCR amplified using primers P1—xxx-sgRNA-F, P2—xxx-sgRNA-R, P3—gam-R, and P4—gam-F. P1 and P2 contain complementary 5’ overhangs encoding the new sgRNA target sequence. After amplification and purification, the linear PCR products are mixed with the DNA-assembly enzymes and incubated at 50 °C. The exonuclease (yellow) degrades the ends of the DNA fragments in the 5’ → 3’ direction generated single-stranded overhangs. The complementary overhangs from each fragment are annealed together and a DNA polymerase (green) extends the annealed fragments. Finally, DNA ligase (purple) seals the remaining nick, resulting in a completed plasmid with the new sgRNA target, pKDsgRNA-xxx](image-url)
1 cycle: 98 °C – 30 s
30 cycles: 98 °C – 10 s
64 °C (−0.2 °C/cycle) – 15 s
72 °C – 2 min (PCR 1); 1 min 30 s (PCR 2)
1 cycle: 72 °C – 2 min
4 °C – hold

5. Add 5 units of DpnI restriction enzyme to each reaction and incubate at 37 °C for at least 15 min to digest template DNA.
6. Prepare a 0.8% agarose 1× TAE gel pre-stained with a blue light transilluminator compatible DNA stain, such as SYBR Safe, or stain the gel after electrophoresis if the DNA stain chosen requires.
7. Add loading dye to each sample and resolve the entire PCR reactions on the gel until the loading dye run approximately 3/4 the length.
8. Observe the DNA under the blue light transilluminator and excise the bands at 4 kb (PCR 1) and 1.5 kb (PCR 2). Gel purify the amplicons according to manufacturer instructions and elute in a minimal volume (≤10 μL).
9. Set up the assembly reaction by adding 1 μL 2× of the assembly master mix, such as NEB HiFi Assembly 2×, and 0.5 μL of each purified PCR product in a single tube. Incubate the reaction at 50 °C for 15 min (see Note 7).
10. Mix the entire 2 μL assembly reaction with 50 μL chemically competent E. coli cells and transform by incubating 15 min on ice, 45 s at 42 °C, and 2 min back on ice. Add 1 mL SOB and recover at 30 °C for 2 h.
11. Plate 100 μL on an LB + spec and incubate at 30 °C until colonies form (~18–20 h).
12. Confirm successful retargeting of the sgRNA by colony PCR and sequencing: Using sterile pipette tips, pick 3–4 single colonies and resuspend each in 100 μL of sterile dH2O. In PCR tubes, set up the following reactions (see Note 8):

| 12.5 μL | PCR Master Mix |
|---------|----------------|
| 0.5 μL  | Primer sgRNAcheck-F (10 μM) |
| 0.5 μL  | Primer sgRNAcheck-R (10 μM) |
| 5.0 μL  | Cell/dH2O resuspension |
| 6.5 μL  | Sterile dH2O |
13. For Taq polymerase, program the thermal cycler with the following conditions:

| Cycle Type | Temperature |
|------------|-------------|
| 1 cycle    | 94 °C – 30 s |
| 30 cycles  | 94 °C – 15 s |
|            | 55 °C – 15 s |
|            | 68 °C – 1 min |
| 1 cycle    | 68 °C – 5 min |
|            | 4 °C – hold  |

14. Sequence the PCR product by Sanger sequencing using primer sgRNASeq.

15. Inoculate 5 mL LB + spec with the remainder of cell resuspensions (see Note 9) and grow overnight at 30 °C. Alternatively, the cells can be spotted or patched onto an LB + spec plate, and then cultured after the sequencing results are returned.

16. Purify the plasmid with the miniprep plasmid purification kit.

**3.4 Preparation of Strain for Recombineering**

This method has been demonstrated to function in both *E. coli* K-12 and BL21, though mutagenesis efficiency appears to decrease in BL21.

1. Obtain the *E. coli* strain with plasmid pCas9CyMutL (Addgene #166981) and grow in a shaking incubator overnight at 37 °C, 200 rpm in 5 mL LB + chlor.

2. Miniprep pCas9CyMutL following the chosen plasmid miniprep purifications kit instructions.

3. To transform pCas9CyMutL into the desired host strain, grow cells in 5 mL LB at 37 °C until they reach OD$_{600}$ 0.5–0.6.

4. Chill cells on ice for 5 min, then pellet 1.5 mL in a microcentrifuge at 5000 × g, 4 °C for 2 min.

5. Remove supernatant and resuspend the cell pellet in 500 μL cold, sterile dH2O. Carefully underlay 500 μL of cold, sterile 20% glycerol/1.5% mannitol solution so that two layers can be seen and spin 2 min at 5000 × g, 4 °C to pellet cells.

6. Aspirate the supernatant from the top down to remove the water layer first, and then carefully remove the glycerol/mannitol layer. Resuspend pellet in 50 μL cold, sterile 20% glycerol/1.5% mannitol solution. Transfer to a prechilled 0.1 cm gap electroporation cuvette (see Note 10).

7. Add approximately 100 ng pCas9CyMutL to cuvette and electroporate with a single 1.8 kV pulse. Recover cells in 1 mL SOB for 1 h at 37 °C, then plate 100 μL on an LB + chlor plate and incubate at 37 °C overnight.
8. The next day, pick 1–3 colonies and grow in 5 mL LB + chlor at 37 °C, 200 rpm in a shaking incubator until the OD_{600} reaches 0.5–0.6.

9. Prepare cells for electroporation as described in steps 4–6 and transform with 100 ng of the retargeted pKDsgRNA-xxx plasmid. Recover cells for 2 h at 30 °C.

10. Spread 50 μL on an LB + spec + chlor plate and incubate at 30 °C.

11. To test the targeting efficiency of the sgRNA prepare tenfold serial dilutions to 10^{−6} and spot 10 μL of each dilution on LB + spec + chlor and LB + spec + chlor + aTc plates. Incubate the plate at 30 °C overnight.

12. Count the number of colonies both with and without aTc. There should be at least 100-fold fewer colonies with aTc induction, indicating successful counterselection against wild-type cells. If there is no difference in cell count between the two plates a different target site should be selected (see Note 11).

3.5 Recombineering and Cas9 Counterselection in E. coli

1. Inoculate 5 mL of SOB + chlor + spec with 1–3 colonies from the 50 μL spread plate and grow at 30 °C, 200 rpm in a shaking incubator until the OD_{600} reaches 0.4–0.5.

2. Add 100 μM cumate and 4 mM arabinose to the cells and continue incubating for an additional 15–30 min to induce expression of mutLE32K and λ-Red, respectively.

3. Chill cells on ice for 10–15 min and make electrocompetent with the glycerol/mannitol method (see Note 12). The whole 5 mL culture or one mL aliquots can be prepared. In both cases, resuspend the final pellet in 1/20 the volume of glycerol/mannitol.

4. Add 1 μg DNA editing oligo (designed in Subheading 3.1) to an electroporation cuvette and electroporate the cells with a single 1.8 kV pulse for a 0.1 cm cuvette or 2.5 kV for 0.2 cm cuvettes. Recover cells in 1 mL SOC + cumate for 2–3 h at 30 °C (see Note 13).

5. After recovery plate 50 μL of cells onto LB + spec + chlor + aTc and incubate at 30 °C overnight.

6. Genotype several colonies from the aTc-induced plate by colony PCR (as described in Subheading 3.3) with primers xxx-MutCheck-F and xxx-MutCheck-R and Sanger sequencing of the product using primer xxx-MutSeq. Adjust the annealing temperature and extension time of the PCR as recommended by the polymerase manufacturer.

7. Once confirmed, inoculate 5 mL LB + chlor with a single recombinant colony and grow at 37 °C for 4–5 h to cure the cells of the pKDsgRNA-xxx plasmid.
8. Streak 10 μL of the culture on an LB + chloramphenicol plate prewarmed to 42 °C, and grow overnight at 42 °C.

9. The following morning, patch 10–20 colonies onto LB + chlor and LB + spec plates and incubate overnight at 30 °C to confirm curing of the pKDsgRNA-xxx plasmid.

10. Strains cured of the pKDsgRNA-xxx plasmid can be used for subsequent mutagenesis if required, otherwise the strain should be cured of the pCas9CyMutL plasmid. If further mutations are desired, obtain new targets as described in the previous sections (Subheading 3.1–3.3) and repeat steps 6–16. If no further mutations are required continue with Subheading 3.6.

3.6 Curing pCas9CyMutL

To remove the pCas9CyMutL plasmid

1. Start a 5 mL culture of strain that possesses pCas9CyMutL and transform with pKDsgRNA-p15 (Addgene #62656) using the glycerol/mannitol density step method and recover in 1 mL SOB at 30 °C for 2 h.

2. Add 100 nM aTc and 50 μg/mL spec to the recovery and incubate for an additional 2 h at 30 °C to induce expression of cas9 and the p15a targeting sgRNA.

3. Plate 50 μL onto LB + spec + aTc and incubate at 30 °C overnight.

4. Screen 10–20 colonies by patching onto LB only and LB + chlor and incubate at 37 °C overnight to check for the loss of chloramphenicol resistance.

4 Notes

1. Benchling is an easy-to-use and free online DNA editing software (https://www.benchling.com) and has built-in features for predicting and analyzing DNA secondary structures, but any DNA editing program and secondary structure prediction tool of choice is sufficient.

2. Targeting the lagging strand in oligo recombineering is more efficient than the leading strand. Therefore, the oligo sequence designed here should correspond to the lagging strand sequence. In E. coli, the origin of replication, oriC, spans positions 3,925,744–3,925,975. As such, the lagging strand sequence can be identified as follows:

   (a) If the target is on replicon 1 (>3,925,975 or <1,588,774) then the oligo sequence should be the same as the (−) strand sequence.
(b) If the target is on replichore 2 (> 1,588,774 and <3,925,744) then the oligo sequence should be the same as the (+) strand sequence.

For example, the *rpoB* gene shown in Fig. 2 is located on replichore 1 starting at position 4,181,245 with its coding sequence on the (+) strand. Thus, to target the lagging strand, the oligo must match the (−) strand sequence.

3. Phosphorothioate bonds are not required, but improve the efficiency of mutagenesis by increasing stability of the DNA inside the cell. Check with preferred vendor for proper notation of phosphorothioate bonds when ordering. For both Integrated DNA Technologies (IDT) and Sigma-Aldrich, phosphorothioate bonds are denoted by an asterisk (*).

4. The *S. pyogenes* Cas9 also has some weak activity with NAG PAM sites. Selecting the NRG PAM will identify both NGG and NAG sites which may produce unwanted off-target effects and reduce genome editing efficiency. When designing the sgRNA target, however, it is recommended to use only NGG PAM sites, which have a higher activity.

5. The “Mismatch Number”, “DNA Bulge Size”, and “RNA Bulge Size” options can be modified to alter the stringency of the search conditions. Generally, it is only necessary to ensure that the 12 bp seed region matches to the target region alone. Strong off-target effects in *E. coli* should result in cell death, and consequently will not be observed; however, they must be avoided since a DSB at a second site will also kill the cells. Weak off-target sites are likely to be repaired by homology-directed repair from intact copies of the chromosome and thus are not a major concern with this method [17].

6. Any overlap cloning technique can be used at this step; including Gibson assembly, SLIC, and CPEC [17–19]. We prefer the NEBbuilder HiFi Assembly MasterMix because it is efficient and allows scaled-down reactions.

7. The manufacturer protocol for NEB HiFi Assembly recommends using 20 μL total reaction volumes; however, we consistently obtain clones with 2 μL reactions, thereby decreasing costs for plasmid construction by tenfold.

8. The 2× OneTaq Master Mix is convenient and works well for colony PCR, but any Taq DNA polymerase should work as well.

9. Do not leave cells in dH₂O longer than a few hours as viability decreases over time.

10. For plasmid transformation for strain preparation any method of transformation can be used, including chemically competent cells with heat shock. We prefer the glycerol/mannitol density
step method for making electrocompetent cells [20] as it is a quick and easy-to-use method that yields consistent time constants with electroporation. Time constants should be >5 ms for efficient transformation to occur.

11. A tenfold decrease in the number of colonies when compared to the uninduced plate may be sufficient if the subsequent recombineering step is highly efficient.

12. Electroporation should be used at this step because high-efficiency transformation of linear DNA is required. Other methods of electrocompetent cell preparation can be used in place of the glycerol/mannitol method.

13. After transforming cells with the DNA editing oligo, cumate is required in the recovery media to keep the mismatch repair system inhibited until the cells have undergone full segregation of their chromosomes.

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Chapter 10

Recombineering in \textit{Staphylococcus aureus}

Kelsi Penewit and Stephen J. Salipante

Abstract

Recombineering has proven to be an extraordinarily powerful and versatile approach for the modification of bacterial genomes, but has historically not been possible in the important opportunistic pathogen \textit{Staphylococcus aureus}. After evaluating the activity of various recombinases in \textit{S. aureus}, we developed methods for recombineering in that organism using synthetic, single-stranded DNA oligonucleotides. This approach can be coupled to CRISPR/Cas9-mediated lethal counterselection in order to improve the efficiency with which recombinant \textit{S. aureus} are recovered, which is especially useful in instances where mutants lack a selectable phenotype. These methods provide a rapid, scalable, precise, and inexpensive means to engineer point mutations, variable-length deletions, and short insertions into the \textit{S. aureus} genome.

\textbf{Key words} \textit{Staphylococcus aureus}, Genome editing, Recombineering, Genetic engineering, Isogenic strain, Mutagenesis

1 Introduction

\textit{Staphylococcus aureus} is an important human and veterinary pathogen with a global distribution \cite{1}, which is responsible for a wide variety of diseases \cite{2–4}. \textit{S. aureus} has historically proven difficult to genetically modify, and it has consequently been challenging to produce isogenic mutant strains that would facilitate an understanding of genes and pathways in that organism. Recognizing these limitations, researchers have invested much effort in developing techniques to introduce directed genetic changes into the \textit{S. aureus} genome. Significant progress has been made towards this end, and multiple protocols to achieve genomic modification of \textit{S. aureus} have now been established \cite{5}, each having its own sets of benefits and drawbacks. Most approaches share a common reliance on rare, homologous recombination events between the genome and large donor fragments that encode the desired genetic change. As such, they involve the individual cloning and optimiza-
tion of each ~1–2 kb homologous repair template, or in the case of engineering gene deletions, their manufacture by splicing overhang extension (SOE) PCR [6, 7].

Recombineering, which incorporates mutagenic oligonucleotides into a host genome through the action of bacteriophage-derived single-stranded DNA (ssDNA) recombinases, presents an attractive alternative to methods rooted in homologous recombination. Recombineering allows point mutations, variable-length deletions, and short insertions to be precisely engineered using mutagenic oligonucleotides that have been specifically designed to encode the desired change [8–11]. Because the oligonucleotide substrates for recombineering can be synthetically produced by commercial suppliers, it is possible to scalably, rapidly, and inexpensively engineer a range of genomic changes into the strain or strains of interest. Perhaps the most well-described recombineering system is λ Red, which was originally developed in *Escherichia coli* but has subsequently been adapted and generalized to other, related species [12–14].

With the goal of developing a recombineering system for *S. aureus*, we assessed the ability of various known and predicted recombinases to mediate ssDNA recombineering in that organism [15]. We evaluated six different recombinases when codon-optimized and expressed from a plasmid: bet, the recombinase utilized in the *E. coli* λ Red recombineering system [9]; EF2132 and OrfC, derived from *Enterococcus faecalis* and *Legionella pneumophila*, respectively, and both previously shown to have cross-species activity in *E. coli* [9]; gp20, a recombinase originating from *S. aureus* having weak activity in *E. coli* [9]; and two putative *S. aureus* recombinases (which we termed *recTS2* and *recTS3*) which we identified on the basis of protein homology to these known enzymes (Fig. 1). Only EF2132 was able to recombineer a *rpoB* H481Y mutation (conferring rifampin resistance [16]) at frequencies significantly greater than the rate of spontaneous drug resistance occurring in mock-transformation and recombinase-negative controls, identifying this enzyme as a suitable catalyst for recombineering in *S. aureus*. As it is generally thought that recombinases function with the highest efficiency in their species background of origin [17], these results additionally suggest either that recombinases native to *S. aureus* lack activity for ssDNA or that *S. aureus* recombinases which are optimal for recombineering are markedly dissimilar in sequence and structure to those which have been previously defined [18]. Interestingly, we also observed that the *S. aureus* strain used for these tests (Fig. 1) and a subset of additional strains tested later (Fig. 2) were measurably recombineigenic without expression of recombinase, indicating that some *S. aureus* isolates are naturally competent to recombineer with ssDNA oligonucleotides, albeit at relatively low frequencies [15].
Here we provide detailed protocols for performing recombineering in *S. aureus* using ssDNA oligonucleotides. It should be noted that the recombineering system described lacks the full functionality of λ Red and orthologous systems in that it does not encode either an exonuclease for converting double-stranded DNA (dsDNA) intermediaries (such as PCR products) to ssDNA recombineering substrates or a protein to inhibit degradation of exogenous DNA templates [12, 13]. As such, recombineering can currently be performed using relatively short ssDNA templates only [15]. Inserting large-scale changes such as gene cassettes or fusion proteins by recombineering through the use of dsDNA templates [12, 13] is therefore not currently possible in *S. aureus*; however, incorporating additional functional components that support these approaches is an attractive goal for future *S. aureus* recombineering systems. To improve the efficiency of mutant identification, we also describe methods to couple recombineering with CRISPR/Cas9-mediated counterselection [15, 19], which facilitates recovery of modified strains even in the absence of an externally selectable phenotype by introducing lethal, double-
stranded DNA breaks into the genomes of unedited cells [20]. Use of temperature-sensitive plasmid vectors to encode these functions ensures that exogenous genetic material can be removed after brief passaging at elevated temperatures [21, 22], resulting in truly isogenic strains. Although the protocols detailed here are specific for *S. aureus*, the general principles are broadly applicable across organisms for which recombineering and CRISPR/Cas9 counter-selection systems have been developed.

![Graph showing recombineering efficiencies across laboratory and clinical strains. Results are shown for wild-type strains and strains expressing recombinase EF2132, compared to paired mock-transformation controls. Recombineering frequencies significantly higher than observed in controls (≤p = 0.008 by a 2-tailed t-test) are indicated by an asterisk for wild-type strains (red asterisk) and strains expressing recombinase (purple asterisk). Sequence type (ST) and clonal complex (CC) are indicated in brackets below each strain name. Strains ATCC29213, N315, and Clinical #5 are capable of low-frequency recombineering in the absence of EF2132 expression. Image copyright © American Society for Microbiology [15].]
2 Materials

2.1 Plasmids

1. pCN-EF2132tet: The conditional recombineering vector, pCN-EF2132tet [15], is available from Addgene (ID 107191). This vector expresses recombinase EF2132 from Enterococcus faecalis, which we have found has high activity in S. aureus, under control of the strong, constitutive p23 promoter [23]. The plasmid is constructed on an E. coli-S. aureus shuttle vector [21]. It is maintained in E. coli at 37 °C under ampicillin selection (100 μg/mL). By virtue of its T181cop-634ts origin of replication [21], the vector is temperature-sensitive in S. aureus, where it must be maintained at 32 °C using chloramphenicol selection (10 μg/mL).

2. pCAS9counter: If coupling recombineering with counterselection, the conditional counterselection vector, pCAS9counter [15], is available from Addgene (ID 107192). This vector expresses self-sufficient small guide RNA (sgRNA) [24] and Cas9 derived from Streptococcus pyogenes SF370 [19]. The specificity of Cas9 for introducing double-stranded DNA breaks is determined through the sequence of targeting DNA oligonucleotides cloned into the sgRNA (see Subheading 3.7.1): in its native state, the sgRNA element contains a nonfunctional ~100 bp spacer, which enables screening for successfully cloned targeting oligonucleotides using fragment length analysis PCR. The plasmid contains a temperature-sensitive E194ts S. aureus origin of replication [22] (which is compatible with T181cop-634ts). It is maintained in E. coli at 37 °C under ampicillin selection (100 μg/mL), and must be maintained in S. aureus at 32 °C using erythromycin selection (10 μg/mL).

2.2 Bacterial Strains

2.2.1 S. aureus Strains

With successful preparation of electrocompetent cells and plasmid preconditioning through the appropriate E. coli plasmid artificial modification strain (Subheading 2.2.2), we are able to recombineer genomes of laboratory strains or primary clinical isolates from a wide variety of genetic backgrounds and lineages, although the efficiency of successful recombineering appears to be strain-specific (Fig. 2) [15].

2.2.2 E. coli Plasmid Artificial Modification Strains

A number of transgenic E. coli strains have been constructed which are able to modify the methylation profiles of plasmids prior to their introduction into the S. aureus strain of interest, which is necessary to bypass the restriction barrier in transferring DNA between those species. Some of the most useful strains for this purpose are summarized below, and each are available from BEI resources (www.beiresources.org):
1. **Cytosine methylase negative E. coli (DC10B):** is a transgenic, cytosine methylase-negative E. coli strain which enables bypass of the *S. aureus* type IV restriction system [6]. The strain is useful in conditioning plasmids for introduction into many *S. aureus* backgrounds, including those for which the clonal complex designation is unusual or is not known, but transformation efficiencies are generally not as high as can be obtained when using a plasmid artificial modification strain that actively mimics the methylation profile of a destination *S. aureus* strain.

2. **Transgenic type I adenine methylation E. coli strains (IMXXB series):** Derivatives of DC10B that actively modify DNA to mimic the methylation profile of specific *S. aureus* clonal complexes have been subsequently developed [25]. These epigenetic modifications enable bypass of the type III restriction system in compatible *S. aureus* strains, and increase transformation efficiencies over what is achievable with DC10B [25]. Strain IM08B carries the methylation machinery of *S. aureus* sequence type 8 (i.e., ST8). We have found that this strain can condition plasmids for their introduction into the majority of *S. aureus* laboratory and clinical isolates, which are largely derived from ST8 or ST8-restriction compatible groups. Specific strains transformable with IM08B-conditioned plasmids include laboratory strain Newman, laboratory strain ATCC29213, laboratory strain N315, USA300 isolates, ST239 isolates, and ST5 isolates [15, 25]. IM30B actively mimics the methylation profile of *S. aureus* ST30. Use of this strain conditions plasmids for introduction into *S. aureus* laboratory strain Cowan and MRSA252, among other ST30 and ST30-restriction compatible strains [25]. Strains IM01B and IM93B similarly condition DNA for transformation into *S. aureus* strains of ST1 and ST93, respectively [25].

### 2.3 Materials for Electrocompetent Cell Preparation

1. B2 broth [26]: 1.0% casein hydrolysate, 2.5% yeast extract, 0.1% K₂HPO₄, 0.5% glucose, 2.5% NaCl, pH 7.5, or LB broth, or TSB broth as appropriate.

2. Sterile 10% glycerol (autoclaved), chilled on ice.

3. Sterile distilled water (autoclaved), chilled on ice.

### 2.4 Electroporation Materials

1. Pellet Paint® NF DNA Co-Precipitant (Millipore, or equivalent DNA co-precipitant).

2. 100% and 70% Ethanol.

3. TE buffer.

4. Sterile 1.5 M sucrose (filter sterilized).

5. Sterile 10% glycerol (autoclaved).

6. 0.1 cm electrode gap electroporation cuvettes.
7. TSB broth or SOC broth, as appropriate.
8. Electroporator (Bio-Rad MicroPulser, or equivalent).
9. LB plates with appropriate selective antibiotics (100 μg/mL ampicillin, 10 μg/mL chloramphenicol, 10 μg/mL chloramphenicol and 10 μg/mL erythromycin, or 25 μg/mL rifampin).

2.5 Antibiotics

1. 100 mg/mL Ampicillin prepared in laboratory-grade water.
2. 34 mg/mL Chloramphenicol prepared in 100% ethanol.
3. 50 mg/mL Erythromycin prepared in 100% ethanol.
4. 50 mg/mL Rifampin prepared in 100% methanol.

2.6 Materials for Counterselection

Vector Preparation

1. bsaI.
2. CIP.
3. T4 Polynucleotide Kinase PNK.
4. T4 Ligase buffer.
5. T4 DNA Ligase.
6. Primers: sgRNA_check_F (5’-AAAAATATGAACACTCTATTG-3’) and sgRNA_check_R (5’-CGGTGCCACTTTTTCAAGTT-3’).
7. KAPA HiFi 2× Hotstart Readymix (KAPA Biosystems) or equivalent.

3 Methods

3.1 Design of Recombineering Oligonucleotides

Recombineering oligonucleotides encode the mutagenic change of interest and are incorporated into the genome through the action of EF2132 recombinase. As recombineering oligonucleotides must be relatively short, they are not suitable for engineering the insertion of entire genes, but can be used to effectively manufacture gene deletions of even relatively large size, single or multiple nucleotide substitutions, and short indels.

3.1.1 General Considerations

1. For optimal recombineering efficiencies in S. aureus, we have found that recombineering oligos should be 90 bp in length and carry a series of 4 phosphorothiate bonds at the 5’ end in order to inhibit exonuclease digestion [15, 27] (Fig. 3). Efforts should be taken to avoid designing recombineering oligonucleotides that contain strong secondary structures [28].

2. In E. coli, recombineering oligonucleotides are preferentially incorporated into the lagging strand during DNA replication [11]. In our experiments in S. aureus, we have found that only some oligonucleotides demonstrate preferential incorporation.
on one strand or the other, nevertheless, both a primary recombineering oligonucleotide and its reverse complement may be designed and empirically tested.

3. The number of substitutions introduced into the recombineering oligonucleotide to encode the directed mutation and to achieve MMR bypass (if required, see Subheading 3.1.3) should be minimized in order to promote hybridization between the oligo and the host genome.

4. Recombineering oligonucleotides are most inexpensively generated as “ultramers” (IDT) with standard salt purification, manufactured on the smallest possible scale (4 nM). If large numbers of recombineering oligonucleotides are required, costs per oligonucleotide can be further reduced by ordering them in a plate-based synthesis format.

5. The efficiency of recombineering is influenced by the sequence context of the oligonucleotide used, although the factors involved are not clearly understood at present. Empiric evaluation of multiple recombineering oligonucleotides may be necessary in practice.

Fig. 3 Oligonucleotide modifications affect recombineering efficiency. The effect of various oligonucleotide lengths, mismatch repair-evading silent substitutions, and phosphorothioate modifications on recombination efficiency are shown. Recombination frequencies significantly higher than observed in the mock transformation control (\( p = 0.002 \) by a 2-tailed \( t \)-test) are indicated by an asterisk. Error bars indicate standard error of the mean. Image copyright © American Society for Microbiology [15]
3.1.2 Gene Deletions

We recommend generating in-frame gene deletions to escape potential polar effects.

1. The junction of the gene deletion should be placed as centrally as possible within the oligonucleotide: as a starting point, the initial 45 bp and the terminal 45 bp of a gene may be joined to generate a central, in-frame gene deletion.

2. We have observed a negative correlation between the size of the engineered deletion and its efficiency by which it can be generated [15, 28], so the 5' and 3' sites of the fusion can be moved more internally for engineering deletions in particularly long genes, if necessary.

3. If CRISPR/Cas9 counterselection is to be performed, the gene deletion must excise the protospacer adjacent motif (PAM) recognition site that is targeted by the counterselection oligonucleotide.

3.1.3 Point Mutations and Small Indels

For engineering point mutations or small indels, separate design considerations are required.

1. The desired change or changes should be placed as centrally as possible within the oligo. At minimum, mismatches should be placed 15 bp or further from the ends of the oligo sequence [29].

2. Measures must be taken to bypass repair of the engineered changes by the bacterial mismatch repair (MMR) system [29]. This may be accomplished through a number of design features in the recombineering oligonucleotide. First, single-base C:C mismatches (i.e., G > C transversions) cannot be efficiently repaired by MMR and can be introduced as singular changes [29]. Second, alteration of four or more consecutive bases (base substitutions or indels) produce lesions which are too large to be corrected by MMR, and can be introduced without additional alterations [29]. Third, four or more wobble positions in adjacent codons can similarly be changed to produce large lesions which do not alter protein coding sequence [29]. This strategy is the most facile for many applications, as it provides the greatest flexibility in design. The desired mutation may either be adjoining or contained within the altered wobble bases. As an alternative approach, an MMR-deficient, mutS deletion strain may be generated for the strain of interest and can subsequently be used to incorporate single nucleotide changes in the absence of additional mutations (silent or otherwise) encoded by the recombineering oligonucleotide [15]. However, it should be noted that MMR deficiency results in strains with elevated rates of spontaneous mutation [15].
3. If CRISPR/Cas9 counterselection is to be performed, and the engineered change does not directly ablate the targeted PAM, then additional mutations must be encoded to destroy the PAM site in engineered cells. These mutations are optimally synonymous so as not to introduce undesired amino acid substitutions with the desired, engineered change. In some cases, introducing silent mutations is not possible, for example, when a PAM overlaps a proline or a glycine codon. In these situations, a conservative amino acid substitution can be used. There are multiple amino acid exchangeability matrices available in the literature which are based on empiric data (for example, [30]) that can be used to inform the choice for the substituted amino acid. If this approach must be taken, we recommend engineering a mutant strain containing the PAM-ablating substitution alone, as well as a mutant strain carrying that substitution in concert with the desired engineered change, so that unintended effects of the substitution can be experimentally assessed. Measures must be taken as above to escape MMR repair of any dedicated PAM-ablating mutations, but these may be integrated with substitutions that protect the desired change in order to minimize mismatches between the oligo and genomic sequence.

3.2 Preparation of Electrocompetent Cells

This basic protocol can be used to prepare electrocompetent cells for either *E. coli* or *S. aureus* strains, as long as appropriate media are used. The protocol essentially follows those of Monk et al. [6] and Ausbuel et al. [31], with minor modifications.

1. Inoculate a single bacterial colony into 3 mL of the appropriate broth. Incubate overnight at 37 °C (or 32 °C for strains carrying temperature-sensitive vectors) with constant aeration. For growth of *S. aureus* the preferred medium is B2 broth [6], although LB or TSB can alternatively be used (see Note 1). If the strain is carrying pEF2131-tet, the media should contain 10 μg/mL chloramphenicol and a reduced temperature should be used. For growth of *E. coli*, use LB media.

2. Add 1.5 mL of the overnight culture to 150 mL of the appropriate, fresh broth in a 1-L flask.

3. Incubate the cells with constant aeration (shaking at 250 rpm) at the appropriate temperature (37 °C or 32 °C) until OD 650 nm reaches 0.5 (typically 130–160 min).

4. Chill the culture on ice for 15 min to stop growth. From this point on, keep cells cold at all times during processing. As the culture chills, transfer ~37.5 mL aliquots into four 50 mL conical centrifuges tubes, on ice.

5. Harvest the cells by centrifugation at 4600 × *g* for 10 min at 4 °C. Remove supernatant.
6. Add ~10 mL ice-cold sterile water to each tube, resuspend pellets, then consolidate all aliquots into a single 50 mL tube. Spin at 4600 × g for 10 min at 4 °C, then remove supernatant.

7. Add ~10 mL of ice-cold sterile water and resuspend. Mostly fill tube with ice-cold sterile water (~30 mL). Centrifuge at 4600 × g for 10 min at 4 °C. Remove supernatant.

8. Repeat for step 7.

9. Add ~10 mL ice-cold sterile 10% glycerol and resuspend pellet. Add an additional 30 mL ice cold sterile 10% glycerol. Centrifuge at 4600 × g for 10 min at 4 °C. Remove supernatant.

10. Repeat step 9.

11. Resuspend the cell pellet in 750 μL ice-cold sterile 10% glycerol. The final cell concentration should be ≥1 × 10¹⁰ CFU/mL.

12. Working on ice, distribute 50 μL aliquots of cell suspension into sterile, prechilled 1.5 mL tubes and freeze immediately at −80 °C (see Note 2).

### 3.3 Transformation of E. coli Plasmid Artificial Modification Strains

Plasmids which will be introduced into *S. aureus* must first be passaged through an appropriate *E. coli* plasmid artificial modification strain in order to enable bypass of the *S. aureus* restriction barrier [6, 25] (see Subheading 2.2.2). If the clonal complex and/or sequence type of the *S. aureus* strain of interest is not known in advance, we recommend empirically testing plasmid modified each of the *E. coli* plasmid artificial modification strains to determine which of them results in the highest transformation efficiency. This protocol essentially follows that of Ausubel et al. [31].

1. Thaw the appropriate electrocompetent *E. coli* cells on ice (~5–10 min). Simultaneously, prechill a 0.1 cm electroporation cuvette on ice.

2. Add up to 2 μL of plasmid DNA to thawed cell suspension. Pipette three times to mix, then incubate on ice for 1 min. Transfer the mixture to the electroporation cuvette, keeping on ice.

3. Place the cuvette in the sample chamber of the electroporator and immediately pulse once at 1.8 kV without using a fixed time constant.

4. Immediately add 1 mL SOC medium to the cuvette, and quickly but gently resuspend cells using a pipette.

5. Transfer the cell suspension to a sterile culture tube, and incubate at 37 °C for 1 h with constant aeration (shaking at 250 rpm).
6. Plate dilutions of the cells on LB containing the appropriate selective medium. Artificially modified plasmids can subsequently be extracted from the transformed cells.

3.4 Establishing a Conditionally Recombinogenic S. aureus Strain

Recombineering plasmid pCN-EF2132tet is introduced into the S. aureus strain of interest to generate a recombineering-competent derivative.

1. Extract plasmid pCN-EF2132tet from an E. coli plasmid artificial modification strain using a commercial plasmid extraction kit. 1 μg of plasmid is required for optimal transformation efficiency.

2. Prepare electrocompetent cells from the S. aureus strain of interest (see Subheading 3.2).

3. Precipitate pCN-EF2132tet plasmid DNA. Bring Pellet Paint NF Co-Precipitant and 3 M Sodium Acetate to room temperature. Invert several times until a uniform suspension is achieved, but do not vortex. Add 2 μL Pellet Paint and 0.1 volume 3 M Sodium Acetate to 1 μg plasmid DNA and mix briefly by pipetting. Add 2 volumes ethanol and briefly vortex to mix. Incubate at room temperature for 2 min. Centrifuge at 14,000–16,000 × g for 5 min. A pink pellet should be visible at the bottom of the tube. Remove the supernatant by pipetting. Rinse pellet with two volumes 70% ethanol, then vortex briefly and centrifuge at 14,000–16,000 × g for 5 min. Remove supernatant and rinse pellet in 100% ethanol. Spin at 14,000–16,000 × g for 5 min, then remove supernatant by pipetting. Any residual ethanol should be removed by air-drying. Finally, resuspend pellet in 2 μL TE buffer.

4. Transform S. aureus by electroporation. Thaw electrocompetent S. aureus cells on ice for 5 min, then transfer to room temperature for an additional 5 min. Centrifuge cells at 5000 × g for 1 min and resuspend in 50 μL 10% glycerol containing 500 mM sucrose. Transfer resuspended cells to the tube containing precipitated DNA, incubate for 1 min, then transfer the mixture to a 0.1 cm electrode gap electroporation cuvette. Place the cuvette in the sample chamber of the electroporator and pulse once at 2.3 kV using a 2.5 ms time constant. Immediately after electroporation, resuspend cells in 950 μL TSB containing 500 mM sucrose.

5. Transfer the cell suspension to a sterile culture tube and incubate at 32 °C for 1 h with shaking at 250 rpm. Plate aliquots of the cell suspension onto LB containing 10 μg/mL chloramphenicol. We recommend plating three aliquots of 250 μL each, and one aliquot of 100 μL. Incubate plates up to 48 h at 32 °C until colonies appear.
6. Once a transformant is isolated, prepare electrocompetent cells of the conditionally recombinogenic *S. aureus* strain (see Subheading 3.2). This batch of cells can be used for many different recombineering experiments.

**3.5 Performing Recombineering**

Recombineering oligonucleotides are electroporated into the conditionally recombinogenic *S. aureus* strain to achieve genome engineering mediated by transgenic recombinase. We recommended that recombineering experiments be performed in parallel with positive and negative controls to assess transformation efficiency and the rate of successful recombineering (see Subheading 3.9 and Note 3). The recombineering efficiency of *S. aureus* strains can vary substantially, although we have had success in recombineering laboratory strains and primary clinical isolates from a variety of different genetic lineages (Fig. 2).

1. Transform *S. aureus* by electroporation as in Subheading 3.4, step 4, except use 2 μL of 100 μM recombineering oligonucleotide instead of plasmid, and incubate transformants 32 °C for 2 h with shaking at 250 rpm.

2. Plate serial dilutions of the electroporation mixtures onto LB media containing 10 μg/mL chloramphenicol or selective media appropriate for the recombineered mutation.

3. Incubate plates a 24–60 h at 32 °C, until colonies appear.

**3.6 Screening for Engineered Strains**

Efficiency of genome engineering may range considerably from target to target (in our experience, from 5% to 50% of colonies screened may contain the desired change, although modification of some genes has not been proven possible by recombineering), so an appropriate number of colonies should be screened. For mutations that confer a selectable change (for example, drug resistance) or an observable phenotypic change (such as pigmentation or slow growth), genetic screening can be used to verify the genotype of colonies which exhibit the expected properties.

1. For deletion mutants, it is sufficient to screen colonies using colony PCR fragment length analysis, using primers flanking the deleted region and appropriate amplification conditions (see Subheading 3.7.2, step 11).

2. For point mutations, it is necessary to assess the success of genome engineering using alternative approaches. Targeted dideoxy-chain termination DNA sequencing offers definitive analysis, and can be used to distinguish successful recombinants from spontaneous, off-target point mutations which confer the same phenotype (Fig. 4). To verify recombineering by sequencing, design primers flanking the intended change, perform colony PCR to amplify the region using appropriate primers and amplification conditions (see Subheading 3.7.2, step 11),
and subject the amplicon to Sanger sequencing. Alternatively, allele-specific PCR [32] may be used to rapidly screen large numbers of colonies for the presence of the intended sequence.

3.7 Recombineering with Counterselection

Introduction of the recombineering oligonucleotide can be performed concurrently with a CRISPR/Cas9 counterselection plasmid targeted to the locus of interest in order to achieve improved efficiency of mutant identification (Fig. 5). Targeting constructs for counterselection are designed such that only cells undergoing successful recombineering are immune to lethal, double-stranded DNA breaks catalyzed by the CRISPR/Cas9 vector. Consequently, the counterselection plasmid’s selectable marker (erythromycin resistance, although alternate resistance genes can be readily substituted [21]) serves as a selectable proxy for the recombineered change.

3.7.1 Design of Counterselection Targeting Oligonucleotides

The specificity of Cas9 counterselection is determined by cloning a 20 bp guide RNA, encoded by synthetic targeting oligonucleotides, into the sgRNA element of pCAS9counter. This element targets Cas9 to a specific protospacer adjacent motif, or PAM, sequence (5′-NGG-3′) and enable its subsequent endonuclease activity.
1. Guide RNAs can be designed by copying the sequence of your gene of interest into a program such as CRISPRscan (http://www.crisprscan.org/) [33], which optimizes the design of targeting oligonucleotides for their activity based on an empiric model. If using CRISPRscan, select the “No Search” option for predicting off-target effects, and “Cas9 – NGG” as the enzyme. A list of possible targeting oligonucleotides will be displayed, along with their accompanying “CRISPRscan score,” a rating of how likely the oligo will be at promoting Cas9-mediated cutting: higher scoring oligos are most likely to have high activity. The sequence of designed guide RNAs are displayed in capital letters within the larger oligonucleotide construct. Other, equivalent programs may also be used for this purpose. If the objective is to engineer a gene deletion, any PAM internal to the site of deletion may be selected, as successful genome editing events will eliminate the PAM site and
consequently eliminate Cas9-mediated DNA cleavage in the engineered strain. If the objective is to engineer a point mutation, the targeted PAM site must be near the desired edit (at most, ~50 bp distant). In general, selecting a PAM closest to the engineered edit is desirable: in ideal cases, the intended mutation will overlap the targeted PAM. However, silent mutations to eliminate the PAM and escape counterselection must ideally avoid altering the protein sequence of the gene, so that a more distal PAM which can be ablated through silent mutation may be favored over a PAM more proximal to the desired edit but which necessitates removal by a nonsynonymous amino acid change (see Subheading 3.2).

2. To be cloned into pCAS9counter, the guide RNA must be reconstituted as double-stranded DNA (dsDNA) with appropriate sticky ends. This can be accomplished by modifying the desired guide RNA sequence as follows in order to separately design the two strands of that element as individual targeting oligonucleotides:

(a) 5'-AGCTC – guide RNA sequence - G'
(b) 5'-AAAAC - reverse complement of the guide RNA sequence - G'.

3. Procure each strand of the targeting oligonucleotides of the required length as standard, salt-purified ssDNA oligonucleotides from Integrated DNA Technologies (IDT), or another commercial supplier. If large numbers of targeting oligos are required, they may be synthesized most inexpensively in a plate-based format.

3.7.2 Preparation of Counterselection Vector

This step guides Cas9 counterselection to a specific site in the genome through the cloning of targeting oligos into the sgRNA element of pCAS9counter. The protocol will result in a large quantity of digested pCAS9counter that can be stored frozen and used to construct many different targeting constructs.

1. Extract plasmid from the *E. coli* strain carrying pCAS9counter using a commercial plasmid extraction kit. At least 1ug of plasmid is recommended.

2. Prepare restriction digest of plasmid as follows:

(a) 1 µg pCAS9counter
(b) 5 µL 10× Cutsmart buffer
(c) 1 µL *bsaI*-HF
(d) water to 50 µL.

3. Incubate reaction at 37 °C for 1 h, followed by a 65 °C heat inactivation for 10 min.

4. Add 1 µL CIP and incubate at 37 °C for 30 min.
5. Perform gel purification of digested plasmid to exclude uncut vector using a 0.8% agarose gel. The digested plasmid will migrate just higher than 10 kb.

6. Prepare a reaction to phosphorylate and subsequently anneal the two targeting oligos (designed in Subheading 3.1) as follows:
   (a) 0.5 μL oligo 1 (100 μM)
   (b) 0.5 μL oligo 2 (100 μM)
   (c) 2.5 μL T4 Ligase Buffer
   (d) 0.5 μL T4 PNK
   (e) 21 μL H₂O.

7. Using a thermocycler, incubate reaction at 37 °C for 40 min, then 95 °C for 5 min, then gradually cool to 20 °C over 42 min.

8. Prepare ligation reaction to insert annealed targeting oligos into pCAS9counter as follows:
   (a) 2.9 μL annealed targeting oligo, diluted 1:50 in water
   (b) 40 ng digested and size selected pCas9counter
   (c) 2 μL T4 Ligase buffer
   (d) 1 μL T4 Ligase
   (e) water to 20 μL.

9. Incubate at room temperature for 30 min to 1 h (up to an overnight incubation).

10. Transform either into commercially available E. coli as per manufacturer instructions or directly into electrocompetent E. coli plasmid artificial modification strains (see Subheading 3.3 and Note 4), selecting on LB media containing ampicillin (100 μg/mL).

11. Screen 10–20 transformants for insert using colony PCR. pCAS9counter natively contains a nonfunctional spacer region between bsaI cut sites in the sgRNA element, which is replaced by the shorter targeting oligonucleotides after successful cloning. Using a sterile pipette tip, pick a transformant colony and gently patch it to a fresh plate. Resuspend the remaining colony in 20 μL reagent grade water, then heat to 95 °C for 10 min to release the DNA. Per colony screened, prepare a PCR cocktail as follows (see Note 5):
   (a) 12.5 μL KAPA HiFi Hotstart readymix (2×)
   (b) 0.75 μL sgRNA_check_F (10 μM)
   (c) 0.75 μL sgRNA_check_R (10 μM)
   (d) 10.35 μL water
   (e) 2 μL colony boiling preparation crude DNA extract.
Amplify using the following conditions: 1 cycle of 95 °C for 3 min; 30 cycles of 98 °C for 20 s, 59 °C for 20 s, 72 °C for 30 s; 1 cycle of 72 °C for 1 min. Resolve PCR products by 2% agarose gel electrophoresis. Unmodified pCAS9counter will yield a 227 bp amplicon, whereas successful cloning products will amplify with a 133 bp band.

12. Extract plasmid DNA from a successful clone using commercially available kits. If a plasmid artificial modification strain has not been used to directly clone the plasmid, transform plasmid into an E. coli plasmid artificial modification strain (see Subheading 3.4) and reextract. At least 1 μg of plasmid is necessary for optimal counterselection.

3.7.3 Performing Recombineering with Counterselection

Recombineering oligonucleotides and counterselection vector can be concurrently electroporated into the conditionally recombinogenic S. aureus strain. We recommend that counterselection experiments be performed in parallel with the transformation of untargeted pCAS9counter plasmid (i.e., vector which does not contain a targeting oligo) or pCAS9counter targeted to a target absent from the genome (such as GFP), in order to control for transformation efficiency and to assess the success rate of counterselection (see Subheading 3.9 and Note 3).

1. Precipitate and concentrate 1 μg counterselection plasmid DNA using pellet paint as in Subheading 3.4, step 3, except use 2 μL of 100 uM recombineering oligonucleotide (rather than TE buffer) to resuspend the precipitated pellet.
2. Transform S. aureus by electroporation as in Subheading 3.4, step 4, except incubate transformants 32 °C for 2 h with shaking at 250 rpm.
3. Plate aliquots of the electroporation mixtures onto LB media containing 10 μg/mL chloramphenicol and 10 μg/mL erythromycin (see Note 6). We recommend plating four volumes of 250 μL each.
4. Incubate plates a 48–60 h at 32 °C, until colonies appear.
5. Screen colonies as in Subheading 3.6.

3.8 Plasmid Curing

Once a successfully engineered colony is identified, the bacteria may be cured of the temperature-sensitive recombineering plasmid (and counterselection plasmid, if used) to produce an isogenic strain.

1. Streak the colony onto LB plates (lacking antibiotics) and incubate overnight at 43 °C.
2. Verify loss of the plasmid in one or two resulting colonies by re-streaking them onto LB media, LB media containing 10 μg/mL chloramphenicol and, if counterselection has been
performed, separately onto LB media containing 10 μg/mL erythromycin. Successfully cured strains will not grow on either antibiotic.

3.9 Positive and Negative Controls for Recombineering and Counterselection

Positive and negative controls are useful in assessing the effectiveness of genome editing or for troubleshooting purposes. We suggest independent controls for recombineering and counterselection, which may be used in combination. Engineering rifampin resistance through the \( rpoB \) H48Y point mutation is useful for testing purposes, as it can be assessed using selective media without the need for molecular analysis.

3.9.1 Recombineering Controls

1. Perform recombineering (Subheading 3.5) using the following recombineering oligonucleotide to introduce the \( rpoB \) H48Y rifampin resistance mutation and a C:C mismatch that eliminates a nearby PAM (* indicate phosphorothioate bonds):

   (a) 5' - A*A*T*T* CATGGACCAAGCAAACCCATTAGCT GAGTTGACCTACAAGCGCCGTCTATCAGCATTAG GACCTGCTGGTTTAACACGTGAACGTG-3'.

2. Separately transform (Subheading 3.5) a nonmutagenic version of the \( rpoB \) recombineering oligonucleotide to serve as a negative control. Alternatively, a TE only transformation control can be used (see Note 7):

   (a) 5' - A*A*T*T* CATGGACCAAGCAAACCCATTAGCT GAGTTAACGCATAAACGTCGTCTATCAGCATTAG GACCTGGTGGTTTAACACGTGAACGTG-3'.

3.9.2 Counterselection Controls

1. As a positive control, prepare counterselection vector as in Subheading 3.7, making use of the following targeting oligonucleotides to target the PAM modified by the mutagenic \( rpoB \) oligonucleotide:

   (a) 5'-AGCTCCGTCTATCAGCATTAGGACCG-3'
   (b) 5'-AAACGGTGCTATGCTAGACGG-3'.

2. A GFP-targeted counterselection vector is generally a good choice as a biologically irrelevant negative control, and can be constructed as in Subheading 3.6 with the following targeting oligonucleotides:

   (a) 5'-AGCTCCCAATTCTTGTTGAATTAGAG-3'
   (b) 5'-AAAACTCTAATTCAACAGAATTGGG-3'.

3.9.3 Assessment of Controls

Transformation of controls for recombineering and counterselection may be performed separately or in conjunction.

1. If recombineering is being performed alone, transform cells separately with the positive and negative control oligonucleotides as in Subheading 3.5. Select the recombinant population
by plating serial dilutions onto LB media containing chloramphenicol (to assess the number of viable cells) and rifampin-containing media (25 μg/mL, to assess the number of recombinants). Relative rates of rifampin-resistant colonies should be greatly elevated compared to spontaneous resistance mutation rates measured when the negative recombineering control is used.

2. Counterselection vectors may be transformed independently to assess their lethality. This step is often helpful in assessing the effectiveness of newly designed counterselection vectors (see Note 3). Transform each counterselection plasmid in parallel with counterselection plasmid positive and negative controls as in Subheading 3.5, except that serial dilutions should be plated on to LB media (to assess the total number of viable cells) and LB media containing erythromycin (to assess the number of transformed cells which are viable). It is expected that positive control transformations and those from other highly active counterselection vectors should yield few to no erythromycin-resistant colonies relative to the transformants obtained with the negative control vector.

3. Testing recombineering in conjunction with counterselection provides a test of the end-to-end process. Transform the negative control counterselection vector concurrently with the negative control recombineering oligonucleotide, which does not encode a functional change (see Note 7), as in Subheading 3.7. The number of colonies recovered on chloramphenicol with erythromycin media represents viable cells that have been transformed with the counterselection vector, while plating to LB media can be used to determine the total number of viable cells present. In parallel, perform transformation with the positive control recombineering oligonucleotide and positive control recombineering vector. It is expected that the fraction of transformed, viable colonies will be substantially less for the positive controls than seen with the negative control transformation, reflecting a high rate of successful counterselection (see Note 3). After colonies appear, replica plate or patch colonies from the positive and negative control transformations to rifampin-containing media (25 μg/mL) to confirm elevated frequencies of rifampin resistance in the positive control population relative to the negative control, which indicates successful recombineering.
4 Notes

1. Our preferred growth medium for growing *S. aureus* for electrocompetent cell prep is B2 medium; however, we have found that some clinical strains are unable to grow in B2 media. In such cases we have achieved good electroporation efficiencies after alternatively growing strains in LB or TSB broth.

2. Electrocompetent cells are stable for several months at −80 °C.

3. If the number of colonies obtained with the targeted counterselection vector is roughly equivalent similar to the control (within a factor of 10), it typically signifies inefficient counterselection. In this situation, redesign of the targeting oligos is advised.

4. We recommend direct cloning into plasmid artificial modification strains only if transformation efficiency of the electrocompetent cell preparation is high (>1 × 10⁸ CFU per μg pUC19 DNA).

5. Use of other PCR enzymes may be used to perform PCR amplification, but appropriate amplification conditions must be empirically established.

6. When recombineering is performed concurrently with counterselection, we have found that additional stress imposed by selective media for the recombineered mutation (such as antibiotics) sometimes prohibits growth of mutants when applied directly after genome engineering. As such, replica plating or patching or counterselected colonies after their growth is advised.

7. Electroporating recombineering oligonucleotides into *S. aureus* decreases the total number of viable cells, to a measurable degree.

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Chapter 11

Genome Editing Methods for Bacillus subtilis
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Abstract

Bacillus subtilis is a widely studied Gram-positive bacterium that serves as an important model for understanding processes critical for several areas of biology including biotechnology and human health. B. subtilis has several advantages as a model organism: it is easily grown under laboratory conditions, it has a rapid doubling time, it is relatively inexpensive to maintain, and it is nonpathogenic. Over the last 50 years, advancements in genetic engineering have continued to make B. subtilis a genetic workhorse in scientific discovery. In this chapter, we describe methods for traditional gene disruptions, use of gene deletion libraries from the Bacillus Genetic Stock Center, allelic exchange, CRISPRi, and CRISPR/Cas9. Additionally, we provide general materials and equipment needed, strengths and limitations, time considerations, and troubleshooting notes to perform each method. Use of the methods outlined in this chapter will allow researchers to create gene insertions, deletions, substitutions, and RNA interference strains through a variety of methods custom to each application.

Key words CRISPR/Cas9, Genetic manipulation, Bacillus subtilis, Genetic engineering, Gram-positive

1 Introduction

The Gram-positive model bacterium Bacillus subtilis is a highly tractable genetic system, and is often more straightforward to manipulate than human pathogens or environmental bacterial isolates from the same phylum. The numerous genetic tools available, the development of genetic competence, and rapid growth rate are a few attributes that have maintained B. subtilis as a model organism for studying a wide variety of conserved biological processes.

B. subtilis can be easily grown under conditions that activate the development of genetic competence for the uptake of exogenous DNA in the laboratory with limited media, tools, and expense [1]. After growing B. subtilis to stationary phase in minimal media, cells induce a genetic program for natural competence allowing for the uptake and integration of extracellular DNA or the uptake, reassembly, and maintenance of plasmids [2]. Growth into stationary phases induces expression of the global competence
regulator ComK, which in turn activates expression of the ComK regulon including genes for DNA uptake, and homologous recombination [3–6]. Once B. subtilis cells nonspecifically bind DNA, the transforming DNA is fed through a channel where it enters the cytosol as single-stranded DNA (ssDNA) ([7] for review [8]). When incoming DNA has sequence homology with the chromosome, RecA catalyzes homology search and pairing with the B. subtilis chromosome [9]. For comprehensive reviews on the mechanism of natural competence and DNA repair, we direct readers to the following reviews [8, 10–12]. Harnessing the natural competency of B. subtilis has led to many methods for genetic manipulation, with several new advances occurring within the last 12 years [13–16]. In addition to natural competence, B. subtilis has an efficient homologous recombination system allowing for straightforward and effective genetic alterations to be built using PCR fragments, plasmids, or genomic DNA [1]. For efficient homologous recombination to occur, template DNA should contain approximately 500 bp of homology to the host chromosome. It is important for users to consider the order of gene deletion and the method of integration used when studying mutant alleles in processes that are dependent on or affected by competence and recombination because integration of transforming DNA uses both the genetic competence and homologous recombination machinery.

There are a number of tools readily available to B. subtilis researchers (Fig. 1). A deletion library containing erythromycin and kanamycin cassette interruptions of nonessential genes and a library containing CRISPR-interference (CRISPRi) strains of essential genes are housed at the Bacillus Genetic Stock Center (http://www.bgsc.org) (Fig. 2). These resources are available for integration of knockout alleles or to knockdown essential genes [14, 16]. Once strains are acquired, researchers can extract genomic

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**Fig. 1** Recombination of DNA into the B. subtilis genome. (a) Recombination of PCR products, (b) Recombination of genomic DNA containing an antibiotic resistance cassette, (c) Recombination of genomic DNA from the CRISPRi library.
DNA (gDNA) and use it to transform their strain background of interest [14]. Double and triple knockouts can be achieved using this method (discussed in the next section). CRISPRi strains contain a single guide RNA (sgRNA) for Cas9 targeting to the chromosomal gene of interest, resulting in RNA polymerase stalling and an inhibition of transcription [16]. The level of gene transcription can also be titrated using CRISPRi, reducing expression to exceptionally low levels without completely eliminating gene function [16]. CRISPRi is beneficial for the study of essential genes or genes that require a conditional knockdown in transcription to examine the resulting phenotype [16].
Markerless mutations in *B. subtilis* can be achieved using several different methods [13–15, 17]. To create a deletion using the *Bacillus* gene knockout library, pDR244 aids in the removal of antibiotic insertions. The plasmid pDR244 contains a temperature-sensitive origin of replication and constitutively expresses Cre recombinase [14]. Cre recognizes *lox* sites flanking an integrated antibiotic cassette in strains from the Bacillus Genetic Stock Center library, allowing for excision of the antibiotic cassettes leaving only a *lox* scar [14] (Fig. 2). A second approach involves the integration and looping out of the pMiniMad plasmid carrying the desired genetic change. The integration vector pMiniMad uses a temperature-sensitive origin of replication to create a markerless change. Cells transformed with pMiniMad are passaged at the nonpermissive temperature to encourage a single crossover event in the host genome, conferring erythromycin resistance [15, 17]. Once transformants are switched to the permissive temperature, the plasmid excises from the host genome resulting in erythromycin sensitivity [14]. This method also allows for the markerless integration of point mutations, insertions, or deletions.

The third and most recent advance in genetic engineering methods is through the use of CRISPR/Cas9. [13, 18]. CRISPR/Cas9 allows researchers to make clean substitutions, deletions, and insertions in multiple backgrounds with high efficiency [13, 18]. In addition to efficiency, the CRISPR/Cas9 system allows for the insertion or deletion of large fragments on the order of 20 kb [13, 18]. Although CRISPR/Cas9 requires more materials than the other methods described above, plasmids engineered for one alteration are easily adapted for the introduction of other genetic alterations.

The aim of this chapter is to provide an in-depth series of methods that will enable researchers to perform genetic manipulation of several different *B. subtilis* strains. We describe five methods for genetic engineering: (1) Traditional gene manipulation, (2) Use of the deletion library, (3) pMiniMad for allelic exchange, (4) CRISPRi, and (5) CRISPR/Cas9. Below, we also provide the resources and protocols for each recombineering method described.

## 2 Materials

### 2.1 Reagents

1. Luria-Bertani (LB) medium: 10 g NaCl, 10 g tryptone, 5 g yeast extract in 1 L H₂O. Autoclave to sterilize.

2. Luria-Bertani (LB) agar plates: 10 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar in 1 L H₂O. Autoclave to sterilize and pour into sterile Petri plates once the mixture cools to 60 °C. If antibiotics are required, add when the mixture reaches 60 °C and mix thoroughly.
3. LB + starch plates: 1 L LB medium, 10 g/L corn starch, 15 g agar. Autoclave to sterilize. Pour ~20 mL into sterile Petri plates once the solution has reached 60 °C.

4. LM medium: LB medium supplemented with 3 mM MgSO₄.

5. PC buffer (10x): 107 g/L potassium hydrate phosphate (anhydrous), 174.2 g/L potassium dihydrate phosphate (anhydrous), 10 g/L trisodium citrate (pentahydrate), up to 1 L H₂O. Filter sterilize.

6. MD medium: 1× PC buffer, 50% w/v glucose, 10 mg/mL L-tryptophan, 2.2 g/mL ferric ammonium citrate, 100 mg/mL potassium aspartate, 1 M MgSO₄, 10 mg/mL phenylalanine. Store protected from light at 4 °C.

7. Iodide solution: 0.5 g iodine, 5.0 g potassium iodide in 100 mL H₂O. Store protected from light at 4 °C.

8. 0.5 μg/mL erythromycin for B. subtilis.

9. 5 μg/mL chloramphenicol for B. subtilis.

10. 100 μg/mL spectinomycin for B. subtilis.

11. 100 μg/mL ampicillin for E. coli.

12. Sterile saline: 0.85% NaCl in H₂O, or phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; adjust the pH to 7.4 using HCl).

13. 50% glycerol.

14. Molecular-grade agarose (to make 0.5–1% final agarose gels for genotyping and purification of amplicons for cloning).

15. 50× TAE buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL EDTA (pH 8.0), H₂O to 1 L.

16. PCR reagents (Taq polymerase for genotyping, High fidelity polymerase for Sanger Sequencing, dNTPs, buffer, nuclease-free H₂O).

17. MC1061 competent E. coli cells or another competent cell line.

18. BsαI-HF.

19. Cutsmart buffer (NEB).

20. T₄ DNA ligase.

21. Calf intestinal phosphatase (CIP).

22. T₄ polynucleotide kinase (PNK).

23. TOP10 or equivalent competent E. coli cells.

24. gDNA or PCR amplified DNA for construct creation.

25. pMiniMad.

26. pDR244.

27. pPB41 or pPB105.
28. Genotyping primers are specific to the user and flank the locus of interest.

29. Optional: genotyping primers flanking *amyE* and/or *lacA* to confirm insertion.
   (a) *amyE*: 5'- TTCTTCGCTTGGCTGAAAAT-3' (forward),
       5'- CACCAGGGTTTTTGGTTTGCT-3' (reverse).
   (b) *lacA*: 5'- TAGACGAAAGGCAAGATT-3' (forward),
       5'- CTGGCGTTTTTCCGTGTAT-3' (reverse).

30. Primers compatible with pPB41 [13]:
   (a) oPEB217: 5'-GAACCTCATTACGAATTCAGCATGC-3'.
   (b) oPEB218: 5'-GAATGGCGATTTTCGTTCGTGAATAC-3'.

31. Primers compatible with CRISPR/Cas9 [13]:
   (a) oPEB232: 5'-GCTGTAGGCATAGGCTTGGTTATG-3'.
   (b) oPEB234: 5'-GTATTCACGAACGAAAATCGCCATTC
       CTAGCAGCAGC CCATAGTGACTG-3'.

2.2 General Equipment

1. 2 mL cryogenic screw top tubes for glycerol stocks.
2. Round bottom plastic tubes (14 mL) or glass tubes.
3. Wooden sticks or sterile loops for picking colonies.
4. Shaking incubator at 37 °C.
5. Shaking incubator at 25 °C.
6. Stationary incubator at 37 °C.
7. Stationary incubator at 42 °C.
8. Heat block at 65 °C (see Subheading 3.4).
9. Thermocycler.
10. Gel electrophoresis apparatus.
11. Sterile spreaders (glass beads, glass or plastic serological pipettes).
12. PCR tubes.

3 Methods

3.1 Traditional Gene Manipulation (2-Day Protocol)

3.1.1 Templates and Genotyping Primers

*B. subtilis* can undergo a double crossover event with DNA containing 100–500 bp (or more) flanking the site of exchange. The template can be plasmid-based, a PCR fragment, or genomic DNA with a selectable marker. For plasmid-based templates, there should be a site of recombination within it (can be the native or ectopic locus). For integration at an ectopic locus, we frequently use the nonessential gene *amyE* coding for starch utilization [19] or *lacA* coding for β-galactosidase [20]. Many different integration vectors using the *amyE* or *lacA* loci are readily available from the
Bacillus Genetic Stock Center (http://www.bgsc.org/_catalogs/Catpart4.pdf). These plasmids and methods described below can be used for a variety of *B. subtilis* strains including JH642, PY79, and NCIB 3610 with a deletion of a competence inhibitor *comI* [21]. Other strain backgrounds can also be found at the Bacillus Genetic Stock Center. To create and confirm the desired strain in 2–3 days (after preparing the template), be sure to order genotyping primers specific to the locus of interest before beginning the competency and transformation protocols. Genotyping primers are used to amplify the locus of interest to easily detect insertions or deletions.

### 3.1.2 Making *B. subtilis* Competent in a Laboratory Setting and Transformation

We recommend making *B. subtilis* competent each time a transformation is desired. For some protocols it is standard to freeze stocks of competent *Bacillus subtilis* using 20% glycerol; however, we prefer freshly prepared competent cells because they are often transformed more efficiently.

1. Streak plates from glycerol or DMSO stocks containing the strain to be manipulated and grow at 30–37 °C for 16 h.

2. Inoculate 2 mL LM medium (LB supplemented with 3 mM MgSO₄) with a single colony in a 14 mL round bottom tube and grow with shaking (200 rpm) for 3 h at 37 °C (until OD₆₀₀ ~ 1). See Note 1a.

3. Using sterile technique, transfer 20 μL of the LM culture to 500 μL MD medium in a test tube and grow for 4 h at 37 °C to reach stationary phase. See Note 1b.

4. Add 1–5 μL of template DNA to turbid MD cultures (up to 100 ng) and let grow for 90 min at 37 °C. Pre-dry antibiotic plates during this time to help with absorption of liquid.

5. Transfer 200 μL of turbid MD cells containing DNA onto respective antibiotic-containing plates and spread until completely dry using sterile glass beads or glass serological pipettes. See Note 2.

### 3.1.3 Genotyping and Storing Transformants

1. After ~16 h of transformation plate incubation, visible colonies will start to form. For clones with multiple antibiotic resistances, this could take 24–48 h. See Note 1c.

2. Restreak at least eight transformations for isolation on fresh antibiotic-containing plates and grow overnight at 30–37 °C. Restreaking of single colonies should be done at least twice. Try to pick a variety of colony sizes from different plate locations.

3. Perform colony PCR on *B. subtilis* colonies that grew following restreak on antibiotic(s). See Notes 3 and 4.
4. Grow positive transformants in LB with respective antibiotics for 4–6 h at 30 °C or 37 °C for glycerol or DMSO stock preparation followed by storage at −80 °C.

5. Add culture with up to 25% glycerol or 10% DMSO to screw top cryogenic tubes and freeze indefinitely at −80 °C.

3.1.4 Considerations

Strengths: After designing appropriate templates, positive transformants are achieved in 2 days. Media can be made in-house.

Limitations: Genetic manipulations using genomic DNA (gDNA) as the template need to be distant (~100,000 bp), or the risk of the WT copy recombining and replacing the altered locus is high, requiring further screening to obtain the correct genotype. All templates using this method rely on antibiotic selection to isolate transformants. When using gDNA, perform DNA dilutions to limit the likelihood for transformation and integration of non-labeled loci by congression (for more information please see ref. 1).

3.2 Deletion Library (Available Through the Bacillus Genetic Stock Center)

3.2.1 History and Mechanism of Deletion Library Strains and pDR244

Koo et al. created a library of erythromycin (BKE) and kanamycin (BKK) single gene interruptions in *B. subtilis* 168, consisting of 3968 and 3970 genes, respectively [14]. These stable, complete libraries allow for creation of multiple mutants with antibiotic markers that can be easily removed by plasmid-borne Cre recombinase driven from pDR244 [14]. Antibiotic cassettes are flanked by *lox*66 and *lox*71 sites, which are acted on by Cre recombinase at permissive temperatures to excise the flanked cassette and create a *lox* scar [14]. Plasmid pDR244 is easily cured from cells following incubation at the nonpermissive temperature, resulting in cells without antibiotic resistance and a clean deletion flanked by a *lox* scar [14]. The double mutant *lox*72 scar is reduced for Cre binding ability, allowing for multiple mutations to be made in the same background [22]. Additionally, the antibiotic cassettes lack a transcriptional terminator to reduce the likelihood of polar effects on downstream genes in an operon [14].

3.2.2 Competency, Transformation, and Recovery

If attempting recombination at a single locus from the deletion library, all protocol elements are the same as in Subheading 3.1.2. The DNA template would be gDNA from a strain obtained from the stock center with the desired change. When making multiple mutations dependent on a single antibiotic resistance cassette, integrate constructs sequentially. For example, transform *B. subtilis* with gDNA for one locus, screen, and make cryogenic stocks for each isolate. Next, remove the antibiotic resistance cassette from the newly created strain using pDR244. After confirmation, make competent cells from the deletion strain and transform with gDNA again and repeat the process. If moving markers from a different genetic background, check all available auxotrophic markers or other genetic markers in the newly created strain.
3.2.3 Use of pDR244 to Create a loxP-Flanked Clean Deletion (4-Day Protocol)

1. Streak cells onto plates from glycerol or DMSO stocks containing the strain to be engineered and grow at 30–37 °C for 16 h (day 1).

2. Inoculate 2 mL LM media (LB supplemented with 3 mM MgSO₄) with a single colony in a 14 mL round bottom tube and grow with shaking (200 rpm) for 3 h at 37 °C (until OD₆₀₀ ~ 1). See Note 1a.

3. Transfer 20 μL of the LM culture to 500 μL MD medium in a test tube and grow for 4 h at 37 °C to reach stationary phase. See Note 1b.

4. Add 1–2 μL of pDR244 (up to 100 ng total) to 500 μL of cells from MD tubes and let grow for 90 min at 37 °C for transformation. Pre-dry antibiotic plates containing spectinomycin (selection for pDR244) and the antibiotic within your gene interruption. See Note 2.

5. Transfer 200 μL of transformation reaction onto plates containing spectinomycin and spread until completely dry using sterile glass beads or glass serological pipettes.

3.2.4 Screening and Excising the Antibiotic Resistance Cassette

1. The next day (day 3), streak at least 8 transformants for isolation on spectinomycin plates and incubate at 37 °C for 16 h. See Note 1c.

2. Streak a single line onto a LB plate and grow at 42 °C for 12 h.

3. Streak a single line from the LB plate onto a new LB plate, and a plate containing spectinomycin+antibiotic (erythromycin if using BKE strain or kanamycin if using BKK strain), and a third plate containing antibiotic but lacking spectinomycin (in that order) and grow at 42 °C overnight. See Note 5.

4. The next day (day 4), assess growth on plates. Transformants containing clean deletions will have grown on LB only. If there is still growth on spectinomycin+antibiotic (erm or kan), then pDR244 was not cured. If there is growth on antibiotic only plates, the cassette remains interrupting the gene and has not recombined out. See Notes 3 and 4.

5. Grow culture and cryogenic stock as in method 1.

3.2.5 Considerations

Strengths: Gene interruption strains are available from the Bacillus Genetic Stock Center at the Ohio State University. Once your order is placed, the Stock Center will next-day ship your strains via UPS.

Limitations: The step-wise nature of making deletions takes >2 days.

3.3 pMiniMAD (6-Day Protocol)

3.3.1 History and Mechanism of pMiniMAD

The pMiniMAD (also known as pMiniMAD2) plasmid was developed by Patrick and Kearns in 2008 for allelic replacement [15]. The plasmid pMiniMAD contains a temperature sensitive origin of replication (ColE1) and an ampicillin and erythromycin...
cassette [15]. In *E. coli*, the plasmid replicates at the permissive temperature and can be selected for using ampicillin resistance. In *B. subtilis* at a nonpermissive temperature, a single crossover event will occur flanking the site of your choice and confer erythromycin resistance. *B. subtilis* transformants are subsequently grown without erythromycin at the permissive temperature, causing the plasmid to replicate and excise from the genome [15]. The result is markerless allelic replacement in your desired *B. subtilis* strain. An *E. coli* strain containing the pMiniMAD plasmid is available at the Bacillus Genetic Stock Center (strain ECE765). Around-the-world PCR amplification [23] of the plasmid can be done using only one primer set, allowing for easy ligation of your desired gene to create the full plasmid [15].

3.3.2 Competency and Transformation

1. The protocol for making competent cells and transformation is the same as in Subheading 3.1.2 (steps 1–5). See Note 1.
2. After plating transformants on erythromycin (Subheading 3.1.2, step 5), grow at 37 °C overnight (day 1). See Note 2.

3.3.3 Recovery and Screening

1. Pick four colonies from transformation plates and use to inoculate 3 mL each of LB. Incubate for 10 h at 25 °C (day 2).
2. Inoculate 30 μL of each culture (may not be visibly turbid, this is OK to continue with) into 3 mL of fresh LB and incubate at 25 °C overnight.
3. The next day (day 3), repeat steps 1 and 2.
4. Inoculate 30 μL of cultures again into 3 mL each of fresh LB. Grow at 37 °C for ~3 h (day 4).
5. Once the cultures reach OD$_{600}$ 1.1–1.3, perform tenfold serial dilutions in sterile saline ($10^{-4}, 10^{-5}, 10^{-6}$). Spread 200 μL of each dilution onto the respective LB plate. At the end of the day, let the plates grow overnight at 37 °C.
6. On day 5, identify which serial dilution for each culture results in the best isolation of individual colonies per plate (approximately 20–80). From that dilution plate, select at least 12 transformants and restreak for single colonies on LB and LB + erm. Grow overnight at 37 °C.
7. The next day (day 6), assess which colonies were sensitive to erythromycin. Perform colony PCR on these colonies to determine if the desired genetic changes is complete. See Notes 3 and 6.
8. Grow transformants in LB at 37 °C for glycerol or DMSO stocks. Sanger Sequence the PCR product of the region of interest from each strain to ensuring the nucleotide substitution or other genetic change is correct.
3.3.4 Considerations

Strengths: Creation of a construct without antibiotic resistance. pMiniMAD is easily changed and available through the Bacillus Genetic Stock Center.

Limitations: Takes 6 days and requires constant passaging and screening of many colonies. This does not work for essential genes or can be challenging for gene disruptions that cause a severe growth phenotype.

3.4 CRISPRi (2-Day Protocol)

3.4.1 History of CRISPRi and Its Application in B. subtilis

Peters et al. created a library of essential gene knockdowns in B. subtilis using CRISPR interference [16]. To perform CRISPRi, constructs have two alterations: lacA::P_{xyl}-dCas9 (erm^R) and amyE::P_{veg}-sgRNA(gene) (cm^R). The sgRNAs target the non-template strand [16]. P_{veg} drives expression of the sgRNA to the gene of interest during vegetative growth. dCas9 is induced with xylose and upon induction, binds the sgRNA, and blocks RNA polymerase from transcribing [16]. This method is important to study B. subtilis because it provides a conditional knockout or knockdown of essential genes or genes that cause a severe growth phenotype when disrupted or depleted.

3.4.2 Competency, Transformation, and Screening

1. Streak plates with glycerol or DMSO stocks containing the strain to be manipulated and grow at 30–37 °C for 16 h.
2. Inoculate 2 mL LM medium (LB supplemented with 3 mM MgSO_4) with a single colony in a 14 mL round bottom tube and grow with shaking (200 rpm) for 3 h at 37 °C (until OD_{600} ~ 1). See Note 1a.
3. Transfer 20 μL of the LM culture to 500 μL MD medium in a test tube and grow for 4 h at 37 °C to reach stationary phase. See Note 1b.
4. Add 1–5 μL your template DNA to 500 μL of cells from MD tubes (up to 100 ng) and let grow for 90 min at 37 °C and pre-dry antibiotic plates.
5. Transfer 200 μL of transformation reaction onto LB + cm + erm plates and spread until completely dry using sterile glass beads, or sterile glass serological pipettes. See Note 2.
6. After ~16 h of transformation plate incubation, visible colonies will start to form. For constructs with multiple antibiotic resistances, this could take 24 h or longer.
7. Restreak at least 8 transformations for isolation on fresh antibiotic-containing plates and grow overnight at 30–37 °C. Try to pick a variety of colony sizes from different plate locations.
8. Perform colony PCR on isolates that grew following restreak on antibiotics at both the amyE and lacA loci. See Note 7.
9. Grow positive transformants in LB with chloramphenicol and erythromycin for 4–6 h at 37 °C for subsequent glycerol or DMSO stock preparation.

10. Add culture with up to 25% glycerol or 10% DMSO to cryogenic screw-top tubes and freeze indefinitely at −80 °C.

3.4.3 Use of Newly Constructed Strains

1. To test interference of your desired gene, we recommend titrating xylose at several different concentrations (or percentages) on LB plates. Start in tenfold increments and then fine-tune within the increments based on your desired result. Alternatively, perform qPCR to measure transcript levels quantitatively.

2. Be sure to keep both erythromycin and chloramphenicol in the media when you use the CRISPRi strains.

3.4.4 Considerations

Strengths: This protocol is fast and includes essential genes. The researcher can fine-tune the level of transcript by carefully modulating gene expression.

Limitations: The desired titration amount is up to the researcher and is determined experimentally. Starting concentrations are usually 0.005% with 1% xylose (w/v) used for maximal induction. In addition, there are two antibiotic cassettes and integrations for a single CRISPRi strain.

3.5 CRISPR/Cas9 (2-Day Protocol)

3.5.1 History of CRISPR/Cas9 Manipulation of Genes in B. subtilis

With the recent application of CRISPR/Cas9 to create mutations in many organisms, an efficient protocol was established for manipulation of B. subtilis by Burby and Simmons [13, 18]. CRISPR/Cas9 allows the user to make deletions, fusions, and point mutations in multiple backgrounds. The plasmids required for this procedure are available at the Bacillus Genetic Stock Center [13, 18]. The editing plasmids pPB41 (SpecR and AmpR) or pPB105 (CmR and AmpR) are modified to insert a proto-spacer sequence to target the locus of interest [13, 18]. The second modification of pPB41 serves as the editing DNA template for introduction into the genome [13, 18]. After constructing both pieces of pPB41 using standard molecular biology methods, the final editing plasmid is assembled. In short, the plasmid backbone containing antibiotic resistance, editing template, Cas9, and protospacer sequences are ligated using Gibson Assembly [24] to create one final plasmid. The resulting plasmid can be used to transform B. subtilis to introduce the desired genetic change and is subsequently cured from cells with ease [13].

3.5.2 Protocol for Creating Plasmid with Proto-Spacer for CRISPR/Cas9 Alteration

1. Digest pPB41 or pPB105 with restriction endonuclease BsaI.

2. Construct a phosphorylated proto-spacer for insertion into pPB41 or pPB105.

3. Ligate plasmid and proto-spacer together, transform E. coli, and isolate plasmid.
3.5.3 Protocol for Creating Editing Plasmid

1. Use oPEB217 and oPEB218 with Q5 DNA polymerase to linearize pPB41. Gel extract and purify PCR product.

2. Use oPEB232 and oPEB234 with Q5 DNA polymerase to PCR amplify CRISPR/Cas9 from plasmid created in step 1. Gel extract and purify PCR product.

3. PCR amplify editing template, gel extract, and purify.

4. Assemble full editing plasmid using Gibson Assembly.

3.5.4 Transformation of Plasmids and Screening of Transformants

1. Make desired *B. subtilis* strain competent using Subheading 3.1.2.

2. After incubation in MD culture, add 200–600 ng of editing plasmid DNA and incubate for 60–90 min at 37 °C.

3. Plate 200 μL transformants on LB + spec and incubate at 30 °C overnight.

4. Restreak single colonies on LB + spec for purity.

5. Cure isolates of plasmid by restreaking on LB for single colonies and incubating overnight at 45 °C.

6. Screen isolates for plasmid loss by restreaking single colonies onto LB and LB + spec and incubate again overnight at 45 °C. There should be no growth on LB + spec plates if cells are cured of the plasmid.

7. Use genotyping primers to confirm alteration of interest.

3.5.5 Considerations

Strengths: It is possible to introduce multiple mutations of your own design in many different backgrounds with high efficiency (80–100% positive clones). This method lacks antibiotic cassettes or remnants of vector DNA used during cloning.

Limitations: Mutations are contingent upon a proto-spacer adjacent motif (PAM) sequence, NGG in *B. subtilis*, near to the desired locus and requires more reagents than the other methods.

4 Notes

1. On competency and transformation: (a) Glass or plastic tubes both work well for incubation of cells for transformation; be sure the culture takes up 1/10 or less of the container volume for proper aeration. Shaking incubation can be performed in a test tube rack in a warm room, water bath, or a rolling rack within an incubator. (b) Prepare MD tube for each transformation. (c) If initial transformation of *B. subtilis* with DNA is unsuccessful or for strains that grow more slowly due to other genetic changes, increasing the incubation in LM media could be extended from 4 h to ~6 h, and incubation with gDNA could be increased from 90 min to 3 h.
2. On plating transformations: Pre-drying plates before spreading transformation mix is important to help the liquid absorb into the plate for growth of single transformant colonies. Any remaining transformation culture can be spread onto another plate (as is or diluted with MD medium to make 1:2, 1:5, and/or 1:10 dilutions).

3. On confirmation of transformants: Use genotyping primers to confirm correct amplicon size of transformants. It is recommended to submit samples for Sanger Sequencing when making substitutions or piecing together constructs using Gibson Assembly [24]. If using the \textit{amyE} locus, add up to 1 mL iodide solution to starch plate “patch” restreaks. If the gene is disrupted, and your construct was successfully inserted, you will no longer see a starch clearing or “halo” around the patch. Please note that iodide treatment is lethal to the cells on the starch plate.

4. On ensuring a double crossover event with exogenous DNA (method 1): Homology between the desired \textit{B. subtilis} locus and amplicon DNA will help increase likelihood of recombination. If homology is sufficient and transformation is unsuccessful, titrating the amount of amplicon DNA in the transformation could lead to the desired recombination event.

5. Notes on curing an antibiotic resistance marker using pDR244 (method 2): It is not necessary to go back to the original LB streak when streaking plates containing antibiotics to assess if pDR244 has been cured. Simply collect some bacteria from the first plate and continue streaking a single line on all plates. Due to the high cell density of bacteria from the original plate during patch plating, there are still enough cells to streak on the last plate of the series.

6. On increasing the number of markerless desired clones (method 3): After transformation and integration of pMiniMad, passaging >4 cultures through room temperature incubations and screening >12 colonies from each could allow the researcher to find additional clones.

7. On CRISPRi (method 4): As the guide RNA is driven by a promoter inserted at \textit{amyE}, streak a single line onto LB plates containing 10 g/L starch to ensure this locus has been disrupted. Streak WT as a control and incubate with the other antibiotic plates. Use genotyping primers to confirm correct amplicon size of transformants. To determine if the \textit{amyE} locus is disrupted, add up to 1 mL iodide solution to starch plate streaks. You will no longer see a starch clearing or “halo” if the insertion at \textit{amyE} occurred.
Acknowledgments

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Highly Efficient Genome Editing in *Clostridium difficile* Using the CRISPR-Cpf1 System

Wei Hong, Jie Zhang, Guzhen Cui, Qian Zhou, Pixiang Wang, and Yi Wang

**Abstract**

*Clostridium difficile* is often the primary cause of nosocomial diarrhea, leading to thousands of deaths annually worldwide. The availability of an efficient genome editing tool for *C. difficile* is essential to understanding its pathogenic mechanism and physiological behavior. Here, we describe a streamlined CRISPR-Cpf1-based protocol to achieve precise genome editing in *C. difficile* with high efficiencies. Our work highlighted the first application of CRISPR-Cpf1 for genome editing in *C. difficile*, which are both crucial for understanding pathogenic mechanism of *C. difficile* and developing strategies to fight against *C. difficile* infection (CDI). In addition, for the DNA cloning, we developed a one-step-assembly protocol along with a Python-based algorithm for automatic primer design, shortening the time for plasmid construction to half that of conventional procedures. Approaches we developed herein are easily and broadly applicable to other microorganisms. Our results provide valuable guidance for establishing CRISPR-Cpf1 as a versatile genome engineering tool in prokaryotic cells.

**Key words** *Clostridium difficile*, *C. difficile* infection, Genome editing, CRISPR-Cpf1

**1 Introduction**

*Clostridium difficile*, recently renamed as *Clostridioides difficile* [1], is a gram-positive strictly anaerobic human pathogen. *C. difficile* infection (CDI) is the leading cause of nosocomial diarrhea, which poses a major threat to health care facilities, including long-term care facilities, nursing homes, and hospitals worldwide [2, 3]. The clinical symptoms of CDI range from mild diarrhea to pseudomembranous colitis, which may result in death.

CRISPR-Cpf1 is an endonuclease-based immune system that has been recently explored for genome engineering purposes [4]. Compared to CRISPR-Cas9, the CRISPR-Cpf1 system has several distinctive features: (1) The Cpf1 nuclease is guided by a
single crRNA to target the DNA locus, and no tracrRNA is needed (Fig. 1b–d); (2) The Cpf1 protein recognizes a T-rich PAM sequence (5'-TTTN-3') located at the 5'-end of the target DNA sequence (Fig. 1c); (3) Cleavage by the CRISPR-Cpf1 RNP complex is staggered, creating a 5-nt 5'-overhang that is 18–23 bp away from the PAM site (Fig. 1c) [5]. In addition, it has been reported that Cpf1 likely has lower toxicity to the host cells [6], and lower off-target effects than Cas9 for genome editing [7]. Recently, the CRISPR-Cpf1 system has been engineered as a robust genome-editing tool with applications to various eukaryotic and prokaryotic...
organisms, including rice [8], soybean [9], mouse [10], zebrafish [11], human cell [7], *Escherichia coli, Yersinia pestis, Mycobacterium smegmatis* [12], and *Corynebacterium glutamicum* [6]. However, compared to the case in eukaryotic systems, the full potential of the CRISPR-Cpf1 system for genome editing in prokaryotic cells has not been well established for large gene deletion and multiplex genome editing. Based on the distinct features of CRISPR-Cpf1 when compared to CRISPR-Cas9 as discussed above, we rationally conjectured that CRISPR-Cpf1 would be a more suitable and easily programmable tool for efficient genome engineering in microbial hosts like *C. difficile* which have naturally low DNA transformation/conjugation efficiencies.

Therefore, in the present work, we developed a streamlined CRISPR-Cpf1-based toolkit for genome engineering in *C. difficile* and demonstrated the application of CRISPR-Cpf1 in a microorganism that is generally recalcitrant to be genetically engineered. We applied the developed CRISPR-Cpf1 system to achieve highly efficient genome editing in *C. difficile* 630. Meanwhile, for the DNA cloning work to construct the CRISPR-Cpf1 vector, we established a one-step-assembly (OSA) approach, in which a new CRISPR-Cpf1 plasmid for genome editing can be obtained within 2 days by assembling all the component fragments into the chassis plasmid in a single step. Moreover, a Python-based algorithm was developed to facilitate the design of the required primers within seconds in a highly semiautomatic fashion. Altogether, these make the implementation of the CRISPR-Cpf1 system highly streamlined and easily applicable to diverse microorganisms.

## 2 Materials

### 2.1 Plasmids and Strains

All the strains and plasmids used in the present protocol are listed in Table 1.

The NEB express *E. coli* strain (New England BioLabs, Ipswich, MA) was used as the general host for plasmid propagation and gene cloning. *E. coli* CA434 was used as the donor strain for the conjugation of *C. difficile*.

### 2.2 Reagents and Antibiotics

1. Luria–Bertani (LB) medium: tryptone (10 g/L), yeast extract (5 g/L), sodium chloride (10 g/L).
2. LB plates: LB with 15 g/L agar.
3. Brain Heart Infusion Supplemented (BHIS) medium: brain heart infusion broth, 0.5% yeast extract, 1.5% agar.
4. 100 μg/mL ampicillin in ddH₂O (Ap).
5. 30 μg/mL chloramphenicol in ethanol (Chl).
6. 100 μg/mL kanamycin in dH₂O (Kan).
7. 15 μg/mL thiamphenicol in N, N-dimethylformamide (Tm).
8. 250 μg/mL D-cycloserine (D-cyc) in ddH₂O.
9. 8 μg/mL cefoxitin in ddH₂O (Fox).
10. Sterilized 75% (v/v) glycerol.
11. 75% (v/v) ethanol.
12. DNAse/RNase-free water.
13. 1.5 mL centrifuge tubes.
14. 15 mL cell culture tubes.

Table 1
Strains and plasmids used in this protocol

| Strains     | Description                                                                 | Sources                                      |
|-------------|------------------------------------------------------------------------------|----------------------------------------------|
| C. difficile 630 | Wild-type strain                                                            | From Dr. Shonna McBride (Emory University)  |
| E. coli CA434  | hsd20(r<sup>R</sup>, m<sup>R</sup>), recA13, rpsL20, leu, proA2, with IncPb conjugative plasmid R702 | Lab stock                                   |
| NEB express competent E. coli (High Efficiency) | fhuA2 mpT gal sulA11 R(mcr-73::miniTn10--Ter<sup>b</sup>)2 [dem]R(zygb-210::Tn10--Ter<sup>b</sup>)endA delta(mcrC- mrr)114::IS10 | NEBa                                      |
| ∆cwp66 | Derived from C. difficile 630, ∆cwp66                                         | This work                                   |
| Plasmids     | Description and Relevant characteristics                                       | References                                  |
| pDEST-hisMBP-AsCpf1-EC | E. coli codon optimized AsCpf1 coding sequence, Am<sup>R</sup> | Addgene, #79007                            |
| pMTL82151<sup>b</sup> | pBP1 ori, Cm<sup>R</sup>, ColE1 ori, TraJ                                   |                                              |
| pWH34S       | Derived from pMTL82151, E. coli–C. difficile shuttle vector, iLacP::AsCpf1, “Chassis” plasmid for gene-targeting plasmid construction, Cm<sup>R</sup>/Tm<sup>R</sup> | This work                                   |
| pWH34        | Derived from pMTL82151, E. coli–C. difficile shuttle vector, iLacP::AsCpf1, iBtI-BtgZI-BtgZI double sites, “Chassis” plasmid for gene-targeting plasmid construction, Cm<sup>R</sup>/Tm<sup>R</sup> | This work                                   |
| pWH37        | Derived from pWH34, iLacP::AsCpf1, sRNAP::crRNA (23-nt repeat), Cm<sup>R</sup>/Tm<sup>R</sup>, PCR template for generating sRNAP::crRNA for retargeting | This work                                   |
| pWH55        | Derived from pWH34, targeting cell wall protein cwp66 (cwp66) gene, Cm<sup>R</sup>/Tm<sup>R</sup> |                                              |

<sup>a</sup>New England Biolabs Inc., Ipswich, MA
<sup>b</sup>J.T. Heap, O.J. Pennington, S.T. Cartman, N.P. Minton. A modular system for Clostridium shuttle plasmids. Journal of Microbiological Methods 78 (2009) 79–85
15. Electroporator.
16. Electroporation cuvettes, 0.1 and 0.2 cm.

2.3 Primers
Primers required used in the present protocol were synthesized are listed in Table 2.

2.4 Molecular Cloning Reagents
1. High-Fidelity DNA polymerase.
2. Long amplicon DNA Polymerase, such as LongAmp (NEB).
3. Gibson DNA cloning kit, such as NEBuilder HiFi DNA Assembly Master Mix or equivalent.
4. Restriction enzymes: BtgZI, NotI, NdeI, and BamHI.
5. Plasmid isolation kit.
6. Gel extraction kit.

3 Methods

3.1 Construction of the Mother Vector pWH34
1. Amplify the lactose inducible promoter (iLacP) from pYW51 [13] using primers YW2817 and YW2818. The 5'-end and 3'-end of the amplicon contain a 30-bp and 35-bp overlapping arm to either the plasmid backbone or the Cpf1 gene, respectively.
2. Amplify E. coli codon optimized Cpf1 gene (from Acidaminococcus sp. BV3L6; AsCpf1) from pDEST-hisMBP-AsCpf1-EC (Addgene, #79007) using primers YW2819 and YW2820. The 5'-end and 3'-end of the amplicon contain 35-bp and 30-bp arm homologous to either iLaP or the plasmid backbone, respectively.
3. Linearize the pMTL82151 plasmid with NdeI restriction digestion site in and assemble the product with iLacP and Cpf1 amplicons by using NEBuilder® HiFi DNA Assembly Master Mix (NEB). The resultant plasmid is denoted pWH34S (pMTL82151::iLacP::Cpf1).
4. Amplify the terminator of thiolase gene (thlT) from C. beijerinckii 8052 genomic DNA using primers YW2823 and YW2824 (Table 2), which contains two oppositely oriented BtgZI sites to facilitate the insertion of exogenous DNA fragments.
5. Digest pWH34S using BamHI. Recover the digestion product and assemble with thlT amplicon; the resultant plasmid is denoted pWH34 (Fig. 3a).

3.2 Construction of pWH37 Containing the “Repeat-Spacer” PCR Template
1. Amplify the small RNA promoter (sRNAP, promoter of sCbei_5830 gene) from C. beijerinckii 8052 genomic DNA using primers YW2821 and YW2822 (Table 2). On the YW2822 primer, the “repeat-spacer” sequence of the
Table 2
Primers used in this protocol

| Primers | Sequences (5’-3’) |
|---------|------------------|
| YW2817  | GGAACACAGCTATGACCCGCGCCGCTGTATCttatagtcttgatttattttgattatt |
| YW2818  | tgcagagctctgaagccctcatacctgtctATCACGACCTCCTGTGAATTTGGTATCGCTCAAA |
| YW2819  | TTGTGAGCGGATAACAATTTCACAGGAGGGCTGAAatgacacagttgaaaagcttcaccaatctctacca |
| YW2820  | gggtaccgagcgttaattcgaatatcattgtttATTAGTTTCTCAGTTCCTGAATGTTAGCCAG |
| YW2821  | tagaatccgaatgctgtacccgggtTATAATTAAGATTTAAAGGTTA |
| YW2822  | CGTGACGTGACTCTAGAtattagaagctctttctatcagttgATCTACAAGAGTAAATTAtagggtgatgattaggtt |
| YW2823  | tagaatccgaatgctgtacccgggtTATAATTAAGATTTAAAGGTTA |
| YW2824  | CGTGACGTGACTCTAGAtgcttgcagtCATCGCtgactcatGCGATGagctaggtcATTTCTATTTTTCCTTATTTTCTTCTT |
| YW3105  | aaagttaaaagaagaaatagaaatATAATCTTTAATTTGAAAAAGATTTA |
| YW3304  | ATTAATTAGCTGCTAAATAACCCACTGACTCAACTCAAGTAGAAATATTATGTTGGAATGATAAGGGTT |
| YW3305  | TTTTGTAAAAAGGTATTTTTAATATTATTTTCCTCCTCTACCTTTCCG |
| YW3306  | CGAAAAAGTATTAAGGTAAGAAATATAATTATAATTAAATCTTTTAAAGGAAAA |
| YW3307  | CTCCATGGACGGCGTCGCTCAACTGCTGAGACAGGTATTGTTGACACTA |
| YW3308  | TGGAGGCTCATGTCTTATC |
| YW2369  | GCTGAGACAGGTATTGTTGACA |
| YW2370  | GCTGAGACAGGTATTGTTGACA |
CRISPR-Cpf1 system downstream of sRNAP (5'-TAATTTCT ACTCTTGTAGATCATCTGATAAGAAGGACTTAATA-3') is added.

2. Digest pWH34 plasmid with BamHI and the digestion product is assembled with “repeat-spacer” amplicon; the resultant plasmid is denoted pWH37.

Fig. 3 The configuration and construction of pWH34, pWH55 by using OSA. (a) A universal chassis plasmid pWH34 was constructed to facilitate constructing a series of gene-targeting plasmids. pWH34 is an assembly of the following fragments: (1) Ndel-linearized pMTL82151 plasmid as backbone (pBP1 replicon, CatP marker, ColE1 + tra gram-negative replicon and MCS); (2) Cpf1 coding sequence driven by a lactose inducible promoter (iLacP::Cpf1); (3) thiola terminator from C. beijerinckii, and (4) a 40-bp synthetic DNA fragment containing two oppositely oriented BtgZI sites. (b) The pWH55 plasmid is a derivative of pWH34, targeting the 1833-bp cell wall protein 66 kD (cwp66) gene (CD630_27890) of C. difficile 630. (c) A schematic representation of the one-step-assembly (OSA) approach. Briefly, the cwp66 specific sRNAP::crRNA-cwp66, the two ~500-bp cwp66-Up-arm and cwp66-Down-arm (flanking the cwp66 gene) are assembled with the BtgZI-linearized pWH34 in one step to generate pWH55.
3.3 Design of Gene-Targeting Primers by Using One-Step-Assembly Primer Finder (OPF) Algorithm

1. Install Python 2.7 (https://www.python.org/) on your computer.

2. Collect the following information: (1) the sequence of the target gene; and (2) the upstream and downstream homology arms to be used.

3. Double-click the “OPF.py” file (Data 1) to launch the algorithm.

4. Input gene sequence into the blank as indicated by the algorithm. All potential protospacers in either sense or antisense strand (based on the User’s selection) of the target gene will be identified and listed. The Primer 0 corresponding to each protospacer will be generated and depicted underneath the protospacer. The position (site, sense/antisense), reverse complementary sequence (RVS), GC content (GC%) and Tm value (Tm) for each protospacer will also be determined and listed. Based on the selected protospacer (depending on the User’s decision), Primers 1, 2, 3, and 4 will be generated (Fig. 2).

3.4 Construction of Gene-Targeting Vector by One-Step Assembly (OSA): Taking cwp66 Gene as an Example

1. The cwp66 (CD630_27890) gene sequence and its upstream and downstream arm sequences are downloaded from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1).

2. The spacer (5’-gcagtgggtgtattagcagctaa-3’) within cwp66 gene sequence is selected as the target.

3. Primer pairs YW3304/YW3105 (Universal primer), YW3305/YW3306, YW3307/YW3308 are designed by OPF algorithm, which are presented as primer 0-YW3105, primer 1-primer 2, and primer 3-primer 4, respectively, by the OPF algorithm.

4. Amplify iLacP::crRNA (iLacP::repeat-spacer-repeat) fragment using primers YW3304/YW3105 and pWH37 as the template with a high-fidelity DNA polymerase according to manufacturer’s instructions.

5. Amplify the upstream and downstream arm of cwp66, by using C. difficile 630 genomic DNA as the template and YW3305/YW3306 (for the upstream arm), YW3307/YW3308 (for the downstream arm) as primers with a high-fidelity DNA polymerase.

6. These three amplicons (iLacP::crRNA, upstream arm, and downstream arm) are purified and assembled with the BtgZI-linearized pWH34 to generate pWH55 plasmid (One Step Assembly, OSA, Fig. 3b, c), which targets the cwp66 gene specifically.

3.5 Conjugation and Mutant Screening

1. Electroporate pWH55 into E. coli CA434 strain and plate onto LB containing chloramphenicol (Chl) and kanamycin (Kan), and incubate at 37 °C overnight.
**Fig. 2** Design primers using the OSA Primer Finder (OPF) to construct the gene-targeting CRISPR-Cpf1 plasmid (taking the plasmid for the deletion of *cwp66* as an example). The *cwp66* gene sequence along with the upstream ~1000 bp and downstream ~1000 bp is first downloaded from NCBI (Accession number: AM180355.1). Before launching OPF, Python 2.7 (https://www.python.org/) should be preinstalled on the computer. Then, the OPF program can be launched by double-clicking the "OPF.py" file (Data 1). The *cwp66* gene sequence can then be input into the appropriate blank as indicated by the algorithm. Then the User will...
2. One to three transformants are picked and inoculated into LB medium (supplemented with Chl and Kan) and incubated for 8–12 h at 200 rpm and 37 ºC.

3. One milliliter of pWH55-harboring *E. coli* CA434 culture is centrifuged at 2841 × g for 3 min and then washed once with sterilized LB medium.

4. Transfer the cell pellet to an anaerobic chamber and mix with 150 μL of an overnight culture of *C. difficile* 630, which has been grown in BHIS medium (with an OD<sub>600</sub> of 0.8, approximately).

5. The mixture is spotted onto the BHIS agar plate (1.5% of agar; prerduced in the anaerobic chamber for at least 12 h) with 20 μL per spot. Then incubate the plate in the chamber at 37 ºC for 10 h.

6. After the 10 h incubation, 1 mL of BHIS liquid medium (prerduced in the anaerobic chamber for overnight) is added onto the plate surface to resuspend the cells.

7. A 100 μL aliquot of the harvested cells is spread onto the BHIS-TDC agar plate, which is BHIS medium containing thiamphenicol (15 μg/mL), D-cycloserine (250 μg/mL) and cefoxitin (8 μg/mL), and incubated at 37 ºC for 36 h.

8. After incubation, conjugants are formed on the plate. Colonies are picked and inoculated into 5 mL BHIS-Tm medium, which is BHIS medium containing 15 μg/mL thiamphenicol. The culture is incubated at 37 ºC anaerobically for 12 h.

3.6 Induce the Expression of Cpf1 and Screen for Positive Mutants

1. Cell culture is spread across the agar plate of BHISL-Tm medium, which is BHIS medium containing 40 mM lactose and 15 μg/mL thiamphenicol, and incubated at 37 ºC for 36 h.

2. Diagnostic primers (YW2369 and YW2370)—flanking the *cwp66* gene—are used to identify clones with desired mutation.

Fig. 2 (continued) be asked whether he/she prefers to identify protospacer sequences on the “Sense or antisense” strand. After inputting the answer and pressing the “enter” key, all potential protospacers in either sense or antisense strand (whichever has been selected by the User) of *cwp66* will be listed. At this point, the Primer 0 corresponding to each protospacer will be generated and listed underneath the protospacer. Furthermore, the position (site; sense/antisense), reverse complementary sequence (RVS), GC content (GC %) and T<sub>m</sub> value (T<sub>m</sub>) for each protospacer will also be determined. Then, the User can select one specific protospacer to use based on his/her own criteria (GC%, site, etc.). After inputting the “Up-arm sequence” and “Down-arm sequence” and defining the ‘Length of overlap regions of adjacent fragments’ (the region on the homology arm that is covered by the primer; the primers are designed to amplify fragments with extended sequence for the Gibson Assembly purpose), the associated Primers 1, 2, 3, 4 will be generated. The primer pairs of 0/YW3105 (the universal primer), 1/2, and 3/4 will be used to generate iLacP::crRNA, upstream arm, and downstream arm, respectively. These three fragments can then be assembled with the *BtgZI*-linearized pWH34 to generate the corresponding plasmid targeting on the specific gene (*cwp66* here) through OSA
The PCR with the wild-type \textit{C. difficile} 630 strain generates 3183-bp amplicon, whereas the PCR with the $\Delta$\textit{cwp66} mutant produces 1350 bp PCR product (Fig. 4).

3. The gene-targeting efficiency is calculated based on the number of positive mutant divided by total screened colonies \[(\text{number of mutants/total screened colonies}) \times 100\%\].

### 3.7 Plasmid Curing

1. The PCR-verified $\Delta$\textit{cwp66} mutant is cultured and transferred in antibiotic-free BHIS medium for several generations (about 10 generations within 5 days) at 37 $^\circ$C anaerobically.

2. The resultant culture (after 10 transfers) is spread on a BHIS agar plate. Each colony grown from the plate is then picked and dotted on both BHIS-Tm plate and BHIS plate (replica plating).

3. The colony that can only grow on BHIS plate is picked and inoculated into the BHIS liquid medium and incubated at 37 $^\circ$C overnight before it is stored (see Subheading 3.9).

### 3.8 Verification and Storage of Mutant

1. YW2369 and YW2370 primers are used to amplify corresponding fragments (flanking the targeted mutation locus) using the genomic DNA of either the wild-type \textit{C. difficile} 630 or $\Delta$\textit{cwp66} strain as the template. Amplicons are subjected to Sanger sequencing to verify the desired gene editing.
3.9 Storage of Mutant Strains

1. The Δcwp66 mutant strain of the mid-log phase grown in BHIS medium is stored in 15% glycerol at −80 °C.

4 Notes

1. Due to the large size of the Cpf1 gene, the amplification could easily fail. Make sure to use a high-fidelity PCR DNA polymerase which is competent to amplify long DNA fragment. We recommend Phanta Max Super-Fidelity DNA Polymerase from Vazyme Biotech Co., Ltd., Nanjing, China (see Subheading 3.1, step 2).

2. Make sure the mother vector pMTL82515 was fully digested by NdeI; undigested plasmid backbone makes the screening process problematic (see Subheading 3.1, step 3).

3. We recommend to use NEBuilder® HiFi DNA Assembly Master Mix from NEB to assemble three amplicons together. It gives stable assembly efficiency for OSA method (see Subheading 3.4, step 6).

4. The conjugation efficiency is low. Make sure Chl and Kan were added to LB medium when propagate CA434 strain (see Subheading 3.5, steps 1 and 2).

5. Make sure BHIS agar plates were prestored in an anaerobic chamber at least for 12 h (see Subheading 3.5, step 5).

6. Do not spread too much harvested cells onto the BHIS-TDC agar plate; the maximum volume we tried was 250 μL (see Subheading 3.5, step 7).

7. When detecting large DNA fragment, an appropriate DNA polymerase that efficiently amplifies long PCR products should be used. In our experience, the LongAmp PCR Master Mix from NEB gives stable performance, though other polymerases may also work (see Subheading 3.6, step 2).

8. We highly recommend that all BHIS medium used to transfer C. difficile cultures should be placed in an incubator (37 °C) in the anaerobic chamber to preclude potential contaminations. The contamination would easily lead to failure of plasmid curing (see Subheading 3.7, step 1).

9. We usually pick cell biomass from the colony formed on the BHIS plate to use directly as the template for the PCR. Please note that too much cell biomass may lead to failure of the PCR (see Subheading 3.8, step 1).
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Genome Editing of *Corynebacterium glutamicum* Using CRISPR-Cpf1 System

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Abstract

*Corynebacterium glutamicum*, as an important microbial chassis, has great potential in industrial application. However, complicated genetic modification is severely slowed by lack of efficient genome editing tools. The *Streptococcus pyogenes* (*Sp*) CRISPR-Cas9 system has been verified as a very powerful tool for mediating genome alteration in many microorganisms but cannot work well in *C. glutamicum*. We recently developed two *Francisella novicida* (*Fn*) CRISPR-Cpf1 assisted systems for genome editing via homologous recombination in *C. glutamicum*. Here, we describe the protocols and demonstrated that N iterative rounds of genome editing can be achieved in 3 \( N + 4 \) or 3 \( N + 2 \) days, respectively.

**Key words** *Corynebacterium glutamicum*, CRISPR-Cpf1 system, ssDNA, Donor DNA, Genome editing, Homologous recombination

1 Introduction

*Corynebacterium glutamicum* is a gram-positive and anaerobic bacterium, which was firstly isolated from soil [1]. It has been widely applied as an industrially important producer of flavorings, cosmetics, vitamins, organic acids, higher alcohols, and polymers [1–4]. Due to its industrial importance, many allelic exchange methods assisted by counterselection system or phage recombination proteins have been developed for precise genome alternation [5–9]. However, low recombineering efficiency and lack of positive selection for mutations lead to laborious work on colony screening [5, 8, 9].

Regularly interspaced short palindromic repeats (CRISPR) system is a disruptive technology to facilitate genome editing, which can increase the frequency of mutants with single or double crossover [10–13]. DNA double strands break created by Cas9 nuclease from *Streptococcus pyogenes* or DNA single-strand break created by Cas9 nickase can filter out most false transformants that did not complete DNA repair by homologous recombination (HR) or
nonhomologous end-joining (NHEJ) [14]. In our hands, genome editing of *C. glutamicum* cannot be achieved using a Class 2 CRISPR-Cas prokaryote immunity system as previously reported [15, 16]. Fortunately, we found that Cpf1 (a single-strand RNA–guided endonuclease of the class 2 CRISPR-Cas system) from *Francisella novicida* (*Fn*) can efficiently cleave targeted DNA in *C. glutamicum* [15], with some very different features from Cas9 as shown in Fig. 1. For example, *Fn*Cpf1 recognizes a T-rich protospacer-adjacent motif (PAM) and cuts in staggered ends at the site distant from PAM. Besides, its guiding RNA is a single RNA, in which 24 nt pair with the target gene [17–19]. These features could make CRISPR-cpf1 system less toxic and easier design than CRISPR-Cas9 to the recipient cells [15].

Here, we demonstrate that genome editing of *C. glutamicum* can be efficiently performed using two CRISPR-Cpf1-assisted systems (Fig. 2). A double-plasmid–based CRISPR-Cpf1 system composed of Cpf1 and RecT expression plasmid, crRNA expression plasmid and single-stranded DNA (ssDNA) can precisely introduce nucleotides mutation or small fragment deletion (Fig. 4); An all-in-one plasmid consisting of FnCpf1, CRISPR RNA, and homologous arms (Fig. 3c) can be used to facilitate large gene deletions and insertions (Fig. 5). N iterative rounds of genome editing can be
achieved in $3N + 4$ or $3N + 2$ days, respectively, using the two CRISPR-Cpf1-assisted systems. Several investigators have also realized genome editing in *Corynebacterium* using similar CRISPR-Cpf1 systems [20–22].

## 2 Materials

### 2.1 Materials for Cell Culture

1. Luria–Bertani (LB) broth: Add the following to about 800 mL of dH$_2$O; 10 g tryptone extract, 5 g yeast extract, 10 g NaCl (and 15 g agar for solidified medium). Dissolve, and adjust final volume to 1 L. Sterilize by autoclaving for 20 min under 115 °C. LB is routinely used for *Escherichia coli* DH5α with purpose of plasmids construction.

2. Brain heart infusion Sorbitol (BHIS) medium: Add the following to about 900 mL of dH$_2$O; 37 g brain heart infusion (BHI; Bacto), 500 mM sorbitol (and 15 g agar for solidified medium). Dissolve and adjust final volume to 1 L. Sterilize by autoclaving for 20 min under 115 °C. BHIS medium supplemented with 10 g/L of glucose (BHISG) is routinely used for *C. glutamicum* ATCC 13032 with purpose of culture, competent cell preparation, electrotransformation, and mutant screening.

3. 100 μg/mL ampicillin (Amp) in agar and liquid medium (from a stock solution of 100 mg/mL in dH$_2$O).

4. 25 μg/mL kanamycin (Kn) in agar and liquid medium (from a stock solution of 50 mg/mL in dH$_2$O).

5. 50 μg/mL spectinomycin (Sp) in agar and liquid medium (from a stock solution of 100 mg/mL in dH$_2$O).

### 2.2 Cell Direct PCR

1. Sterile water: Autoclave.

2. KOD-plus-neo polymerase set (TOYOBO BioTechnology, CO., LTD, Japan): containing KOD-plus-neo polymerase (1.0 U/μL), 10× PCR buffer, MgSO$_4$ (25 mM) and dNTP mixture (2 mM each). Or equivalent robust DNA polymerase.

3. Taq DNA Polymerase set: containing Taq DNA Polymerase (5 U/μL), 10X PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), MgSO$_4$ (50 mM), and dNTP mixture (2 mM each).

### 2.3 DNA Electrophoresis

1. TAE Electrophoresis buffer (50× stock): 242 g Tris–base, 57.1 mL glacial acetic acid (17.4 mol/L), 100 mL 0.5 M EDTA, adding dH$_2$O to 1000 mL, pH 8.0.

2. TAE Electrophoresis buffer (0.5×): 20 mM Tris–acetate, 0.5 mM EDTA, pH 7.9. Prepare from a 50× stock.
3. Agarose gels: 1% (w/v) ultra-pure agarose in 0.5 × TAE. The suspension is then brought to a microwave oven boil to dissolve the agarose, cooled down to 50 °C and poured onto 20- or 30-well horizontal gel casting plates. 0.5 μg/mL ethidium bromide (EB) is added in the gel and shake well prior to casting or used for post-electrophoresis staining of the gel.

2.4 Molecular Biology Reagents

1. Strains used are *E. coli* strain DH5α (F- cnDA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupGΦ80dlacZΔM15 Δ(lacZYA-argF) U169, hsdR17 (rK-mK), λ−, cloning host, purchased from Takara Biotechnology Co., Ltd) and *C. glutamicum* ATCC13032 (Type strain, purchased from American Type Culture Collection, ATCC).

2. Plasmids used are pJYS1Ptac (is based on a temperature-sensitive replicon, pBLts, carries Knr and confers resistance to Kn in *E. coli* and *C. glutamicum*, Addgene: 85545), pJYS2_crtYf (is based on the pMB1 replicon, carries Sp† and confers resistance to Sp in *E. coli* and *C. glutamicum*, Addgene: 85544.) and pJYS3_crtYf (is based on the pBLts replicon, carries Knr and confers resistance to Kn in *E. coli* and *C. glutamicum*, Addgene: 85542).

3. The oligonucleotides and primers in Table 1 are synthesized and should be used at 25 μM.

4. Genomic DNA of *C. glutamicum* ATCC13032 is used as PCR template for generation of the upstream and downstream homologous arms, ~200 bp Psod fragment (a strong constitutive promoter in *C. glutamicum*) [23] and crRNA; pJYS2_crtYf is used as PCR template for generation of plasmid pJYS2_argR; *E. coli* MG1655 genomic DNA is used as PCR template for generation of 1.2 kb tdcB gene fragment.

5. KOD-plus-neo DNA polymerase and/or Taq DNA polymerase.

6. DNA Ligase.

7. Restriction endonucleases ApaI and XbaI.

8. ClonExpress® II One Step Cloning Kit and ClonExpress® MultiS One Step Cloning Kit or equivalent used for isothermal assembly.

9. Genomic DNA purification kit.

10. Plasmid DNA purification kit.

11. PCR cleanup kit.

12. DNA gel-extraction kit.

13. 0.4 mm electroporation cuvettes.
### Table 1
Oligonucleotides and primers used in the work

| Primers/oligonucleotides/crRNA | Sequences (5’-3’) | Characteristics |
|-------------------------------|------------------|-----------------|
| crRNA_crtYf | GAATTCTACTGTTGTAGATCAGGCAACCA<br>TTGGCAGGAA | PAM:TTTC (loop>N21) |
| crRNA_argR | GAATTCTACTGTTGTAGATTACAGATCA<br>TTCCGGCAACATCGC | PAM:TTTC (loop>N21) |
| P51 | GGAATGATCTGTAATCTACAACAGTAGAAA<br>TTCGGATCCATTATACCTAGGACTGAG | to clone pJYS2_argR<br>(N21>loop>Pj23119) |
| P52 | GGGTAGATTACAGATCATTTCCGGCAACACA<br>TCGCTGTACAAATAAAAACGAAAGGCTCAG | to clone pJYS2_argR<br>(loop>N21>terminator) |
| P59 | CATTATACTAGGACTAGCTAGCTGTCAA<br>TCTAGCCTATCCAGCAGTCTTTCTGTC | to clone upstream homologous arms for pJYS3_crtYf or pJYS3_crtYf::tdcB |
| P67 | GTTCTTCCCTATCCGCGCAACCCAAATGAGAAC<br>TTGAGAG | to clone upstream homologous arms for pJYS3_crtYf::tdcB |
| P68 | GAATAATTGGCGAGCTAGA<br>TCCAGCAATACAGTCTGAG | to clone downstream homologous arms for pJYS3_crtYf::tdcB |
| P62 | CTAACACACAAAAGTAGAAGAACAATCTG<br>TTCCGGGCGTGGGTAGGTCG<br>TAGGCAAGTTAC | to clone downstream homologous arms for pJYS3_crtYf::tdcB |
| P69 | AGTTCTCATTGGGTGCCGGA<br>TAGGGAAGAACAGCACC | to clone tdcB for pJYS3_crtYf::tdcB |
| P70 | AAGGATTTCATTTACATAGTACATACGAC<br>TCTGCC | to clone tdcB for pJYS3_crtYf::tdcB |
| P71 | TATGTTATATGCATGGGTAAACATCC<br>TTCCGTAGGTTTC | to clone Pssd for pJYS3_crtYf::tdcB |
| P72 | GCGTGGTGGGTGATCTAGCTGGAATTAG<br>TTCCGGGCTTTC | to clone Pssd for pJYS3_crtYf::tdcB |
| O_argR59-1 | CAGCAGCATGCTGTTGCCCGAATGATC<br>TAGAGAACCAGCAGTTCATCAAG<br>CATGCGGC | lagging strand, 59-nt, argR324/325AC>CT (Q109AMB) |
| O_argR59-2 | CGGCCGCTTGGCGCACTGCGATGTTGCC<br>---<br>CCAGCAGTTCATCAAGCATGCGGC | lagging strand, 59-nt, 17-bp deletion from 318–336 positions of argR reading frame containing PAM region |
3 Methods

The basic principle of CRISPR-cpf1 system is to make DNA double strand break at specific locus using a single-strand RNA-guided endonuclease Cpf1, facilitating subsequent DNA repair by homologous recombination (HR) assisted by donor single-stranded DNA (ssDNA) or double-stranded DNA homologous arms, thus introducing genome alterations [15, 17]. The genome editing cycle starts with guide RNA design, continues with plasmids construction and transformation, and finalizes with positive mutant identification. In certain instances, further mutations may be desirable, requiring plasmid curing and iterative genome editing [15].

We are now able to use two CRISPR-Cpf1-assisted systems for efficient genome editing in *C. glutamicum* [15], as shown in Fig. 2. The double-plasmid–based CRISPR-Cpf1 system includes a FnCpf1 and RecT expression plasmid pJYS1 series (indicated as pJYS1Ptac in Fig. 3a) cleaving and repairing DNA, and a crRNA

![Diagram](https://www.nature.com/articles/ncomms15179/)

**Fig. 2** Overview of CRISPR-Cpf1-assisted genome editing in *C. glutamicum* using double-plasmid–based CRISPR-Cpf1 system assisted by ssDNA (a) and an All-in-one CRISPR-Cpf1 plasmid system (b). The figure has been published in reference 15 (https://www.nature.com/articles/ncomms15179/) and this figure is licensed under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/)
Fig. 3 Basic plasmids used in the work. pJYS1Ptac is a Cpf1 expression plasmid, in which Cpf1 is driven by an IPTG inducible promoter Plac and recT is driven by an IPTG inducible promoter Ptac (a); pJYS2_crtYf is a crRNA expression plasmid. A new crRNA plasmid pJYS2_argR can be constructed based on the plasmid by PCR (b); pJYS1Ptac, pJYS2_crtYf, and ssDNA can compose a double-plasmid–based CRISPR-Cpf1 system used for small genome alterations; pJYS3_ΔcrtYf consists of FnCpf1 and crRNA, and the upstream and downstream homologous arms that served as the donor DNA for DSB repair (c). The new plasmid pJYS3_ΔcrtYf::tdcB can be constructed based on pJYS3_ΔcrtYf by replacing homologous arms and an insertion fragment Psod-tdcB. The contents of the figure have been published in reference 15 (https://www.nature.com/articles/ncomms15179/) and they are licensed under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/)
expressing plasmid pJYS2 series targeting different loci in the genome (indicated as pJYS2_crtYf in Fig. 3b). pJYS1 was firstly introduced to C. glutamicum competent cell. Then the pJYS2 series plasmid was cotransformed with donor ssDNA into competent cells harboring pJYS1 expressing RecT and FnCpf1. The positive mutant can be identified using colony PCR, enzyme digestion or DNA sequencing according to its feature. Finally, the pJYS2 series plasmids can be cured by overnight incubation in liquid medium without spectinomycin but with kanamycin, followed by sub-culturing the next day for the next round of editing. The desired strain can be obtained by overnight culturing in antibiotic-free medium at 34 °C. The total editing time is 3 N + 4 days for N rounds of genome editing. During genome editing, plasmid pJYS1 can be directly adapted, while a new pJYS2 plasmid targeting new loci need to be generated by changing its crRNA.

As for the all-in-one CRISPR-Cpf1 plasmid system, pJYS3 series plasmid consists of FnCpf1 and crRNA (indicated as pJYS3_ΔcrtYf in Fig. 3c), and the upstream and downstream homologous arms that served as the donor DNA for DSB repair. Only one plasmid transformation is needed using this system followed by positive mutant screening. The pJYS3 series can be cured by overnight culturing in liquid medium without antibiotics at 34 °C, followed by subculturing the next day for the next round of editing. The desired strain can be obtained by overnight culturing in antibiotic-free medium at 34 °C. The total editing time is 3 N + 2 days for N rounds of genome editing. During genome editing, a new pJYS3 plasmid targeting new loci and harboring new upstream and downstream homology arms as well as inserted fragment need to be constructed.

Generally, the double-plasmid–based CRISPR-Cpf1 system is used for small genome alterations and all-in-one CRISPR-Cpf1 plasmid is used for large gene deletions and insertions. Their procedures are basically similar, only differing in some individual steps, which will be specially emphasized.

3.1 crRNA Design and Synthesis

1. The easiest way to design the crRNA is to manually select a T-rich protospacer-adjacent motif (PAM) such as TTTC exemplified here (see Note 1, Table 1) in the target DNA sequence [24], and then choose its downstream 20–24 bp sequence as targeting RNA (ACAGATCATTCCGGCAACATCGC).

2. The targeting RNA and an RNA loop structure of 20 bp (5'-GAATTTCTACTGTTGTAGAT-3') compose a complete crRNA sequence of 40–44 bp, in which the loop structure locates at upstream (5' end).

3. The complete crRNA used in the work (GAATTTC-TACTGTTGTAGAT ACAGATCATTCCGGCAACATCGC)
is driven by Pj23119 (see Note 2), a synthetic constitutive expression promoter (5′-TGACAGCTAGCTCAGTCC\ TAGGTATAAT-3′) [25].

4. To facilitate construction of plasmid harboring new crRNA (pJYS2_argR), we introduced the crRNA by PCR primers (P51/P52) (Table 1). Plasmids pJYS2_argR can be constructed by direct PCR amplification using plasmid pJYS2_crtYf as template, and subsequent assembly using ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China).

3.2 **E. coli Competent Cell Preparation**

1. 10 mM CaCl\(_2\) in 200 mL dH\(_2\)O; sterilize the solution by autoclaving. Precool at 4 °C.
2. 10 mM CaCl\(_2\) and 20% (w/v) glycerol in 10 mL dH\(_2\)O; sterilize the solution by autoclaving. Pre-cool it at 4 °C.
3. Pick a single clone of *E. coli* DH5\(\alpha\) and incubate overnight in a test tube containing 4 mL LB medium at 37 °C.
4. Add 500 μL of the broth to a shake flask containing 50 mL of LB medium.
5. Incubate at 37 °C on a shaker to an OD\(_{600}\) of 0.4–0.6 and then place the flask on ice; simultaneously, precool a high-speed centrifuge and some 50 mL centrifuge tubes.
6. Transfer the broth in the shake flask to the centrifuge tube and centrifuge at 4000–5000 × g for 10 min. Remove supernatant, wash the cells three times with 10 mM CaCl\(_2\).
7. Resuspend the competent cells using 2 mL of a mixture of 10 mM CaCl\(_2\) and 20% glycerol; Aliquot the cell suspension in 100 μL volumes into a precooled 1.5 mL EP tube, and store at −80 °C until use.

3.3 **DNA Fragments Amplification and Assembly for Construction of crRNA Expression Plasmid pJYS2_argR and All-in-One Plasmids pJYS3_ΔcrtYf::tdcB**

1. After obtaining the necessary oligonucleotides, centrifuge the tubes with low speed for 1 min, and dilute the primers with dH\(_2\)O to 100 μM and stored for use. Dilute the stored solution to 25 μM as a working solution ready for use.
2. KOD-plus-neo polymerase set is used for proofreading PCR to obtain fragment for plasmids construction or DNA sequencing. These PCR reaction and temperature program are set as shown in Table 2.
3. To demonstrate how the double-plasmid–based CRISPR-Cpf1 system work, a new plasmid pJYS2_argR is constructed using plasmid pJYS2_crtYf as a template for whole-plasmid PCR using primers P51/P52.
4. After clean-up, the PCR product is self-assembled via the isothermal assembly method [26].
5. To demonstrate how the all-in-one plasmid CRISPR-Cpf1 system work, a new plasmid pJYS3_ΔcrtYf::tdcB is constructed
based on pJYS3_ΔcrtYf (The crRNA expression cassette in pJYS3_ΔcrtYf::tdcB can also be changed as need).

6. pJYS3_ΔcrtYf is digested using ApaI and XbaI to remove upstream and downstream homologous fragments and linearize to be a skeleton fragment. Primers P59/P67 and P68/P62 are used to amplify upstream and downstream homologous fragments, respectively, using the C. glutamicum ATCC13032 genome as template.

7. The ~1.2 kb tdcB fragment is amplified using the E. coli MG1655 genome as a template and primers P69/P70.

8. The ~200 bp Psod fragment is amplified using the C. glutamicum ATCC13032 genome as a template and primers P71/P72.

9. The Psod-tdcB fragment is obtained by overlap-extension PCR using the Psod and tdcB fragments [27].

10. These three fragments and the skeleton fragment are assembled via the isothermal assembly method using a ClonExpress® MultiS One Step Cloning Kit (Vazyme, Nanjing, China).

### Table 2

**KOD-plus-neo PCR system and temperature program for proofreading PCR amplification**

| PCR system                          | Temperature program |
|-------------------------------------|---------------------|
| KOD-plus-neo polymerase set (50 μL) |                     |
| 10× PCR buffer                      | 5 μL                |
| dNTP (25 μM)                        | 4 μL                |
| Mg²⁺ (25 μM)                        | 4 μL                |
| Template                            | 1 μL                |
| Forward primer (25 μM)              | 1 μL                |
| Reverse primer (25 μM)              | 1 μL                |
| KOD-plus-neo (1.0 U/μL)             | 1 μL                |
| ddH₂O                               | 33 μL               |

| Step   | Temp   | Time |
|--------|--------|------|
| 1      | 95 ℃   | 10 min |
| 2      | 95 ℃   | 5 s   |
| 3      | 50–65 ℃*| 30 s   |
| 4      | 68 ℃   | 2 min* |
| 5      | Step 2–4| 35 cycles |
| 6      | 68 ℃   | 10 min |

* indicates the annealing temperature depends on the specific primer;
* indicates that the extension time depends on the length of the target fragment, basically 1 kb per minute

### 3.4 Transformation and Screening for Construction of crRNA Expression Plasmid pJYS2_argR and All-in-One Plasmids pJYS3_ΔcrtYf::tdcB

1. **E. coli** DH5α competent cells are taken out from the −80 °C freezer and placed on ice for 5 min.

2. Add the pJYS2_argR or pJYS3_ΔcrtYf::tdcB ligation mixture to the competent cells, with no addition of enzymes from kits as control.

3. Mix and place on ice for 20 min. Heat at 42 °C for 90 s and then place on ice for 2 min.
4. Add 600 μL of LB medium and incubate for 40–60 min at 37 °C, 200–220 rpm in a shaker.

5. Centrifuge at 4000 × g for 5 min, remove the supernatant, and resuspend the cells to about 100 μL.

6. Transfer the resuspension to a solid LB medium supplemented with 50 μg/mL of Sp (for pJYS2_argR) or 25 μg/mL of Kn (for pJYS3_ΔcrtYf::tdcB) and spread evenly.

7. Incubate at 37 °C for 10–12 h until a single colony is large enough (0.5 mm of diameter) to pick by a toothpick.

8. The positive mutants can be verified by colony PCR using P51/P52 primers for pJYS2_argR), or P59/P67, P68/P62, and P69/P72 for pJYS3_ΔcrtYf::tdcB) using Taq polymerase set as Table 3. After confirming the putative positive mutants using colony PCR, incubate as least three independent colonies of them in 4 mL LB medium for overnight at 37 °C.

9. Extract the plasmid DNA using a standard Miniprep kit for enzyme digestion confirmation or sequencing confirmation.

3.5 Electrocompetent Wild C. glutamicum Cells Preparation

Electrocompetent C. glutamicum is prepared as described with modifications (see Note 3) [28].

1. 0.4 cm gap electroporation cuvettes is precooled at 4 °C.

2. 10% (w/v) glycerol in 200 mL dH2O. Sterilize the solution by autoclaving. Precool it at 4 °C.

3. Pipet 4 μL wild C. glutamicum from the bacteria-preserving tube; streak on the agar BHIS plate supplemented with 10 g/L glucose (BHISG), and incubate the plate at 30 °C for 2–3 days;

### Table 3
Taq PCR system and temperature program for colony PCR

| PCR system            | Temperature program |
|-----------------------|---------------------|
| 10× PCR buffer        | 2 μL Step Temp Time |
| dNTP (25 μM)          | 1.6 μL 1 95 °C 10 min |
| Template              | 0.5 μL 2 95 °C 5 s |
| Forward primer (25 μM)| 0.5 μL 3 50–65 °C* 30 s |
| Reverse primer (25 μM)| 0.5 μL 4 72 °C 2 min* |
| ThermoTaq (20 μM)     | 0.3 μL 5 Step 2–4 35 cycles |
| ddH2O                 | 15.1 μL 6 72 °C 10 min |
|                       | 7 4 °C preservation |

* indicates the annealing temperature depends on the specific primer; * indicates that the extension time depends on the length of the target fragment, basically 1 kb per minute
4. Pick up a single clone and inoculate it in a tube containing 4 mL antibiotic-free BHISG and incubate overnight at 220 rpm, 30 °C.

5. Transfer the broth in tube to a 500 mL shake flask containing 100 mL antibiotic-free BHIS medium supplemented with 1 mL/L Tween 80 and 4 g/L glycine, and incubate at 30 °C until the optical density at 600 nm (OD600) reaches about 1.0;

6. The flask is chilled on ice for 20 min. The cells are collected by centrifugation at 4 °C, 2600 × g for 8 min. The cells are washed twice with 50 mL of 10% glycerol;

7. Resuspend Competent cells are resuspended using 1 mL 10% glycerol; Aliquot the cell suspension in 90 μL volumes into a precooled 1.5 mL EP tube, and store at −80 °C until transformation.

3.6 Transformation of Cpf1 Expression Plasmids pJYS1Ptac to Wild C. glutamicum

1. The wild C. glutamicum competent cells are taken out from the −80 °C freezer and thawed on ice for 5 min.

2. 5 μL (~500 ng) of the pJYS1Ptac plasmid is added and mixed with the competent cells.

3. Transfer the mixture into a 4 °C precooled electroporation cuvette.

4. Electroporation is performed at 25 mF, 200 Ω and 2.5 kV using a Bio-Rad Gene Pulser Xcell Electroporation System.

5. Immediately after the pulse, 900 mL of 46 °C prewarmed BHISG medium is added to the mixture and heat-shock for 6 min at 46 °C.

6. The mixture is grown to recover for 1–2 h at 30 °C, 170 rpm in a shaker.

7. Centrifuge at 4000 × g for 5 min, remove the supernatant, and resuspend the cells to about 100 μL.

8. Transfer the resuspension to a solid BHISG medium supplemented with 25 μg/mL of Kn and spread evenly;

9. Incubate the plate at 30 °C for 2 days for c.f.u. determinations and identification of mutant harboring pJYS1Ptac.

3.7 Electro-competent Recombinant C. glutamicum Cells Preparation

Electrocompetent C. glutamicum harboring pJYS1Ptac (C. glutamicum/pJYS1Ptac) is prepared as described with modifications [28].

1. A single colony of C. glutamicum/pJYS1Ptac is picked up and transferred to a tube containing 4 mL BHISG supplemented with 25 μg/mL of Kn and incubate overnight at 220 rpm, 30 °C.

2. Transfer the broth in tube to a 500 mL shake flask containing 100 mL BHISG medium supplemented with 25 μg/mL of Kn,
0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG), 1 mL/L Tween 80 as well as 4 g/L glycine, and incubate at 30 °C until the optical density at 600 nm (OD_{600}) reaches about 1.0.

3. The flask is chilled on ice for 20 min. The cells are collected by centrifugation at 4 °C, 2600 × g for 8 min. The cells are washed twice with 50 mL of 10% glycerol.

4. Resuspend Competent cells are resuspended using 1 mL 10% glycerol; Aliquot the cell suspension in 90 μL volumes into a precooled 1.5 mL EP tube, and store at −80 °C until transformation.

3.8 Transformation of crRNA Expression Plasmid pJYS2-argR and ssDNA to C. glutamicum/pJYS1Ptac

1. The C. glutamicum/pJYS1Ptac competent cells are taken out from the −80 °C freezer and thawed on ice for 5 min.

2. The competent cells are mixed with 5 μL (~500 ng) of the pJYS2-argR plasmid and 5 μL (1–10 μg) of ssDNA O_argR59-1 (for bases mutation) or O_argR59-2 (for 17 bp deletion).

3. Transfer the mixture into a 4 °C precooled electroporation cuvette.

4. Electroporation is performed at 25 mF, 200 Ω and 2.5 kV using a Bio-Rad Gene Pulser Xcell Electroporation System.

5. Immediately after the pulse, 900 mL of 46 °C prewarmed BHISG medium is added to the mixture and heat-shock for 6 min at 46 °C.

6. The mixture is grown to recover for 1–2 h at 30 °C, 170 rpm in a shaker.

7. Centrifugue at 4000 × g for 5 min, remove the supernatant, and resuspend the cells to about 100 μL.

8. Transfer the resuspension to a solid BHISG medium supplemented with 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG), 25 μg/mL of Kn as well as 50 μg/mL of Sp, and spread evenly;

9. Incubate the plate at 30 °C for 2 days for c.f.u. determinations and identification of mutant of interest.

3.9 Desired Mutant Screening and Confirming

1. Once transformant/transconjugant colonies are big enough to pick (approx. 1 mm diameter), colony PCR is adapted to screen for the positive mutant. Five individual colonies generated from pJYS2_argR/O_argR59-1 and pJYS2_argR59-2- respectively, are picked up for colony PCR and subsequent sequencing.

2. Visualize the PCR product on a 1% (w/v) agarose gel and cut the band (of all three reactions) that corresponds to the expected ~700-bp fragment. Purify the DNA and send for sequencing.
3. The results of sequencing indicated that the system of pJYS1/pJYS2_argR/O_argR59-1 successfully made all colonies mutate from AC to CT at base 324/325 (Fig. 4a); parallelly, the system of pJYS1/pJYS2_argR/O_argR59-2 realized 17 bp deletions at positions of argR in 2 of 5 colonies (Fig. 4b).

4. The positive mutants are then picked up and inoculated into a tube containing 4 mL antibiotic-free BHISG medium for all plasmids curing and subsequent preservation in 20% glycerol in −80 °C refrigerator (see Note 4).
5. In certain instances, further mutations may be desirable. The positive mutants can be transferred into BHISG medium supplemented with 25 μg/mL of Kn to keep pJYS1Plac plasmid but drop pJYS2_argR plasmid for the next round competent cells preparation (Subheading 3.7) and genome editing (Subheadings 3.8 and 3.9).

3.10 Genome Editing Using All-in-One Plasmid System

1. The protocol of genome editing using all-in-one plasmid system is similar to that of double-plasmid–based CRISPR-Cpf1 system assisted by ssDNA, including crRNA design (Subheading 3.1), generation of plasmids (Subheadings 3.2, 3.3, and 3.4), wild \textit{C. glutamicum} competent cells preparation (see \textbf{Note 6}, Subheading 3.5).

2. Subheadings 3.6 and 3.7 are not needed for genome editing using all-in-one plasmid system.

3. In Subheadings 3.8, competent cells and plasmids are replaced with wild \textit{C. glutamicum} competent cells and pJYS3_ΔcrtYf::tdcB.

4. In Subheading 3.9 for positive mutant identification, PCR product visualization on a 1% (w/v) agarose gel is an important approach to screen the putative colonies as shown in Fig. 5a, while PCR product sequencing becomes a method to double check (Fig. 5b).

4 Notes

1. crRNA design is critical for genome editing efficiency. Different crRNA may lead to very different genome editing efficiency [17]. It is advisable to parallelly design at least two crRNA sites for one gene, which may significantly increase the probability of successful editing.

2. Except crRNA, other factors can also affect genome editing efficiency, including but not limited to RecT induction, amount of ssDNA oligo, oligo length, oligo position (lagging oligo generally better than leading oligo), strength of promoter driving Cpf1 [15].

3. The growth state of the harvested cells for competent cells preparation has an important effect on their transformation efficiency. Generally, it is better that the optical density at 600 nm (OD600) reaches about 1.0. The transformation efficiency decreased significantly when OD600 exceed 1.2 [15, 28]. If preparation of competent cells is well done, resultant electroporation time constant values should be about 6 ms.
4. In general, after free-antibiotic incubation in liquid BHISG medium overnight, nearly 100% of the strains can achieve plasmid curing [15]. However, loss of the plasmid needs to be confirmed. The strain used for next round genome editing should not be able to grow on medium containing the antibiotic appropriate to the marker present on the lost vector backbone.

5. The length of PCR product is not always reliable, because it contains extensive secondary structure which can lead to errors during PCR. It is advisable to perform a double-check using DNA sequencing [15].

6. The double-plasmid–based CRISPR-Cpf1 system assisted by ssDNA and all-in-one plasmid system can also be applied to other strains of Corynebacterium genus [15], such as
C. acetoacidophilum B230, C. acetoacidophilum B299, C. glutamicum B1, C. pekinense B3, and C. crenatum B6, but operation conditions may vary for each strain.

7. Although Cpf1 nucleases having a longer PAM and longer targeting RNA may also help reduce off-target effects, it is still necessary to minimize off-target activity or carefully check that [15, 17]. Gene complementation experiments are commonly necessary and helpful; Off-target detection and even genomic resequencing can also be carried out as needed.

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CRISPR/Cas-Mediated Genome Editing of *Streptomyces*

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Abstract

*Streptomyces* are an important source and reservoir of natural products with diverse applications in medicine, agriculture, and food. Engineered *Streptomyces* strains have also proven to be functional chassis for the discovery and production of bioactive compounds and enzymes. However, genetic engineering of *Streptomyces* is often laborious and time-consuming. Here we describe protocols for CRISPR/Cas-mediated genome editing of *Streptomyces*. Starting from the design and assembly of all-in-one CRISPR/Cas constructs for efficient double-strand break-mediated genome editing, we also present protocols for intergeneric conjugation, CRISPR/Cas plasmid curing, and validation of edited strains.

Key words *Streptomyces*, CRISPR/Cas, Genome editing, Genetic engineering, Double-strand break, Deletion, Knock-in, Substitution

1 Introduction

*Streptomyces* species are prolific producers of bioactive secondary metabolites that are important to pharmaceutical, food, and agricultural industries. Many of the bioactive chemical scaffolds used in the development of immune modulators, antimicrobial, anticancer, and anthelmintic drugs are derived or inspired by secondary metabolites from streptomycetes [1, 2]. Recent progress in next-generation sequencing and data processing have predicted a much greater biosynthetic potential of streptomycetes than previously appreciated [3]. Advanced synthetic biology and metabolic engineering strategies to realize this potential often rely on genetic manipulation of *Streptomyces* spp. [4], which is oftentimes challenging and time-consuming.

Development of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) tools for genome editing of *Streptomyces* offers unprecedented
opportunities to harness the biosynthetic potential of these industrially important bacteria. Employing a programmable nuclease to generate targeted double-strand breaks in genomes for homology-directed repair or non-homologous end joining in the presence and absence of repair templates, respectively, CRISPR/Cas enables efficient, scarless genome editing of *Streptomyces* in a single step [5–8]. CRISPR/Cas-mediated deletions, insertions, and substitutions facilitated pleiotropic and pathway-specific approaches to discover, diversify and produce bioactive natural products from biosynthetic gene clusters of interest in *Streptomyces* [9]. At the same time, the application of CRISPR/Cas genome editing in *Streptomyces* is expected to accelerate chassis strain development. Engineered strains of several *Streptomyces* species such as *Streptomyces lividans*, *Streptomyces avermitilis*, and *Streptomyces albus* are proving useful for functional metagenomics [10, 11], as well as for heterologous production of target metabolites or enzymes at laboratory and industrial scales [12–14]. By enabling large deletions of entire biosynthetic gene clusters of up to 82 kb in a single step at high efficiencies [7], CRISPR/Cas tools facilitate the generation of genome-reduced strains for improved heterologous production of various chemicals [15]. Notably, CRISPR/Cas-mediated base editing that does not require the generation of double-strand breaks has been recently demonstrated in *Streptomyces* [16].

Here in this chapter, we discuss the protocols for CRISPR/Cas-mediated genome editing in *Streptomyces*. We describe the design and construction of an all-in-one CRISPR/Cas system, which includes the Cas protein, single guide RNA (sgRNA), and homology repair template to generate insertions, deletions, and substitutions at the genomic locus of interest. The protocols of intergeneric conjugation, CRISPR/Cas plasmid curing, and validation of edited strains are also presented. To cater to the diverse physiological characteristics of different *Streptomyces* spp., possible protocol modifications are mentioned in Subheading 4. However, as handling of *Streptomyces* strains is highly varied, we recommend optimizing methods for culuring and introducing foreign DNA for the strains of interest [17, 18].

## 2 Materials

### 2.1 Equipment and Tools

1. 0.2 mL polymerase chain reaction (PCR) tubes.
2. 1.5 mL microcentrifuge tubes.
3. Thermocycler.
4. Minicentrifuge.
5. Sterile 90 mm petri dishes.
6. Sterile 14 mL culture tubes.
7. Benchtop centrifuge (2500 \times g).
8. Benchtop microcentrifuge (21,000 \times g).
9. Biological safety cabinet.
10. 30 °C and 37 °C shakers.
11. 30 °C and 37 °C incubators.
12. Sterile glass microscope slides.
13. Sterile 50 mL centrifuge tubes.
14. Vortex mixer.
15. Sterile syringe 10 cc Luer-Lok.
16. Sterile cotton balls.
17. Parafilm.
18. Inoculating loops.
19. Spectrophotometer.

2.2 Plasmids
1. pCRISPyces-Sth1Cas9 (Addgene #129552).
2. pCRISPyces-SaCas9 (Addgene #129553).
3. pCRISPyces-FnCpf1 (Addgene #129554).

2.3 Media and Solutions
1. 10× oligo annealing buffer: 100 mM Tris–HCl, pH 7.5–8.0, 500 mM NaCl, 10 mM EDTA pH 8.0.
2. Nuclease-free water.
3. T4 DNA ligase buffer.
4. 10 mM ATP.
5. Quick ligase (New England Biolabs, USA).
6. Restriction endonucleases and their corresponding 10× digestion buffers:
   (a) BbsI (see Note 1).
   (b) HindIII.
   (c) SpeI.
   (d) XbaI.
7. Lysogeny Broth (LB) Miller: For 1 L, add 5 g yeast extract, 10 g tryptone, and 10 g NaCl to deionized water and autoclave at 121 °C for 20 min.
8. 50 mg/mL apramycin stock, sterile-filtered (0.22 μm) (see Note 2).
9. LB with apramycin: 1 mL of apramycin stock is added to 1 L of LB.
10. Thermosensitive alkaline phosphatase (TSAP).
Enzymatic lysis buffer: 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% (v/v) Triton X-100. Add lysozyme to a final concentration of 20 mg/mL before use.

0.7–1.0% Tris–Acetate–EDTA (TAE) agarose for gel electrophoresis.

ISP2 broth: For 1 L, add 10 g malt extract, 4 g Bacto yeast extract, 4 g glucose to deionized water. Autoclave at 121 °C for 20 min.

Sterile water.

TX buffer: 50 mM Tris–HCl, pH 7.4 with 0.001% (v/v) Triton X-100, sterile-filtered (0.22 μm).

Flooding solution: Add 20 mg nalidixic acid sodium salt to 16 mL sterile water and add 10 M NaOH (see Note 3) with stirring until dissolved. The pH should measure around 9. Add 400 μL of apramycin stock solution and adjust pH to ~7.4. Top up mixture with sterile water to final volume of 20 mL so that final nalidixic acid concentration is 1 mg/mL. Sterile filter (0.22 μm) and store at −20 °C.

Exonuclease I (ExoI, 20 U/μL).

Shrimp alkaline phosphatase (SAP, 1 U/μL).

2.4 Bacterial Strains

1. High efficiency competent Escherichia coli (E. coli) cells.
2. Streptomyces spores.
3. Conjugal E. coli donor strain.

2.5 Media Plates

(See Note 4)

1. Apramycin LB plates: For 1 L, add 5 g yeast extract, 10 g tryptone, 10 g NaCl, 15 g Bacto agar to deionized water and autoclave at 121 °C for 20 min. Cool to 60 °C and add 1 mL apramycin stock to 1 L of media before pouring the agar into 90 mm petri dishes.

2. X-gal/IPTG/apramycin plates: Spread 40 μL of 20 mg/mL X-gal and 5 μL of 1 M IPTG evenly on apramycin LB plates and leave to dry before use (see Note 5).

3. ISP2 plates: For 1 L, add 10 g malt extract, 4 g Bacto yeast extract, 4 g D-glucose, 20 g Bacto agar to deionized water and autoclave at 121 °C for 20 min. Cool to 60 °C before pouring plates.

4. Apramycin ISP2 plates: Before pouring ISP2 plates, add 1 mL apramycin stock to 1 L of media.

5. R2 without sucrose plates: For 1 L, add 0.25 g K₂SO₄ (0.025% w/v), 10.12 g (1.012% w/v) MgCl₂·6H₂O, 10 g (1% w/v) D-glucose, 0.1 g (0.01% w/v) Bacto casamino acids, 5.73 g (0.573% w/v) TES, 20 g (2% w/v) Bacto agar to deionized water and autoclave at 121 °C for 20 min. Let it cool to 60 °C,
then add: 1 mL KH$_2$PO$_4$ (50 mg/mL and 0.22 μm sterile-filtered), and freshly prepared 2.94 g CaCl$_2$·2H$_2$O and 3 g l-proline dissolved in 5 mL of 1 M NaOH (0.22 μm sterile-filtered) before pouring plates (see Note 6).

### 2.6 Kits (See Note 7)

1. Plasmid DNA miniprep kit.
2. KOD Xtrement™ Hot Start DNA Polymerase (Merck, Germany), or equivalent robust DNA polymerase.
3. PCR purification kit.
4. NEBuilder® HiFi DNA Assembly Master Mix or equivalent.
5. Genomic DNA isolation kit.

### 3 Methods

#### 3.1 Design of All-in-One CRISPR/Cas Plasmid

Here we focus on three CRISPR-Cas constructs that have been previously developed and are available on Addgene (Table 1). Derived from pCRISPomyces-2 that utilizes the canonical *Streptococcus pyogenes* Cas9 protein (spCas9), these constructs employ alternative Cas proteins; *Streptococcus thermophilus* CRISPR1 Cas9 (Sth1Cas9), *Staphylococcus aureus* Cas9 (SaCas9), and *Francisella tularensis* subsp. novicida U112 Cpf1 (FnCpf1). When correctly assembled, these all-in-one constructs consist of the Cas protein, sgRNA transcription cassette, homology repair template, and transformation of a single plasmid is sufficient for genome editing. To generate these all-in-one constructs, the protospacer is first introduced into the CRISPR/Cas plasmid via Golden Gate assembly [26] of synthesized oligonucleotides (Fig. 1). Successful insertion of the protospacer disrupts the lacZ gene, allowing blue-white screening of recombinant bacteria. Subsequent insertion of the homology repair template by isothermal assembly is facilitated by the presence of adapter sequences in pCRISPomyces-Sth1Cas9, pCRISPomyces-SaCas9, and pCRISPomyces-FnCpf1 (Fig. 2).

##### 3.1.1 Protospacer Design

1. Depending on the Cas protein selected (see Note 9), protospacers within the genomic locus of interest can be selected based on the following criteria.
   (a) Protospacer should have a protospacer adjacent motif (PAM) for the selected CRISPR/Cas protein (see PAM, PAM location, Table 1, Fig. 1).
   (b) Protospacer should be 20–25 nucleotides depending on the CRISPR/Cas protein selected (see protospacer length, Table 1).
   (c) To ensure specificity of the chosen protospacer, the PAM-proximal seed sequence and its PAM should be
Table 1
All-in-one CRISPR/Cas plasmids and parameters for construct design

| Name                     | Cas protein                          | PAM     | PAM location (relative to guide) | Protospacer length (nt) | Seed sequence length (nt) | Addgene ID, references |
|--------------------------|--------------------------------------|---------|----------------------------------|--------------------------|---------------------------|------------------------|
| pCRISPomyces-Sth1Cas9    | *Streptococcus thermophilus* CRISPR1 Cas9 | NNAGAA  | 3'                               | 20–25                    | 5                         | #129552 [19–21]        |
| pCRISPomyces-SaCas9      | *Staphylococcus aureus* Cas9          | NNG R RT| 3'                               | 20–21                    | 8                         | #129553 [19, 22]       |
| pCRISPomyces-FnCpf1      | *Francisella tularensis subsp.* novicida U112 Cpf1 | TTN     | 5'                               | 23–25                    | 5                         | #129554 [19, 23, 24]   |
| pCRISPomyces-2            | *Streptococcus pyogenes* Cas9         | NGG     | 3'                               | 20                       | 12                        | #61737 [5, 19, 25]     |

N is any nucleotide, R is A or G

Fig. 1 Protospacer design. (a) Cas9 and (b) Cpf1 PAMs are located differently. The PAM for SpCas9 (NGG) is shown as representative of Cas9 PAMs. PAMs for SthCas9 and SaCas9 are in the same location as SpCas9. After the protospacer sequence is selected, 4 nucleotides overhangs are incorporated into the sense and antisense guide oligonucleotides for insertion of the protospacer into the CRISPR/Cas plasmid via Golden Gate assembly

unique within the genome. This can be done via a NIH nucleotide BLAST search for the [seed sequence + PAM] against the target *Streptomyces* genome [27] (see Notes 10–12).
2. To facilitate Golden Gate assembly incorporation, 4 nucleotides are added to the oligonucleotides containing the protospacer sequence. These will form the required overhangs for ligation after annealing of the oligonucleotides. For Cas9 constructs, add “ACGC” and “AAAC” to the 5’ ends of the sense and antisense guide oligonucleotides respectively (Fig. 1a). For Cpf1 constructs, add “AGAT” and “GAGC” to the 5’ ends of the sense and antisense guide oligonucleotides respectively (Fig. 1b).
3.1.2 Homology Repair Template Design for Direct Cloning

1. Typically, the homology repair template for *Streptomyces* contains the desired genome edit(s) (i.e., deletion, insertion, or substitution) flanked by 2 kb homologous sequences at each of the 3′ and 5′ ends (we refer to these homologous regions as flanks in this protocol) (see Note 13).

2. An adapter sequence is present in pCRISPomyces-Sth1Cas9, pCRISPomyces-SaCas9, and pCRISPomyces-FnCpf1 to facilitate cloning of homology repair templates by DNA assembly. This adapter improves isothermal DNA assembly efficiencies by providing optimal overlaps for annealing (Fig. 2a).

   Design primers to incorporate overlaps for isothermal assembly on the 3′ and 5′ ends of the homology repair template by PCR (Fig. 2b) (see Notes 14–16).

3.2 Construction of All-in-One CRISPR/Cas Plasmid for Genome Editing

3.2.1 Insertion of Protopspacer by Golden Gate Assembly

1. Into a 0.2 mL PCR tube, add 1 μL of each of the sense and antisense guide oligonucleotides (100 μM) to 1 μL of 10× oligo annealing buffer and 7 μL of nuclease-free water to achieve a final concentration of 10 μM of each guide oligonucleotide in 1× oligo annealing buffer and mix well.

2. Anneal the complementary oligonucleotides by heating the tubes to 95 °C for 5 min, then ramping to 4 °C at 0.1 °C/s in a thermocycler.

3. For protospacer insertion by Golden Gate assembly, add the components listed in Table 2 into a 0.2 mL PCR tube and mix gently by pipetting up and down.

4. Briefly spin in a minicentrifuge and place in a thermocycler with the program listed in Table 3.

5. Transform 3 μL of Golden Gate assembly reaction into 40 μL of competent *E. coli* cells and plate cells onto X-gal/IPTG/ampicillin LB plates for blue-white screening (see Note 17).

6. Incubate plates at 37 °C overnight.

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**Table 2**

Golden Gate assembly reaction

| Components                              | Amount  |
|-----------------------------------------|---------|
| CRISPR/Cas plasmid (Table 1)           | 50 ng   |
| Annealed oligonucleotides (from step 2 in 3.2.1) | 0.3 μL  |
| T4 DNA ligase buffer                    | 1 μL    |
| 10 mM ATP                               | 1 μL    |
| Quick ligase                            | 0.5 μL  |
| BbsI                                    | 0.5 μL  |
| Top up with nuclease-free water to a total volume of 10 μL |
7. Pick white colonies into 5 mL LB with apramycin in 14 mL culture tubes.

8. Incubate cultures with shaking at 37 °C overnight.

9. Isolate plasmids from cultures using a plasmid DNA miniprep kit.

10. Sequence recovered plasmids to verify the insertion of protospacer (see Note 18).

### 3.2.2 PCR Amplification of Homology Repair Template

To insert the homology repair template into CRISPR/Cas plasmids using isothermal assembly, appropriate overlaps are incorporated into the homology repair template using PCR (Fig. 2).

1. Add the following components (Table 4) in a 0.2 mL PCR tube and mix by gentle pipetting.

2. Place tubes in the thermocycler with the program in Table 5 (see Note 20).

| Stage | Steps | Temperature (°C) | Time (min) | Cycles |
|-------|-------|-----------------|------------|--------|
| 1     | Restriction enzyme (BbsI) activation | 37 | 10 | 10 |
|       | Ligase activation | 16 | 10 | |
| 2     | Inactivation of restriction enzyme (BbsI) | 50 | 5 | 1 |
| 3     | Inactivation of ligase | 65 | 20 | 1 |
| 4     | Hold | 4 | ∞ | |

### Table 3
Golden Gate assembly cycling conditions

| Components | Amount |
|------------|--------|
| 2× Xtreme buffer | 25 μL |
| 2 mM dNTPs | 10 μL |
| 10 μM primers (forward & reverse) | 1.5 μL each |
| Homology repair template (see Note 19) | 10–100 ng |
| KOD Xtreme™ Hot Start DNA Polymerase (1 U/μL) | 1 μL |
| Top-up with nuclease-free water to a total volume of 50 μL | |

### Table 4
PCR amplification of homology repair template using KOD Xtreme™
3. Run PCR reactions on a 0.7–1% TAE agarose gel to ensure PCR product is of the desired size without secondary amplification products (see Note 21).

4. Clean up reaction with a PCR purification kit (see Note 22).

### 3.2.3 Insertion of Homology Repair Template by Isothermal Assembly

1. Linearize the protospacer-containing CRISPR/Cas plasmid from Subheading 3.2.1 with *Spe*I and *Hind*III in a 0.2 mL PCR tube (Table 6).

2. Place the tube in 37 °C for ≥4 h. Add 1 μL of TSAP at the final 1 h of incubation.

3. Clean up the reaction with a PCR purification kit.

4. Set up a 2-fragment isothermal assembly reaction in a 0.2 mL PCR tube (Table 7).

5. In a thermocycler, incubate the reaction at 50 °C for 0.5–1 h.

6. Transform 2 μL of reaction mixture into 40 μL of competent *E. coli* cells and plate cells onto apramycin LB plates (see Note 17).

7. Incubate plates at 37 °C overnight.

8. Pick colonies into 5 mL LB with apramycin in 14 mL culture tubes.

9. Incubate cultures with shaking at 37 °C overnight.

### Table 5
**PCR cycling conditions using KOD Xtreme™**

| Stage | Step                        | Temperature (°C) | Time      | Cycles |
|-------|-----------------------------|-----------------|-----------|--------|
| 1     | Polymerase activation       | 94              | 2 min     | 1      |
| 2     | Denaturation                | 98              | 10 s      | 30     |
|       | Annealing                   | Lowest primer T_m | 30 s      |        |
|       | Extension                   | 68              | 1 min/kb  |        |
| 3     | Hold                        | 4               | ∞         |        |

### Table 6
**Restriction digest of protospacer-containing CRISPR/Cas plasmid**

| Components                                                                 | Amount   |
|---------------------------------------------------------------------------|----------|
| Protospacer containing CRISPR/Cas plasmid (from Subheading 3.2.1)         | 1 μg     |
| 10× digestion buffer                                                      | 1 μL     |
| *Spe*I                                                                    | 0.5 μL   |
| *Hind*III                                                                 | 0.5 μL   |

*Top-up with nuclease-free water to a total volume of 10 μL.*
10. Isolate plasmids from the overnight cultures using a plasmid DNA miniprep kit.

11. Check isolated plasmids by restriction digest with XbaI (Table 8, see Note 23).

12. Run a 0.7–1% TAE agarose gel to check for clones with expected fragment sizes (see Note 21).

13. Sequence plasmids with the expected digest patterns to verify insertion and sequence integrity of the homologous repair template (see Note 18).

3.3 Introduction of CRISPR/Cas Plasmid into Streptomyces Strain by Conjugation (See Note 24)

3.3.1 Preparing Streptomyces Spore Suspension

All work involving Streptomyces cultures is performed in a biological safety cabinet.

1. Grow up Streptomyces spores in 2 mL ISP2 broth in 14 mL culture tubes and incubate in 30 °C shaker (see Note 25).

2. Dilute 2 μL of the saturated culture into 50 μL of sterile water and spread evenly on ISP2 plate (see Notes 26–28).

3. Incubate plate at 30 °C for 4–5 days until heavy sporulation is visible (see Note 29).

4. Gently scrape spores off the surface of the plate using a sterile glass microscope slide (see Note 30).
5. Wash the spores off the glass slide into a 50 mL centrifuge tube using 10–15 mL sterile TX buffer.

6. Vortex suspension for 2 min (see Note 31).

7. Pass suspension through a 10 mL syringe containing a sterile cotton ball into a new 50 mL centrifuge tube.

8. Centrifuge the filtered suspension in a benchtop centrifuge at 2500 \( \times g \) for 10 min.

9. Discard the supernatant and resuspend the pellet in 1 mL sterile water. Transfer the suspension into a 1.5 mL microcentrifuge tube.

10. Centrifuge the suspension for 5 min at maximum speed (21,000 \( \times g \)) in a benchtop microcentrifuge and discard supernatant.

11. Add sterile water to cover the pellet and resuspend the spores (see Note 32).

12. Aliquot spore preparation and store at –80 °C (see Note 33).

13. Determine spore preparation quality and density by plating serial dilutions of the spore preparation (see Note 34).

---

### 3.3.2 Culturing E. coli Donor Strains for Conjugation

1. Transform the assembled all-in-one CRISPR/Cas plasmid from Subheading 3.2 into a conjugal E. coli donor strain (see Note 35).

2. Inoculate single colonies of the E. coli donor containing the assembled CRISPR/Cas plasmid into 2 mL LB with apramycin in 14 mL culture tubes.

3. Incubate cultures at 37 °C overnight with shaking.

---

### 3.3.3 Intergeneric Conjugation in Streptomyces

1. Dry R2 without sucrose media plates in the biological safety cabinet.

2. Dilute the overnight E. coli donor cultures 1:100 in 2 mL LB with apramycin and incubate at 37 °C with shaking until an OD\(_{600}\) of about 0.4–0.6 is achieved. OD\(_{600}\) can be measured using a spectrophotometer.

3. Pipet 400 \( \mu \)L of culture into a 1.5 mL microcentrifuge tube.

4. Pellet cells at 21,000 \( \times g \) in a benchtop microcentrifuge for 30 s and discard supernatant.

5. Wash in 1 mL non-selective LB.

6. Repeat steps 4 and 5 two more times.

7. Resuspend cells in 1 mL non-selective LB.

8. Mix washed E. coli cells with spores of the recipient Streptomyces strain. We typically add 5 \( \mu \)L washed E. coli cells for every 25 \( \mu \)L spore preparation (see Note 36).
9. Gently pipet to mix and then spot the entire mixture in the form of 2 μL spots onto R2 without sucrose plates.
10. Wait for the spots to dry completely before incubating the plate at 30 °C.
11. After 16–20 h, overlay plates with 3 mL of flooding solution, making sure to cover the plate evenly and leave the plate in the biological safety cabinet.
12. After 4–8 h, pipet off excess flooding solution and flip the plates right-side-up and parafilm the plates (see Note 37).
13. Incubate plate at 30 °C until exconjugants are visible (see Note 38).
14. Using an inoculating loop, streak exconjugants onto apramycin ISP2 plates and incubate at 30 °C until colonies appear.
15. To cure the CRISPR/Cas plasmid, restreak single colonies onto pre-warmed non-selective ISP2 plates and incubate at 37 °C until colonies appear. This will select against the temperature-sensitive pSG5 replication origin in the CRISPR/Cas plasmids (see Note 39).
16. To verify plasmid curing, restreak the same colony onto both non-selective and apramycin ISP2 plates (see Note 40).
17. Incubate plates at 30 °C until colonies appear on the non-selective plates.
18. Clone(s) that have successfully lost the CRISPR/Cas plasmid will not grow on apramycin ISP2 plates. Select the corresponding colony on the non-selective ISP2 plate for sequence validation (see Note 41).

### 3.4 Validation of Edited Strains

All work involving Streptomyces cultures is performed in a biological safety cabinet.

#### 3.4.1 Genomic DNA Extraction

1. Pick individual colonies from Subheading 3.3.3 into 2 mL ISP2 broth in 14 mL culture tubes. Incubate with shaking at 30 °C until culture is saturated (see Note 28).
2. Pellet 0.3 mL of culture in a benchtop microcentrifuge at maximum speed (>16,000 × g) for 30 s and discard the supernatant.
3. Resuspend bacterial pellet in 180 μL enzymatic lysis buffer.
4. Incubate at 37 °C for 30 min.
5. Isolate genomic DNA using a genomic extraction kit.
6. Measure DNA concentration with a spectrophotometer (see Notes 42 and 43).
3.4.2 PCR Amplification of Target Genomic Locus for Sequencing Validation

Target genomic locus are PCR amplified from the isolated genomic DNA (see Notes 14, 44, and 45). As a control, the same locus from wild-type strains are also amplified.

1. Set up PCR reactions using genomic DNA from Subheading 3.4.1 according to Table 9.
2. Place tubes in the thermocycler with the program described in Table 5 (see Note 20).
3. Run a 0.7–1% TAE agarose gel to ensure the PCR fragment is of the desired size (see Note 21).

3.4.3 Enzymatic PCR Clean-up for Validation by Sanger Sequencing (See Notes 46 and 47)

1. Add 0.5 μL of ExoI and 1 μL of SAP directly to 5 μL of the PCR products from Subheading 3.4.2 in a 0.2 mL PCR tube.
2. In a thermocycler, incubate at 37 °C for 15 min, followed by 80 °C for 15 min.
3. Sanger sequencing can be performed directly on the treated PCR product to validate the genome edits (see Note 48).

4 Notes

1. BbsI is best stored at −80 °C for long-term storage. Isochizomer BpiI may be used in place of BbsI.
2. The working concentration of apramycin in media and agar is 50 μg/mL.
3. Store in 50 mL centrifuge tubes instead of glass bottles since the solution will react with glass.
4. These media are general recommendations; they can be substituted for suitable media depending on Streptomyces of interest.
5. Plates should be freshly prepared before use.
6. Be careful when handling NaOH, heat will be produced upon mixing.
7. Other equivalent kits can be used.
8. We have successfully used a less preferred Sth1Cas9 PAM (NNGGAA) for genome editing in *Streptomyces* [19, 20].
9. Selection of Cas protein to be used for genome editing is often guided by the availability of PAM motifs within the target site and toxicity of the Cas protein to the strains [19].
10. Although these Cas have varying seed sequence length, we typically use 12 nucleotides as seed sequences for all four CRISPR/Cas constructs.
11. Include all combinations of the PAM for the BLAST search. For example, for a given spCas9 protospacer, one will have to check that there is only one perfect match when searching [12 nucleotides seed sequence] + NGG against the *Streptomyces* genome, where N can be A, G, C, or T.
12. In type II CRISPR systems, the seed region has been defined as the 5–12 nucleotides located on the 3' end of the spacer sequence proximal to the PAM. Seed sequence mismatches can severely or completely interfere with target DNA binding and cleavage, and therefore genome editing. On the other hand, high homology in the seed region can lead to unwanted off-target binding events despite mismatches elsewhere in the protospacer.
13. Homology flanks routinely used for *Streptomyces* are approximately 2 kb in length. Shorter flanks (~1 kb) will work for some strains but will result in significantly lower editing efficiencies [28]. Both flanks in a homology repair template would have to be of similar lengths to each other (<0.1 kb difference).
14. In general, primer design (excluding the overlaps to be introduced) should have following properties: length ≥ 20 bases, melting temperature (Tm) of 60–70 °C, and GC content of 50–70%.
15. Standard ligation cloning can also be employed to incorporate the homology repair template using *Spe*I, *Hind*III, or *Xba*I restriction sites in the CRISPR/Cas plasmids.
16. The homology repair template on the final CRISPR/Cas plasmids should be sequenced completely as any PCR-acquired mutations will introduce unintended mutations during genome editing.
17. Other chemical or electrocompetent *E. coli* strains, including the widely used DH5α, DH10B, TOP10 strains, may be used.
18. Sequencing primers: 5' - ACTCCATCTGGATTGGTTCGAACGCTCG-3' (For homology template sequencing) and
5’-GCGGCCTTTTTACGGTTCCTGGCCTCTAGA-3’ (For both homology template and sgRNA sequencing).

19. Homology repair template can be linear or circular DNA. The amount of template DNA used for PCR varies with the quality and nature of the template.

20. We find that touchdown PCR helps reduce secondary amplification products. In a typical touchdown PCR program, initial cycles start with a high annealing temperature and the annealing temperature is decreased gradually for following amplification cycles [29].

21. 0.7–1% TAE agarose gel is suitable for 500 bp to 6 kb fragment sizes. If the desired fragment is out of this range, adjust the agarose percentage accordingly.

22. Gel extraction should be performed if multiple PCR products are present.

23. Depending on the insert sequence, other restriction enzymes may be used to determine the insertion.

24. There are variations to this intergeneric conjugation protocol. Also, depending on the Streptomyces species, direct transformation, electroporation or protoplast transformation may be more efficient in introducing DNA [17, 18].

25. Colonies from plates may be also used to seed cultures.

26. Depending on the Streptomyces species, different growth and sporulation media will be preferred [18].

27. Agar should be thick enough to withstand prolonged periods (weeks) of incubation without drying out. We routinely pour 30 mL of agar media per 90 mm petri dish.

28. It usually takes 3–4 days to get a sufficiently dense culture depending on the Streptomyces strain and age of the inoculum.

29. Some strains can take up to 2 weeks to a month to reach maximum sporulation.

30. Sterile glass beads can also be used to pick up spores from the plate. Roll them around on the surface of the plate until they are coated with spores before transferring them into a 50 mL centrifuge tube.

31. If clumps remain, use a sterile 1 mL pipette tip to break them up.

32. The amount of water for resuspension can vary depending on the quality and quantity of the spores.

33. We usually prepare 50–100 μL aliquots of spore preparations for conjugation experiments and 10 μL aliquots for cell propagation.
34. A good spore preparation typically yields about $10^8$ to $10^9$ spores/mL.

35. Different conjugal \textit{E. coli} donors should be used depending on the restriction-modification systems present in the recipient strain, which can significantly impair transformation efficiencies. We routinely use the DNA methylation-deficient WM3780 strain [30] or the DNA methylation-proficient WM6026 strain, both obtained from Professor William Metcalf at the University of Illinois Urbana-Champaign, for conjugation. Other \textit{E. coli} donors commonly used for \textit{Streptomyces} conjugation include ET12567/pUZ8002 (DNA methylation-deficient) and S17-1 (DNA methylation-proficient).

36. Depending on the \textit{Streptomyces} strain, the ratio of \textit{E. coli} donor cell number to recipient spore number can be optimized to improve conjugation efficiencies.

37. Ensure plates are dry before sealing with parafilm.

38. Exconjugants usually appear near the edges of the spots.

39. Not pre-warming the plates may result in initial growth with plasmid propagation and thus reduce efficiency of plasmid loss.

40. If using the same inoculating loop, the first restreak should be onto the non-selective ISP2 plate before the apramycin ISP2 plate to prevent accidental transfer of antibiotics.

41. For apramycin-resistant clones still retaining the CRISPR/Cas plasmid, repeat the plasmid curing process from \textbf{step 15}.

42. About 10 \textmu g genomic DNA can be obtained from 0.3 mL of saturated culture.

43. Genomic DNA is stable and may be stored at 4 °C for weeks. Aliquot and freeze for long-term storage to avoid freeze–thaw cycles.

44. Primers should not be found in flanking homologous regions of the repair template to avoid amplifying from the CRISPR/Cas plasmids.

45. Colony PCR may be performed to circumvent the need for genomic DNA extraction [31]. Success and consistency of colony PCR is highly dependent on the \textit{Streptomyces} strain and pretreatments (e.g., detergents, microwave). In general, we find PCR reactions using genomic DNA to be more reproducible.

46. Genotyping can also be performed by comparing the restriction digests of PCR products from wild-type and edited strains, provided that the desired edit yields a distinct restriction digest pattern from the wild-type sequence.

47. Enzymatic clean-up of PCR products gives us good quality sequencing results. PCR product purification or gel extraction
kits can also be used to clean up PCR products for sequencing. The latter is preferred for PCR reactions yielding multiple bands.

48. To evaluate off-targets, the relevant genomic loci can also be PCR amplified and sequenced.

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Chapter 15

Cas9 Nickase-Based Genome Editing in *Clostridium cellulolyticum*

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Abstract

*Clostridium cellulolyticum* is a model mesophilic, cellulolytic bacterium, with the potential to produce biofuels from lignocellulose. However, the natural cellulose utilization efficiency is quite low and, therefore, metabolically engineered strains with increased efficiency can decrease both the overall cost and time required for biofuel production. Traditional genetic tools are inefficient, expensive, and time-consuming, but recent developments in the use of CRISPR-Cas genetic editing systems have greatly expanded our ability to reprogram cells. Here we describe an established protocol enabling one-step versatile genome editing in *C. cellulolyticum*. It integrates Cas9 nickase (Cas9n) which introduces a single nick that triggers repair via homologous recombination (SNHR) to edit genomic loci with high efficiency and accuracy. This one-step editing is achieved by transforming an all-in-one vector to coexpress Cas9n and a single guide RNA (gRNA) and carries a user-defined homologous donor template to promote SNHR at a desired target site. Additionally, this system has high specificity and allows for various types of genomic editing, including markerless insertions, deletions, substitutions, and even multiplex editing.

**Key words** *C. cellulolyticum*, CRISPR, Cas9 nickase, Genome editing, Microbial engineering

1 Introduction

As an adaptive RNA-guided immune system in prokaryotes, the clustered, regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) protein system is able to recognize target sites (known as protospacers) in invader genomes through base-pairing complementarity and then cleave these invasive DNAs removing the threat of infection [1, 2]. These systems have now been repurposed by researchers and many novel, programmable CRISPR/Cas based platforms have been developed for targeted genome editing and regulation of gene expression in both prokaryotes [3–5] and eukaryotes [6–15]. While diverse CRISPR/Cas systems have been identified in a variety of microorganisms, the

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type II Cas9 endonuclease has been the most widely used to develop genetic systems. Compared to traditional genetic tools which are usually time-consuming and technically challenging [16], Cas9-based genome editing tools have a higher efficiency, broader targeting density, and stronger adaptability for both gene knock-out and gene knock-in studies [17].

The type II CRISPR-Cas system only requires three components for targeted DNA cleavage, including a mature CRISPR RNA (crRNA), a trans-activating crRNA (tracrRNA), and the DNA endonuclease Cas9 [18–22]. Cas9 is a large multidomain protein with two nuclease domains, RuvC and HNH [22, 23]. Cas9 recognizes a protospacer flanked by a short protospacer adjacent motif (PAM) at the 3′ end and then cuts both strands of the DNA [19, 20, 24]. Orthogonal Cas9 nucleases from different microorganisms require different PAM sequences [24, 25]. Cas9 from *Streptococcus pyogenes* (SpCas9) recognizes a PAM with 5′-NGG-3′ and creates double-stranded DNA breaks (DSBs) 3 bp upstream of the 3′-terminal complementary region formed between the crRNA and protospacer [22, 23, 26]. When either the RuvC or the HNH motif is inactivated via mutation, SpCas9 is transformed to SpCas9 nickase (Cas9n) and will either cut only the noncomplementary or complementary strand, respectively [15, 22, 23]. The other necessary component is the tracrRNA-crRNA duplex [25, 27, 28], which forms a nucleoprotein complex with Cas9 (or Cas9n) and recognizes the protospacer by complementary base-pairing [17, 20]. To simplify the application of type II CRISPR-Cas systems, the tracrRNA-crRNA duplex has been engineered into a single molecule, named chimeric guide RNA (gRNA) [9, 13, 23]. The gRNA contains the 20-nt complementary protospacer sequence and a hairpin structure (or gRNA scaffold) that retains the base-pairing interaction between the tracrRNA:crRNA duplex and provides proper structure [9, 13, 23]. The cleavage site created by Cas9 or Cas9n can be repaired by nonhomologous end-joining or homologous recombination [29, 30].

As a model mesophilic cellulolytic bacterium, *Clostridium cellulolyticum* can directly convert lignocellulose to biofuels including ethanol and hydrogen, making it a potential candidate for consolidated bioprocessing [31]. To enhance its overall performance for industrial use [32, 33], microbial engineering of *C. cellulolyticum* is necessary to improve cellulose catabolism and increase biofuel production. However, genome editing in *C. cellulolyticum* is challenging due to technical limitations of traditional genome manipulation methods and low transformation efficiency of *C. cellulolyticum* [16]. To overcome these challenges, we adapted the type II CRISPR-SpCas9 system for genome editing in *C. cellulolyticum*, which allows for markerless gene delivery and versatile editing in a single step with high editing efficiency and precision [16].
Although *C. cellulolyticum* has a predicted native type II CRISPR-Cas system, it has not been extensively characterized and, therefore, would require significant work to understand its biochemical mechanism, including the enzymatic activity of Cas9 and characteristics of protospacer, PAM, and gRNA. In our previous study, a codon optimized *cas9* gene from *S. pyogenes* along with a synthesized gRNA were tested for the ability to edit the genome of *C. cellulolyticum*. Unfortunately, it was discovered that Cas9 was toxic to *C. cellulolyticum*, which was likely caused by the inefficiency of an endogenous DNA repair system capable of repairing a Cas9-induced DSB. However, when Cas9 was replaced with Cas9n (D10A mutant) to reduce toxicity, successful genomic editing was achieved. Our established protocol, demonstrated here, also provides a donor template to promote homology-directed repair at the target site allowing for customized genetic changes to be introduced. All three components (e.g., *cas9n* gene, customized gRNA, and homologous donor template) are assembled into an all-in-one vector for one-step editing in *C. cellulolyticum* (Fig. 1). The all-in-one vector is a shuttle vector, capable of replication in both *Escherichia coli* and *C. cellulolyticum*. A synthetic promoter (named P4) and the ferredoxin (Fd) promoter (derived from *Clostridium pasteurianum*) are used to drive expression of the gRNA and *cas9n*, respectively. The *catP* gene is an antibiotic resistance gene used for selection in both *E. coli* and *C. cellulolyticum*. To program the all-in-one vector for retargeting, the user only needs to change the gRNA cassette and donor template. Procedures to obtain mutants of interest are illustrated in a general flowchart (Fig. 2).

![Fig. 1](image)

**Fig. 1 (a)** Design of the “all-in-one” vector for Cas9n-triggered homologous recombination. The 20-bp protospacer (red), PAM (purple) and homologous donor fragments (black) on the vector are customized for targeted editing. **(b)** Mechanism of Cas9n-based nick formation. SpCas9n (D10A mutation in the RuvC domain) is guided by the gRNA to cut the noncomplementary strand. A nick is made at a position (indicated by red arrow) 3 bp upstream of the PAM site (purple).
Fig. 2 Workflow of Cas9n-triggered homologous recombination for targeted genome editing. An appropriate protospacer is selected for the target gene; a donor template is designed to carry the desired genetic change. This could be a precise deletion, insertion, or substitution. All of the pieces are generated (P4::gRNA cassette, the left and right homology arms, and the linearized vector backbone) and assembled into an all-in-one vector via Gibson assembly. Successful transformants are selected by plating on the appropriate antibiotic agar medium and then identified by amplicon sequencing.

2 Materials

2.1 Preparation of an All-in-One Vector

1. Vectors and primers used here are listed in Tables 1 and 2.
2. Phusion high-fidelity DNA polymerase.
3. Phusion HF Buffer.
4. Deoxynucleotide (dNTP) Solution Mix (10 mM).
5. DNA Clean & Concentrator purification kit.
6. Nuclease-free water.
7. Gel purification kit.
8. Plasmid Miniprep Kit.
9. Agarose.
10. LB medium.
11. PvuI.
12. KpnI.
13. DpnI.
14. Gibson Assembly® Master Mix (New England Biolabs, Ipswich, MA).
15. MspI Methyltransferase.
16. MspI Methylase Reaction Buffer.
17. S-adenosylmethionine (SAM).
18. Chloramphenicol.
19. Agar.
Table 1
Vectors and strains used in this protocol

| Strain or vector | Phenotype or genotype<sup>a</sup> | Source |
|------------------|----------------------------------|--------|
| **Strains**      |                                  |        |
| C. cellulolyticum H10 | ATCC 35319                      | [16]  |
| **Vectors**      |                                  |        |
| pFdCas9n-pyrF-donor<sup>b</sup> | Cmp<sup>R</sup> in *E. coli*, Tmp<sup>R</sup> in *C. cellulolyticum* H10 | [16]  |

<sup>a</sup> Abbreviations: Cmp chloramphenicol, Tmp thiamphenicol
<sup>b</sup> This vector was originally designed to inactivate the pyrF gene in *C. cellulolyticum*

Table 2
Primers used for all-in-one vector construction

| Primer name<sup>a</sup> | Sequences 5’-3’ | Description |
|-------------------------|----------------|-------------|
| 3FF<sup>b</sup>         | CTCGTTGCAACAAATTGATGAG | Forward primer for P4 promoter amplification |
| GRR<sup>b,c</sup>       | CTAAGACATACCTCTAATT AAATCAGCTTAGGCAATT | Reverse primer for P4 promoter amplification with overlapping end with gRNA scaffold. |
| GRF<sup>b,c</sup>       | GTTGTATACCTACCTAT GTTCTGGTAGCTAGAATAGCAAGT | Forward primer for gRNA scaffold amplification with overlapping end with P4 promoter. |
| 2RR<sup>b</sup>         | CTGCCTACTGTGGGAAGGCAGTACGCGGC | Reverse primer for gRNA scaffold amplification with overlapping end with vector backbone. |
| LF<sup>b</sup>          | GAATTTTATTATGTTACCCGGG AAGTCTGTAG CAACAGATTCTAGTTGTCCTC | Forward primer for left homologous arm amplification with overlapping end with vector backbone. |
| LR                      | CCATTTTTAATTGTCCTTTCCAGTTTGGGTGTAATCCATATATTTCCAATTACAGTTAGTTT | Reverse primer for left homologous arm amplification with overlapping end with right homologous arm. |
| RF                      | GCTAATAATCCTTTAAAAATGAATTAGGGATTTAATACGAAATTAAATCAGGAAAGCAATTAAATGG | Forward primer for right homologous arm amplification with overlapping end with left homologous arm. |
| RR<sup>b</sup>          | CTCATCAATTTGTCACAGGAG GAC CACGCTTTTTGCTTGATGATAATGCTCC | Reverse primer for left homologous arm amplification with overlapping end with P4::gRNA cassette. |

<sup>a</sup> All primers presented here were used for inactivation of the *mspI* gene (*Ciel_2866*) by deletion of a 23-bp DNA fragment
<sup>b</sup> Users can change the GRF, GRR, LF, LR, RF, and RR primers for other target sites. Underlined sequences are fixed and do not need to be changed for retargeting
<sup>c</sup> Italicized sequences represent the protospacer sequence and its reverse complement
20. Competent *E. coli* (High Efficiency) cells.
21. SOC Outgrowth Medium.
22. Gel loading dye.

### 2.2 Preparation of Transformation

1. Modified VM medium [34]: Dissolve 1.0 g of KH$_2$PO$_4$, 3.4 g of KH$_2$PO$_4$, 2.14 g of Urea, 1.0 g of MgCl$_2$$\cdot$6H$_2$O, 0.15 g of CaCl$_2$$\cdot$6H$_2$O, 1.25 mg of FeSO$_4$, 10 g of MOPS, 2.0 g of yeast extract in 1 L of dd H$_2$O (*see Note 1*).
2. Electroporation buffer: 270 mM sucrose, 1 mM MgCl$_2$ and 5 mM sodium phosphate buffer, pH 7.4 (*see Note 1*).
3. D-(+)-Cellobiose (*see Note 1*).
4. Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap (Bio-Rad Laboratories, Hercules, CA).
5. Bacterial Genomic DNA Kit.
6. Thiamphenicol.
7. Glycerol.
8. Gene Pulser Xcell microbial system (Bio-Rad, Hercules, CA).

### 3 Methods

As an example of the general workflow, this protocol will disrupt the *mspI* gene encoding the restriction endonuclease MspI in *C. cellulolyticum* by deletion of a 23-bp DNA fragment. The model vector used here is pFdCas9n-pyrF-donor which was previously used for disruption of the *pyrF* gene in *C. cellulolyticum* [16]. This protocol can be modified for other genome editing purposes, such as DNA insertion, substitution, and multiplex editing.

#### 3.1 Construction of the P4::gRNA Expression Cassette (*See Note 2*)

1. Identify a qualified target site with the format of 5’-N20 protospacer + NGG PAM-3’ within the target gene. Here we will select 5’-ATTAAAGAAGGGTACTCTATAGG-3’ found in the *mspI* gene (*see Note 3*).
2. Swap the protospacer sequence in GRF and GRR to contain the new protospacer (5’-ATTAAAGAAGGGTACTCTAT-3’) via primer design and synthesis (*see Fig. 3 and Note 4*).
3. Start an overnight culture of pFdCas9n-pyrF-donor *E. coli* strain in 5 ml of LB medium with 35 μg/ml chloramphenicol, grow at 37 °C with 200 rpm shaking.
4. Purify pFdCas9n-pyrF-donor vector DNA with the Plasmid Spin Miniprep Kit following the manufacturer’s protocol. The purified vector DNA will also be used as template in Subheading 3.3.
5. PCR amplify the P4 promoter and gRNA fragments using pFdCas9n-pyrF-donor as the template. The PCR reaction mix and program parameters are listed in Tables 3 and 4.

**Fig. 3** Primer design and generation of the components for the construction of the all-in-one vector. Primers are represented by arrows and colors represent the overlapping sequences required for Gibson assembly. LF and LR are used to amplify the left homologous arm; RF and RR are used to amplify the right homologous arm. The customized sequence (green diamond) that represents the desired genetic change (e.g., deletion, point mutation, or substitution) can be introduced into overlapping region (purple) of the LR and RF primers. 3FF and GRR are used for P4 promoter amplification. GRF and 2RR are used for gRNA scaffold amplification. The target protospacer (red) is added in the overlapping region of the GRR and GRF primers. KpnI and Pvul are used to digest the original vector to generate a linearized backbone. Finally, all fragments can be assembled together to generate the all-in-one vector by Gibson assembly.

**Table 3**
**PCR mixes for amplification of P4 promoter and gRNA fragments**

|                      | PCR mix for P4 promoter | PCR mix for gRNA scaffold |
|----------------------|-------------------------|---------------------------|
| 5× HF buffer         | 10 µl                   | 10 µl                     |
| Forward Primer (20 mM)| 1 µl (3FF primer)      | 1 µl (GRF primer)         |
| Reverse Primer (20 mM)| 1 µl (GRR primer)      | 1 µl (2RR primer)         |
| Phusion DNA Polymerase (2000 U/ml) | 0.5 µl | 0.5 µl |
| dNTP mix (2.5 mM)    | 2.5 µl                  | 2.5 µl                    |
| Template DNA         | 1–10 ng                 | 1–10 ng                   |
| ddH₂O                | to a total volume of 50 µl | to a total volume of 50 µl |
6. Join the P4 promoter and the gRNA scaffold using splicing by overlap extension (SOEing) to generate a P4::gRNA expression cassette. PCR reactions should be prepared in three replicates (see Note 5). The PCR reaction mix and program parameters for SOEing are listed in Tables 5 and 6 (see Note 6).

7. Pool the PCR products from the three replicates and determine the amplification specificity on a 1% agarose gel (see Note 7).

8. Directly mix 3 μl of DpnI (20,000 U/ml) with the PCR product to digest vector template at 37 °C for 1 h.

9. Purify the resulting product with the DNA Clean & Concentrator purification kit. Elute DNA with 10–15 μl nuclease-free water and adjust the final concentration of the purified P4::gRNA cassette to 100–200 ng/μl. Store the purified products at −20 °C.

---

**Table 4**

| Step               | Temp       | Time  |
|--------------------|------------|-------|
| Initial denaturation  | 98.0 °C    | 45 s  |
|                     | 98.0 °C    | 10 s  |
| 30 cycles          | 53.0 °C    | 20 s  |
|                     | 72.0 °C    | 10 s  |
| Final extension     | 72.0 °C    | 8 min |
| Hold               | 4 °C       | ∞     |

**Table 5**

| PCR mix for P4::gRNA |
|----------------------|
| 5× HF buffer         | 10 μl      |
| Forward Primer (20 mM) | 1 μl (3FF primer) |
| Reverse Primer (20 mM) | 1 μl (2RR primer) |
| Phusion DNA Polymerase (2000 U/ml) | 0.5 μl |
| dNTP (2.5 mM)        | 2.5 μl    |
| Template DNA         | 1 μl (p4 promoter amplified in Subheading 3.1, step 5) + 1 μl (gRNA scaffold amplified in Subheading 3.1, step 5) (see Note 6) |
| ddH₂O                | to a total volume of 50 μl |
### 3.2 Construction of User-Defined Donor Templates

1. Design primers LF, LR, RF, and RR to amplify the left and right homologous arms of the donor template (*see Fig. 3 and Note 8*).

2. PCR amplify the left and right homologous arms using *C. cellulolyticum* genomic DNA as the template. Both PCR reactions should be prepared in three replicates (*see Note 5*). The PCR reaction mixes and program parameters are listed in Tables 7 and 8.

3. Pool the PCR products from the three replicates and determine the amplification specificity on a 1% agarose gel. If there are no nonspecific products, purify the PCR product with the DNA Clean & Concentrator kit and elute DNA in 10–15 µl nuclease-free water (100–200 ng/µl). Otherwise, the desired fragments should be excised from the agarose gel and purified with the Gel purification kit. Store the purified DNA at −20 °C until use.

---

**Table 6**  
PCR cycling parameters for SOEing

| Step               | Temperature | Time     |
|--------------------|-------------|----------|
| Initial denaturation| 98.0 °C     | 45 s     |
|                    | 98.0 °C     | 10 s     |
| 30 cycles          | 53.0 °C     | 20 s     |
|                    | 72.0 °C     | 12 s     |
| Final extension     | 72.0 °C     | 8 min    |
| Hold               | 4 °C        | ∞        |

**Table 7**  
PCR mixes for amplification of homologous arms

|                              | PCR mix for left homologous arm | PCR mix for right homologous arm |
|------------------------------|---------------------------------|----------------------------------|
| 5× HF buffer                 | 10 µl                           | 10 µl                            |
| Forward Primer (20 mM)       | 1 µl (LF primer)                | 1 µl (RF primer)                |
| Reverse Primer (20 mM)       | 1 µl (LR primer)                | 1 µl (RR primer)                |
| Phusion DNA Polymerase (2000 U/ml) | 0.5 µl      | 0.5 µl                            |
| dNTP (2.5 mM)                | 2.5 µl                          | 2.5 µl                           |
| Template DNA                | ~200 ng (gDNA)                  | ~200 ng (gDNA)                  |
| ddH₂O                       | to a total volume of 50 µl      | to a total volume of 50 µl      |
### 3.3 Preparation of the Linearized All-in-One Vector

1. Digest purified pFdCas9n-pyrF-donor vector DNA (from Subheading 3.1) with PvuI and KpnI at 37 °C for 2 h. The reaction mix is listed in Table 9.

2. Add 8 μl of Gel Loading Dye (6×) to the digestion product and load the entire sample into a 0.8% agarose gel. Purify the linearized product (~10,000 nt) with the Gel purification kit and elute in 30 μl of elution buffer.

3. If the DNA purity is low, that is, 260/280 > 1.9 or < 1.8 and 260/230 ratio < 2.0, repurify the linearized backbone DNA with the DNA Clean & Concentrator purification kit and elute with ~10 μl nuclease-free water (~100 ng/μl final). Store the purified backbone at −20 °C for subsequent use.

### 3.4 Construction of the Customized All-in-One Vector

1. Assemble the P4::gRNA cassette, left homologous arm, right homologous arm, and linearized vector backbone, in a single reaction using the Gibson Assembly® master mix following the manufacturer’s protocol and incubate the reaction mixture at 50 °C for 1 h. The reaction mix is listed in Table 10 (see Note 9).

---

**Table 8**

| Step               | Temp  | Time |
|--------------------|-------|------|
| Initial denaturation | 98.0 °C | 45 s |
| 30 cycles          | 98.0 °C | 10 s |
|                    | 53.0 °C | 20 s |
|                    | 72.0 °C | 20 s a |
| Final extension     | 72.0 °C | 8 min |
| Hold               | 4 °C   | ∞    |

*a The extension time depends on the length of the homologous arms (15–30 s/kb)*

**Table 9**

| Reaction mix                          | Reaction mix |
|---------------------------------------|--------------|
| 2× CutSmart® Buffer                   | 4 μl         |
| PvuI-HF® (20,000 U/ml)                | 2 μl         |
| KpnI-HF® (20,000 U/ml)                | 2 μl         |
| pFdCas9n-pyrF-donor                   | 4 μg         |
| ddH₂O                                 | to a total volume of 40 μl |

**Table 10**

**Table 11**

| Reaction mix        | Reaction mix |
|---------------------|--------------|
| 2× CutSmart® Buffer | 4 μl         |
| PvuI-HF® (20,000 U/ml) | 2 μl      |
| KpnI-HF® (20,000 U/ml) | 2 μl      |
| pFdCas9n-pyrF-donor | 4 μg         |
2. Add 5 μl of the assembly product to 50 μl of chemically competent E. coli cells. Mix gently by flicking 4–5 times and incubate the mixture on ice for 30 min.

3. Heat shock at 42 °C for 30 s and then incubate on ice for 2 min.

4. Add 250 μl SOC medium to the cells and incubate with 200 rpm shaking at 37 °C for 1 h.

5. Spread different amounts (e.g. 50 μl and 150 μl) of the recovered cells on LB agar plates containing 35 μg/ml chloramphenicol. Incubate the plates at 37 °C overnight.

6. Pick several colonies from the selective agar plate and grow them separately in 2 ml of liquid LB with 35 μg/ml chloramphenicol.

7. Extract vector DNA with the Plasmid Spin Miniprep Kit.

8. Verify the all-in-one vector by Sanger sequencing and store the strain at −80 °C for subsequent use.

### 3.5 Methylation of the All-in-One Vector (See Note 10)

1. Grow the E. coli strain harboring the all-in-one vector in 5 ml of LB medium with 35 μg/ml chloramphenicol overnight at 37 °C.

2. Extract the all-in-one vector DNA with the Plasmid Spin Miniprep Kit. Elute the DNA with 35 μl nuclease-free water. Make sure the purity of the vector is qualified (260/280 should be between 1.8 and 1.9; 260/230 > 2.0). If not, purify with DNA Clean & Concentrator kit.

3. Use MspI methyltransferase to methylate vector DNA at 37 °C for 2 h. The reaction mix is listed in Table 11.

4. After 1 h, add an additional 0.5 μl SAM to the mixture and incubate at 37 °C for an additional 1 h (see Note 11).

5. Stop the reaction by heating to 65 °C for 20 min. Purify the methylated vector with the DNA Clean & Concentrator kit and check the DNA purity (260/280 should be between 1.8 and 1.9; 260/230 > 2.0). The purified methylated vector can be stored at −20 °C for a few days.

### Table 10

The reaction mix for all-in-one vector assembly

| Reaction mix                  |   |
|-------------------------------|---|
| Gibson Assembly Master Mix (2×)| 10 μl |
| Total amount of each fragment | X μl (0.2–1 pmols) (see Note 9) |
| ddH2O                          | to a total volume of 20 μl |
3.6 Transformation of C. cellulolyticum and Confirmation of Mutant

1. Inoculate *C. cellulolyticum* in 10 ml of modified VM medium with 5 g/l cellobiose and grow overnight at 34 °C.

2. Subculture the strain into 10 ml of fresh modified VM medium with 5 g/l cellobiose in a Hungate tube and incubate at 34 °C.

3. Monitor cell growth. When cells reach early to mid-exponential phase (OD \(_{600} = 0.3–0.5\)), chill the culture on ice for 30 min (see Note 12).

4. Transfer 10 ml of the culture to a 15 ml anoxic conical centrifuge tube, and then spin the cells at 3400 \(g\) at 4 °C for 7 min.

5. Discard the supernatant and resuspend the pellet with 5 ml of ice-cold anoxic electroporation buffer. Spin down the cells at 3400 \(g\), 4 °C for 7 min.

6. Repeat the wash steps four more times resuspending in 2 ml, 2 ml, 1 ml, and 1 ml of ice-cold anoxic electroporation buffer, respectively (see Note 13). Meanwhile, transfer 2 \(\mu\)g of methylated all-in-one vector (~10 \(\mu\)l) to a prechilled 1.5 ml anoxic microfuge tube and keep on ice.

7. After the final wash, resuspend the cell pellet in 100 \(\mu\)l of ice-cold anoxic electroporation buffer. Mix gently by pipetting up and down for 4–5 times.

8. Add 50 \(\mu\)l of the electrocompetent cells to the tube containing the methylated vector (2 \(\mu\)g in ~10 \(\mu\)l) and mix gently by flicking the tube 3–4 times. Incubate the mixture on ice for 5 min.

9. Transfer the mixture to a prechilled electroporation cuvette (2 mm gap). Electroporate the cells using the Gene Pulser Xcell microbial system at 1250 kV (square wave) for 5 ms.

10. Resuspend the electroporated cells in 10 ml of modified VM medium with 5 g/l cellobiose and transfer to a Hungate tube. Recover the cells at 34 °C for 24 h (see Note 14).

11. Transfer 1.5 ml of the recovered cell culture to 10 ml of VM medium with 5 g/l cellobiose and 15 \(\mu\)g/ml thiamphenicol. Incubate at 34 °C and record OD\(_{600}\) daily (see Note 15).

### Table 11

| Reaction mix                              | 5 µg    |
|-------------------------------------------|---------|
| All-in-one vector                         |         |
| Methyltransferase Reaction Buffer (10×)   | 3 µl    |
| SAM (3.2 mM)                              | 0.75 µl |
| MspI (5 U/µl)                             | 1.5 µl  |
| ddH\(_2\)O                                | to a total volume of 30 µl |
12. When the OD<sub>600</sub> reaches 0.3 or higher, spread 10–20 μl of cells on selective VM agar plates with 15 μg/ml thiamphenicol and incubate at 34 °C.

13. Design a set of genomic DNA-specific primers to screen the mutant by amplicon sequencing (see Note 16).

14. Pick several colonies from the selective plate and inoculate each into 10 ml of modified VM medium with 5 g/l cellobiose and 15 μg/ml thiamphenicol.

15. Extract gDNA from mid-exponential phase (OD<sub>600</sub> = 0.4–0.6) cell cultures with the Millipore Sigma GenElute Bacterial Genomic DNA Kit.

16. Use the genomic DNA-specific primers (Subheading 3.6, step 13) to amplify the target DNA sequence from the genome for Sanger sequencing.

17. Based on amplicon sequencing results, cryopreserve the correct strain in 25% glycerol for long term storage at −80 °C.

### 3.7 Vector Curing of Mutant Strains

1. Grow the mutant at 40 °C in 10 ml of nonselective VM medium with 5 g/l cellobiose.

2. Passage the mutant in nonselective VM medium with 5 g/l cellobiose and incubate at 40 °C. Repeat this step four to five times.

3. Spread 10–20 μl of the final subculture on nonselective VM agar plates with 5 g/l cellobiose and incubate at 34 °C.

4. Pick several colonies and inoculate in 10 ml of nonselective VM medium with 5 g/l cellobiose.

5. Screen the selected colonies by PCR amplification using crude cell lysate as the template with vector-specific primers 3FF and 2RR. The absence of PCR products indicates the vector is cured. Include appropriate positive (vector DNA) and negative (no template) control.

6. Further confirmation can be conducted by extracting the genomic DNA from the vector cured colonies identified in step 5 with the Millipore Sigma GenElute Bacterial Genomic DNA Kit and repeating the PCR from Subheading 3.7, step 5. Store verified mutants at −80 °C for future use.

### 4 Notes

1. The anoxic VM medium is prepared by boiling while purging with 100% nitrogen to remove oxygen and supplemented with 0.1% cysteine hydrochloride (wt/vol). The electroporation buffer is made by boiling while purging with 100% nitrogen
to remove oxygen. A 10% (wt/vol) stock of cellobiose should be prepared by boiling while purging with 100% nitrogen to remove oxygen.

2. As the price of oligo synthesis continues to decrease, an alternative option is to have the P4::gRNA expression cassette containing the necessary overhangs for Gibson assembly synthesized (e.g., as a gBlock from Integrated DNA Technologies).

3. In theory, all 5'-N20-NGG-3' sites (N represents any nucleotide and N20 represents the protospacer with the PAM (NGG) at the 3' end) can be a potential target site for Cas9n from *S. pyogenes*. However, the specificity of the 23-bp target site affects targeting precision; unwanted off-target mutations will occur if low-specificity target sites are used [8, 35, 36]. Therefore, only unique target sites should be selected for further use. The filtering criteria for unique target sites is as follows: (1) at least 3 mismatches between the protospacer and any other genomic site [13]; (2) a string of five or less T’s in the 23-mer sequence and no T3 in the 6-mer region directly upstream of NGG [13, 37]; (3) not an extremely low or high GC content (<25% or >80%).

4. In order to minimize polar effects on downstream genes, we prefer to select a protospacer close to the 5' end of the target gene.

5. We have found that pooling three PCR reactions will yield enough DNA for purification and Gibson assembly.

6. PCR products from Subheading 3.1, step 5 can be used without additional purification as DNA templates for Subheading 3.1, step 6.

7. The P4::gRNA cassette should be ~250 bp in length. If non-specific products are present, gel purification should be performed.

8. The donor template can be PCR amplified as two pieces (the left homology arm and the right homology arm). This allows for the desired genetic alteration to be designed as part of the primers used to amplify each piece of the donor template. The length of the donor template significantly affects editing efficiency. Based on our study [16], if each homologous arm is greater than 0.2 kb, the editing efficiency of our single-nick-triggered homologous recombination is higher than 95%. Additionally, the donor template should be designed in a way that the protospacer is altered so that the site is not continuously cut by Cas9n. This can be accomplished by removing part or all of the protospacer as part of a gene inactivation or making a few synonymous single nucleotide changes (we have found that changing 3 nucleotides is sufficient). Primer design for the
homologous arms should adhere to the following criteria (Fig. 3): (1) the protospacer should be in the middle of the donor template; (2) design LR and RF primers spanning the protospacer region and add the customized sequence that represents the desired genetic change (e.g. deletion, point mutation, or substitution) into the primer design; (3) LR and RF are partially complementary with at least 20-bp overlap for Gibson assembly; (4) The LF and RR can be designed to anneal to different distances from LR and RF to alter the homologous arm size; (5) For proper Gibson assembly LF contains at least 20-bp overlap with the vector backbone and RR contains at least 20-bp overlap with the P4::gRNA cassette. An alternative option is to have the entire donor template (containing the desired genetic alteration and the proper overlaps on either end for Gibson assembly) synthesized (e.g. as a gBlock from IDT).

9. We follow the suggestions provided in the NEB Gibson Assembly protocol to determine the amount of each fragment for assembly of the all-in-one vector. Specifically, the DNA molar ratio for the backbone and P4::gRNA expression cassette should be 1:5 and 1:3 for the backbone and each homologous arm. We suggest using 100 ng of linearized backbone for assembly, which is ~0.0144 pmols for pFdCas9n-pyrF-donor.

10. DNA methylation is required for wild-type C. cellulolyticum transformation. If a ΔmspI mutant is used as the parental strain, the methylation step can be skipped.

11. As SAM is unstable at 37 °C, we suggest adding an additional 0.75 μl SAM (3.2 mM) to the reaction after 1 h incubation.

12. All steps for transformation should be performed on ice and under anaerobic conditions. Oxygen should be removed from the 1.5 ml microfuge tubes and electroporation cuvettes used for the transformation experiment. We have found that 12 h in an anaerobic chamber is sufficient.

13. It is necessary to wash the cells at least 5× to remove excess salts and to avoid arcing.

14. An alternative carbon source containing a mixture of cellobiose (4 g/l), D-glucose (2 g/l), D-xylene (2 g/l), and L-arabinose (2 g/l) can be used instead of cellobiose alone when targeting genes involved in carbon metabolism.

15. After inoculation into the selective medium, the OD$_{600}$ may go up to ~0.2 but will drop back to 0.1 or less. The transformed cells should grow to an OD$_{600}$ of 0.3 after 3–7 days. If the OD$_{600}$ never increase, it indicates the transformation may have failed or the inactivation of the target gene is lethal. In this case, the transformation should be repeated.
16. To avoid amplification from the all-in-one vector, at least one primer is designed to anneal outside of the donor template region in the genome.

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Chapter 16

Genome Editing with Cas9 in Lactobacilli

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Abstract

The bacterial genus *Lactobacillus* comprises a vast range of strains with varying metabolic and probiotic traits, with genome editing representing an essential tool to probe genotype–phenotype relationships and enhance their beneficial properties. Currently, one of the most effective means of genome editing in bacteria couples low-efficiency recombineering with high-efficiency counterselection by nucleases from CRISPR-Cas systems. In lactobacilli, several CRISPR-based genome editing methods exist that have shown varying success in different strains. Here, we detail a fast and simple approach using two shuttle vectors encoding a recombineering template as well as the *Streptococcus pyogenes* Cas9, a trans-activating RNA, and a CRISPR array. We provide a step-by-step procedure for cloning the shuttle vectors, sequentially transforming the vectors into lactobacilli, screening for the desired edit, and finally clearing the shuttle vectors from the mutant strain. As CRISPR-based genome editing in bacteria can fail for various reasons, we also lay out instructions for probing mechanisms of escape. Finally, we include practical notes along the way to facilitate each stage of genome editing, and we illustrate the technique using a representative edit in a strain of *Lactobacillus plantarum*. Overall, this method should serve as a complete guide to performing genome editing in lactobacilli.

Key words Recombineering, SpCas9, Lactobacillus, Genome editing, Shuttle vector

1 Introduction

*Lactobacillus* represents an important genus of bacteria with vast genomic and metabolic diversity [1–3] that has contributed to different sectors of biotechnology. Due to their natural ability to produce lactic acid and survive in harsh environments, lactobacilli have traditionally been used in fermented foods, including fruits, vegetables, grains, meat, and milk products. Ingesting lactobacilli-related foods has also been found to promote skin and gut health, resulting in research efforts to elucidate the bacteria’s probiotic potential and their potential as cell-based therapeutics to treat diseases such as obesity [4], diabetes [5, 6], autoimmunity [7], and cancer [8, 9]. Furthermore, lactobacilli have been found to inhibit or prevent infections by different bacterial pathogens, including *Helicobacter pylori* [10], *Salmonella* sp. [11], and...
Clostridium difficile [12]. Finally, the metabolic diversity of lactobacilli species has made them intriguing candidates to use as cell factories to produce biochemicals and biofuels [13] including alcohols [14], aldehydes [15], and amino acids [16]. Taken together, the probiotic efficacy and diversity of lactobacilli species render this genus widely important. However, further advancing applications of lactobacilli require enhanced genetic tool development to effectively study, interrogate, and modulate their important physiological traits.

Genome editing represents a powerful tool that can be used to interrogate genotype–phenotype relationships and enhance metabolic and physiological traits. In bacteria, genome editing often relies on recombineering between the genome and a transformed editing template through endogenous or heterologously expressed recombinases. In many cases, recombineering efficiencies tend to be very low, necessitating the need for the introduction of large selectable marker cassettes to isolate mutants [17, 18]. Furthermore, efforts to improve recombineering efficiencies or extend recombineering to other bacteria often require extensive screening and characterization to find the best available recombinase. In lactobacilli, the ssDNA recombinase RecT has been used to introduce point mutations in strains of L. reuteri and L. lactis using oligonucleotide donors [19], although the low-ranging efficiencies (0.4–19%) required substantial screening to isolate mutants. To address these issues, the authors combined ssDNA recombineering with counterselection by nucleases from CRISPR-Cas systems to remove unedited cells, which improved their previous genome editing efficiencies in L. reuteri to 90–100% and allowed for selection of chromosomal deletions as well as point mutations [20].

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated) systems are prokaryotic adaptive immune systems whose interference functions are carried out by Cas nucleases and CRISPR RNAs (crRNAs) processed from a transcribed CRISPR array. These crRNAs can be reprogrammed to target any sequence on the genome flanked by a distinct protospacer adjacent motif (PAM) recognized by the nuclease. While there are a vast range of Cas nucleases in nature [21], the nuclease Cas9 associated with type II CRISPR-Cas systems has been most thoroughly investigated and harnessed [22, 23]. In these systems, the guide RNA is processed from the transcribed CRISPR array with the help of a trans-activating CRISPR RNA (tracrRNA) and the endoribonuclease RNase III [24]. To simplify exogenous expression, the processed crRNA and tracrRNA can be fused into a single-guide RNA (sgRNA) [23]. Cas9-based genome editing was first demonstrated in Escherichia coli, where a double-stranded linear DNA template was coupled with the λ-Red system for recombineering, and the Cas9 nuclease from S. pyogenes (SpCas9) was used to select against unedited cells [25]. Since then, CRISPR-
based genome editing methods with Cas9 or the type VI nuclease Cas12a have been slowly implemented in other bacteria, although several barriers to editing have limited consistent success across strains and targets [26]. As a result, methods often vary based on the type of CRISPR nuclease, the recombinering template donor, the number of plasmids, and the inclusion of an exogenous recombinase. In lactobacilli, several different methods of genome editing have been developed since the original demonstration of RecT-mediated ssDNA recombinering and counterselection with SpCas9. For instance, a plasmid recombinering template but no heterologous recombinase was utilized with SpCas9 to achieve genomic deletions in *L. lactis* [27], a species where RecT-ssDNA recombinering has also been demonstrated [28]. Plasmid-based recombinering without a heterologous recombinase has also been coupled with the nickase version of SpCas9 to achieve genomic deletions and insertions in *L. casei* [29]. Finally, the RecE/T recombinase system was used to assist dsDNA recombinering with a plasmid template and counterselection by SpCas9 in *L. plantarum* and *L. brevis* [30].

Here, we outline a fast and simple procedure for SpCas9-based genome editing in lactobacilli. Our method utilizes two *E. coli*–lactobacilli shuttle vectors (Fig. 1): one harboring SpCas9, the tracrRNA, and a single spacer CRISPR array; and the other containing the dsDNA recombinering template [31]. A distinct advantage of using a plasmid-based recombinering template over a linear template is that every cell is given the chance to undergo editing before SpCas9 is introduced. The use of shuttle vectors further supports cloning in *E. coli*. We used this method to edit multiple strains of *L. plantarum* and in *L. paracasei*. At the same time, we found that the editing approach could fail for different reasons [26]. We therefore provide instructions to probe mechanisms of escape. Throughout this detailed protocol, we include tips and suggestions for optimizing cloning, transformation, and screening of putative mutants. We also suggest a timeline for each step in the genome-editing pipeline (Fig. 2). Finally, to illustrate the process of designing and performing editing, we detail the insertion of a premature stop codon into the *ribB* gene of *L. plantarum* WJL as a representative edit. Overall, this protocol should serve as a complete guide for performing genome editing in lactobacilli with the help of CRISPR-Cas9.

## 2 Materials

### 2.1 Growth Media

*See Note 1*

1. LB broth (per liter of water: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride).
2. LB agar (per liter of water: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride, 15 g agar).
3. SOB broth (per liter of water: 20 g tryptone, 5 g yeast extract, 0.5 g sodium chloride).

4. SOC broth: Add 20 mM MgCl₂ and 20 mM glucose to SOB broth.

5. MRS broth (per liter of water: 10 g proteose peptone No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate).

6. MRS agar (per liter of water: 10 g proteose peptone No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 2 g dipotassium phosphate, 15 g agar).

7. MRS-G (2.5%): Add 0.625 g of glycine for every 25 mL of MRS broth. Filter sterilize.

8. SacGly: Dissolve 85.55 g sucrose in 400 mL water. Combine with 100 mL of 50% glycerol. Mix thoroughly.

9. MMRS (optional): MRS supplemented with 500 mM sucrose, 20 mM MgCl₂, and 2 mM CaCl₂.
Fig. 2 Approximate timeline for generating edited strains. Genome editing can be separated into three stages: cloning the recombineering template (blue), cloning the crRNA (CR) (green), and performing genome editing (GE) (orange). Cloning the RT shuttle vector harboring the designed edit requires two cloning steps before transferring the RT into a nonmethylating (NM) *E. coli* strain and can be performed in approximately 6 days. Cloning the CR requires a single digestion and ligation step before transferring to a NM *E. coli* strain, where this step can be performed in approximately 6 days in parallel with RT cloning. Genome editing requires two sequential transformations into the lactobacilli strain before screening colonies for correct mutants and can be achieved in 10 days. Clearing the RT and CR plasmids may require multiple rounds of nonselective growth, although this process typically can be completed within a week.

### 2.2 Strains

1. Chemically competent *E. coli* (or other cloning strain).
2. *E. coli* strain without methylation pattern or multiple strains with varying methylation patterns (e.g., *E. coli* EC135 [32]).
3. Lactobacilli strain of interest.

### 2.3 Antibiotics

1. Erythromycin: 300 μg/mL for LB medium, 10 μg/mL for MRS medium.
2. Chloramphenicol: 34 μg/mL for LB medium, 10 μg/mL for MRS medium.
3. Ampicillin: 50 μg/mL for LB medium.

### 2.4 Molecular Reagents

1. T4 polynucleotide kinase (PNK).
2. T4 DNA ligase buffer.
3. Shrimp alkaline phosphatase (rSAP).
4. Instant Sticky-end Ligase Master Mix or equivalent.
5. PvuI enzyme.
6. NotI enzyme.
7. SphI enzyme (optional).
8. DpnI enzyme.
9. Restriction digestion buffer.
10. 100% ethanol.
11. 3 M Sodium Acetate, pH 5.2: Dissolve 20.42 g sodium acetate in 20 mL water. Add 9 mL glacial acetic acid and remainder water to 50 mL.
12. GlycoBlue (optional).
13. NaOH.
14. Lysozyme.
15. Lysozyme lysis buffer: 20 mM Tris–HCl pH 8, 2 mM sodium EDTA, 1.2% Triton® X-100.
16. DNA polymerase for PCR (Taq and/or high-fidelity polymerase).

2.5 Kits
1. Gibson DNA assembly kit (Such as NEBuilder HiFi DNA Assembly Mastermix).
2. Site-directed mutagenesis kit (Such as Q5 site-directed mutagenesis kit from New England Biolabs).
3. PCR purification kit.
4. DNA mini prep kit.
5. DNA midi prep kit.

2.6 Primers and Plasmids
(See Tables 1 and 2)

3 Methods

3.1 Clone Targeting Spacer into SpCas9 Shuttle Vector to Generate crRNA (CR) Plasmid
1. Design forward and reverse oligos containing the new spacer to clone into pCB578 (Addgene # 141095). The forward oligo should be 64 nts and the reverse oligo should be 70 nts (Fig. 3a). These oligos, once annealed, will include the 3’ repeat, a 20-nt target sequence, and additional bases to regenerate the PvuI and NotI enzyme motifs for subsequent cloning of new spacers.

2. Setup digestion of pCB578 with NotI and PvuI restriction enzymes. To a PCR tube, add pCB578 (1 μg), buffer (1×), NotI (1 μL), PvuI (1 μL), and remainder water to 50 μL (see Note 2). Incubate at 37 °C for 1 h, then heat-inactivate the restriction enzymes at 80 °C for 5 min.
3. Add 1 μL of rSAP to the digested backbone and incubate at 37 °C for 1 h (see Note 3).

4. Clean and concentrate digested backbone with a PCR purification kit, eluting in 21 μL of water or elution buffer (see Note 4).

5. Phosphorylate forward and reverse oligos with T4 PNK. To a PCR tube, add 30 μL of 10 μM oligo, 5 μL T4 DNA Ligase Buffer, 14 μL water, and 1 μL T4 PNK. Incubate at 37 °C for 1 h, then heat inactivate 80 °C for 20 min to deactivate the enzyme.

### Table 1
Shuttle vector screening primers used in this method

| Oligo name | Sequence | Purpose |
|------------|----------|---------|
| RL323      | CTGTAATTTGTTTAATTGCCA TTTCAATT | Forward primer to screen new spacer |
| RL324      | GGAACCTACAAAATAATTA TAAGGAGGC | Reverse primer to screen new spacer |
| RL402      | TTTTGCTCACATGTCTTTTC | Forward primer to screen recombineering template |
| RL403      | CTGCTTTTTGGCTATCAATC | Reverse primer to screen recombineering template |
| JV121      | TAATTATTAGGGGGAAGG | Forward primer to screen Cas9 cassette |
| JV123      | TTTCGTTTATCCATAGTTGC | Reverse primer to screen Cas9 cassette |
| JV117      | TATTTTATAACTTTTATAAACAATAA TCAAG | Sequencing primer 1 for SpCas9 |
| JV126      | AAACCCCTATTAACGCAAGTGGAGTA | Sequencing primer 2 for SpCas9 |
| JV127      | GGTGCTTCAGCTCAATCATT | Sequencing primer 3 for SpCas9 |
| JV128      | GCATCCTGTGGAAAATACTCAATGTGC | Sequencing primer 4 for SpCas9 |
| JV129      | GCCCCAAGTCAATATTGTCAAGAAA | Sequencing primer 5 for SpCas9 |

### Table 2
Base *E. coli*–lactobacilli shuttle vectors used in this method

| Plasmid | Description | Resistance | Source |
|---------|-------------|------------|--------|
| pCB578  | *E. coli*–lactobacilli shuttle vector containing SpCas9, tracrRNA, and a repeat-spacer-repeat array | Erm | [31] |
| pCB591  | *E. coli*–lactobacilli shuttle vector for recombineering template cloning | Amp (*E. coli*) Cm (Lactobacillus) | [31] |
6. Anneal forward and reverse oligos by combining 25 μL of each phosphorylated oligo in a PCR tube and running the following program on a thermocycler.

(a) 95 °C for 5 min.
(b) −1 °C every 15 s, until reaching 15 °C.
(c) Hold at 15 °C.

7. Set up ligation of annealed oligos and digested backbone with Instant Sticky-end Ligase (see Note 5). To a PCR tube, add digested pCB578 (50 ng), annealed oligos (3 × molar), Instant Sticky-end Ligase Master Mix (1×), and remainder water to 10 μL. Pipet up and down 10 times and place the ligation reaction on ice.

8. Transform ligation reaction into chemically competent E. coli cells. Add 2 μL of ligation reaction to 50 μL E. coli cells that have been thawed on ice. Incubate for 30 min on ice then heat shock at 42 °C for 30 s. Incubate on ice for 5 min, then add 950 μL SOC media to E. coli cells and shake at 250 rpm and 37 °C to allow cells to recover for 1 h. Spin down cells at 12,000 × g for 2 min to collect the cell pellet. Resuspend cell pellet in 20 μL SOC media and plate entire 20 μL of cells on LB agar with erythromycin.

Fig. 3 Cloning Cas9 and recombineering template shuttle vectors. (a) Design of oligonucleotides encoding the targeting spacer. The forward oligo should be 64 nts and the reverse oligo should be 70 nts (red lines). Oligos include a 20-nt spacer (green), a 36-nt repeat (yellow) and nucleotides that regenerate PvuI and NotI restriction sites (purple). To clone the new crRNA, the pCB578 backbone is digested with PvuI and NotI enzymes and the designed oligos are phosphorylated, annealed, and ligated into the digested backbone. The final construct will contain a 30-nt spacer, including 10 nts already present in the base construct that do not participate in DNA targeting (green box). (b) Generation of the recombineering template shuttle vector. Starting with shuttle vector pCB591, Gibson cloning is first performed to insert the gene of interest and flanking homology arms (HA) into a multicloning site (MCS). Q5 mutagenesis can be performed to insert the desired mutation into the gene and generate the final RT.
9. Screen CR colonies with cPCR using Taq polymerase (see Note 6). Using a pipette tip, add a small piece of the colony to the cPCR reaction and stir vigorously to mix. Streak out the remainder of each colony on a fresh LB agar plate with erythromycin to obtain a replica plate. Run the following program on a thermocycler:
   (a) 94 °C for 5 min.
   (b) 94 °C for 20 s.
   (c) 48 °C for 45 s.
   (d) 68 °C for 1 min.
   (e) Repeat steps (b)–(d). 30×.
   (f) 68 °C for 5 min.
   (g) Hold at 16 °C.

   Check cPCR reaction using gel electrophoresis (see Note 7). Submit samples that yield the expected PCR product for Sanger sequencing with primer RL324 to check if the correct spacer is present.

3.2 Clone Recombineering Template (RT) into Shuttle Vector to Generate RT Plasmid

1. Design necessary primers for cloning a recombineering template harboring the desired mutation starting with shuttle vector pCB591 (Addgene # 141096, Fig. 3b).

2. Perform Gibson assembly to insert the desired genomic region into multicloning site (MCS) located in pCB591. Amplify pCB591 and the lactobacilli gDNA with primers containing at least 20 bp of homology to each other. Check the PCR reactions on a gel to confirm the expected size before proceeding.

3. Add 1 μL of DpnI enzyme to the PCR reactions to digest the leftover pCB591 backbone and gDNA. Clean and concentrate the amplified backbone and gDNA insert with a PCR purification kit, eluting each in 10 μL water.

4. Set up Gibson DNA assembly reaction in a PCR tube with 0.04 pmol backbone, 0.16 pmol insert, Gibson DNA assembly 2× MM (1×), and remainder water to 20 μL (see Note 8). Incubate at 50 °C for 15 min to 1 h. Transform 2 μL into competent E. coli by heat shock cells as described in Subheading 3.1 step 8. Plate cells on LB agar supplemented with ampicillin.

5. Screen colonies with cPCR using Taq polymerase (see Note 9) using primers RL402 and RL403. Add a small piece of the colony to the cPCR reaction and stir vigorously to mix. Streak out the remainder of each colony on a fresh LB agar plate with ampicillin to obtain a replica plate. Run the following program on a thermocycler:
(a) 94 °C for 5 min.
(b) 94 °C for 20 s.
(c) 46 °C for 45 s.
(d) 68 °C for 1 min per kb template.
(e) Repeat steps (b)–(d). 30×.
(f) 68 °C for 5 min.
(g) Hold at 16 °C.

Check cPCR reaction using gel electrophoresis. Submit samples that yield the expected PCR product for Sanger sequencing with primer pair RL402 and RL403 to sequence junctions.

6. Inoculate a correct clone from replica plate in 4 mL LB media supplemented with ampicillin to make a glycerol stock and to mini-prep the plasmid.

7. Aliquot 1 mL of culture and add 333 μL of 60% glycerol to a cryogenic storage vial and store at −80 °C. Mini-prep the remainder of the culture to collect the plasmid.

8. Perform Q5 mutagenesis to add desired mutations to the recombineering template (see Note 10). Amplify plasmid from Gibson assembly with site directed mutagenesis primers following manufacturer’s protocol. Check PCR reaction on gel before proceeding. Set up KLD reaction following manufacturer’s protocol. Transform 5 μL from KLD reaction into competent E. coli cells as previously described in Subheading 3.1 step 8. Plate cells on LB agar supplemented with ampicillin.

9. Screen colonies with cPCR using Taq polymerase and primers RL402 and RL403 as previously described.

3.3 Prepare Sequence-Verified Shuttle Vectors for Transformation into Lactobacilli

1. Inoculate a correct CR clone and a correct RT clone from the replica plates in 4 mL LB media supplemented with appropriate antibiotic to make a glycerol stock and to mini-prep the plasmids.

2. Aliquot 1 mL of each culture and add 333 μL of 60% glycerol to a cryogenic storage vial and store at −80 °C. Mini-prep the remainder of each culture to collect the plasmids.

3. Transform the plasmids into other strain(s) of E. coli without methylation or with varying methylation patterns (see Note 11).

4. Inoculate E. coli strain harboring correct clones in 50 mL LB supplemented with appropriate antibiotic.

5. Midi-prep the plasmids from the desired E. coli strain. Elute in 200 μL of water and check the concentration on a Nanodrop or Qubit (see Note 12).
6. If necessary, ethanol-precipitate the plasmids to increase the concentration prior to transforming lactobacilli. To a microcentrifuge tube, add 200 μL plasmid, 500 μL 100% ethanol, 20 μL 3 M sodium acetate, and 1.5 μL GlycoBlue. Vortex briefly, then incubate at −80 °C for at least 30 min. Spin down at 4 °C and 12,500 × g for 15 min to collect the DNA pellet. Remove supernatant and add 200 μL of ice-cold 70% ethanol. Spin down at 4 °C and 12,500 × g for 10 min. Remove supernatant and repeat this wash step. Discard the supernatant, and add 10 μL of water to the side of the microcentrifuge tube to avoid disturbing the DNA pellet. Incubate at 65 °C for 10 min or until the DNA is dissolved, then vortex briefly and spin down.

3.4 Transform Shuttle Vectors into Lactobacilli (See Note 13)

1. Grow up a single colony of the lactobacilli strain in MRS broth at 37 °C without shaking for 24 h.
2. Dilute 1 mL lactobacilli culture in 25 mL of 2.5% MRS-G (see Note 14) using a 50 mL Falcon tube.
3. Grow up culture at 37 °C without shaking until OD\textsubscript{600} = 0.8 (see Note 15). Place cells on ice for 10 min, then spin down the culture at 2200 × g and 4 °C for 10 min to collect the cell pellet (see Note 16).
4. Discard the supernatant. Add 5 mL ice-cold 10 mM MgCl\textsubscript{2} to resuspend the cell pellet. Spin down at 2200 × g and 4 °C for 10 min. Discard the supernatant and repeat this wash step.
5. Discard the supernatant. Add 5 mL of ice-cold SacGly solution to resuspend the cell pellet. Transfer the cells to a fresh 50 mL Falcon tube to avoid making additional contact with MgCl\textsubscript{2} salt solution. Spin down at 2200 × g and 4 °C for 10 min.
6. Discard the supernatant. Add 1 mL ice-cold SacGly solution and transfer cells to a microcentrifuge tube. Spin down at 12,000 × g and 4 °C for 2 min.
7. Discard the supernatant. Add 500 μL of ice-cold SacGly to resuspend pellet and use cells immediately (see Note 17).
8. Set electroporator to the following values: 1800 V, 25 capacitance, 1-mm tubes, 200 Ω.
   Add 2.5–5 μg of plasmid DNA suspended in water (5 μL max) to a precooled 1-mm electroporation cuvette tube. Add 60 μL of ice-cold electrocompetent cells. Perform electroporation, and immediately add 1 mL of MRS or MMRS broth to the cells and recover at 37 °C for 3–4 h (see Note 18).
9. Plate 250 μL on MRS plates containing appropriate antibiotics. Grow for 3 days before counting CFU (see Note 19).
3.5 Follow Genome-Editing Pipeline

1. Design oligos to screen mutant lactobacilli strains (Fig. 4a).

2. Prepare electrocompetent cells of lactobacilli strain as described above (see Note 20).

3. Transform RT plasmid harboring the desired edit into electrocompetent lactobacilli cells. Plate transformations on MRS agar supplemented with chloramphenicol. Once colonies are large enough (~3 days), inoculate a single colony in 5 mL MRS media supplemented with chloramphenicol.

4. Prepare electrocompetent cells of lactobacilli strain harboring recombineering template (see Note 21).

5. Transform CR plasmid into lactobacilli recombineering-template strain (see Note 22). Plate transformations on MRS agar supplemented with erythromycin. Count CFU after 3 days.
6. Perform colony PCR on putative mutant colonies. Using a pipette tip, add a small piece of lactobacilli colony to a PCR tube containing 20 μL of 20 mM NaOH and stir vigorously. Incubate at 98 °C for 20 min. Spin down PCR tubes to collect liquid at the bottom. Microwave PCR tubes with cap open for 1 min, then spin down again. Add 1 μL to a PCR reaction with Taq polymerase and designed primers that will only amplify from the genome. Submit PCR reaction for Sanger sequencing to validate desired mutant.

7. Begin plasmid clearance by inoculating a correct mutant strain in MRS media without antibiotics. Then plate the culture on MRS agar without antibiotics and on MRS agar supplemented with either erythromycin or chloramphenicol to check for plasmid clearance.

If there is growth on either antibiotic plate, inoculate a single colony from MRS agar in MRS media to perform a second round of nonselective growth. Once there is no growth on either antibiotic plate, inoculate a colony from MRS agar into MRS media to make a stock. Also inoculate the same colony in MRS supplemented with either erythromycin or chloramphenicol to confirm plasmid clearance. Proceed with downstream applications.

3.6 Screen Lactobacilli Escaper Colonies for Modes of Escape (See Note 23)

1. Mini-prep CR plasmid from lactobacilli escaper colony (Fig. 4b). Inoculate escaper colony in 3 mL MRS media containing erythromycin (see Note 24). Spin down the culture to collect the cell pellet, and resuspend in 200 μL of 20 mg/mL lysozyme solution. Incubate at 37 °C while shaking at 250 rpm for 30 min. Remove lysozyme and proceed with mini-prep following manufacturer’s protocol. Perform two PCR reactions on CR plasmid with Taq polymerase to amplify SpCas9 and the repeat-spacer-repeat array.

(a) To amplify SpCas9, use primers JV121 and JV123 which will anneal at 45 °C and yield an expected band size of 5424 bp. Sequence PCR product with primers JV117, JV126, JV127, JV128, JV129, JV123 to check for disruptions (Table 1).

(b) To amplify repeat-spacer-repeat array, use primers RL323 and RL324 as previously described. Sequence PCR product with RL324 to check for disruptions.

2. Mini-prep RT plasmid from lactobacilli RT strain (stock without transformed CR plasmid) as previously described. Perform PCR reaction on RT plasmid with Taq polymerase using primers RL402 and RL403 as previously described. Sequence PCR product with both primers, and, if necessary, additional primers to check for reversion of the desired mutations in the recombineering template.
3. If original colony PCR (Subheading 3.5 step 6) did not yield a PCR product, design wider PCR primers that amplify from the lactobacilli genome to check for a genomic excision that includes the target site (see Note 25). Repeat colony PCR as described previously until a PCR product is obtained.

4 Notes

1. Growth media and molecular reagents listed are most suitable for *L. plantarum* and may need to be changed depending on the lactobacilli strain.

2. It is recommended to set up a control digestion containing either *PvuI* or *NotI* with *SphI* to verify that both enzymes are active before proceeding.

3. Adding rSAP maximizes cloning efficiency by de-phosphorylating the backbone to prevent reannealing.

4. Digestion should be visualized using gel electrophoresis, and DNA concentration can be determined using a Nanodrop or Qubit.

5. T4 DNA Ligase may be used as an alternative following manufacturer’s protocol.

6. It is recommended to screen at least 5 putative colonies to ensure obtaining a correct clone.

7. The cPCR should yield a band size of 522 bp.

8. Varying molar ratios of backbone to insert may be used according to the size of the insert.

9. It is recommended to screen at least 4 putative colonies to ensure obtaining a correct clone.

10. Site-directed mutagenesis works well for generating deletions, mutations, and small insertions. For larger insertions, perform a second Gibson assembly instead.

11. Passaging shuttle vectors through *E. coli* strains with varying methylation patterns can enhance transformation efficiencies in lactobacilli.

12. If the concentration is lower than 1000 ng/μL, proceed with ethanol precipitation to further concentrate the DNA.

13. The outlined protocol is for transforming *Lactobacillus plantarum*. Transformation protocol may vary if using other lactobacilli strains.

14. For best results, make MRS-G solution fresh each time by adding 1.25 g of glycine for every 50 mL of MRS broth and filter sterilizing. The amount of glycine required for optimal transformation efficiency may vary with the strain.
15. Transformation efficiency may be reduced when cells do not reach the appropriate OD\textsubscript{600}. Typically OD\textsubscript{600} should reach ~0.8 after 3–4 h of outgrowth. Lower OD\textsubscript{600} (0.4–0.8) works well for some strains, although, for optimal results, do not exceed OD\textsubscript{600} = 1.0 before making cells electrocompetent.

16. For best results, keep cells on ice throughout wash steps.

17. Transformation efficiency may drop significantly after storing cells at \(-80 °C\).

18. MMRS recovery medium has been shown to boost transformation efficiency for some lactobacilli strains.

19. Colonies could start forming after 2 days of incubation. For nonmodel strains of \textit{L. plantarum}, grow for up to 4 days before counting CFU.

20. For many lactobacilli strains, it may be necessary to make fresh electrocompetent cells prior to each transformation.

21. When back diluting the recombineering template strain of lactobacilli, add 10 \(\mu\)g/mL chloramphenicol to MRS-G to ensure the recombineering template shuttle vector is maintained.

22. It is recommended to also transform the cloned targeting CR as well as pCB578 (nontargeting CR) into wild-type lactobacilli strain to validate counterselection.

23. Screening for escape modes can elucidate why editing failed in cases where colony PCR of surviving colonies fails to yield a product or Sanger sequencing reveals the wildtype sequence.

24. For best results, it is recommended to first clear the RT plasmid from the escaper colony by performing growth cycles in MRS medium with erythromycin until a colony is sensitive to chloramphenicol.

25. It is recommended to systematically design primer pairs 500 bp apart.

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