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A Practical CRISPR-Based Method for Rapid Genome Editing in *Caulobacter crescentus*

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Abstract

The RNA-guided Cas enzyme specifically cuts chromosomes and introduces a targeted double-strand break, facilitating multiple kinds of genome editing, including gene deletion, insertion, and replacement. *Caulobacter crescentus* and its relatives, such as *Agrobacterium fabrum* and *Sinorhizobium meliloti*, have been widely studied for industrial, agricultural, and biomedical applications; however, their genetic manipulations are usually characterized as time-consuming and labor-intensive. *C. crescentus* and its relatives are known to be CRISPR/Cas-recalcitrant organisms due to intrinsic limitations of *Sp*Cas9 expression and possible CRISPR escapes. By fusing a reporting gene to the C terminus of *Sp*Cas9M and precisely manipulating the expression of *Sp*Cas9M, we developed a CRISPR/*Sp*Cas9M-reporting system and achieved efficient genome editing in *C. crescentus* and relatives. Here, we describe a protocol for rapid, marker-less, and convenient gene deletion by using the CRISPR/*Sp*Cas9M-reporting system in *C. crescentus*, as an example.

Key features

- CRISPR-*Sp*Cas9M-reporting system overcomes the limitation of CRISPR escape and achieves a high apparent editing efficiency.
- The method enables multiple kinds of genome editing, generating in-frame and marker-less chromosomal modifications.
- The method completes a cycle of genome editing within one week.
- The method could be readily applied for genome editing in *C. crescentus*, *A. fabrum*, and *S. meliloti*.

Keywords: CRISPR, *Sp*Cas9M, *Caulobacter crescentus*, Genome editing, Reporting system

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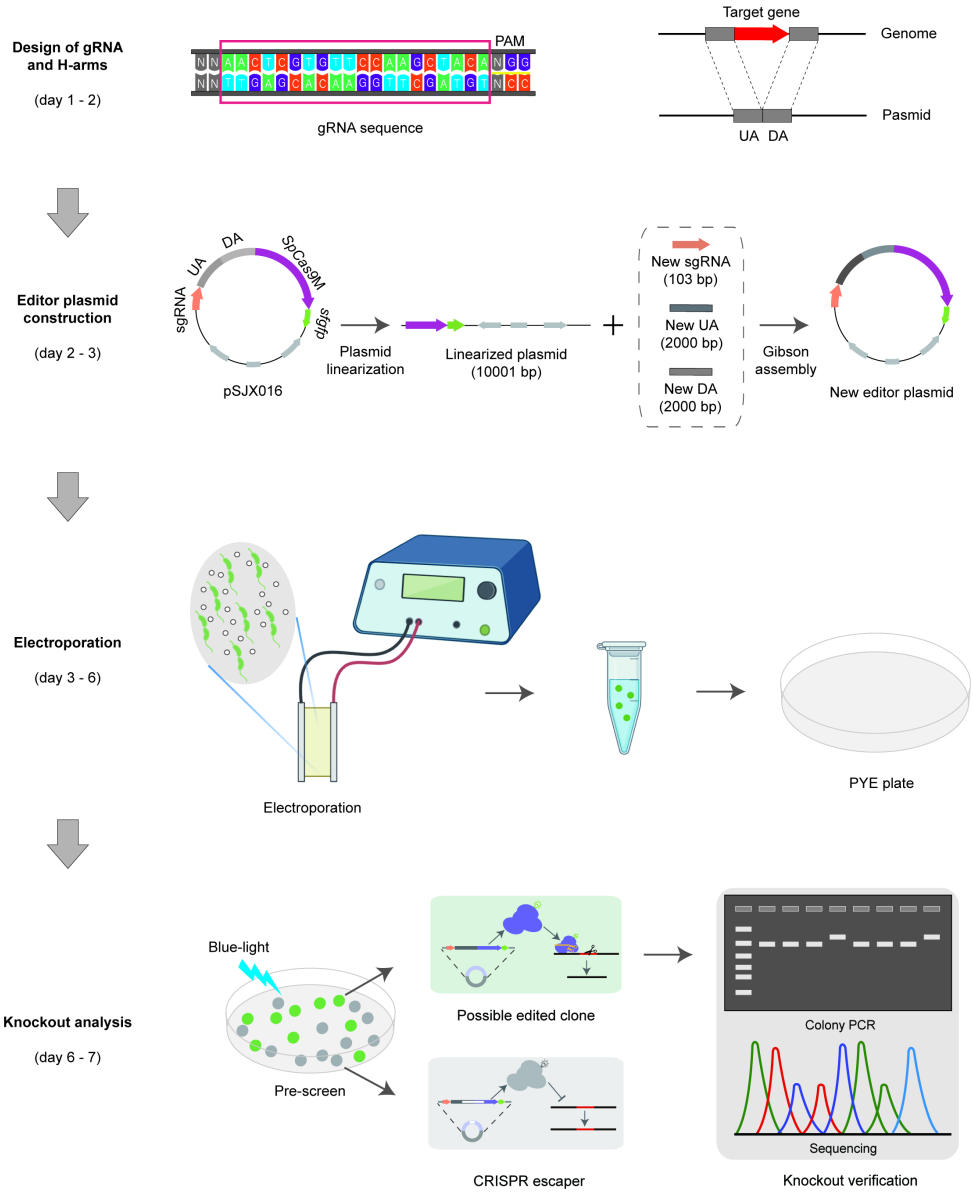
Graphical overview



Workflow of gene deletion using the CRISPR/*Sp*Cas9M-reporting system. Step 1: Design of gRNA and H-arms. All the gRNA sequences were designed in CHOPCHOP [1]. H-arms consist of the upstream arm (UA, 2,000 bp) and the downstream arm (DA, 2,000 bp) of the targeted gene. Step 2: Editor plasmid construction. The starting plasmid pSJX016 was used as a starting point by replacing the sgRNA and H-arms in just one round of Gibson assembly. The corrected editor plasmids were checked by PCR and sequencing. Step 3: Electroporation. The new editor plasmid was transferred into the competent cells of *C. crescentus* through electroporation, and the possible edited cells were first selected on the PYE plates with the corresponding resistance. Step 4: Knockout analysis. The clones normally expressing *Sp*Cas9M were prescreened by the indication of fluorescent sfGFP under a blue-light lamp, and edited clones were finally confirmed by colony PCR and sequencing.

Background

Caulobacter crescentus, a member of Caulobacterales within α-proteobacteria, is a free-living bacterium widely distributed in freshwater environments [2,3]. Due to its tightly regulated cell cycle and asymmetric cell division, *C. crescentus* serves as an excellent model for studying cell polarity and cell differentiation [4–6]. The current genetic tool for chromosomal

deletions and insertions in *C. crescentus* was first established in 1991 by Ely et al., utilizing sacB-based counterselection and allelic exchange via homologous recombination (HR) [7]. However, this method is labor-intensive and exhibits a relatively low editing efficiency [8]. In 2006, Martin Thanbichler and Lucy Shapiro et al. reported a single-crossover integration strategy that enables efficient gene insertion in *C. crescentus* [9,10]. Despite its greatly improved efficiency, this approach integrates the entire editing plasmid (including the selection marker) into the genome and is unsuitable for gene knockout.

CRISPR technology enables precise genome editing by utilizing a vector encoding the Cas enzyme and single-guide RNA (sgRNA). Guided by the sgRNA, the Cas enzyme induces a double-strand break (DSB) at the target sequence [11,12]. The corresponding cells then repair the DSB through either non-homologous end joining (NHEJ) or homology-directed repair (HDR), facilitating targeted gene knockout or knock-in [13]. Unlike the first-generation zinc finger nucleases (ZFNs) and the second-generation transcription activator-like effector nucleases (TALENs), CRISPR relies on the base pairing between gRNA and target DNA rather than protein–DNA binding interactions [14]. This enables CRISPR to exhibit distinct advantages, including both accuracy (editing specificity) and practicality (speed and cost-effectiveness).

For decades, scientists have attempted to develop efficient genome-editing tools for *C. crescentus* and its relatives via HR-based approaches. However, these efforts have largely been unsuccessful [15–17]. To address the challenges, we developed a CRISPR/Cas-assisted HR method for *C. crescentus* using an all-in-one editor plasmid, named CRISPR/*Sp*Cas9M-reporting system [18]. The reporting system facilitates the identification of CRISPR escape events and increases the apparent editing efficiency in *C. crescentus*. This method enables targeted gene deletion and insertion within less than a week, producing in-frame and marker-less edits on the chromosomes. Moreover, it has been successfully applied to two *C. crescentus* relatives, *Agrobacterium fabrum* and *Sinorhizobium meliloti*, establishing it as a general editing strategy [18]. We anticipate that this practical tool could be applicable to other difficult-to-edit organisms, facilitating both basic and applied research in α-proteobacteria.

Materials and reagents

Biological materials

1. *C. crescentus* NA1000 (Lucy Shapiro lab, Department of Developmental Biology, Stanford University School of Medicine)
2. Trans5α chemically competent cell (Transgen Biotech, catalog number: CD201-01)
3. pSJX016 plasmid (our lab, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology)

Reagents

1. PCR primers (Sangon Biotech), standard 25–50 nmol scale
2. 2× Phanta Flash Master Mix (Vazyme, catalog number: P520-01/02/03)
3. KOD One™ PCR Master Mix (TOYOBO, catalog number: 450800)
4. Agarose (Baygene Biotech, catalog number: 232475)
5. Bacto™ peptone (BD, catalog number: 211677)
6. Bacto™ yeast extract (BD, catalog number: 212750)
7. Bacto™ agar (BD, catalog number: 214010)
8. Magnesium sulfate ($MgSO_4$) (Sigma, catalog number: 10034-99-8)
9. SYBR™ Safe DNA gel stain (Invitrogen, catalog number: 2978645)
10. Ethanol absolute (Macklin, catalog number: 64-17-5)
11. 50× TAE (Sangon Biotech, catalog number: B548101-0500)
12. LB broth (Huankai Microbial, catalog number: 028324)
13. LB agar (Huankai Microbial, catalog number: 028334)
14. Gel Extraction kit (Omega, catalog number: D2500020000E20Y090)
15. EasyPure Plasmid MiniPrep kit (TransGen Biotech, catalog number: EM101-02)
16. ClonExpress MultiS One Step Cloning kit (Vazyme, catalog number: C113-01/02)
17. FastDigest DpnI (Thermo Scientific, catalog number: FD1704)
18. 10× FastDigest buffer (Thermo Scientific, catalog number: FD1704)
19. DNA marker (Accurate Biology, catalog number: AG11909)

20. Kanamycin (Sangon Biotech, catalog number: A600286-0025)
21. ddH_2O
22. Vanillic acid (Sigma, catalog number: 34770-10G)
23. $MgSO_4$ (Sigma, catalog number: 10043-52-4)
24. $CaCl_2$ (Sigma, catalog number: 10034-99-8)

Solutions

1. Kanamycin stock solution (50 mg/mL) (see Recipes)
2. LB agar medium (see Recipes)
3. LB liquid medium (see Recipes)
4. Peptone yeast extract (PYE) agar medium (see Recipes)
5. PYE liquid medium (see Recipes)

Recipes

1. Kanamycin stock solution (50 mg/mL)

a. Dissolve 0.5 g of kanamycin (powder) in 10 mL of distilled water at room temperature and filter the solution through a 0.2 μm filter. Then, aliquot the solution into 1 mL fractions and store in a -20 °C freezer.

b. When necessary, supplement media with kanamycin at the following concentrations: 5/25 and 50/50 (liquid/solid media for *C. crescentus* and for *E. coli*, respectively, in μg/mL).

2. LB agar medium

Reagent	Final concentration	Quantity or volume
Bacto tryptone	1% (w/v)	10 g
Yeast extract	0.5% (w/v)	5 g
Agar	1.5% (w/v)	15 g
NaCl	0.5% (w/v)	5 g
H_2O	n/a	Fill up to 1 L
Total	n/a	1 L

3. LB liquid medium

Reagent	Final concentration	Quantity or volume
Bacto tryptone	1% (w/v)	10 g
Yeast extract	0.5% (w/v)	5 g
NaCl	0.5% (w/v)	5 g
H_2O	n/a	Fill up to 1 L
Total	n/a	1 L

4. PYE agar medium

Reagent	Final concentration	Quantity or volume
Peptone	0.2% (w/v)	2 g
Yeast extract	0.1% (w/v)	1 g
Bacto agar	1.5% (w/v)	15 g
1 M $MgSO_4$	1 mM	1 mL
1 M $CaCl_2$	0.5 mM	0.5 mL
H_2O	n/a	Fill up to 1 L
Total	n/a	1 L

5. PYE liquid medium

Reagent	Final concentration	Quantity or volume
Peptone	0.2% (w/v)	2 g
Yeast extract	0.1% (w/v)	1 g
1 M $MgSO_4$	1 mM	1 mL

1 M $CaCl_2$	0.5 mM	0.5 mL
H_2O	n/a	Fill up to 1 L
Total	n/a	1 L

Laboratory supplies

1. Petri dishes (Fisher Scientific, catalog number: FB0875712)
2. 0.22 μm Millipore filter (Millipore, catalog number: R7MA69670)
3. 0.2 mL PCR tube (Corning Life Sciences, catalog number: PCR-0208-C)
4. 1.5 mL microtube (Axygen, catalog number: MCT-150-C)
5. Electroporation cuvette (Bio Rad, catalog number:1652082)
6. Polypropylene round-bottom tube (Falcon, catalog number: 352059)
7. Disposable cell spreader (BKMAMLAB, catalog number: 110305003)
8. Erlenmeyer flask (HEQI GLASS, catalog number: B-000204)
9. High-temperature resistant tissue sealing film (BKMAMLAB, catalog number: 110104005)
10. Pipette tips (KIRGEN, catalog number: KG1333)
11. Cuvettes (Loikaw, catalog number: B-002016)
12. Spectrophotometer (SHST, Model: SH-NanoOne)

Equipment

1. Pipettes (any source)
2. PCR thermocycler (Thermo Fisher Scientific, model: ProFlexTM Base)
3. DNA electrophoresis apparatus (Guangzhou Dao Yi Science and Technology Co., Ltd, model: EPHC 400)
4. Gel image analysis system (Tanon, model: MINI SPACE 960)
5. Nanodrop spectrophotometer (Shenhua Science Technology, model: SH-NnoOne)
6. Centrifuge (Eppendorf, model: 5425)
7. Bacterial incubator (Shanghai Bluepard Instruments)
8. Shaker (Shanghai Chuzhi Biotechnology, model: ZQLY-180ES)
9. Water bath (Shanghai Bluepard Instruments, model: DK-8D)
10. Electroporator (Bio-Rad, model: MicroPulser)
11. Blue-light lamp (MIULAB, model: DUT-48)

Software and datasets

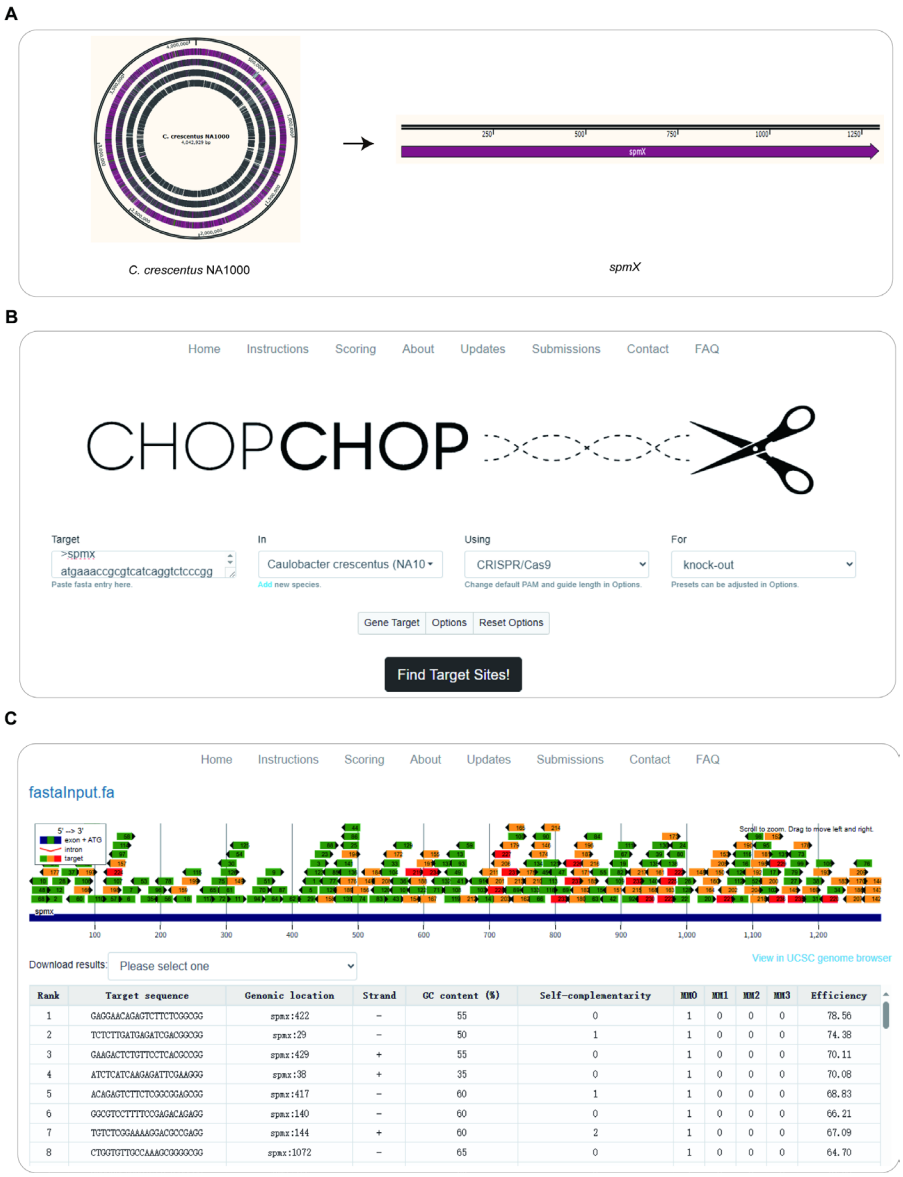
1. Snapgene®4.3.6 (GSL Biotech)
2. Adobe Illustrator, 2021
3. GraphPad Prism 8
4. CHOPCHOP (https://chopchop.cbu.uib.no/)
5. TargetFinder algorithm (https://github.com/ ECBCgit/targetFinder)

Procedure

A. gRNA design

1. Design gRNA in CHOPCHOP sgRNA designer (https://chopchop.cbu.uib.no/) [1] using *C. crescentus* NA1000 as the target organism.
2. Search for the gene of interest in the genome of *C. crescentus* NA1000. Here, we selected *spmX* as the gene of interest (Figure 1).
3. Click the *Target* box and paste the gene sequence of *spmX* in FASTA format.

4. Click the *In* box and select *Caulobacter crescentus (NA1000)*.
5. Click the *Using* box and select *CRISPR/Cas9*.
6. Click the *Using* box and select *knockout*.
7. Click the button *Find Target Sites!* (All the parameters in the *options* are default parameters).
8. The length of the gRNA sequence is 20 bp, and the protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9 (*Sp*Cas9) is 5'-NGG-3' (Table 1).
9. The spacer sequence may be located on the non-transcribed strand or the transcribed strand.
10. Select gRNAs based on computational predictions of high on-target editing efficiency (efficiency score > 60), while excluding sequences with potential self-complementarity or off-target genomic binding.
11. The three most promising gRNAs should be selected.



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GAGGAACAGAGTCTTCTCGGCGG	spmx:422	-	55	0	1	0	0	0	78.56
2	TCTCTTGATGAGATCGACGGCGG	spmx:29	-	50	1	1	0	0	0	74.38
3	GAAGACTCTGTTCCTCACGCCGG	spmx:429	+	55	0	1	0	0	0	70.11
4	ATCTCATCAAGAGATTCGAAGGG	spmx:38	+	35	0	1	0	0	0	70.08
5	ACAGAGTCTTCTCGGCGGAGCGG	spmx:417	-	60	1	1	0	0	0	68.83
6	GGCGTCCTTTTCCGAGACAGAGG	spmx:140	-	60	0	1	0	0	0	66.21
7	TGTCTCGGAAAAGGACGCCCGAGG	spmx:144	+	60	2	1	0	0	0	67.09
8	CTGGTGTTGCCAAAGCGGGGCGG	spmx:1072	-	65	0	1	0	0	0	64.70

Figure 1. Design gRNAs in CHOPCHOP. (A) *C. crescentus* NA1000 was selected as the target organism, and *spmX* was selected as the gene of interest. (B, C) The gene sequence of *spmX* was pasted in the *target* box in fasta format on the CHOPCHOP web interface (https://chopchop.cbu.uib.no/). The following parameters were used: "*Caulobacter crescentus* (NA1000)" was selected in the *In* field, "CRISPR/Cas9" was chosen in the *Using* field, and "Knockout" was specified in the *For* field. After clicking the *Find Target Sites!* button, a list of all potential gRNA sequences was generated within *spmX*. From these output results, the most promising gRNAs with relatively high predicted on-target editing efficiencies (>60) were selected for further use.

Table 1. gRNAs for *spmX* editing, designed by CHOPCHOP

Target sequence	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
GAGGAACAGAGTCTTCTCGG**CGG**	0	1	0	0	0	78.56
GAAGACTCTGTTCCTCACGC**CGG**	0	1	0	0	0	70.11
ATCTCATCAAGAGATTCGAA**GGG**	0	1	0	0	0	70.08

MM0 = 0 mismatches, MM1 = 1 mismatches, MM2 = 2 mismatches, MM3 = 3 mismatches. The regions in bold font represent the PAM sequences of the target DNA.

B. Editor plasmid construction

1. Design H-arms according to the target gene. H-arms consist of upstream (UA) and downstream arms (DA). Select the upstream sequence (2,000 bp) of the start codon as the UA and the downstream sequence (2,000 bp) of the stop codon as DA.
2. Preparation of linearized vector, H-arms, and sgRNA fragments: The primers used to amplify the linearized fragments are listed in Table 2.

Table 2. Primers used in editor plasmid construction

Fragment	Forward primer	Reverse primer	Size
Vector	tccaagctggcgcgcccagcggcgcgga	ccgagaagactctgttcctcactagtattatacctaggactgagctagctg	11,011 bp
sgRNA	gaggaacagagtcttctcgggttttagagctagaaatagcaagttaaaataaggctagtc	gggtgattgtaaaaaaagcaccgactcggtg	113 bp
UA	accgagtcggtgctttttttacaatcacccgatcgccc	ggcaagaaaccgcctagattgacccagtttgag	2,020 bp
DA	aatctaggcggtttcttgcctcgccacc	gctgggcgcgccagcttggaccgatcgt	2,020 bp

a. Amplify the linearized vector via PCR using pSJX016 (Figure 2) as the template (Figure 3).

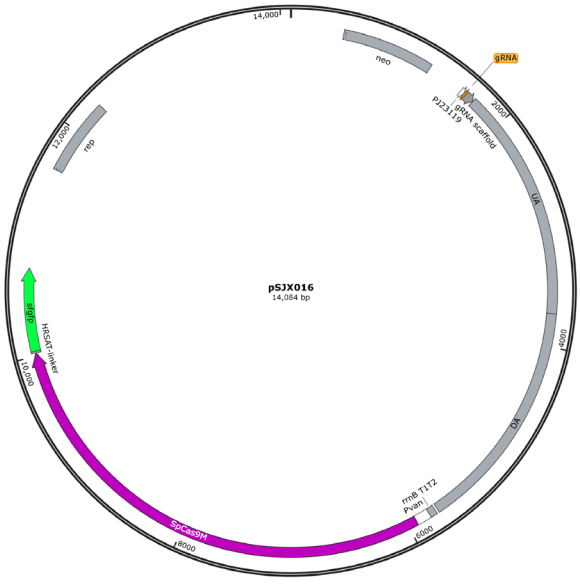


Figure 2. Map of starting editor plasmid pSJX016. pSJX016 is used for genome editing in *C. crescentus* and confers kanamycin resistance in the host cells. It contains the codon-optimized *SpCas9*M that is driven by an inducible promoter (P_{van}, working concentration: 50 μM vanillic acid), the reporter gene, *sfgfp*, which is fused to the C terminus of *SpCas9*M via a flexible HRSAT linker to prescreen the clones normally expressing *Sp*Cas9M, the sgRNA that is driven by a constitutive promoter (P_{J23119}), and the H-arms, which are used for homologous recombination. To generate new knockout constructs, both gRNA and H-arms can be efficiently replaced using Gibson assembly.

b. Amplify the linearized UA and DA fragments via PCR using *C. crescentus* NA1000 genomic DNA as the template (Figure 3).
c. Amplify the linearized sgRNA fragment via PCR using pSJX016 (Figure 2) as the template. Add gRNA sequence as the homologous region to the upstream of the gRNA scaffold (Figures 2 and 3).
d. Conduct the PCR in a 40 μL reaction mixture consisting of 20 μL of KOD One™ PCR Master Mix, 2 μL of forward primer (10 μM), 2 μL of reverse primer (10 μM), 1 μL of template DNA, and 15 μL of H_2O. Amplification is conducted under the following PCR program: 98 °C for 5 min; 28 cycles of 98 °C for 10 s, 60 °C for 5 s, and 68 °C for 1–6 min (2 kb/min); and a final 68 °C for 5 min.

3. Construct the editor plasmids through the Gibson assembly method [19] (Figure 3) in a 20 μL reaction mixture: 0.03 pmol linearized vector, 0.03 pmol linearized sgRNA, 0.03 pmol linearized UA and DA, 4 μL of 5× CE MultiS Buffer, 2 μL of Exnase MultiS, and H_2O up to 20 μL. Then, incubate the 20 μL mixture at 37 °C for 50 min.

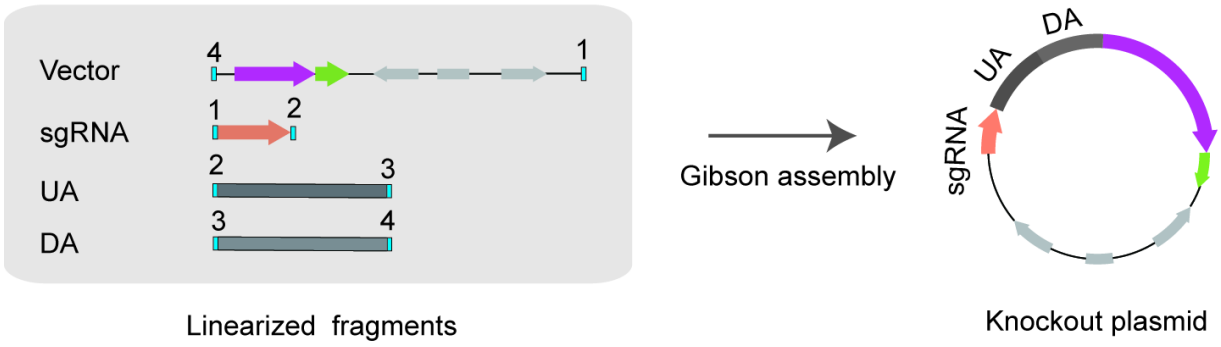


Figure 3. Diagram of constructing editor plasmid by Gibson assembly. By introducing homologous sequences at the 5' end of specific primers, linearized fragments with homologous regions at both ends could be amplified via PCR. Using Gibson assembly, the four linearized fragments could be assembled into a new editor plasmid by sequential homologous recombination with one another. The box filled with blue represents homologous regions (20 bp), and the homologous regions between adjacent fragments are represented by the same number.

C. Competent cells preparation

1. Inoculate 5 μL of bacterial culture of *C. crescentus* NA1000 onto a PYE plate at 30 °C for 2–3 days.
2. Pick the mono-clone, inoculate it in 3 mL of PYE liquid medium, and grow overnight at 30 °C, 200 rpm.
3. Transfer 1 mL of culture into 100 mL of PYE liquid medium and culture it to a final OD_{600} of 0.7–1.0 at 30 °C.
4. Collect the culture into a sterilized ice-cold 50 mL centrifuge tube and centrifuge it at 6,000× *g* for 10 min at 4 °C.
5. Discard the supernatant and resuspend the pellets in 40 mL of cold distilled water; then, centrifuge it at 6,000× *g* for 10 min at 4 °C.
6. After washing three times, resuspend the pellets in 1 mL of cold distilled water and then separate it into 100 μL aliquots.

D. Electroporation

1. Mix 1 μg of editor plasmid into 100 μL of competent cells for 20 min on ice.
2. Transfer the mixture to an ice-cold Bio-Rad Gene Pulser cuvette and subject it to a 2.5 kV electric shock.
3. Set the capacitor to 25 μF and the resistance on the pulse controller to 200 ohms.
4. After the electric shock, transfer the cells from the cuvette to a 1.5 mL tube containing 1 mL of PYE liquid medium for recovery (30 °C). During the recovery period, add the inducer of P_{van}, vanillic acid, to the PYE liquid medium. The working concentration of vanillic acid is 50 μM.
5. After a 3-h recovery period at 30 °C, plate the *C. crescentus* cells onto the appropriate selective PYE plates.

E. Genome editing analysis

1. Pick a single clone from plates and suspend it in 20 µL of reaction mixture, containing 10 µL of 2× Phanta Flash Master Mix, 1 µM primer, and 8 µL of H_2O.
2. Design the primer pairs for genome editing analysis (Figure 4B). The forward primer was located within the UA region, and the reverse primer was located downstream of the DA region.
3. Separate the PCR products by 1% agarose gels and stain with SYBR Safe. Negative controls (usually wild-type cells) and experimental samples were analyzed in parallel to identify nonspecific bands.
4. Verify the PCR products by Sanger sequencing to further confirm the knockout results.

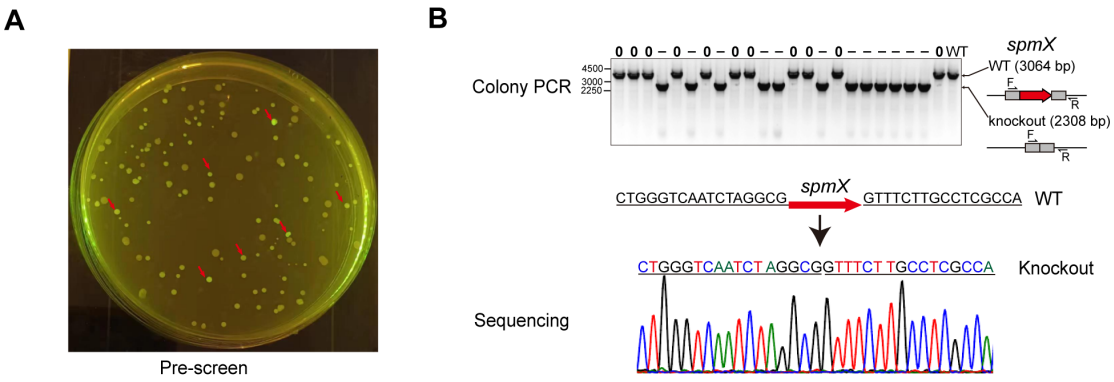


Figure 4. Analysis of *spmX* knockout. (A) The clones normally expressing *Sp*Cas9M (indicated by red arrows) were prescreened by the indication of fluorescent sfGFP under a blue-light lamp. (B) The edited clones were finally confirmed by colony PCR and Sanger sequencing.

F. Editor plasmid curing

1. Culture the correct knockout clone of *C. crescentus* in 5 mL of PYE liquid medium (without any antibiotics) at 30 °C, 200 rpm.
2. After 15–18 h, collect the cells and dilute into 1:1,000 in fresh PYE liquid medium.
3. Spread 50 µL of diluted culture on PYE plates without antibiotics and then incubate at 30 °C for about 2 days.
4. Pick the single clones and replica-spot them on PYE plates with no antibiotics and on PYE plates with antibiotics.
5. Select the clones that grew only on the antibiotic-free plates as the cured strains.

Validation of protocol

This protocol has been used and validated in the following research article:

- Sun et al. [18] A CRISPR/SpCas9M-reporting system for efficient and rapid genome editing in *Caulobacter crescentus*. *Nucleic acids research* (Figure 1, Figure 2, and Figure 3).

General notes and troubleshooting

General notes

1. Editing efficiency varies from 10% to 80% depending on the target, with some genes being far more difficult to edit than others. For essential genes, the conventional knockout is not possible because their loss results in non-viable cells.
2. Due to the significant variation in editing efficiency among different gRNAs, three or more gRNAs need to be selected to ensure successful knockout.
3. To minimize CRISPR escape events, clones exhibiting brighter green fluorescence could be prioritized for colony PCR detection. This pre-selection strategy can improve apparent editing efficiency.

4. The method is still effective for large gene deletions, including a gene as large as 7.7 kb, which was successfully knocked out in this study.
5. Curing efficiencies were usually high, up to 100% within one round.
6. Several common pitfalls, such as low electroporation efficiency, weak GFP expression, and PCR false positives, also influence the results of genome editing, and the corresponding troubleshooting advice is provided in Table 3.

Troubleshooting

Table 3. Troubleshooting advice for common pitfalls

No.	Step	Problem	Suggestion
1	Electroporation	Low electroporation efficiency	Increase the concentration of competent cells and prepare the editor plasmid with high quality.
2	Electroporation	Weak GFP expression	Increase the concentration of P_{van} inducer appropriately in PYE liquid medium.
3	Genome editing analysis	PCR false positives	Confirm the results by PCR again after curing the editor plasmid or change the checking primers.

Acknowledgments

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Competing interests

The authors declare no competing financial interests.

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