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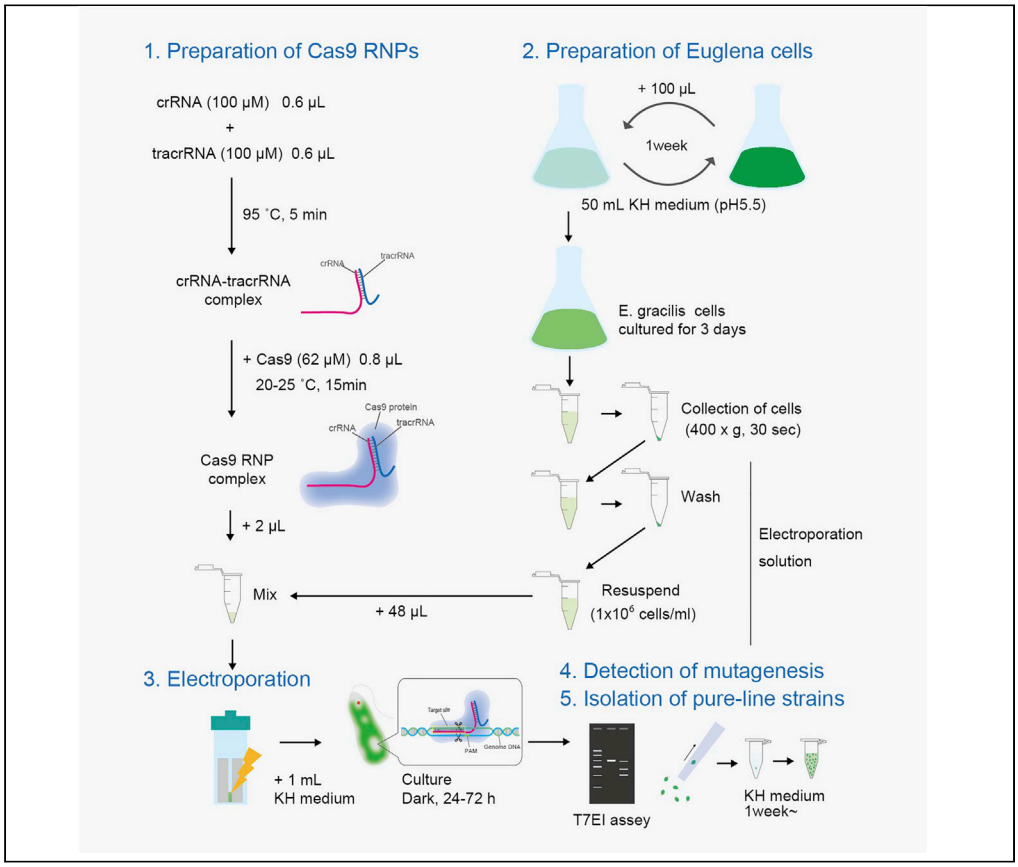
Highly Efficient CRISPR-Associated Protein 9 Ribonucleoprotein-Based Genome Editing in *Euglena gracilis*



Euglena gracilis, a unicellular phytoflagellate microalga, is a promising biomaterial for foods, feeds, and biofuels. However, targeted mutagenesis in this species has been a long-standing challenge. We recently developed a transgene-free, highly efficient, genome editing method for *E. gracilis* using CRISPR/Cas9 ribonucleoproteins (RNPs). Our method achieved mutagenesis rates of approximately 80% or more through an electroporation-based direct delivery of Cas9 RNPs. Therefore, this method is suitable for basic research and industrial applications, such as the breeding of *Euglena*.

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HIGHLIGHTS

Protocol for an efficient genome editing method using Cas9 RNPs in *Euglena gracilis*

Transgene-free targeted mutagenesis is possible via direct delivery of Cas9 RNPs

Highly efficient knock-in of short sequences into the target site is possible

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Highly Efficient CRISPR-Associated Protein 9 Ribonucleoprotein-Based Genome Editing in *Euglena gracilis*

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SUMMARY

***Euglena gracilis*, a unicellular phytoflagellate microalga, is a promising biomaterial for foods, feeds, and biofuels. However, targeted mutagenesis in this species has been a long-standing challenge. We recently developed a transgene-free, highly efficient, genome editing method for *E. gracilis* using CRISPR/Cas9 ribonucleoproteins (RNPs). Our method achieved mutagenesis rates of approximately 80% or more through an electroporation-based direct delivery of Cas9 RNPs. Therefore, this method is suitable for basic research and industrial applications, such as the breeding of *Euglena*.**

For complete details on the use and execution of this protocol, please refer to Nomura et al. (2019).

BEFORE YOU BEGIN

⚠ CRITICAL: All procedures in this protocol are performed aseptically on a clean bench.

Culture of *Euglena gracilis*

TIMING: 3–7 days

1. *Euglena gracilis* Z strain was cultured using KH medium (Koren, 1967) adjusted to pH 5.5 with potassium hydroxide on a rotary shaker (120 rpm) at 28°C under continuous light (50 μmol photons m^{-2} s^{-1}) conditions.
2. The culture was maintained by inoculating 100 μL into 50 mL of fresh KH medium every week (Figures 1A and 1C).
3. For the direct delivery of Cas9 ribonucleoproteins (RNPs) by electroporation, *E. gracilis* cells that have been cultured for 3 days are used (Figure 1B).

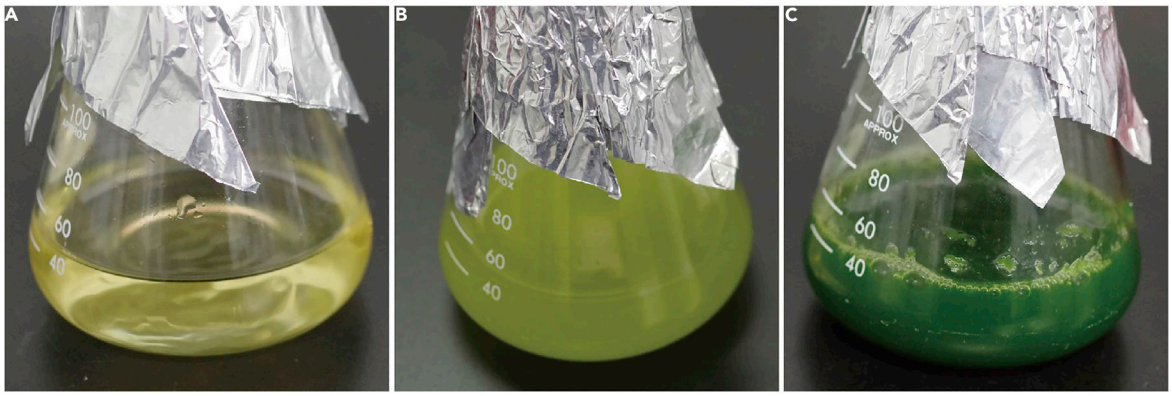

Figure 1. Liquid Culture of *Euglena gracilis*

Just after the *E. gracilis* cells were transferred to fresh KH medium (A), after culturing for 3 days (B), and 7 days (C).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Alt-R CRISPR-Cas9 tracrRNA	Integrated DNA Technologies	1072532
Alt-R CRISPR-Cas9 crRNA	Integrated DNA Technologies	N/A
Alt-R S.p. Cas9 Nuclease V3	Integrated DNA Technologies	1081058
Ultramer DNA Oligos	Integrated DNA Technologies	N/A
Kaneka Easy DNA Extraction Kit v.2	Kaneka	KN-T110005
Tks Gflex DNA Polymerase	Takara Bio	R060A
CloneJET PCR Cloning Kit	Thermo Scientific	K1231
Alt-R Genome Editing Detection Kit	Integrated DNA Technologies	1075931
$(NH4)_2HPO_4$	FUJIFILM Wako Pure Chemical Corporation	016-03325
KH_2PO_4	FUJIFILM Wako Pure Chemical Corporation	169-04245
$MgSO_4 \cdot 7H_2O$	FUJIFILM Wako Pure Chemical Corporation	131-00405
$CaCl_2$	FUJIFILM Wako Pure Chemical Corporation	038-24985
$Fe_2(SO_4)_3 \cdot nH_2O$	FUJIFILM Wako Pure Chemical Corporation	091-02832
$MnCl_2 \cdot 4H_2O$	FUJIFILM Wako Pure Chemical Corporation	139-00722
$ZnSO_4 \cdot 7H_2O$	FUJIFILM Wako Pure Chemical Corporation	264-00402
$CoSO_4 \cdot 7H_2O$	FUJIFILM Wako Pure Chemical Corporation	032-03802
$Na_2MoO_4 \cdot 2H_2O$	FUJIFILM Wako Pure Chemical Corporation	196-02472
$CuSO_4 \cdot 5H_2O$	FUJIFILM Wako Pure Chemical Corporation	039-04412
Vitamin B_1 (Thiamin)	FUJIFILM Wako Pure Chemical Corporation	201-00852
Vitamin B_{12}	FUJIFILM Wako Pure Chemical Corporation	226-00343
10% Sulfuric acid	FUJIFILM Wako Pure Chemical Corporation	198-11705
Potassium hydroxide	FUJIFILM Wako Pure Chemical Corporation	168-21815
Experimental Models: Organisms/Strains		
Euglena gracilis Z	Institute of Applied Microbiology (IAM) culture collection	IAM E-6, NIES-48

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
BioTRON	NK systems	LPH-411SP
Shake-LR	TAITEC	0054809-000
T100 Thermal Cycler	Bio-Rad	#1861096
NEPA21 Super Electroporator	Nepa Gene	N/A

MATERIALS AND EQUIPMENT

Design of the Target Sequence for crRNA Synthesis

Normally, the target sequence used is 20 bp upstream of the protospacer adjacent motif sequence (5′-NGG-3′). The GC content of the target sequence should be 40%–60%. It is recommended to design two or more target sequences for one target gene.

Preparation of Stock crRNA and tracrRNA Solutions (100 μM) with Nuclease-free Duplex Buffer

Alt-R CRISPR-Cas9 crRNA (CRISPR RNA) and tracrRNA (trans-activating crRNA) were synthesized by Integrated DNA Technologies (IDT), and a 100-μM solution was prepared using nuclease-free duplex buffer (IDT).

Note: The stock solution can be stored at −20°C.

Preparation of the Modified CM Medium (Cramer and Myers, 1952)

- Prepare a modified CM medium with the following composition.

Composition of Modified CM Medium

Reagent	Weight per L
$(NH4)_2HPO_4$	1.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	0.02 g
$Fe_2(SO_4)_3 \cdot nH_2O$	3 mg
$MnCl_2 \cdot 4H_2O$	1.8 mg
$ZnSO_4 \cdot 7H_2O$	0.4 mg
$CoSO_4 \cdot 7H_2O$	1.5 mg
$Na_2MoO_4 \cdot 2H_2O$	0.2 mg
$CuSO_4 \cdot 5H_2O$	0.02 mg
Vitamin B_1	0.1 mg
Vitamin B_{12}	0.0005 mg

Adjust the pH to 5.5 with 10% sulfuric acid.
Autoclave at 121°C for 15 min.

Note: CM medium can be stored at 20°C–25°C.

Preparation of Electroporation Solution

- Prepare a 0.3-M sucrose solution and sterilize using a 0.2-μm pore size syringe filter.
- Mix the modified CM medium and the filter sterilized sucrose solution at a ratio of 3:2 (v/v).

Table 1. Alt-R CRISPR-Cas9 crRNA Reagent Volumes Used in Each Electroporation Reaction

Reagent (μM)	Volume (μL)
Alt-R CRISPR-Cas9 crRNA (100)	0.6
Alt-R CRISPR-Cas9 tracrRNA (100)	0.6
Total volume	1.2

Note: The electroporation solution can be stored at 20°C–25°C.

Alternatives: This protocol can be implemented using other thermal cyclers, plant growth chambers, and orbital shakers with equivalent device performance as the above equipment.

STEP-BY-STEP METHOD DETAILS

Preparation of Cas9 RNPs

TIMING: 30 min

This step describes the procedure to prepare Cas9 RNP complexes for electroporation.

1. Equal amounts of crRNA and tracrRNA solution (100 μM stock) were mixed in a 0.2-mL PCR tube. An example of the reagent amounts used for one electroporation reaction is shown in Table 1.
2. The mixture was heated at 95°C for 5 min and then cooled to 20°C using a thermal cycler with a ramp rate setting of −0.1°C/s.

Alternatives: Heated crRNA-tracrRNA complex can be cooled on the bench at 20°C–25°C.

3. Alt-R S.p. Cas9 Nuclease V3 (IDT, 62 μM solution) was added to the cooled gRNA complex. Examples of the amount of reagent used for one electroporation reaction are shown in Table 2.
4. To form the Cas9 RNP complexes, the mixture was incubated for 15 min at 20°C–25°C.

PAUSE POINT: The Cas9 RNP complex can be stored at −80°C for 6 months.

Direct Delivery of Cas9 RNPs by Electroporation

TIMING: 10–20 min

This step describes the procedure to deliver Cas9 RNPs to *E. gracilis* cells by electroporation.

5. *E. gracilis* was cultured in KH medium (pH 5.5) for 3 days (Figure 1B), and 1 mL of the cell culture was transferred to a 1.5 mL tube (Figure 2A).

Note: The optical density at 600 nm of 10-fold dilution of *E. gracilis* after 3 days of culture is approximately 0.5–0.7.

6. The cell culture sample was centrifuged at 400 × *g* for 30 s (Figure 2B), and the supernatant was removed.

Table 2. crRNA-tracrRNA Complex and Alt-R CRISPR-Cas9 crRNA Reagent Volumes Used in Each Electroporation

Reagent (μM)	Volume (μL)
crRNA-tracrRNA complex	1.2
Alt-R S.p. Cas9 Nuclease V3 (62)	0.8
Total volume	2.0

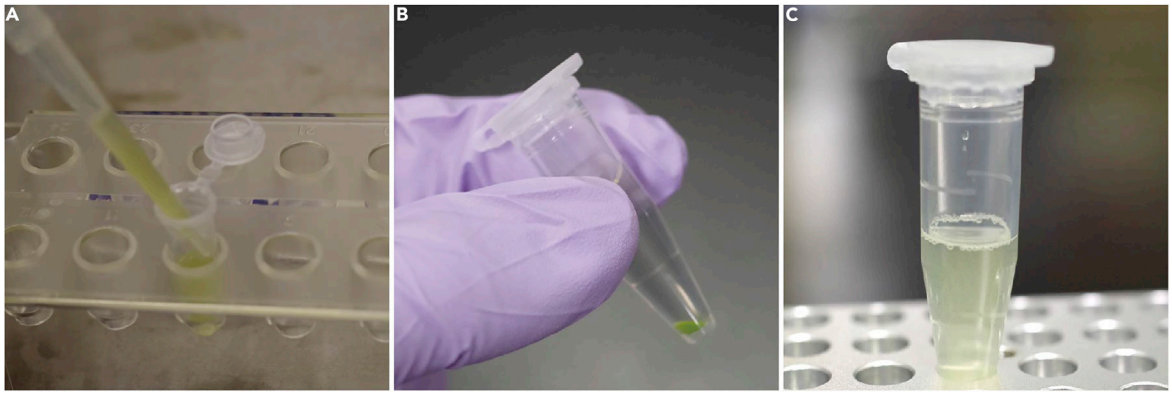

Figure 2. Preparation of the Suspension of E. gracilis Cells for Electroporation

Transfer of cultured *E. gracilis* cells (A), *E. gracilis* cells pellet after centrifugation (B), Resuspended *E. gracilis* cells (C).

7. The cells were then resuspended with the addition of 1 mL of electroporation solution.
8. The cell suspension was centrifuged at 400 × *g* for 30 s, and the supernatant was removed.
9. The cells were resuspended in electroporation solution at a concentration of 1 × 10^6 cells/mL (Figure 2C).
10. Then, 48 μL of the *E. gracilis* suspension was transferred to 0.2 mL new tube (Figure 3A).
11. Finally, 2 μL of Cas9 RNP complex solution was added and the solution was mixed several times by pipetting (Figure 3B). Alternatively, to prevent the loss of Cas9 RNPs solution, the *E. gracilis* suspension can be directly transferred to a tube containing 2 μL of the RNP complex solution.

⚠ CRITICAL: The amount of Cas9 RNPs is important. If the RNP amount is reduced, the genome editing efficiency will decrease.

12. The Cas9 RNPs and *E. gracilis* suspension mixture was transferred to a 2-mm gap cuvette (EC-002; Nepa Gene) (Figure 3C).
13. The cuvette was tapped to drop the Cas9 RNP and cell suspension mixture to the bottom of the cuvette (Figure 3D).
14. A NEPA21 Super Electroporator (Nepa Gene) was used for the introduction of the RNP complexes. The electroporation conditions are shown in Table 3 and Figure 4A.
15. The cuvette was inserted into the electrode chamber (Figure 4B), and the "Ω" switch was pressed. The impedance value was usually approximately 0.4–0.5 kΩ (Figure 4C).

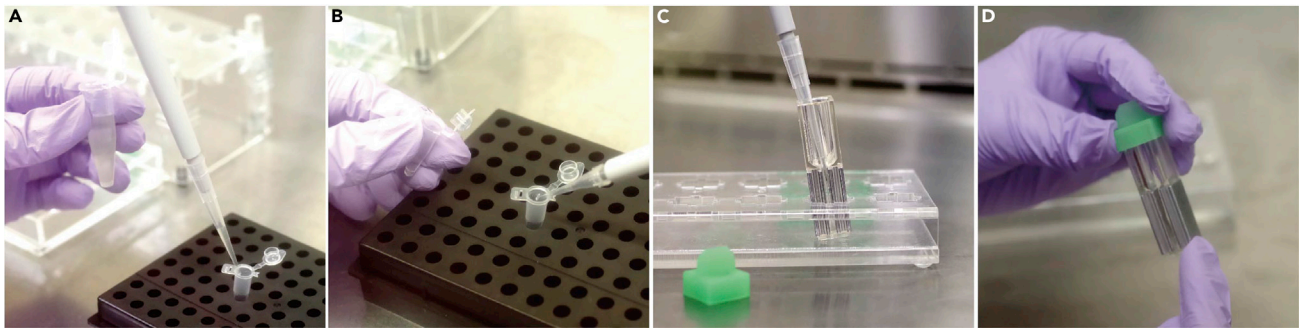

Figure 3. Preparation Procedure for Cas9 RNPs Introduction by Electroporation

Transfer of resuspended *E. gracilis* cells (A), Addition of Cas9 RNPs (B), Transfer of Cas9 RNPs and cell suspension mixture to cuvette (C), Tapping of the cuvette (D).

Table 3. Settings for the NEPA21 Super Electroporator

	Poring Pulse	Transfer Pulse
Voltage (V)	300	20
Pulse length (ms)	5	50
Pulse interval (ms)	50	50
Number of pulses	2	5
Decay rate (%)	40	40
Polarity switching	+	±

16. The "Start" switch was pressed to initiate electroporation (Figure 4D).
17. After electroporation, 1 mL of KH medium (pH 5.5) was added to the cuvette (Figures 5A and 5B).
18. The cell suspension was transferred to a 12-well plate (Figure 5C) and cultured on a rotary shaker (120 rpm) at 28°C under dark conditions (by covering with aluminum foil) for 24–72 h (Figures 5D and 5E).

Note: Seal the plate with parafilm and surgical tape to prevent evaporation of the culture medium.

Single-Stranded Oligodeoxynucleotide-Mediated Knockin (Optional)

TIMING: 5 min

The high-efficiency knockin of short sequences (~50 bp) is possible by introducing the Cas9 RNPs and single-stranded oligodeoxynucleotides (ssODNs) with 50 bp upstream and downstream homology sequence arms of the target site (Nomura et al., 2019).

19. ssODNs were synthesized by IDT, and a 200 μM solution was prepared with nuclease-free duplex buffer (IDT)
20. For electroporation, 2 μL of RNP complex solution and 1 μL of 200 μM ssODN stock solution were added to 47 μL of the *E. gracilis* suspension.

Note: It is possible to rewrite the target base using an ssODN containing the nucleotide substitutions. In this case, to prevent re-cleavage by Cas9 RNPs, two or more base mismatches are required in the seed region of the target sequence in ssODN.

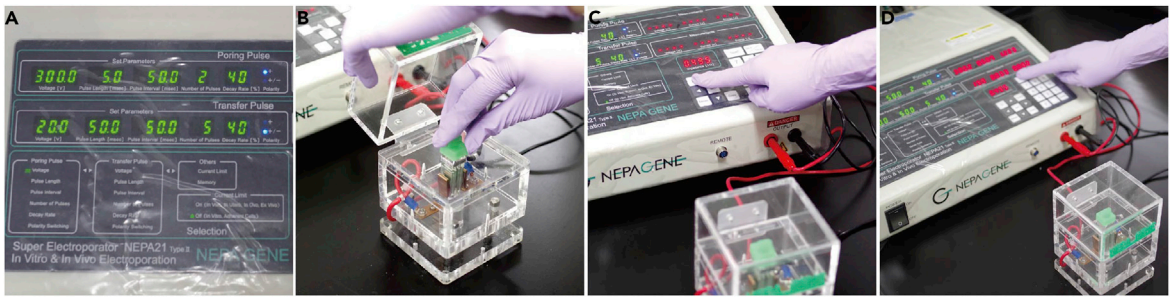

Figure 4. Operation Procedure for the Electroporator

Settings of the electroporator (A), Insertion of the cuvette into the electrode chamber (B), Measurement of the impedance value (C), Initiation of electroporation (D).

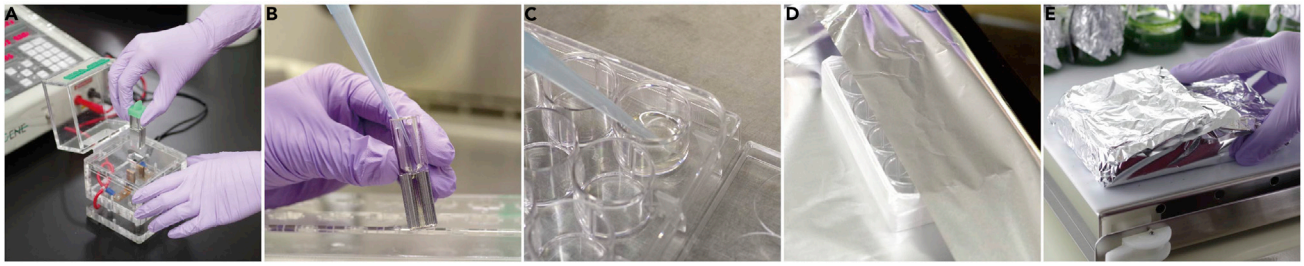

Figure 5. Procedure of Recovery Culture after Electroporation

Ejection of the cuvette from the electrode chamber (A), Addition of KH medium (B), Transfer of cell suspension to well plate (C), Covering of culture plate with aluminum foil (D) and recovery culture on a rotary shaker (E).

Detection of Targeted Mutagenesis

TIMING: 3 h or more

For the detection of mutagenesis at on-target sites in Cas9 RNP-introduced *E. gracilis*, a T7 endonuclease I (T7EI) mismatch cleavage assay was performed using an Alt-R Genome Editing Detection Kit (IDT).

21. The DNA template was extracted from the small *E. gracilis* cells pellet (Figure 6A) using a Kaneka Easy DNA Extraction Kit v.2 (Kaneka).

    Note: Alternatively, another DNA extraction kit can be used.

22. DNA fragments, including the target sites, were amplified by Tks Gflex DNA Polymerase (Takara Bio) using the specific primer set.
23. Using the amplified DNA fragments, the T7EI treatment was performed according to the product manufacturer's instructions.
24. The digested DNA fragments were monitored using 1% agarose gel electrophoresis (Figure 6B).

    Note: Because T7EI does not recognize single-base indels, the T7EI assay estimates a lower editing efficiency rate.

Pure-Line (Single-Cell-Derived Clone) Isolation from a Single *E. gracilis* Cell

TIMING: 1 week or more

This step describes how to obtain a single-cell-derived clone in *E. gracilis*.

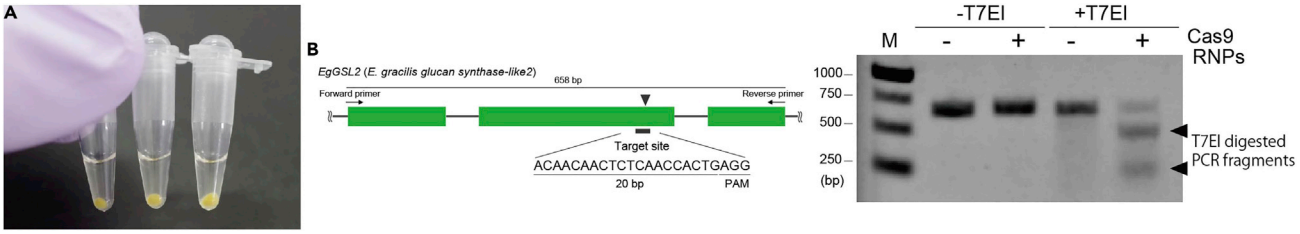

Figure 6. Example of T7 Endonuclease I Mismatch Cleavage Assay

Small *E. gracilis* cells pellet after recovery culture for DNA extraction (A) and example of T7E1 assay results (B).

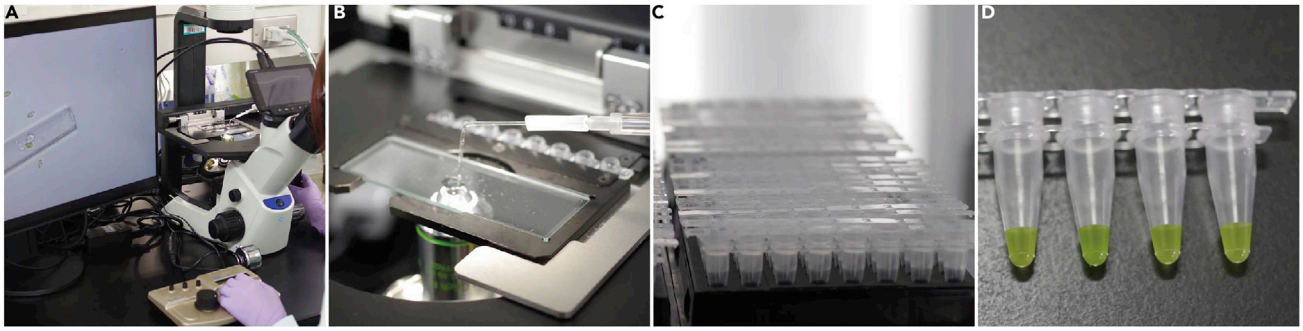

Figure 7. Procedure of Pure-Line Isolation from Single Cell of *E. gracilis*

A single *E. gracilis* cell isolation using a micro pick-and-place system (A and B), Culture from isolated single *E. gracilis* cell (C and D).

25. Following electroporation after 24–72 h culture in KH medium, a single cell was isolated using a micro pick-and-place system (Nepa Gene) (Figures 7A and 7B).

    Alternatives: A single cell of *E. gracilis* can be manually isolated using a glass capillary pipette or cell sorter.

26. The isolated single cell was cultured for ≥7 days using KH medium to obtain a pure line (Figures 7C and 7D).

    Note: Contamination can be suppressed by using KH medium at pH 3.5 for culture.

Genotyping by Sanger Sequencing

TIMING: 3 days

This step describes Sanger sequencing for genotyping of *E. gracilis*.

27. The DNA template was extracted from a 5–10-μL cell pellet volume of *E. gracilis* using the Kaneka Easy DNA Extraction Kit v.2 (Kaneka).
28. DNA fragments, including the target sites, were amplified by Tks Gflex DNA Polymerase using the specific primer set for target site.

    Note: A PCR product length of approximately 500–1000 bp is suitable for genotyping.

29. To clone the PCR products, we used a CloneJET PCR cloning kit (Thermo Fisher Scientific).
30. Colonies were then selected with the vector containing the insert DNA by colony PCR.
31. Each plasmid was extracted using a plasmid DNA extraction Mini Kit (FAVORGEN), and sequences were determined using Sanger sequencing.

EXPECTED OUTCOMES

Euglena gracilis, a unicellular phototrophic protist, is a promising material for foods, feeds, and biofuels. However, the development of a targeted mutagenesis method in this species has been a long-standing challenge. Among the current genetic manipulation technologies, genome editing by the direct delivery of RNPs has various advantages, including time effectiveness, a low cytotoxicity, a high efficiency, and the reduction of off-target effects (Jeon et al., 2017). In our method, insertion and/or deletion (Indel) mutations rate of 77.7%–90.1% has been detected by amplicon sequencing in two different target sequences in the *EgGSL2* gene (Nomura et al., 2019). Therefore, our developed RNP-based genome editing in *E. gracilis* opens up new avenues to reveal the functions of genes.

In addition, based on our developed method, it will be possible to develop convenient CRISPR-based technology for the basic research of *E. gracilis*, such as chromosome visualization and epigenome editing (Doudna and Charpentier, 2014; Wang et al., 2016). Furthermore, RNP-based genome editing without transgenes is suitable for industrial use because it can potentially bypass the regulation of genetically modified organisms. Thus, RNP-based genome editing in *E. gracilis* paves the way for improving its industrially relevant traits to promote production of bio-based material.

LIMITATIONS

Because the published genome sequence information of *E. gracilis* strain Z1 appears to be fragmented (Ebenezer et al., 2019), it has been difficult to precisely assess the off-target effects of genome editing in *E. gracilis*. This limitation will be resolved by improving the genome assembly of *E. gracilis*. To confirm a phenotypic change by on-target in genome-edited strains, several target sites for a target gene should be examined. Off-target effect can be suppressed by reducing the amount of RNPs and using a truncated (~18 bp) target sequence (Fu et al. 2014). Furthermore, off-target can be reduced by the use of S.p. HiFi Cas9 Nuclease V3 (IDT).

In case a different strain of *E. gracilis* is used, efficiency may decrease due to differences in cell properties, such as growth rate. Therefore, in such cases, the protocol for each strain needs to be optimized. In addition, there is a possibility that parts of the genomic DNA sequence vary depending on the strain of *E. gracilis* used. Before designing the target sequence, it is also necessary to obtain information on the surrounding DNA sequence of the target site by cloning and sequencing.

The maximum length of single-stranded DNAs that can be knocked in in our protocol remains undetermined. It is generally considered that the homology arm needs to be lengthened according to the length of the DNA to be knocked in. Although the present method has enabled us to precisely knockin a DNA fragment of at least 50 bp in length, an improvement in our method for the knockin of longer DNAs in *E. gracilis* will provide opportunities to engineer *E. gracilis* cells to enhance its productivity and function.

TROUBLESHOOTING

Problem

Low mutagenesis efficiency

Potential Solution

In cases when the genome-edited cells exhibit a slow growth phenotype, the mutagenesis efficiency could be decreased due to the faster growth of wild-type cells. This problem can be solved by earlier cell isolation (e.g., 24 h) after the introduction of Cas9 RNPs. On the other hand, if the performance of the designed target sequence is poor it can be improved by doubling the amount (4 μL/total 50 μL reaction) of Cas9 RNPs used for electroporation.

ACKNOWLEDGMENTS

We thank Aya Ide and Hiromi Ojima for their support in the acquisition of the photos in this experimental protocol.

AUTHOR CONTRIBUTIONS

Methodology, T.N.; Investigation, T.N. and M.Y.; Resources, K.S., Writing – Original Draft, T.N. and K.M.

DECLARATION OF INTERESTS

K.S. is an employee and shareholder of euglena Co., Ltd. T.N., K.S., and K.M. have applied for a patent for this method.

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