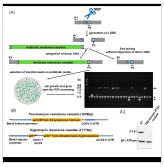

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TIM, a Targeted Insertional Mutagenesis method utilizing CRISPR/Cas9 in *Chlamydomonas reinhardtii*

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bdcki2uw

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ABSTRACT

Generation and subsequent analysis of mutants is critical to understanding the functions of genes and proteins. Here we describe TIM, an efficient, cost-effective, CRISPR-based targeted insertional mutagenesis method for the model organism *Chlamydomonas reinhardtii*. TIM utilizes delivery into the cell of a Cas9 ribonucleoprotein (RNP) together with exogenous double-stranded (donor) DNA. The donor DNA contains gene-specific homology arms and an integral antibiotic-resistance gene that inserts at the double-stranded break generated by Cas9. After optimizing multiple parameters of this method, we were able to generate mutants in six out of six different genes in two different cell-walled strains with mutation efficiencies ranging from 40% to 95%. Furthermore, these high efficiencies allowed simultaneous targeting of two separate genes in a single experiment. TIM is flexible with regard to many parameters and can be carried out using either electroporation or the glass-bead method for delivery of the RNP and donor DNA. TIM achieves a far higher mutation rate than any previously reported for CRISPR-based methods in *C. reinhardtii* and promises to be effective for many, if not all, non-essential nuclear genes.

MATERIALS TEXT

Media

Tris-acetate-phosphate (TAP) medium [1]

M (minimal) medium I [2] modified to contain 0.0022 M KH_2PO_4 and 0.00171 M K_2HPO_4 [3]

M-N medium (modified M medium lacking NH_4NO_3)

Plasmids containing drug-resistance cassette

The paromomycin-resistance cassette can be amplified from the pKS-aphVIII-lox aphVIII plasmid.

The hygromycin-resistance cassette can be amplified from the pHyg3 plasmid [4].

Note: Both plasmids are available from the Chlamydomonas Resource Center https://www.chlamycollection.org/.

Reagents and equipment

IDT Alt-R® S.p.Cas9 Nuclease V3 (10 µg/µL)

IDT Alt-R®CRISPR-Cas9 tracrRNA, 5 nmol

IDT Alt-R®CRISPR-Cas9 crRNA, 2 nmol

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Paromomycin

Hygromycin

Bio-Rad 0.2-cm gap electroporation cuvettes

BTX electroporation Electro cell manipulator ECM-600 (or equivalent) OR glass beads (0.45-0.52mm diameter)

Selected citations for materials above

1. Gorman DS, Levine RP. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. Proc Natl Acad Sci U S A. 1965;54(6):1665-9.
2. Sager R, Granick S. Nutritional studies with *Chlamydomonas reinhardi*. Ann N Y Acad Sci. 1953;56(5):831-8.
3. Witman GB. Isolation of *Chlamydomonas* flagella and flagellar axonemes. Methods Enzymol. 1986;134:280-90.
4. Berthold P, Schmitt R, Mages W. An engineered *Streptomyces hygroscopicus aph 7"* gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. Protist. 2002;153(4):401-12.

gRNA design

1 gRNA should target an exon in the first 2/3 of the gene of interest to increase the chance of generating a null mutation.

2 gRNA design websites: IDT: https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM -or- CRISPR direct: http://crispr.dbcls.jp.

Donor DNA preparation

3 A first set of PCR primers is designed to amplify the appropriate antibiotic-resistance cassette from plasmid DNA.

4 A second set of primers is designed to add homology arms to the amplified antibiotic-resistance cassette. These homology arms are specific for the gene of interest. Gene-specific homology should be upstream and downstream of the SpCas9 cut site.

5 PCR products are gel purified and eluted in water.

Transformation with SpCas9/gRNA RNP and donor DNA

6 Grow cells synchronously in a 14:10 light:dark cycle on tris-acetate phosphate (TAP) + 1.5% agar plates until confluent (confluency is usually achieved in 3-5 days).

7 Transfer cells from one plate to 6 mL of gametic autolysin solution and leave for 1.0 h at room temperature with gentle shaking. Cell-wall removal by autolysin treatment is critical for achieving high mutation rates. To test the effectiveness of the autolysin treatment, mix a small aliquot of cells (~10 µL) with an equal volume of 0.5% Triton-X100 and observe under a phase microscope using a 16x-objective lens. If the autolysin treatment was effective, the majority of cells should have undergone lysis. Multiple autolysin treatments might be needed to achieve effective cell-wall removal.

8 Following cell-wall removal, incubate the cells (still in autolysin solution) at 40°C for 30 minutes with gentle agitation.

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9 During incubation in autolysin, generate the gRNA and the ribonucleoprotein complex (RNP).

10 To assemble the gRNA, add 10 µL of 40 µM tracrRNA in IDT RNA duplex buffer to 10 µL of 40 µM crRNA in RNA duplex buffer and anneal by heating for 2 min at 95°C followed by slow cooling to room temperature on the bench-top.

11 To form the RNP, incubate 2 µL of gRNA complex with 5 µg of IDT Alt-R® S.p.Cas9 Nuclease V3 (10 µg/µL) in RNA duplex buffer in a total volume of 10 µL for 15 min at 37°C.

12 Wash the autolysin-treated cells in TAP + 2% sucrose and centrifuge at 1819 x g for 5 minutes. Resuspend the cells to a concentration of 2.0-7.0x10^8 cells/mL in TAP + 2% sucrose (usually between 500 µL to 1 mL).

13 Mix approximately 110 µL of cell suspension + 10 µL RNP mix + 2 µg donor DNA (volume of cell suspension will vary depending on concentration of donor DNA) to give a final volume of 125 µL.

14 Transfer the mixture to a Bio-Rad 0.2-cm gap electroporation cuvette. Electroporate cells at 350 V, 25 Ω, and 600 µF.

15 Immediately following electroporation, cool the cuvette (containing cells) in a 16°C water bath for 1 h.

16 Transfer cells to 10 mL of TAP + 2% sucrose (total volume) and incubate for 24 h at room temperature with gentle rocking under dim light.

17 The following day, collect cells by centrifugation at 1819 x g for 5 minutes, resuspend the cell pellet in 3.5 mL of TAP + 0.5% agar (< 42°C), and pipette onto TAP + 1.5% agar plates containing the appropriate selection agent (10 µg/mL of paromomycin or hygromycin).

18 Grow plated cells with a 14:10 light:dark cycle at 23°C for approximately 1 week, or until colonies are observed.

19 Transfer individual colonies to 150 µL M medium in 96-well plates and grow for 2-3 days with a 14:10 light:dark cycle.

20 Perform initial screening of cell lines by PCR with primers designed to flank the SpCas9 cut site.

If using the glass-bead method instead of electroporation, replace steps 14 and 15 of the above protocol with the following:

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21 Transfer the mixture to a 15-mL tube with 0.3 g of glass beads (0.45-0.52 mm diameter). In contrast to Kindle [1], we do not add PEG to the mixture.

22 Vortex immediately at the top speed on a Vortex Genie 2 (Scientific Industries) for 15 seconds, rest the tube for 10 seconds, and then vortex again for 10 seconds.

Reference

23 1. Kindle KL. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci U S A. 1990;87(3):1228-32.

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