Homology-directed transgene-free gene editing in Chlamydomonas reinhardtii

Chapter 14

Cover page

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Chapter 14

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Running head

Gene editing in *Chlamydomonas reinhardtii*

Abstract

Chlamydomonas reinhardtii is a microalgal model organism with a suite of molecular and genetic techniques, but routine editing of its nuclear genome is yet to be realised. DNA-based transformation techniques are prohibitively inefficient and lead to predominantly non-homologous (i.e. off-target) integration. Standard CRISPR-based gene editing protocols have proved too ineffective to enable routine application. We have found that the use of CRISPR/Cpf1 in conjunction with single-stranded DNA (ssODN) repair templates achieves nuclear gene editing efficiencies as high as 30% [1]. This produces edits with predictable outcomes in a transgene- and selection marker-free manner. The possibility to purchase all necessary reagents commercially with no preparation time (besides design) facilitates rapid and routine genetic engineering in this organism. Here we describe the use of this technique to knockout locus *FKB12*, which leads to rapamycin resistance and lends itself to an easy assay when adopting this gene-editing protocol.

Keywords: CRISPR, Cpf1, *Chlamydomonas reinhardtii,* ssODN, homology-directed repair (HDR)

14.1 Introduction

Chlamydomonas reinhardtii is the most thoroughly studied microalga [2]. It has been quintessential to the fields of photosynthesis, cilia biology, lipid metabolism, carotenoid

biosynthesis, and more [2]. It's acutely tractable owing to a suite of well-developed molecular and genetic techniques. Remarkably however, gene editing of its nuclear DNA is yet to become routine practice.

Nuclear gene targeting in *C. reinhardtii* has historically been precluded by inefficient DNA-mediated transformation with predominantly non-homologous (off-target) outcomes [3-5]. The emergence of gene editing enzymes like Cas9 now enables circumventing these attributes of *C. reinhardtii*. Critically, however, transgenic expression of gene editing enzymes is still subject to low transformation efficiency, pervasive transgene fragmentation [5], and transgene silencing [6,7], which necessitates the use of positive selection markers [8-10]. This approach hinders successive rounds of edits being performed in a single strain and involves cloning for targeting each new locus. An alternative strategy, which bypasses transgenesis, is to deliver Cas9 protein as ribonucleoproteins (RNPs). Prior endeavours delivering Cas9 RNPs have achieved imprecise edits with poor gene editing efficiencies even when coupled with selection marker-assisted gene disruption (<1%), unconducive to routine editing applications [10-13].

Our gene editing technique for *C. reinhardtii* combines three key advantages: 1) it uses a homologous repair template to achieve precise predictable edits, 2) it is transgene- and selection marker-free, and 3) it is potentially efficient enough to enable mutant detection simply by colony PCR. The technique relies on inducing targeted, double-stranded breaks in the nuclear DNA using a CRISPR endonuclease called Cpf1 (or Cas12a) [14] and the use of single-stranded DNAs (ssDNAs or ssODNs) as repair templates to guide DNA repair and achieve precise, predictable gene edits. The increased gene editing efficiencies conferred through the use of the ssODNs enables a selection marker-free approach, whereby mutants can be identified by colony PCR [1]. Cpf1 is delivered as a protein, bypassing transgenic expression and cloning, making the technique non-transgenic.

Here we describe knocking out of gene *FKB12*, which leads to rapamycin-resistance [15]. Editing of *FKB12* can, therefore, be readily assayed by plating cells on media supplemented with rapamycin [1]. We believe that this simple positive selection system will facilitate adoption of the technique both in CC-1883 and other strains. An overview of the method is provided in Figure 14.1a.

14.2 Materials

Use deionized H_2O where H_2O is required. All solutions are autoclaved unless indicated otherwise.

14.2.1 TAP medium

The following recipe has been accessed through the *Chlamydomonas* Resource Center website (https://www.chlamycollection.org/) in January of 2015, where it was referenced to the following publications [16,17]. The current recipe on this website is for a slightly more dilute version of TAP, but we believe this to be interchangeable with the recipe described here.

a) Salts solution: 15 g NH_4Cl, 4 g $MgSO_4.7H_2O$, 2 g $CaCl_2.2H_2O$, water up to 1 L.

b) Phosphate solution: 5.76 g K_2HPO_4, 2.88 g KH_2PO_4, water to 20 mL.

c) Hutner's trace elements: prepare the following solutions using H_2O (final volumes in brackets), 5.5 g $ZnSO_4.7H_2O$ (25 mL), 2.85 g H_3BO_3 (50 mL), 1.265 g $MnCl_2.4H_2O$ (12.5 mL), 0.403 g $CoCl_2.6H_2O$ (12.5 mL), 0.393 g $CuSO_4.5H_2O$ (12.5 mL), 0.275 g $(NH_4)_6Mo_7O_{24}.4H_2O$ (12.5 mL), 1.25 g $FeSO_4.7H_2O$ (12.5 mL), 12.5 g disodium EDTA (62.5 mL). Mix all solutions except EDTA, bring to boil, then add EDTA. The mixture should turn from an orange-brown colour to blue-green. Cool to 70°C, then add 21.25 mL KOH (20% w/v). Cool to room temperature and bring volume to 250 mL with H_2O. Oxidize for 1 to 2 weeks by bubbling with air or orbital shaking. During this process, the

solution will turn dark purple and form a brown precipitate. Filter through Whatman #1 filter paper until the solution is clear of this precipitate. Do not autoclave. Aliquot and store at -20°C. Keep a working stock at 4°C (*see* **Notes 1**).

d) Tris (tris(hydroxymethyl)aminomethane)

e) Glacial acetic acid

f) Deionized H_2O

g) TAP medium (per L): 2.42 g Tris, 25 mL salts solution, 0.375 mL phosphate solution, 1 mL Hutner's trace elements, 1 mL glacial acetic acid, pH should be around 7.0-7.5 (*see* **Notes 2**).

14.2.2 *Chlamydomonas reinhardtii* cultures

a) *C. reinhardtii* strain CC-1883 (*Chlamydomonas* Resource Center) (*see* **Notes 3**).

b) Incubation facilities with 100 μmol m^{-2} s^{-1} light and 28-30°C (these are temperatures measured under the light where the cells will be incubated).

c) Orbital shaker.

d) Laminar flow hood.

e) TAP media: 5 × 10 mL aliquots (this includes 1 spare) in 100 mL Erlenmeyer flasks sealed with foam bungs, covered with aluminium foil, then autoclaved (*see* **Notes 4, 5**).

f) TAP plates: TAP with 1.5% agar (*see* **Notes 6**), autoclaved and poured into 90mm Petri dishes sealed with parafilm. Prepare in batches of 10 or 20.

g) Cell stain: 0.25 g iodine in 100 mL absolute ethanol. Do not autoclave. Alternative stains are described in [18].

h) Filtered pipette tips (1 mL).

i) Haematocytometer.

j) Light microscope with 200× total magnification.

k) Handheld tally counter.

l) Optional: inoculation loops.

14.2.3 *C. reinhardtii* electroporation

a) Cpf1 (Cas12a) from *Lachnospiraceae* bacterium ND2006 (LbCpf1). LbCpf1 can be purchased from NEB [EnGen® Lba Cas12a (Cpf1)] or purified [1,19,20] (see **Notes 7**).

b) gRNA: UAAUUUCUACUAAGUGUAGAUUGCACUACACGGGCACCCUGACCG (the target-specific sequence is underlined). Dissolve the gRNA using DEPC-treated, nuclease-free H_2O (Table 14.1) and aliquot in batches enough for 10-20 electroporation reactions, store at -80°C (*see* **Notes 8, 9**).

c) Single-stranded oligodeoxynucleotide (ssODN): GGTATACAGGCGTGAGCTTCCCCAAGACTGGCCAGACCGTG**TTTG**CTAGAGTA**TAA**C**TAG**AATTCCCACACGGCAAGAAGTTCGACAGCTCCCGCGACCGTGGCGAGCCCTTCT (the PAM is bold, the 24 nt sequence replacing the target site is underlined and within it the stop codons are bold) (*see* **Notes 10**) (Figure 14.1b). This can be ordered from commercial suppliers like Sigma Aldrich or IDT. Note that the concentration of the ssODN should be 1 mM (Table 14.1).

d) 2 M sterile sucrose solution (*see* **Notes 11**).

e) TAP media with 40 mM sucrose: 5 mL TAP, 0.1 mL sucrose (2 M), prepare in a 25 mL Erlenmeyer flask sealed with a foam bung, covered with aluminium foil, then autoclaved.

f) Bio-Rad Gene PulserXcell with PC module.

g) 4 mm electroporation cuvettes (we use CELL PROJECTS Ltd, BR-204).

h) Filtered pipette tips (1 mL, 200 µL, 10 µL).

i) Parafilm.

14.2.4 Cell plating

a) One plate of TAP agar and one of TAP agar supplemented with 10 µM rapamycin (*see* **Notes 12, 13**).

b) Optional: sterile Duran bottle (*see* **Notes 13**)

c) 50 mL TAP.

d) Starch (corn/potato).

e) Sterile Eppendorf tubes (*see* **Notes 14**).

f) Absolute ethanol.

g) 100 mL sterile H_2O.

14.2.5 Colony PCR

a) Thermal Cycler.

b) Phire Plant Direct PCR kit (Thermo Fisher).

c) PCR primers to amplify the *FKB12* target; forward: ATGGGTGTCGACGTCGCGACTA; reverse: CTGACCATCAGCCACGACTTC.

d) QIAGEN MinElute PCR Purification Kit (or any alternative PCR purification kit).

e) BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher).

f) Agarose gel running equipment.

14.3 Methods

All steps are performed at room temperature. Work with cells is performed aseptically under a laminar flow hood (*see* **Notes 15**). *C. reinhardtii* is cultured under 70-100 µmol m^{-2} s^{-1} white light at 28 °C-30 °C (*see* **Notes 16**). Liquid cultures are shaken at 100-130 rpm (Stuart SSL1 Orbital Shaker).

14.3.1 *Chlamydomonas reinhardtii* culture

a) Streak out CC-1883 onto a TAP plate to obtain a freshly growing culture.

b) After growth is observed (typically 3 days), inoculate a 10 mL TAP aliquot using a 1 mL pipette tip or inoculation loop (*see* **Notes 17**).

c) The next day, measure the cell density using a haematocytometer. To do this, aliquot 3 × 20 µl cell stain into Eppendorf or PCR tubes. Add 200 µl of culture to each stain aliquot, ensuring the culture is well mixed by swirling prior to taking each sample. A standard haematocytometer has a central 5 × 5 square with a volume of 0.1 mm^3, with each square subdivided into smaller 4 × 4 squares. Count the cells in the 5 × 5 portion, average the counts (removing outliers), multiply by 1.1 to account for the dilution caused by the stain (*see* **Notes 18**), and multiply by 10^4 to obtain the density in cells/mL (e.g., a cell count of 100 equates to a 1.1 × 10^6 cells/mL).

d) Calculate the dilution required to obtain a density of 2 × 10^6 cells/mL by the next day. Assume a generation time of 8-10 hours and the division of each cell into 2 daughter cells (*see* **Notes 19**). Proceed to make this dilution by subculturing into a new 10 mL TAP aliquot. Make two further dilutions, one less and one more dilute to ensure that at least one culture will be near target density the next day (*see* **Notes 20**). It is optional to check for contamination prior to making the dilutions (*see* **Notes 21**).

14.3.2 Electroporation

This procedure takes typically ~2 hours.

a) Set up the laminar flow hood.

b) Start defrosting the Cpf1, gRNA and ssODN on ice.

c) Count the cell densities of the three overnight cultures using a haematocytometer as described above (*see* **Notes 22**)**.** Pick the culture that is closest to 2 × 10^6 cells/mL (*see*

Notes 23). It is optional to check for contamination prior to proceeding with this culture (*see* **Notes 21**).

d) Prepare 2.5×10^5 cells per electroporation reaction in a volume of 125 µL (this equates to a density of 2×10^6 cells/mL). This may involve diluting or concentrating the culture. To dilute, add 2.5×10^5 cells into the electroporation cuvette and supplement with fresh TAP (*see* **Notes 24**). To concentrate, aliquot 2.5×10^5 cells into a sterile Eppendorf tube (*see* **Notes 14**), centrifuge (0.4 g, 15 min), remove and discard the surplus volume from the top, then gently resuspend the cells in the remaining 125 µL by pipetting.

e) In the laminar flow hood, add 2.68 µL of sucrose (2 M) to the bottom of an electroporation cuvette (Table 14.1). Note that the volume of sucrose will need to be adjusted if the reagent concentrations deviate from those specified in Table 14.1 (*see* **Notes 25**).

f) Add the cells to the cuvette and mix by flicking the cuvette.

g) In an Eppendorf or PCR tube, mix the 1.9 µL Cpf1 (138 µM) and 1.13 µL gRNA (698 µM) (Table 14.1) (*see* **Notes 26**). To catalyse the formation of ribonucleoproteins (RNPs), incubate for 10 min at 37°C (*see* **Notes 27**).

h) Add 2.63µL ssODN (1 mM) to the RNPs, then add this to the electroporation cuvette and mix by flicking (*see* **Notes 28**).

i) Electroporate using the following exponential decay pulse ("Exponential protocol") settings: 600 V, 50 µF, 200 Ω, 4 mm. Flick the cuvette prior to electroporation to ensure mixture homogeneity (*see* **Notes 29**).

j) Using the contents of a fresh 5 mL aliquot of TAP-sucrose, add 800 µL TAP-sucrose into the cuvette immediately after electroporation (*see* **Notes 30**).

k) Transfer the cuvette's contents into the TAP-sucrose aliquot that was used in the previous step (*see* **Notes 31**). Recover the culture for 24 h by shaking on an orbital shaker.

14.3.3 Plating

Depending on the number of cultures that require plating, this step typically takes 2-4 hours.

a) Prepare fresh, sterile 30% (w/v) starch solution for plating (*see* **Notes 32**). This step takes 15-20 minutes. Each plate requires 0.5 mL to be spread, prepare volumes accordingly (*see* **Notes 33**). Weigh the starch in Eppendorf or 15 mL Falcon tubes. Wash the starch twice with absolute ethanol, three times with sterile H_2O, once with TAP, and finally resuspend in TAP (use the 50 mL TAP aliquot for this purpose). Washing consists of vortexing, centrifuging (maximum speed, 1 min) then decanting (*see* **Notes 34**). After decanting the first ethanol wash, only open the starch solution in a laminar flow hood.

b) To spread one electroporated culture onto two plates, aliquot 1 mL starch solution into a sterile Eppendorf tube (*see* **Notes 14**). To this, add 50µL of overnight-recovered culture (*see* **Notes 35**). This will result in approximately 200-800 colonies on the TAP plate (*see* **Notes 36**).

c) Mix the starch-cell solution by pipetting up and down several times using a 1mL pipette (*see* **Notes 37**). Spread the 1 mL volume as 2 × 450 µL onto a TAP and TAP-rapamycin plate (*see* **Notes 38, 39**).

d) Dry the plates with the lids partially removed for 15-30 min, then seal with parafilm (*see* **Notes 40**).

e) Grow the plates under low light (5 – 15 µmol m^{-2} $s^{-}1$) to prevent the photodegradation of rapamycin (*see* **Notes 41**). Colonies typically appear in 4 days, but we always wait 7 days to analyze colony counts and perform colony PCR.

14.3.4 Calculating rapamycin resistance

Determine the efficiency of gene editing by dividing the number of cells on the TAP-rapamycin plate by the number of cells on the TAP plate (*see* **Notes 42**). We typically obtain 25%-30% rapamycin resistant cells.

14.3.5 Colony PCR

a) Aliquot 10 µL of the Phire Plant Direct PCR kit' s dilution buffer into PCR tubes.

b) Pick cells with a 10 µL pipette tip, touch onto the surface of a new TAP plate and place into the dilution buffer aliquot (*see* **Notes 43, 44**). When removing the tip from the dilution buffer, ensure the cells have transferred into it.

c) Centrifuge the PCR tubes for a few seconds and add 0.5 – 1 µL of the supernatant into a 10 µL Phire PCR reaction as per manufacturer's instructions. Cycling conditions: 98°C, 5 min > (98°C, 5 s > 67°C, 5°C > 72°C, 10s) ×35 > 72°C, 2 min (primers under Materials) (*see* **Notes 45**).

d) Separate 5 µL of the PCR product on a 1.5% agarose gel to confirm specific PCR amplification.

e) Clean the PCR product using the QIAGEN MinElute PCR Purification kit as per manufacturer's instructions.

f) Sequence the cleaned PCR product using the BigDye Terminator v3.1 Cycle Sequencing Kit as per manufacturer's instructions using the forward primer.

g) Send sequenced reactions for capillary analysis at your local sequencing facility (Figure 14.1c).

14.4 Notes

1. Aliquot stocks in batches of 50 mL and store 5 mL refrigerated as the working stock.

2. We do not routinely check for or correct the pH.
3. This is a cell wall-deficient strain with the *cw15* genotype. As such, it requires starch plating as described in the Methods.
4. The aluminium foil serves to keep the neck of the flask sterile, therefore ensure it sufficiently covers it.
5. The purpose is to have single-use sterile TAP aliquots to minimize the potential for contamination. We have previously autoclaved several hundred millilitres of liquid TAP and aliquoted it into sterile Erlenmeyer flasks in a laminar flow hood as it was required but found it difficult to determine contamination prior to starting experiments. We prepare TAP aliquots in batches of 20 and store them at 4°C.
6. Anecdotal evidence suggests that *C. reinhardtii* can be sensitive to the quality of the agar. We use Duchefa Phyto agar P1003.1000 sold by Melford.
7. We purify LbCpf1 from *E. coli* as described in [1]. We have not described its purification here as we felt it is a separate technique covered in numerous other publications [1,19,20].
8. Notes on gRNA design. First, identify a protospacer-adjacent motif (PAM) of TTTV [14,21] within the gene's coding sequence (CDS) and as close as possible to the translation start site. The 24-nucleotide sequence downstream of the PAM is the gRNA target site (Figure 14.1b). The LbCpf1-induced double-stranded DNA cut occurs 18 – 22 nt downstream of the PAM [14]. Ensure this cut is within the CDS. Check for potential (homologous) off-targets in the *C. reinhardtii* genome using BLAST [22]. It is best to avoid targets with any hits, but if one has no alternative target site, consider what is known about Cpf1's gRNA mismatch tolerances to evaluate the risk of these off-targets being targeted [14, 21, 23]. Finally, to turn the target sequence into a full-length gRNA, replace all occurrences of T with U, then concatenate it downstream of the LbCpf1 gRNA repeat sequence (UAAUUUCUACUAAGUGUAGAU). The end-result is a 45-nt gRNA.

If disruption of a gene's coding sequence (CDS) is desired and there is a lack of PAMs in the CDS, consider intronic PAM sites with the cut site falling within the exon. In addition, consider the design flexibility offered through the ssODN design, whereby cut sites just outside of exons can still be useful to achieve disruption of the CDS. In such cases where ssODNs will overlap intron-exon boundaries, take care to preserve the splice junction motif (*see* **Notes 10**).

9. We order gRNAs from Sigma Aldrich and Synthego, but they can also be generated through *in vitro* transcription (IVT) [1]. In our experience, as a function of the time input requirements and yields, IVT costs considerably more than purchasing gRNAs. *In vitro* transcription using our method [1] takes 3 days, could be performed in a maximum of 200 µL volumes (bottlenecked by the step of polyacrylamide gel purification), and yielded 50 – 100 µg (~3-7 nmol) per 100 µL reaction. We recommend purchasing *FKB12*-targetting gRNA in order to hone the editing assay described here.
10. Notes on ssODN design. The ssODNs consist of 45 nt homology arms that are directly upstream and downstream of the target site (the upstream arm includes the PAM), and a central 24 nt sequence replacing the target site ('replacement sequence') (Figure 14.1b). For the replacement sequence, we modified a 24 nt eGFP sequence to include stop codons in every reading frame as well as an *Eco*RI site. We add stop codons in every frame to capture editing events that have additional insertions or deletions [1]. It is not necessary for the replacement sequence to match the target site in size, though we design all ssODNs this way. What is key is that the replacement sequence contains stop codons. For universal applications, we have designed a 12 nt sequence that contains stop codons in each reading frame in both directions: **C<u>TAA</u>T<u>TAA</u>C<u>TAA</u>** (sense and antisense stop codons are underlined and bold, respectively). ssODN design flexibility also allows use of gRNAs where the cut sites are in intronic sequences. In this case, position the

replacement sequence appropriately to place the stop codons within the gene's CDS, taking care to preserve the splice junction motif (usually a few nucleotides long).

11. During filter sterilization, we aliquot 2 M sucrose solution in batches of few hundred microlitres. These are subsequently opened only under sterile conditions in a laminar flow hood.
12. We typically prepare TAP-rapamycin plates on the day that they are required (the day of cell plating). Ahead of time, we prepare and autoclave the volume of TAP agar that will be required for the TAP-rapamycin plates (20 mL/plate) and use this to prepare the TAP-rapamycin on the day of cell plating. Alternatively, we prepare TAP-rapamycin plates no more than one week before use in order to prevent the degradation of rapamycin. In this case, store TAP-rapamycin at 4°C and in the dark to prevent rapamycin's photodegradation.
13. With certain batches of rapamycin, we experience precipitation when adding the rapamycin stock (10 mM, in DMSO) directly into the sterile, molten TAP agar. This can be mitigated by adding rapamycin into a sterile Duran bottle and diluting it by adding lukewarm, sterile TAP agar (30-50°C) in increments with swirling to mix in between each addition. Even this way, very fine grains of rapamycin precipitate can form, which may lead to a speckled pattern of condensation on the media surface as it solidifies. In our experience, this does not affect rapamycin-mediated counter selection of wildtype cells. Note that this requires a separate sterile Duran bottle.
14. We autoclave sealed containers of Eppendorf tubes that are only opened under the flow hood for sterile applications. This minimizes the potential for contamination.
15. Contamination while working with *C. reinhardtii* can ruin experiments. To minimize the chances of contamination, observe sterile technique, including: wiping all surfaces of the laminar flow hood with 70% ethanol, letting the hood run for 30 min prior to starting

work inside it, spraying all items that enter the hood with 70% ethanol (and each time with items that enter it multiple times), use of filtered pipette tips, spraying gloves and lab coat sleeves with 70% ethanol. In addition, it is recommended to keep a waste container for pipette tips inside the flow hood rather than outside of it.

16. Stocks may be maintained under lower light (e.g., 10-30 µmol m^{-2} s^{-1}) to limit growth and thereby minimize the re-streaking frequency.
17. The culture should be pale green after inoculation for densities to be 1×10^5 to 1×10^6 cells/mL by the next day, which is desirable for the purposes of cell counting.
18. Settling of stained cells occurs in minutes. Adequate mixing of the stained cells is, therefore, paramount for accurate cell counts. Before loading each sample onto a haematocytometer, pipette up and down 5 – 10 times using a volume of 100 – 200 µl to mix.
19. These values work well in our lab under the culture conditions described here. Lab-to-lab variation may require these values to be adjusted and can be easily determined from one overnight culture where densities 24 h or 48 h apart are known.
20. For the additional subcultures, we subculture volumes that are half and double the calculated volume. e.g., if the calculated dilution requires adding 2 mL culture into 10 mL fresh TAP, make the two subcultures using 1 mL and 4 mL of culture.
21. Check for contamination by centrifuging 1 mL of culture (17k g, 1 min). A white surface layer on top of the green algal pellet indicates bacterial contamination.
22. Set up triplicate stains for all 3 cultures (a total of 9 samples) and count one sample of each culture. Of the culture that is closest to the target density, proceed to count the other two samples. Future experience with culture colours will help guide this process to minimize cell counting.
23. We typically use cultures between 1×10^6 and 3×10^6 cells/mL.

24. Use a spare 10 mL aliquot of TAP for this purpose. It is recommended to discard this aliquot of TAP after use, though an experienced experimenter may choose to do otherwise.
25. At this point, loosening but not removing the cuvette lid allows for easy handling during subsequent steps.
26. We do not perform this step in a laminar flow hood, nor do we use dedicated sterile Eppendorf tubes as both the Cpf1and gRNA are non-sterile solutions, though we very rarely experience contamination of the subsequently plated cells.
27. When using desalt-purified gRNAs from Sigma Aldrich, we sometimes observe precipitation during incubation. We have anecdotal evidence that precipitation is eliminated by PAGE purification of the gRNAs. However, we have not noticed this precipitation to be detrimental to the efficacy of editing but is never-the-less not desirable.
28. On occasion, the ssODN suspension is sticky and difficult to pipette. This is the result of the ssODN being insufficiently dissolved. To remedy this, heat the ssODN at 65°C for 2 min, vortex, and cool on ice prior to adding to the RNPs. If this does not completely dissolve the ssODN, try incubating for longer or at a higher temperature.
29. After the electroporation is completed, the electroporator will display two values: the time constant and delivered voltage. These are typically around 6.5 ms and 585 V, respectively.
30. We believe the immediate addition of TAP-sucrose after electroporation increases cell survival rate post-electroporation but have not designed experiments to test this.
31. If using a 1 mL pipette tip, a good technique is to pipette from the side of the electroporation cuvette and to simultaneously tilt the cuvette as the pipette's plunger is being depressed. Surface tension will draw out all of the liquid from the cuvette.
32. Using starch is vital for improved and consistent plating efficiencies of cell wall-deficient (*cw*) strains of *C. reinhardtii* such as CC-1883 [24]. Without it, cells struggle to form

colonies and colony counts can be confounded by conditions that affect colony formation (e.g., agar moisture content).

33. It is better to prepare 1 mL of starch solution per plate since this step is inexpensive, yet time-consuming. Occasional mistakes in later steps will benefit from a surplus of the starch solution having been prepared.
34. Some loss of starch and hence a white hue of the decanted supernatants is normal.
35. The cell culture is colourless at this point.
36. Initially, it is recommended to spread additional plates with more and/or fewer cells (e.g., quarter, half, double, quadruple volumes of cells) to account for lab-to-lab variation in colony numbers. For example, we have noticed the amount and composition of the Cpf1's storage buffer to affect cell survival during electroporation (perhaps exerting some osmo-protective effect), which in turn affects colony numbers.
37. This pipetting is vital to ensure the cells are evenly distributed on the two plates.
38. When spreading the cells, leave a 0.5 cm margin along the edge of the plate. This serves two functions. First, in case of condensation inside the plate during the subsequent incubation period, it prevents cells around the edge from being washed together as the condensate typically swirls around the edges of the plate. Second, if counting cells using a software like Open CFU [25] or ImageJ, labelling around the edge of the plate will not overlap with the colonies.
39. The starch solution spreads easier if the agar medium is moist. Starch will stick to over-dry plates, making it difficult to spread evenly. It is, therefore, beneficial to dry plates that will be used for spreading cells for a minimum amount of time after pouring.
40. Dryness of the starch solution is seen by the glossy (wet) surface turning matt.

41. We place plates (upside down) under 70-100 µmol m^{-2} s^{-1} and overlay them with 3 – 6 layers of white tissue roll as described by Shimogawara *et al.* [24]. A superior method would be to use neutral density filters.
42. We photograph the plates (without opening them, plates being upside down) and count cells using the free Open CFU software [25]. The software has a function to draw a mask to exclude false positives. This is under 'ROIs and Mask'> 'Draw Mask'. If processing many plate images, it helps to take plate images in a fixed position using a stand. This way, the mask does not have to be re-drawn for each image.
43. Using 0.5% agar TAP speeds up colony growth of CC-1883, presumably due to its cell wall-deficient phenotype (*cw15*).
44. Our dilution buffer tends to be colourless to pale green after the addition of cells. We occasionally notice inconsistencies in PCR amplification and have suspected cell concentration to be an issue. Despite this, we have not noticed a correlation between the green colouration of the dilution buffer and PCR success.
45. Where the mutant colony phenotype is unknown, we recommend pooling colonies and performing PCR with a mutation-specific primer. To do this, after placing picked cells into 10 µL dilution buffer, centrifuge, then use 1 µL of each to create pools of 8 samples. Add 1 µL of the pooled sample into a 10 µL PCR reaction. In pools where there is specific PCR amplification, screen the individual samples. After identifying mutants, repeat the PCR using primers that are suitable for sequencing.

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Caption for Table 14.1

Reagent stock concentrations, quantities and volumes required for an electroporation reaction. Note that the final sucrose concentration should be 40 mM; sucrose volumes will need to be adjusted if the reagents concentrations deviate from those specified in the table.

Caption for Figure 14.1

a. Schematic overview of gene editing protocol up to the point of cell plating. **b.** Schematic overview of the design of the gRNA (top panel), ssODN (middle panel) and primers (bottom panel) for editing and analysing *FKB12*. The central bulge of the ssODN (the 'replacement sequence', dark-red) represents non-homology with the target site (black). Cpf1-mediated, double-stranded DNA cleavage is indicated with a dark red line inside the 24 nt gRNA target (top panel). **c.** Sanger chromatograms of the wildtype (top) and edited (bottom) *FKB12* target site. The diagram is adapted from SnapGene Viewer.

Table 14.1

	Molecular weight (kDa)	Concentration (mg/mL)	Concentration (μM)	Amount (nmol)	Volume (μL)
LbCpf1	145	20	138	0.262	1.90
gRNA	14.3	10	698	0.786	1.13
ssODN	35	35	1000	2.623	2.62
Cells	-	-	-	-	125

Sucrose (2M)	-	-	2000	-	2.68
Total volume	-	-	-	-	133

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