



A quick-to-implement and optimized CRISPR-Cas9 protocol to obtain insertional and small indel mutants in *Chlamydomonas reinhardtii* [☆]



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ABSTRACT

Chlamydomonas reinhardtii is a leading model organism in algal research, widely used to study photosynthesis, chloroplast and cilia biology, and more recently, metabolism, light signaling, the cell cycle, and algal biotechnology. Its sequenced genome has significantly accelerated research in the field, while improved genome-editing tools are key to advancing reverse genetics and genetic engineering. Building on previous advances, we present a streamlined and efficient CRISPR-Cas9 protocol for generating knockout mutants in *Chlamydomonas* via non-homologous end joining (NHEJ), using only commercially available reagents. Additionally, we introduce a cost-effective, PCR-based screening method capable of detecting mutants with large insertions as well as short indels -as small as one base pair- thereby enhancing overall CRISPR efficiency.

- This protocol is easy to setup and can be fully executed using commercially available reagents.
- This protocol allows for quick implementation and generation of mutants: 5 weeks from design to sequencing of candidate mutants.
- This protocol describes a novel PCR-based strategy to identify mutants containing short indels. Screening is designed to identify large insertion mutants and the often overlooked small indel mutants.

Specifications table

This table provides general information on your protocol.

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	CRISPR Cas9 mediated Genome Editing

(continued on next page)

[☆] **Related research article:** None.

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Name of your protocol:	Quick-to-implement and optimized protocol for the generation of CRISPR-Cas9 knockout mutants that allows detection of short indels in a cell-wall featuring <i>Chlamydomonas reinhardtii</i> strain.
Reagents/tools	<p>While we provide the source of the materials used, reagents and tools listed in this box can be purchased from any supplier.</p> <p>1. MATERIALS</p> <p>TAP-agar refreshed 1-week-old <i>Chlamydomonas</i> cells (CC-124 - cell wall containing, <i>Chlamydomonas</i> Resource Center)</p> <p>10 mM EDTA pH 8</p> <p>Primers</p> <p>PCR Master Mix Kit (NZYTaQ II 2x Green Master Mix - NZYTaQ II DNA polymerase, NZYtech)</p> <p>GeneRuler DNA Ladder Mix (Thermo Fisher)</p> <p>Dimethylsulfoxid (DMSO)</p> <p>Nuclease Free Water</p> <p>TAE 1X</p> <p>Agarose</p> <p>Electrophoresis loading buffer (6X Loading Dye, Thermo Fisher)</p> <p>PCR clean-up kit (NZYGelpure, NZYtech)</p> <p>crRNA (Alt-R™ CRISPR-Cas9 crRNA - 2 nmol, IDT)</p> <p>tracrRNA (Alt-R™ CRISPR-Cas9 tracrRNA - 5 nmol, IDT)</p> <p><i>Streptococcus pyogenes</i> Cas9 (Alt-R™ S.p. Cas9 Nuclease V3, IDT)</p> <p>CRISPR buffers (Nuclease Free Duplex Buffer; IDTE pH 7.5 (1X TE Solution), IDT)</p> <p>Buffer O (Thermo Fisher) {can also be homemade prepared following manufacturers specifications}</p> <p>MAX Efficiency™ Transformation Reagent for Algae (Thermo Fisher) {can be replaced with autolysin solution as shown by Picariello et al. [12]}</p> <p>Sterilized 1 M Sucrose</p> <p>2 mL, 1.5 mL and 200 µL sterile tubes</p> <p>2 mm gap electroporation cuvette</p> <p>24-well plates</p> <p>TAP liquid medium</p> <p>TAP agar plates with paromomycin (10 µg mL⁻¹)</p> <p>Linearized resistance-carrying vector (pSL18 vector, <i>pAPHVIII</i> cassette)</p> <p>Parafilm</p> <p>Hemocytometer</p> <p>Iodine (0.25 % w/v in EtOH)</p> <p>50 mL tubes</p> <p>Aluminum foil</p> <p>Razor blades</p> <p>Square 120 × 120 mm Petri dishes</p> <p>Round 60 × 60 mm Petri dishes</p> <p>Paromomycin sulfate (Merck)</p> <p>2. LABORATORY EQUIPMENT</p> <p>Thermoblock</p> <p>Thermoshaker</p> <p>Thermocycler</p> <p>Centrifuges for 1.5, 2 and 50 mL tubes</p> <p>Electrophoresis equipment</p> <p>Transilluminator</p> <p>Growth Chamber (with programmable light-dark cycles)</p> <p>Electroporator (NEPA21 from NEPAGENE or other)</p> <p>Laminar flow hoods</p> <p>Water bath (with cooling system)</p> <p>Spectrophotometer (Nanodrop or other)</p> <p>Rotary Shaker</p> <p>Optical microscope</p> <p>3. OPTIONAL</p> <p>Vacuum Concentrator</p> <p>Cell Counter</p> <p>TOPO-TA cloning kit for Sequencing (Thermo Fisher)</p>
Experimental design	Cell wall featuring <i>Chlamydomonas</i> cells, grown in synchronizing conditions, were electroporated with pre-assembled Cas9-gRNA ribonucleotides (RNPs) and a linearized resistance-carrying vector. Electrophoresis screening for mutants allowed detection of both large insertions and small indels.
Trial registration	N/A
Ethics	N/A
Value of the Protocol	<ul style="list-style-type: none"> • Easy to reproduce. Our protocol can be fully executed using commercially available reagents. • Quick implementation and generation of mutants. From CRISPR design to sequencing of candidate mutants this protocol can be completed in 5 weeks. • Detection of mutants containing short indels. Electrophoresis screening was designed to identify both large insertion mutants and often overlooked small indel mutants, increasing overall CRISPR efficiency.

Background

In *Chlamydomonas*, a fundamental requirement in both basic and applied research is the ability to inactivate specific genes. To address this, several techniques have been developed in recent years, with CRISPR-Cas9 emerging as the most widely used tool. However, its robustness and efficiency remain limited [1,2]. The principle of the Cas9 gene-editing technique is straightforward: a Cas9 endonuclease binds to a guide RNA (gRNA), which is designed to target a specific genomic site, forming a ribonucleoprotein complex (RNP). These RNPs are pre-assembled *in vitro* and subsequently introduced into cells via electroporation. Guided by the gRNA, Cas9 induces a double-strand break (DSB) at the target site, which is then repaired by the cell's intrinsic repair machinery [3–5]. This repair process can lead to mutations at the target site. In *Chlamydomonas*, DSBs are typically repaired via non-homologous end joining (NHEJ), a process that can result in short insertions or deletions (indels) or in large insertions in the presence of exogenous DNA (such as a resistance cassette) [4]. Alternatively, *Chlamydomonas* can repair DSBs through homologous recombination (HR) if DNA fragments with homology regions flanking the cut site are provided [6], or through single-strand templated repair (SSTR) in the presence of single-stranded oligodeoxynucleotides (ssODNs) [7–9].

Recent CRISPR protocols for *Chlamydomonas* have primarily focused on taking advantage of the HR mechanism, as it enables precise gene modifications such as creating custom knockout mutations, introducing resistance markers or other tailored cassettes, performing single-base substitutions, or adding tags [10–14], or the SSTR for scar-less gene editing with a transgene-free approach [7–9]. While these advanced modifications are highly desirable, they involve time-consuming procedures including cloning optimizations and the often costly purchase of custom cassettes for HR or ssODNs for SSTR. Despite these efforts, the overall efficiency of these approaches remains lower compared to non-homologous end joining (NHEJ)-based mutagenesis [9–11]. Higher efficiencies have been reported when HR is employed to disrupt a gene using a resistance marker cassette [12,14]. This strategy, however, has two key limitations: (1) the resistance marker cannot be crossed out, and (2) it does not allow for certain types of gene editing such as single base substitutions [11]. Given its simplicity, cost-effectiveness, and higher efficiency, NHEJ remains the preferred strategy for generating knockout mutants.

Another common challenge in performing CRISPR in *Chlamydomonas* is the screening for mutants with unknown or subtle phenotypes. Typically, this is done by amplifying a short section of the target gene that includes the Cas9 cut site and analyzing it via electrophoresis. The goal is to identify larger amplicons compared to the wild type, indicative of an insertion [11–13]. However, this method often overlooks mutants with small indels, leading to their misclassification as false negatives.

Here, we present a streamlined and efficient CRISPR-Cas9 protocol for generating knockout mutants in *Chlamydomonas*. This approach eliminates the need for complex additional procedures and can be fully carried out using standard laboratory equipment and commercially available reagents. Additionally, we introduce a cost-effective screening strategy capable of detecting mutants with short indels, thereby enhancing overall CRISPR efficiency.

Description of protocol

The following is a quick and simple protocol for the generation of *Chlamydomonas* knockout mutants in any gene of interest (*GOI*) using CRISPR-Cas9, followed by a PCR-based screening strategy that allows detection of large insertional mutants as well as short indels.

The protocol was established and optimized using the *PHYTOENE SYNTHASE 1 (PSY1)* gene as a target. *psy1* knockout mutants are known to have severely impaired carotenoid biosynthesis, which is essential for chlorophyll stabilization [16]. As a result, *psy1* mutants exhibit a distinct pale coloration. During our initial PCR screens for large insertions, we observed that some pale colonies did not show the expected PCR bands, suggesting they might carry small indels in *PSY1* that were undetectable with the original method. This realization prompted us to optimize the protocol and develop a PCR-based approach to detect small indels. Importantly, this protocol enables detection of both large insertional and small indel mutations in any *GOI*, regardless of whether the mutation produces a visible phenotype. While the *psy1* phenotype facilitated the development of the method, a visible phenotype is not required for broader application.

CRISPR-Cas9 ribonucleoproteins (RNPs) are composed of a Cas9 nuclease and a guide RNA (gRNA): the latter is an RNA duplex composed of a complementary base pairing CRISPR RNA (crRNA), which is custom designed to target the *GOI*, and a transactivating CRISPR RNA (tracrRNA), a constant sequence that forms a secondary structure and serves as a scaffold recognized by the Cas9. Here, gRNA design refers to the specific design of crRNAs (as the tracrRNA is constant in all gRNA). The gRNA is designed immediately upstream of an NGG sequence, called protospacer adjacent motif (PAM), which is required by the SpCas9 (from *Streptococcus pyogenes*) for DNA recognition.

The entire protocol takes about 5 weeks from the design (Step 1) to mutant detection (Step 5). A general overview of the method is presented in the Graphical Abstract, while the CRISPR design process (Steps 1–3) is schematically summarized in Fig. 4.

Step 1 – gRNA and primer design

Time of execution: one day.

1.1 Design of custom single guide RNA (gRNA) for your *GOI*.

Use CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>) [17] to choose the most suitable gRNA in the region of your *GOI* where you would like to have your mutation. Use the following settings: choose NGG as PAM sequence, 20 as guide length,

and “Chlamydomonas v5.5” as target genome. Copy and paste the *GOI* sequence, and leave default values for the rest of the options; select the gRNA with the highest on-score (>0.5, if possible) and with low off-score levels for the identified off-targets. *Note 1.1.1. To generate knockout mutants, we recommend to have the Cas9 target site localized in the first exon of the coding sequence (CDS) and as close to the start codon as possible, to minimize the possibility of generating functional truncated proteins.*

Note 1.1.2. when ordering gRNA from IDT, make sure these do not include the NGG sequence at the 3', as indicated by the company.

Note 1.1.3. We recommend to design and test two different gRNA to maximize the options of generating mutations, given that different gRNA might display different efficiencies.

Note 1.1.4. After the design, we do not recommend purchasing the gRNA until successfully completing the primer tests (Step 2).

1.2 Design of primers.

The protocol uses a total of 6 different primers (see Graphical Abstract and Fig. 1–4):

(a) Four Screening (S) primers to be used in the mutant screen:

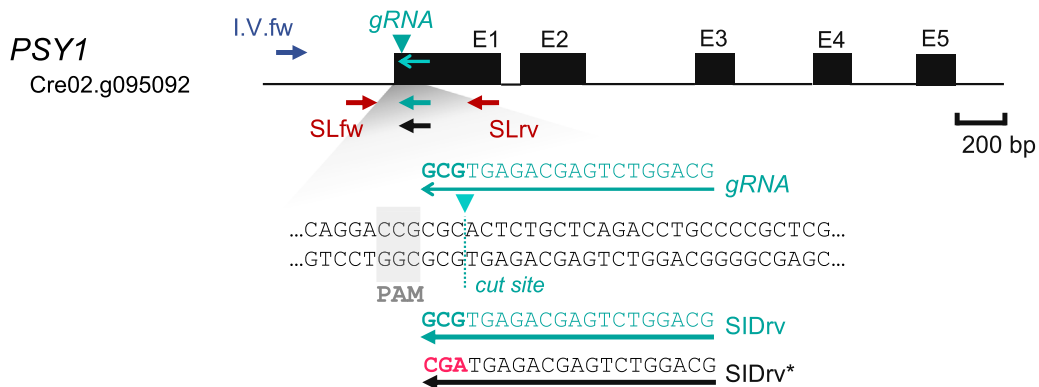


Fig. 1. CRISPR-Cas9 design to target *PSY1* gene. Scaled representation of the *PSY1* gene is shown with its five exons (black boxes, E1–5). A gRNA was designed in the beginning of the first exon of this gene: the sequence is shown below (light blue), with the predicted cut site by the Cas9 (triangle) and the PAM region (grey). In this example, 4 screening primers were designed, two for the screening of large insertions (SLfw and SLrv, red) and two for the screening of short indels (SID). SID primers are both reverse primers which are paired with SLfw to detect short indels by PCR: SIDrv (light blue) features the same sequence as the gRNA, while SIDrv* (black) features the 3 bases at 3' changed (light red), and serves as a negative control, to check that mismatches caused by the introduction of short indels at this *locus* do not result in PCR amplification. For the *in vitro* test a single forward primer was designed (I.V.fw, blue), and SLrv was employed as reverse primer for this test.

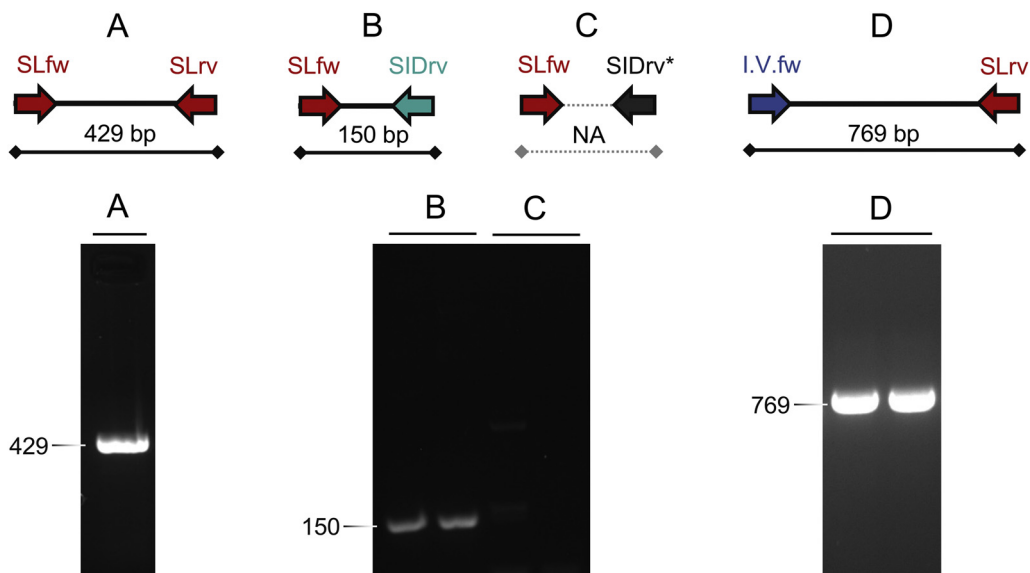


Fig. 2. PCR test of all the designed primers for *PSY1*. All PCR reactions were performed at 64 °C and yielded bands with the length indicated on top or no band (NA), as expected. A) PCR for the screening of large insertions (SLfw+SLrv), B) PCR for the screening of short indels (SLfw+SIDrv), C) negative control of short indels screening (SLfw+SIDrv*), D) *in vitro* test PCR (I.V.fw+SLrv). Two technical replicates are shown for each amplicon. Uncropped electrophoresis gel images including the DNA ladder are provided in Supplemental Fig. 1.

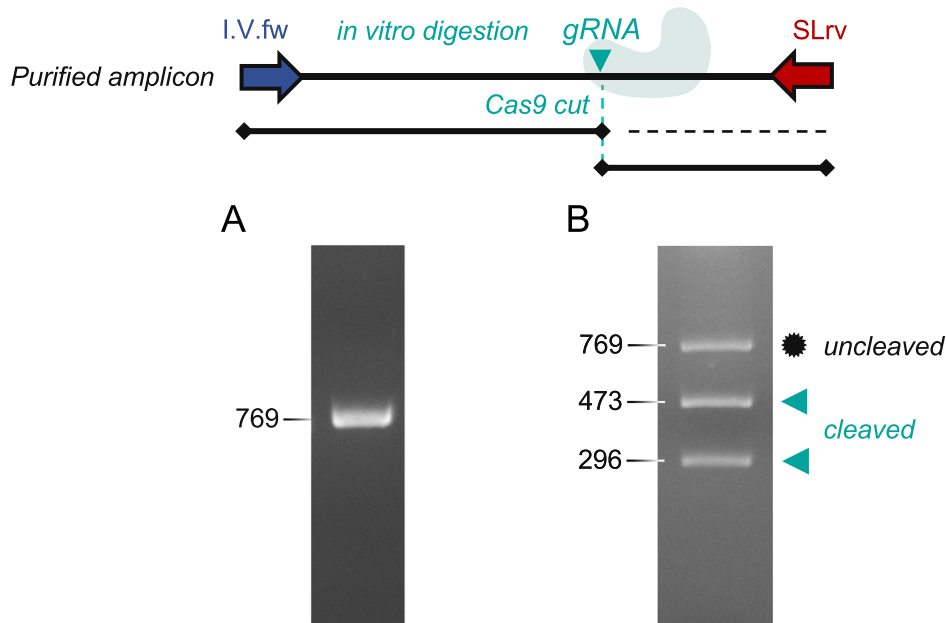


Fig. 3. *In vitro* test of CRISPR machinery designed to target *PSY1*. PCR was performed as indicated in Fig. 1 (I.V.fw+SLrv) and yielded amplicons of 769 bp; these were afterwards purified (left electrophoresis gel). *In vitro* digestion gave the two expected fragments of 473 and 296 bp (cleaved, right electrophoresis gel). The additional largest band corresponds to rests of the original undigested (uncleaved) amplicons. Uncropped electrophoresis gel images including the DNA ladder are provided in Supplemental Fig. 2.

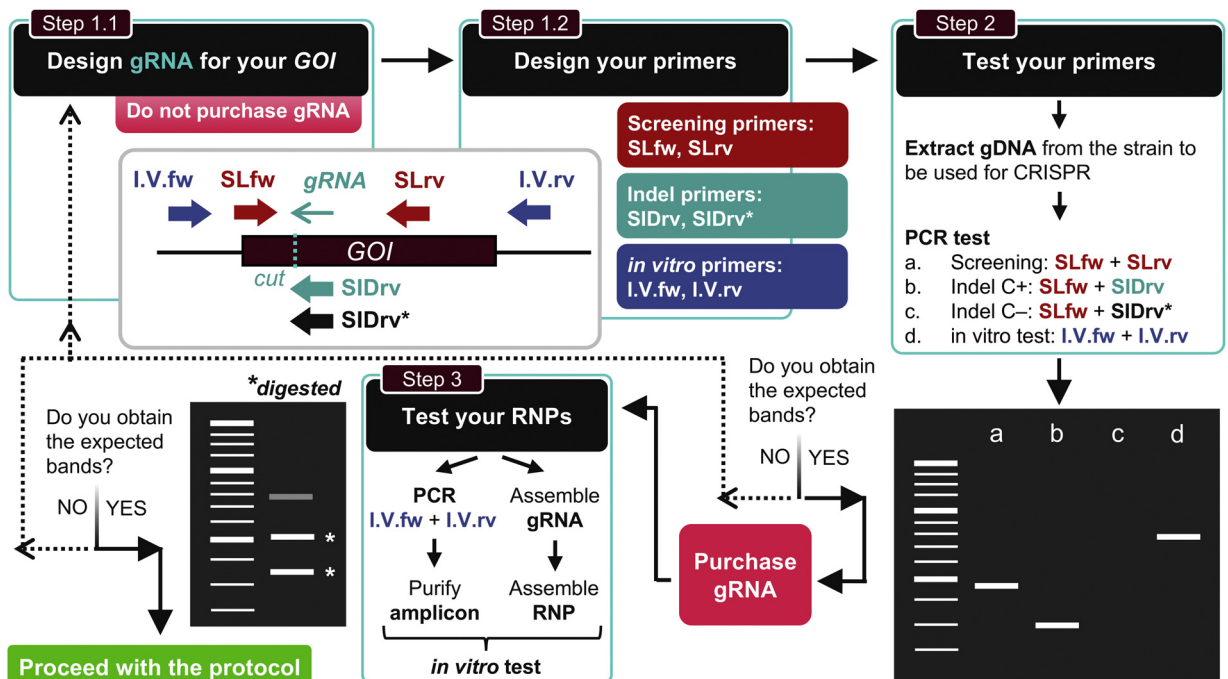


Fig. 4. General overview of the CRISPR design (Step 1–3). Step 1.1. Design gRNA(s) for your gene of interest (GOI), but do not purchase gRNA(s) before successfully testing all primers. Step 1.2 Design all the primers required for this protocol, which will depend on the target site of the chosen gRNA(s): screening primers (SLfw, SLrv), indel primers (SIDrv, SIDrv*) and *in vitro* primers (I.V.fw, I.V.rv). Step 2. Test the designed primers (Step 2), by performing 4 different PCRs with gDNA from the strain to be used for CRISPR (follow indications in Table 1). If PCRs give the expected bands then purchase the designed gRNA(s); if not, go back to Step 1.1 and design new gRNA(s). Step 3. Test your RNPs (*in vitro* test): for this purpose, perform a PCR using I.V. primers and purify the resulting amplicon; also, assemble RNPs as indicated in “Assembly of the CRISPR-Cas9 machinery” following *iv* (*in vitro* test) reagent proportions; finally, *in vitro* digest the purified amplicon with the assembled RNPs, purify the reaction and perform an electrophoresis run. *PSY1*, as described here, can be used as positive control in Step 3.

- a.1-Two S primers to screen for Indels (id): Indel primer wild-type reverse (SIDrv), with the same sequence as the gRNA, and Indel primer mutated reverse (SIDrv*), with 3 mismatches at the 3'-end. SIDrv is designed to detect the presence of mutations at the Cas9 cleavage site (if these are present it won't produce amplification), while SIDrv* serves as a negative control, to check that mismatches caused by the introduction of short indels at this *locus* do not result in PCR amplification (see Primer test).
- a.2-Two S primers to screen for large (L) insertions: SL forward (fw) and SL reverse (rv), which combined should give a 100–500 bp amplicon flanking the Cas9 cleavage site.
- (b) Two *in vitro* (I.V.) test primers (I.V.fw and I.V.rv), which combined should give a 600–800 bp amplicon asymmetrically flanking the Cas9 cleavage site (Figs. 1, 3). Cas9 cleavage in the *in vitro* test of the resulting amplicon will give two different sized bands.

Note 1.2. Depending on the Cas9 site and the overall design and strategy, one of the SL and I.V. primers can have the same sequence.

We use Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) [18] to design SL and I.V. primers, with the following parameters: optimal primer melting temperature 63 °C (min 60 °C and max 66 °C),

- optimal primer size of 23 bp (min 22 and max 24 bp);
- choose primer pairs with the least number of unspecific amplifications (<3) and with at least a G or a C in the two last bases at 3'.

Oligo Calc can be used to check primer self-complementation (<http://oligocalc.eu/>) [19].

For *PSY1*, the designed gRNA had a 0.78 on-score and maximum off-score of 0.23 (Fig. 1). The gRNA and the indel primer sequences are shown in Fig. 1. SLfw and SLrv primer pair was designed to give an amplicon of 429 bp. I.V.fw and I.V.rv (which was the same as SLrv) primer pair was designed to give an amplicon of 769 bp (Fig. 1).

Step 2: primer test

Time of execution: one week.

- 2.1 Obtain a crude gDNA extract. Scrape *Chlamydomonas* cells (approximately the size of a lentil) from a recently refreshed plate (1–3 weeks) using a sterile toothpick and transfer to a 1.5 mL tube. Resuspend cells in 30–50 µL of 10 mM EDTA pH 8 with vigorous vortexing until the suspension is homogenous. Heat suspension at 100 °C for 5 min, and transfer to ice for 5 min. Centrifuge at max speed for 3 min, and collect the supernatant into a new 1.5 mL tube: this is your crude gDNA extract.

Note 2.1. The use of 10 mM EDTA as buffer for gDNA crude extracts is standard [10,12,20], but failed PCR amplifications could be caused by EDTA chelating effects on Mg. In this case, alternative buffers can be used for gDNA extraction (see Troubleshooting).

- 2.2 Primer test: Perform a PCR using the designed primer pairs choosing the most suitable annealing temperature (T_m) according to the primer pair specifications (typically 3–5 °C lower than the lowest T_m of the primers). Both the content of a single 10 µL PCR reaction and the PCR cycling conditions are indicated in Table 1. Perform an electrophoresis run of PCR reactions on a 1 % agarose gel: the optimum T_m temperature should give the expected band without unspecific amplification.

Note 2.2. In case of failed PCRs (no amplification or unspecific amplification) see Troubleshooting.

Table 1

Single PCR reaction in 10 µL volume. For the annealing step, choose the most suitable T_m according to the primer pair specifications (typically 3–5 °C lower than the lowest T_m of the primers): the optimal T_m should yield the highest amount of amplicon without unspecific amplification.

Single PCR reaction in 10 µL volume		
Reagent	Volume	
NZYTaq II Green Master Mix	2X	5 µL
DMSO	-	1 µL
Primer Fw	10 µM	0.6 µL
Primer Rv	10 µM	0.6 µL
gDNA crude extract	-	1 µL
Nuclease free water	-	1.8 µL
TOTAL		10 µL
Cycling conditions		
Step	Temperature	Time
Initial Denaturation	95 °C	3 min
Denaturation	95 °C	30 s
Annealing	X	30 s
Extension	72 °C	1 min
Final Extension	72 °C	5 min
Store	4 °C	∞

X40
cycles

2.3 Screening Indel primer test. Perform a PCR combining the SLfw primer with either the SIDrv or the SIDrv* primer. If your indel primers are suitable for detecting short indels, the PCR performed using SLfw+SIDrv should amplify a band, whereas the PCR performed using SLfw+SIDrv* should not amplify anything.

2.4 Screening Large insertions primer test. Perform a PCR using the SLfw+SLrv primer combination.

2.5 *In vitro* primer test. Perform a PCR using the I.V.fw+I.V.rv primer combination.

Note 2.5. Once successfully confirmed, proceed with purchasing gRNAs and with Step 3. If test PCRs give unspecific bands, even after correcting with troubleshooting adjustments, repeat using different primer sequences. If the indel primer test is unsuccessful, even after correcting with troubleshooting adjustments, repeat from Step 1.1 using a different gRNAs sequence.

For PSY1, 64 °C T_m was employed for all PCRs in the protocol (Fig. 2A-C). Primer pair SLfw+SLrv gave a unique band of the expected size (Fig. 2A). PCR with indel test combination SLfw+SIDrv showed the presence of the expected size amplicon (Fig. 2B), whereas the indel test combination SLfw+SIDrv* did not amplify (Fig. 2C). These results indicated that the designed indel primers were suitable for detecting small base mismatches resulting from the presence of short indels in the gRNA target site. Primer pair I.V.fw+I.V.rv gave a unique band of the expected size (Fig. 2D).

Step 3: Cas9 *in vitro* cleavage test

*Time of execution: two days (one day to prepare and purify PCR amplicons and one day to perform the *in vitro* digestion and electrophoresis run of the digested fragments).*

This step is recommended to test functionality of Cas9 and a given gRNA when used for the first time. PSY1 can be used as a positive control.

3.1 Prepare PCR amplicons for *in vitro* digestion. For each gRNA prepare a 60 µL PCR using *in vitro* test primers (I.V.fw+I.V.rv) and scaling up the reagents of Table 1. Purify the PCR product with a PCR cleanup kit and elute in nuclease free H₂O. Measure DNA concentration. For the *in vitro* test, a total of 750 ng of amplicon is required, in a concentration of minimum 75 ng/µL (lower concentrations can be concentrated using a vacuum concentrator).

3.2 Assemble CRISPR-Cas9 RNPs. Prepare RNPs following the procedure explained in “Assembly of the CRISPR-Cas9 machinery” (*in vitro* test).

3.3 Digest amplicons with the assembled RNPs. Combine RNPs with the purified amplicons and Buffer O (ThermoFisher) and follow the reaction procedure specified in Table 2.

3.4 Clean digested amplicons and visualize bands. Purify the PCR product with a PCR cleanup kit and elute in 20 µL of Elution Buffer or nuclease free water. Combine this volume with 6X Loading Dye (Thermo Fisher). Perform an electrophoresis run of 20 µL of this mix on a 1 % agarose gel: 2 bands corresponding to the digested fragments from the original amplicon and with the expected length should be observed. It is likely that original undigested amplicon will also be visible. The higher the amount of digested fragments compared to the original undigested amplicon the more efficient the digestion *in vitro*.

Note 3.4. A PCR clean up step is necessary to ensure that Cas9 is released from DNA and will not interfere with gel visualization of the DNA.

To test the functionality of our designed gRNA to target PSY1, first an amplicon of 769 bp was generated using primers I.V.fw+SLrv and cleaned up (Fig. 3A). After *in vitro* digestion the expected digested fragments were obtained (fragments of 473 and 296 bp) (Fig. 3B, cleaved). A higher size band corresponding to the original undigested amplicon was also observed (Fig. 3B, uncleaved).

Prior to transformation

Time of execution: five days (time needed to grow Chlamydomonas cells in synchronizing conditions).

- Grow Chlamydomonas cells in synchronizing conditions. Inoculate a tris-acetate phosphate (TAP) liquid culture with scraped cells from an agar plate of the strain you wish to transform, and grow in alternating light and dark conditions (12:12), under 22–23 °C in white light (~50 µmol m⁻² s⁻¹) for at least 5 days and under mild shaking (150 rpm). Dilute culture on day 3 so that cells are

Table 2
in vitro digestion reaction. See “Assembly of the CRISPR-Cas9 machinery” to obtain RNPs.

<i>in vitro</i> digestion		
Reagent		<i>in vitro</i> test
RNPs	2.75 µM	3.4 µL
Purified amplicon	75 ng µL ⁻¹	10 µL
Buffer O	10 X	1.5 µL
Nuclease free water	-	0.6 µL
TOTAL	-	15 µL

Reaction condition: 37 °C for 60 min, 98 °C for 5 min and store at 4 °C.

- in logarithmic growth phase at day 5 ($2^{-4} \times 10^6 \text{ mL}^{-1}$). Keep track of the cell concentration of your culture, preferably by cell counting, using a cell counter or manually: take a 200 μL sample, add 20 μL of iodine (0.25 % w/v in EtOH) to immobilize cells, load 10 μL of sample in an hemocytometer and count cells with the help of an optical microscope.
- Prepare linearized pAPHVIII-containing plasmid. Digest a pAPHVIII (paromomycin resistance)-containing plasmid using a restriction enzyme that performs a single cut outside of the paromomycin cassette. Ensure successful digestion by running an aliquot on a 1 % agarose gel. Inactivate restriction enzyme at 80 °C for 10 min, and keep it in sterile conditions. For each transformation, a total of 2 μg of linearized plasmid at a concentration of 200 $\text{ng } \mu\text{L}^{-1}$ is required.
 - Prepare TAP-agar $\pm$ paromomycin plates. Combine melted TAP-agar with filter-sterilized paromomycin sulphate (for a final concentration of 10 $\mu\text{g mL}^{-1}$) and spread into square Petri dishes (120 $\times$ 120 mm) with approximately 100 mL selection medium each.

The pAPHVIII containing plasmid pSL18 (Chlamydomonas Resource Center) was employed to generate *psy1* mutants. This was linearized using EcoRV (Thermo Fisher) which performs a single cut in this plasmid. The digestion reaction was carried out following manufacturer’s instructions.

Step 4: Transformation

Time of execution: 2 days (one day for electroporation and one day for recovery and plating).
Important. The following steps (except the last one) should be done during the same day. All steps should be performed under sterile conditions.

- 4.1 Cell preparation and heat shock. Determine the concentration of cells growing at logarithmic growth phase. Calculate the amount of cells that you will need to perform the planned transformations, and transfer the necessary volume of culture into a 50 mL tube. Perform a mild centrifugation (6 min at 2500 rpm). Resuspend in the appropriate amount of MAX Efficiency™ Transformation Reagent for Algae (Thermo Fisher, ME) plus sterilized 1 M sucrose (for a final concentration of 40 mM) to obtain a suspension of $2 \times 10^8 \text{ cells mL}^{-1}$, and transfer cells into a 1.5 mL sterile tube. Apply heat shock using a thermoshaker at 40 °C for 30 min with mild shaking (350 rpm). Let the cells recover for 30 min at RT (22–25 °C), always with mild shaking.
Note 4.1.1. See Troubleshooting (h) for best time to harvest cells during the day cycle.
Note 4.2.2. Autolysin solution can be used instead of ME as shown by Picariello et al. [12].
- 4.2 Assemble CRISPR-Cas9 RNPs. Prepare RNPs following the procedure explained in “Assembly of the CRISPR-Cas9 machinery” (transformation). Keep RNPs on ice.
- 4.3 Transform by electroporation. Combine 120 μL of heat-shocked cells (at $2 \times 10^8 \text{ cells mL}^{-1}$) with 10 μL of RNPs and 10 μL of linearized plasmid (200 $\text{ng } \mu\text{L}^{-1}$). This 140 μL mix will be enough to transform the gRNA in 3 independent electroporations (40 μL each). Let the suspension sit for 10 min on ice. Prepare a 24-well plate with 600 μL of liquid TAP medium in each well. Next, wash the inside of a 2 mm-gap electroporation cuvette with TAP from one of the wells, using a sterile Pasteur pipette with thin and long tip. Completely remove the excess medium from the cuvette, and load 40 μL of the mix. Make sure there are no air bubbles and the volume is evenly distributed at the bottom of the cuvette. Load the cuvette in the electroporation chamber of the NEPA21 (NEPAGENE). Measure impedance: the value should be between 0.2 and 0.4 K Ω . Setup electroporation conditions as shown in Table 3. Electroporate. With the help of the pipette, transfer the 40 μL containing the electroporated cells into a sterile 1.5 mL tube. The same cuvette can be used to perform electroporation of the other 2 replicates ($2 \times 40 \mu\text{L}$) from the same mix.
Note 4.3.1. It is important that the volume of 40 μL is exact.
Note 4.3.2. The electroporator needs to be setup in a sterile hood to keep conditions sterile during transformation.
Note 4.3.3. In case of using a different electroporator see Troubleshooting (f).

Table 3
Components of the electroporation mix and NEPA21 electroporator settings. These settings might require optimization depending on the electroporator and strain employed (see Troubleshooting (f)).

Electroporation						
Reagent				<i>in vitro</i> test		
Treated Chlamydomonas		2 10 ⁸ cells mL ⁻¹			120 μL	
RNPs		3.125 μM			10 μL	
Linearized plasmid		200 ng μL ⁻¹			10 μL	
TOTAL		-			140 μL	
NEPA21 Settings						
Pulse	Voltage	Pulse Length	Interval	N° of pulses	Decay Rate	Polarity
Poring Pulse	300 V	8 ms	50 ms	1	40 %	+
Transfer Pulse	20 V	50 ms	50 ms	5	40 %	+ /-

Reaction condition: 95 °C for 2 min, ramp 95-to-20 °C at 0.1 °C s⁻¹.

- Note 4.3.4. We recommend including a control consisting of heat-shocked cells with linearized plasmid, but not RNPs (which should give colonies on a selection plate), and a control consisting of heat-shocked cells only (which should not render any colonies on the selection plate). These controls are only needed once for each strain and plasmid employed.*
- Note 4.3.5. The use of the 24-well plate is recommended given that the capacity of the wells (600 µL) is a convenient volume for all the described procedures. Combining the necessary washes, control and triplicates, one 24-well plate is sufficient for 5 transformation mixes (each in triplicate, therefore a total of 15 transformations plus 2 controls if using one strain and marker plasmid). Each gRNA transformation (triplicates) can use the same cuvette.*
- 4.4 16 °C incubation. Incubate tubes containing the electroporated cells at 16 °C for 1 h (as indicated by Picariello et al. [12]).
- 4.5 Recovery: Transfer the 40 µL electroporated cells into the 600 µL TAP-filled 24-well plate (do not employ wells used for cuvette washes of Step 4.3). Seal the plate with parafilm and incubate cells at 24 °C and gentle shaking (150 rpm), under constant light (50 µmol m⁻² s⁻¹) overnight. If the mutation might confer photosensitivity (i.e. *psy1* mutants), wrap the plate in aluminum foil and perform recovery in the dark.
- 4.6 Plating. Gently plate the recovered electroporated cells (640 µL from one well) onto TAP-agar + paromomycin plates with the help of a sterile Drigalski spreader. Air dry plates for 1 h. Seal plates with parafilm, incubate plates upside down at 24 °C in continuous light (50 µmol m⁻² s⁻¹) or in the dark if the mutation might confer photosensitivity. Colonies should appear after 2–4 weeks depending on the strain and light conditions. Using the impedance range of this protocol, 5–20 colonies should appear on each plate.
- Note 4.6. In our experience for PSY1, the % of mutants was higher when impedance values were low (i.e. above 0.5 KΩ we did not generate any mutant, whereas with low values -below 0.35 KΩ- very few colonies were obtained but many contained mutations).*

Cell-wall containing CC-124 strain (Chlamydomonas Resource Center) was employed to generate *psy1* mutants. Transformation was performed using a NEPA21 electroporator (NEPAGENE): the settings displayed in Table 3 will likely work for most cell-wall containing strains, but cell-wall less strains require different electroporation settings (Kelterborn et al. [11]). Also, other electroporators have been shown to be successful for transformation using pre-assembled CRISPR-Cas9 RNPs [4,12].

Assembly of the CRISPR-Cas9 machinery

- Under sterile conditions:
- Resuspend and aliquot crRNA and tracrRNA. Resuspend lyophilized crRNA and tracrRNA in IDTE pH 7.5 (1X TE Solution) (IDT) at 40 µM. Prepare single use aliquots in sterile 0.2 mL tubes and store at –80 °C, as several cycles of freezing and thawing have been shown to decrease efficiency of mutant generation [12].
 - Prepare gRNAs. Perform the reaction indicated in Table 4. Less concentration is used for *in vitro* tests.
- Note gRNAs. Although gRNAs can be ordered already assembled, we have always purchased gRNAs separately and assembled them ourselves as indicated.*
- Prepare RNPs. Dilute (if necessary) SpCas9 (IDT) at 11 µM in DUPLEX Buffer. Perform the reaction indicated in Table 5 (Transformation).
- Note RNPs. The volume of Buffer O used highly affects impedance values (due to its BSA content): the higher the amount of this buffer the lower the impedance values.*
- Important. If CRISPR reagents are purchased from a different company it might be preferable to use CRISPR buffers specified by the manufacturer (all buffers can be replaced except Buffer O, which can also be homemade prepared following manufacturers specifications). In this case, keep concentrations of CRISPR components as indicated in these tables.*

Table 4
Assembly of gRNAs. Depending on the use of RNPs (*in vitro* / transformation) different amounts of the indicated reagents are required. Final concentration of gRNA is indicated at the bottom for both *in vitro* test (iv) and transformation (T).

gRNA assembly			
Reagent		<i>in vitro</i> test	Transformation
crRNA	40 µM	1.05 µL	1.5 µL
tracrRNA	40 µM	1.05 µL	1.5 µL
DUPLEX Buffer	-	3.9 µL	3 µL
TOTAL	7 µM (iv) / 10 µM (T)	6 µL	6 µL

Reaction condition: 37 °C for 15 min, keep at 4 °C.

Table 5

Assembly of RNPs. Depending on the use of RNPs (*in vitro* / transformation) different amounts of the indicated reagents are required. Final concentration of RNPs is indicated at the bottom for both *in vitro* test (iv) and transformation (T).

RNP assembly			
Reagent		<i>in vitro</i> test	Transformation
gRNA	7 μ M (iv) / 10 μ L (T)	2 μ L	4.4 μ L
Cas9	11 μ M	1 μ L	3 μ L
DUPLEX Buffer	-	1 μ L	1.4 μ L
Buffer O	10X	-	2.2 μ L
TOTAL	2.75 μ M (iv) / 3.125 μ M (T)	4 μ L	11 μ L

Reaction condition: 37 °C for 15 min, keep at 4 °C.

Step 5: Screening for large-insertion and short-indel mutants

Time of execution. Colonies will appear 2–4 weeks after electroporation (depending on the strain used and light conditions). Screening takes approximately two days.

5.1 Isolate colonies and extract gDNA. Wait until colonies reach 3–4 mm of diameter (2–4 weeks depending on the strain and light conditions). For large colonies, pick colonies with a sterile toothpick: transfer a small amount to a fresh TAP-agar plate and use the rest to perform a crude gDNA extract as explained in 2.1. For smaller colonies even after 4 weeks, streak on a fresh TAP-agar plate and once grown take a small amount following instructions in 2.1.

Note 5.1. This procedure can be optimized to obtain crude gDNA from a high number of colonies at the same time, by performing the extractions in a 96-well plate as explained in Kelterborn et al. [11].

5.2 Screening for long insertions and short indels. For each colony, perform two PCRs as indicated in Step 2: a PCR to detect large insertions using primers SLfw+SLrv, and a second PCR to detect indels using primers SIDfw+SIDrv. Run PCRs on a 2 % agarose gel. If no mutations are present in the target gene, both PCR reactions will yield a band of wild-type (wt) size; if a large insertion is present at the target site, PCR with SL primers yield a band of higher size; in the case of a small indel, PCR with SL primers will yield a band with the same size as the wt, but PCR with SID primers will not show amplification.

Note 5.2. If the insertion is large or if it involves rearrangement of the site for primer annealing, it is possible that none of the 2 PCRs yield a band. In this situation, PCR conditions and/or primer sequence will need to be adjusted. We also recommend running a control with a primer pair to amplify in a different region to control for the quality of the DNA extract.

5.3 Isolate colonies. Pick the original colony carrying a detected mutation and streak onto a fresh plate to obtain isolated colonies. Repeat SL PCR on a few isolated colonies to confirm analysis. This step controls for heterogeneity in the original colony and ensures singling of the mutant of interest.

5.4 Sequence of mutants. Perform a PCR with SL primers of a singled mutant colony in 40 μ L total volume following the concentrations indicated in Step 2. Run 5 μ L of this reaction on an agarose gel to confirm presence of the expected amplicon. Perform a PCR clean-up with the rest of the reaction, elute in the appropriate volume and sequence using the same SL primers.

Note 5.4. Sequencing of the obtained amplicon may fail using SL primers. This can be solved by cloning the amplicon into a TOPO type vector (Thermo Fisher) and using a vector primer for sequencing.

Fig. 5 shows colonies obtained after targeting *PSY1*. A number of successful *psy1* mutants could be detected by the pale green coloration of the colonies (Fig. 5A). PCR for these colonies using the SL and SID primer combinations detected both large insertion as well as indel mutants (Fig. 5B,C). The sequenced insertional mutant feature incorporation of a fragment of the marker plasmid through non-homologous end joining. Indel mutant sequencing revealed a mutant carrying 1 base insertion, and a mutant carrying a 11-base pair deletion, both at the Cas9 cutting site (Fig. 5C).

Protocol validation

This protocol was employed to successfully generate *psy1* knockout mutants carrying large insertions or small indels. Importantly, this could be achieved in just 5 weeks from CRISPR design to the sequencing of the isolated mutants, using tools and reagents which are commercially available. Compared to other recent CRISPR methods for *Chlamydomonas*, our protocol is easy and quick to implement as it does not require specific procedures such as cloning or homemade production of CRISPR reagents (Table 6).

Here, we used *PSY1* due to the characteristic pale color of *psy1* mutant colonies (Fig. 5A). Initial screening with SL primers revealed that some *psy1* mutants did not carry large insertions and therefore were instrumental in setting up the strategy to screen for small indel mutants, a valuable feature of our protocol. Our designed indel primers do not allow amplification by PCR in the presence of modifications at the Cas9 target site, that result not only from large insertions but also from the presence of short indels (Fig. 5B). Remarkably, we found this strategy to be able to detect modifications at the target site as small as an insertion of a single nucleotide (Fig. 5C).

We have used this protocol to generate and identify mutants for GOIs other than *PSY1* that did not feature a visible phenotype. At the impedance range indicated (0.2–0.4 K Ω) we usually obtained a small number of colonies (<50), and gDNA extraction and

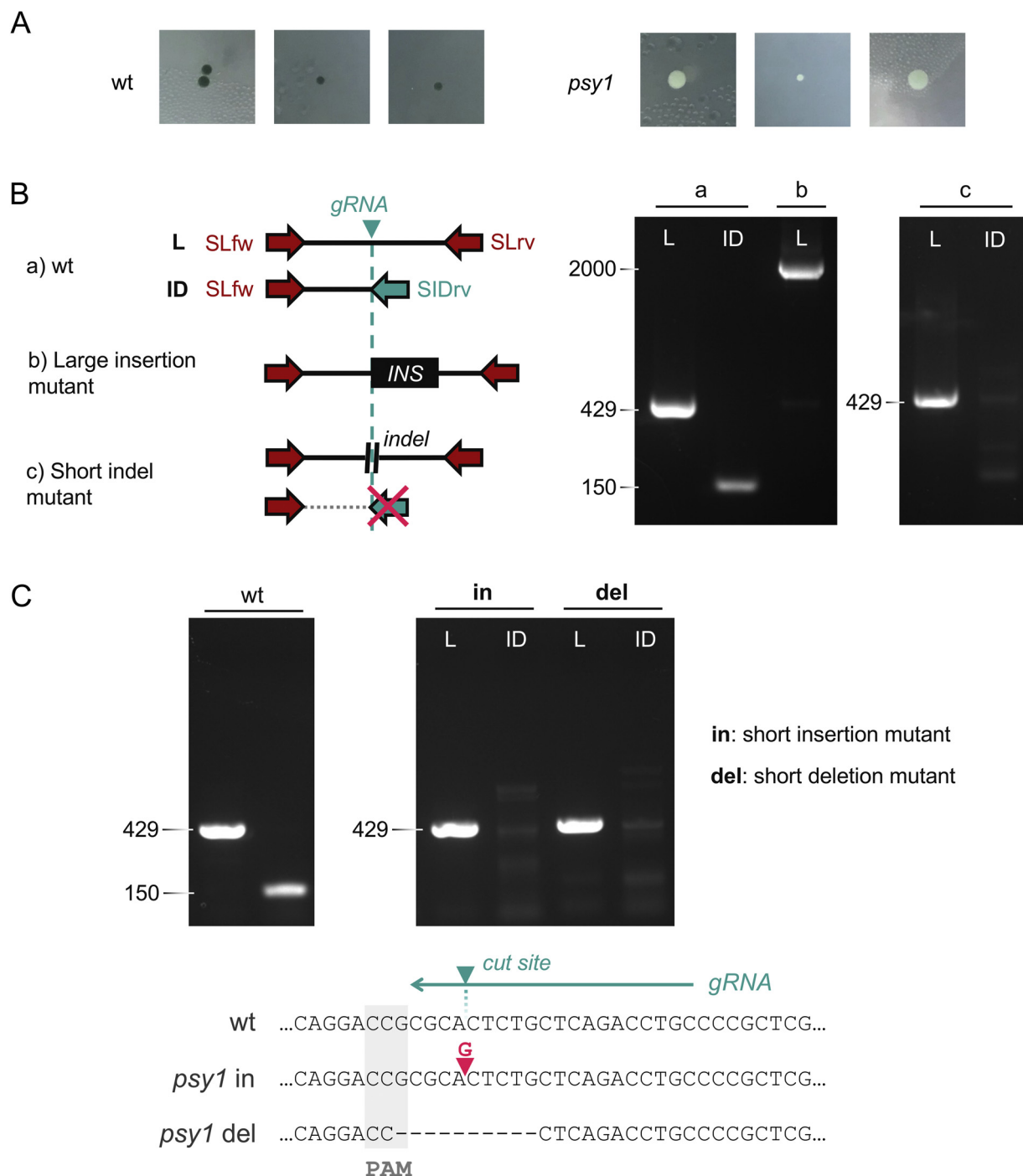


Fig. 5. A. Screening of *psy1* mutants by phenotype: *psy1* mutants exhibit pale green coloration. B. Screening of *psy1* mutants by genotype was carried out using primers to detect large insertions (SLfw+SLrv, giving L band) and short indels (SLfw+SIDrv, giving ID band). Screening by genotype resulted in three possible scenarios: a) wt colonies displayed both L and ID bands with the length expected in the absence of mutations at the cut site; b) large insertion mutants displayed L bands with much higher length; c) short indel mutants displayed the same L band with the same size as the wt, but didn't display the ID band. C. Screening and sequencing of *psy1* mutants from the latter scenario, one with a short insertion (in) and one with a short deletion (del). Uncropped electrophoresis gel images including the DNA ladder are provided in Supplemental Fig. 3.

Table 6

Comparison of recent CRISPR protocols developed for *Chlamydomonas*. All protocols (ours included) share three common steps: 1) *in vitro* assembly of RNPs, 2) transformation by electroporation and 3) mutant selection.

Previous protocols	Genome editing strategy	Screening method	Mutation efficiency ¹	Genes tested	Specific procedures required ²
Kelterborn et al. 2022	HR of a custom cassette (NHEJ fallback)	PCR screen on 96-well plates	<i>Not specified</i>	<i>SNRK2.2</i>	-Synthesis and purification of SpCas9
Picariello et al. 2020	HR of a resistance marker cassette (NHEJ fallback)	PCR screen on 96-well plates	-Total (including NHEJ): 30–93 % -Double gene targets: 3–15 %	<i>IFT81, FAP70, MOT17, CDPK13, CEP131, IFT43</i>	-Cloning of resistance marker cassette -Autolysin preparation
Angstenberger et al. 2020	HR of a resistance marker cassette (NHEJ fallback)	PCR screen of phenotype-positive colonies	-Total (including NHEJ): 13.8–14.3 % -HR: 30–40 % of mutant colonies	<i>FTSY</i>	-Cloning of resistance marker cassette -Synthesis and purification of recombinant Cas9 - <i>in vitro</i> transcription of gRNAs
Dhokane et al. 2020	NHEJ and HR of a resistance marker cassette	PCR screen of phenotype-positive colonies	<i>Not applicable</i>	<i>MAA7</i>	- <i>in vitro</i> transcription of gRNAs (For HR: cloning of resistance marker cassette)
Ferenczi et al. 2024	SSTR (NHEJ fallback)	PCR screen of phenotype-positive colonies	<i>Not applicable</i>	<i>FKB12, FTSY, SRP43</i>	-Synthesis and purification of Cas9 and/or Cas12a
Chen et al. 2023	Custom gene editing by HR	Pooled colony PCR screen	-Precise gene editing: 1.4–16 % depending on the gene and editing strategy	<i>CrKU80, FTSY, VIPP1, IFT46, CrTET1, FLA3, FLA10, MAA7, IFT46</i>	-Cloning of custom donor DNA -Synthesis and purification of SpCas9 - <i>in vitro</i> transcription of gRNAs
Nievergelt et al. 2024	Custom gene editing by HR	qPCR screen on 96-well plates	-Overall editing frequency: 65–90 % -Precise gene editing: 20 %	<i>IFT46</i>	-Cloning of custom donor DNA -Autolysin preparation
This protocol	NHEJ	PCR screen (large insertions and short indels detection)	-Total: 7–60 % depending on the gene -Short indels: 15–50 % of mutants depending on the gene	<i>PSY1</i> (other <i>GOI</i> discussed)	NO

¹ Mutation efficiency refers to the percentage of mutant colonies out of all the screened colonies (resistance-selected). “*Not applicable*”, when screened colonies were selected based on phenotype and numbers compared to total colonies are not provided; “*Not specified*”, when efficiency is not discussed in original publication.

² Requirement of specific procedures in the protocol such as time-consuming cloning or homemade production of CRISPR reagents. “*PCR screen on 96-well plates*”: gDNA extraction + PCR screening of multiple selected colonies is performed individually. “*Pooled colony PCR screen*”: gDNA extraction + PCR screening of multiple combined colonies. “*qPCR*”: quantitative PCR.

screening PCRs were performed one-by-one. For larger number of colonies, the procedure can be performed in 96-well plates as described by Kelterborn et al. [11] to reduce protocol times. In addition, although our protocol requires PCR analysis of single colonies to detect small indels, a pooled-PCR screening strategy might be implemented in *Chlamydomonas* based on the protocol described by Angelopoulou et al. in mouse cell cultures [21] based on PCR-induced mutagenesis-restriction fragment polymorphism (PIM-RFLP).

The overall efficiency of mutant generation for *PSY1* shown here (number of positive mutants vs total number of colonies) was 34 % and 60 % in two separate transformation runs (11 out 32 colonies, and 9 out of 15 colonies). In accordance to other reports, efficiency can highly vary among targeted genes and gRNA sequences. Indeed, we experienced a variety of efficiencies when targeting other genes using this same protocol (ranging 7–60 %, comparable to other recent CRISPR protocols, see Table 6) and *PSY1* was our highest efficiency gene. Frequency of indel mutants ranged between 13–50 % of all detected mutants.

In summary, our protocol provides a quick to implement and easy method for the generation of CRISPR-Cas9 knockout mutants in *Chlamydomonas*, including a strategy to identify short indels at the Cas9 target site and thus increasing mutant detection efficiency.

Troubleshooting

a) No PCR amplification or unspecific bands when using SL primers

Perform a gradient PCR to evaluate alternative annealing temperatures (T_m). DMSO amount can also be increased (up to 20 % of final PCR reaction volume). When performing gDNA extractions, test different amounts of starting material and/or replace 10 mM EDTA pH 8 with alternative buffers as indicated by Kelterborn et al. [11], or Dhokane et al. [15]. If none of these adjustments work, design a new primer pair combination.

b) No PCR amplification or unspecific bands when using SIDrv primer

Follow the recommendations indicated in a). If none of these adjustments work, redesign both gRNA and indel primers. Remember to successfully complete Steps 1 and 2 before purchasing CRISPR-Cas9 reagents.

c) PCR amplification when using SIDrv* primer

Perform a gradient PCR to evaluate alternative annealing temperatures (T_m). If amplification is still observed, redesign gRNA and indel primers. Remember to successfully complete Steps 1 and 2 before purchasing CRISPR-Cas9 reagents.

d) We use a different DNA polymerase for PCR reactions

Several DNA polymerases and PCR kits have been successful for performing PCRs in *Chlamydomonas*, as reported by other CRISPR protocols [10–14]. When performing PCRs in this species for the first time, or in case of failed PCRs using a different DNA polymerase, we recommend to employ the PCR reagents and the proportions we indicate in this protocol.

e) Impedance values are not in the range of 0.2–0.4 K Ω

If impedance values are above this range, slightly increase the amount of Buffer O when preparing RNPs, or increase the amount of RNPs during the electroporation step. Impedance values up to 0.5 K Ω might be used, but above 0.5 K Ω we were not able to generate any mutant.

If impedance values are below the indicated range, decrease the amount of Buffer O and/or purify the linearized resistance-carrying plasmid after enzymatic digestion. Also, abnormally low levels of impedance can be an indication of cell lysis which can critically affect the generation of mutant colonies; check cells are in good health when harvesting them for electroporation and, if necessary, adjust growth conditions.

Typically, the higher the impedance values the more colonies obtained, but with decreased mutant generation efficiency. Values can also be adjusted by slightly changing the volume of electroporation mix inside the cuvette (2–3 μ L at most) as indicated by Kelterborn et al. [11]: higher electroporation volumes decrease impedance values, and *vice versa*.

f) We have a different electroporator which does not measure the impedance value

Perform an electroporation trial by electroporating with treated cells and the linearized resistance-carrying plasmid. Test the effect of adding 10 μ L of DUPLEX Buffer (or the employed CRISPR Buffer) to the electroporation mix with increasing amounts of Buffer O to setup the conditions to get 10–20 colonies/plate. Additional indications on the use of alternative electroporators can be found in Shin et al. [4], Picariello et al. [12] or Chen et al. [13] to mention a few.

g) No colonies obtained after electroporation

Check that cells are in good health before starting the transformation step. Make sure impedance values are within the range indicated in e). Perform an electroporation control using heat-shocked treated cells and the resistance-carrying plasmid, which should give much higher impedance values (>0.7 K Ω), due to the absence of the BSA-containing Buffer O. In this test, values <0.7 K Ω might be indicative of cell lysis, and/or that the linearized resistance-carrying plasmid needs to be purified after enzymatic digestion, then follow recommendations indicated in e). Much higher impedance values (>1.2 K Ω) do not represent an issue in these tests if the impedance is in the correct range in the presence of RNPs.

h) Transformed colonies are obtained but no mutants are detected.

Make sure to perform the *in vitro* cleavage test to test functionality of the Cas9 and the gRNA. Check impedance values are in the indicated range and cells are in good health before starting the transformation step. The time of transformation during the day cycle can also impact CRISPR efficiency. As described by Angstenberger et al. [10], 4 h after the switch from dark to light would be the best option, given that cells are in G1 phase and NHEJ is the dominant DNA repairing mechanism. In addition, the accessibility of the locus to be edited might also have an impact, and editing might be more favorable in the time window when your *GOI* is expressed (can be checked in Strenkert et al. [22]). If none of these recommendations work, design new gRNAs.

i) Contamination on the selection plates

Make sure all components are sterile and manipulation of reagents is performed in strict sterile conditions. Check that the strain and cell culture do not carry any contamination. Make sure that the linearized resistance-carrying plasmid is heated at 80 °C before transformation. The electroporation cell should be cleaned with 70 % ethanol before its use and opened in sterile conditions. When re-utilizing electroporation cuvettes, clean them with ethanol and UV-sterilized them before using. If it is still a problem, ampicillin against bacteria (100–500 μ g/mL) or cefotaxime against fungi (100 μ g/mL) can be added to the media and the plates [11].

Limitations

Our protocol features the following limitations:

- **Random insertion of the resistance cassette in the genome.** To assign mutant phenotypes to the loss-of-function of a particular *GOI*, we recommend generating multiple mutants and/or complementing the mutant.

- **Variable efficiency of gene editing.** Mutant generation efficiency for different *loci* and/or gRNAs might vary, although a high gene editing efficiency is possible using this protocol as exemplified here for *PSY1*.
- **No high-throughput screening for indels.** High-throughput screening methods (pooling colonies) can be used to screen for large insertion mutants. Detection of small indels by PCR as described in this protocol need to be performed on single colonies.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.mex.2025.103416](https://doi.org/10.1016/j.mex.2025.103416).

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