

## DNA-free Genome Editing of *Chlamydomonas reinhardtii* Using CRISPR and Subsequent Mutant Analysis

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**[Abstract]** We successfully introduced targeted knock-out of gene of interest in *Chlamydomonas reinhardtii* by using DNA-free CRISPR. In this protocol, the detailed procedures of an entire workflow cover from the initial target selection of CRISPR to the mutant analysis using next generation sequencing (NGS) technology. Furthermore, we introduce a web-based set of tools, named CRISPR RGEN tools (<http://www.rgenome.net/>), which provides all required tools from CRISPR target design to NGS data analysis.

**Keywords:** Genome editing, CRISPR-Cas9, Microalgae, Ribonucleoproteins, *Chlamydomonas reinhardtii*, DNA-free transformation

**[Background]** We recently reported (Baek *et al.*, 2016) a one-step transformation of the model green microalga *Chlamydomonas reinhardtii* (Harris, 2001) using preassembled Cas9 protein-guide RNA ribonucleoproteins (RNPs). The manner of DNA-free CRISPR-Cas9 delivery has several advantages such as no need for codon optimization and specific promoters in different species of microalgae. Furthermore, it reduces off-target effects and may also be less cytotoxic in cells because the Cas9 protein is transiently active and then degraded by endogenous proteases in cells (Kim *et al.*, 2014). In addition, the resulting gene-edited microalgae could be exempt from genetically modified organism (GMO) regulations due to the absence of foreign DNA sequences. In this protocol, the detailed procedures of an entire workflow are contained from the initial target selection of CRISPR to the mutant analysis using NGS technology (Bae *et al.*, 2014a and 2014b; Park *et al.*, 2015 and 2017).

### Materials and Reagents

- A. Cas9 protein purification
  1. 1.5 ml Eppendorf tubes
  2. Sterile 50 ml conical tubes
  3. 10 ml syringe
  4. 0.45  $\mu$ m syringe filter

5. Protein concentrator–100K MWCO (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 88533)
6. Poly-Prep Chromatography columns (Bio-Rad Laboratories, catalog number: 7311550)
7. BL21-Pro cells
8. pET28 plasmid containing 6xHis-SpCas9 (Addgene)
9. Kanamycin
10. Lysozyme
11. PMSF
12. Ni-NTA agarose beads (QIAGEN, catalog number: 30210)
13. Bradford reagent (Bio-Rad Laboratories, catalog number: 5000205)
14. Bovine serum albumin (BSA)
15. Sodium chloride (NaCl)
16. Tryptone
17. Yeast extract
18. IPTG
19. Sodium phosphate monobasic (NaH<sub>2</sub>PO<sub>4</sub>)
20. Imidazole
21. Sodium hydroxide (NaOH)
22. HEPES
23. Ethylenediaminetetraacetic acid (EDTA)
24. DL-dithiothreitol (DTT)
25. Sucrose
26. Glycerol
27. LB medium (see Recipes)
28. 1 M IPTG stock (see Recipes)
29. Lysis buffer (see Recipes)
30. Wash buffer (see Recipes)
31. Elution buffer (see Recipes)
32. Cas9 storage buffer (see Recipes)

B. *In vitro* transcription and library PCR

1. 1.5 ml Eppendorf tubes
2. Forward and reverse oligos (see Tables 1 and 2)

**Table 1. Pre-index primers**

Pre-index forward primer	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT gDNA target-3'
Pre-index reverse primer	5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT gDNA target-3'

**Table 2. Index primer sequences**

	index sequence [i5]	Index forward primer sequence
D501	tatagcct	AATGATACGGCGACCACCGAGATCTACACtatagcctACACTCTTTCCTACACGAC
D502	atagaggc	AATGATACGGCGACCACCGAGATCTACACatagaggcACACTCTTTCCTACACGAC
D503	cctatcct	AATGATACGGCGACCACCGAGATCTACACcctatcctACACTCTTTCCTACACGAC
D504	ggctctga	AATGATACGGCGACCACCGAGATCTACACggctctgaACACTCTTTCCTACACGAC
D505	aggcgaag	AATGATACGGCGACCACCGAGATCTACACaggcgaagACACTCTTTCCTACACGAC
D506	taatctta	AATGATACGGCGACCACCGAGATCTACACtaatcttaACACTCTTTCCTACACGAC
D507	caggacgt	AATGATACGGCGACCACCGAGATCTACACcaggacgtACACTCTTTCCTACACGAC
D508	gtactgac	AATGATACGGCGACCACCGAGATCTACACgtactgacACACTCTTTCCTACACGAC
	index sequence [i7]	Index reverse primer sequence
D701	cgagtaat	CAAGCAGAAGACGGCATAACGAGATcgagtaatGTGACTGGAGTTCAGACGTGT
D702	tctccgga	CAAGCAGAAGACGGCATAACGAGATtctccggaGTGACTGGAGTTCAGACGTGT
D703	aatgagcg	CAAGCAGAAGACGGCATAACGAGATaatgagcgGTGACTGGAGTTCAGACGTGT
D704	ggaatctc	CAAGCAGAAGACGGCATAACGAGATggaatctcGTGACTGGAGTTCAGACGTGT
D705	ttctgaat	CAAGCAGAAGACGGCATAACGAGATttctgaatGTGACTGGAGTTCAGACGTGT
D706	acgaattc	CAAGCAGAAGACGGCATAACGAGATacgaattcGTGACTGGAGTTCAGACGTGT
D707	agcttcag	CAAGCAGAAGACGGCATAACGAGATagcttcagGTGACTGGAGTTCAGACGTGT
D708	gcgcata	CAAGCAGAAGACGGCATAACGAGATgcgcataGTGACTGGAGTTCAGACGTGT
D709	catagccg	CAAGCAGAAGACGGCATAACGAGATcatagccgGTGACTGGAGTTCAGACGTGT
D710	ttcgcgga	CAAGCAGAAGACGGCATAACGAGATttcgcggaGTGACTGGAGTTCAGACGTGT
D711	gcgcgaga	CAAGCAGAAGACGGCATAACGAGATgcgcgagaGTGACTGGAGTTCAGACGTGT
D712	ctatcgct	CAAGCAGAAGACGGCATAACGAGATctatcgctGTGACTGGAGTTCAGACGTGT

3. dNTP mix
4. Phusion DNA polymerase (Thermo Fisher Scientific, Thermo Scientific™, catalog number: F530L)
5. PCR purification kit
6. ATP, CTP, GTP, UTP, 100 mM MgCl<sub>2</sub>, DEPC-treated water
7. T7 RNA polymerase (New England Biolabs, catalog number: M0251L)
8. DNase I (RNase-free) (New England Biolabs, catalog number: M0303L)
9. RNase inhibitor murine (New England Biolabs, catalog number: M0314L)
10. RNeasy MinElute Cleanup Kit (QIAGEN, catalog number: 74204)
11. Illumina Miseq Reagent Kit (v2)

### C. Transfection

1. 1.5 ml Eppendorf tubes
2. Sterile 15 ml and 50 ml conical tubes
3. Purified Cas9 protein and sgRNA
4. *Chlamydomonas reinhardtii* strains CC-4349 cw15 mt-(Chlamydomonas Resource Center)

5. PCR DNA purification kit (MG Med, catalog number: MD008)
6. TAP medium (Harris, 1989 or Thermo Fisher Scientific, Gibco™, catalog number: A1379801)
7. TAP sucrose solution (see Recipes)
8. TAP agar plates (see Recipes)
9. Top agar (see Recipes)

## **Equipment**

### A. Cas9 protein purification

1. Centrifuge: swing rotor (LaboGene, model: 1580R)
2. Sonicators (Qsonica, model: Q125)
3. Pipettes
4. Shaking incubator (JS Research, model: JSSI-300C)

### B. *In vitro* transcription and library PCR

1. Incubator (JS Research, model: JSGL-050T)
2. Thermal cycler (Bio-Rad Laboratories, model: C1000 Touch™ Thermal Cycler)
3. Micro centrifuge (LaboGene, model: 1730R)
4. Spectrophotometer

### C. Transfection

1. 100 ml flask
2. Spectrophotometer (GE Healthcare, Amersham Biosciences, model: Ultrospec 2100 pro)
3. Hemocytometer (Marienfeld-Superior, catalog number : 0650030)
4. Microscope (Olympus, model: CH30)
5. Micro high speed centrifuge (Hanil, model: MICRO 17TR)
6. Orbital shaker (N-biotek, model: NB-101M)
7. Clean bench (BioFree, model: BF-150BSC)
8. Electroporation device (Bio-Rad Laboratories, model: Gene Pulser Xcell™ Electroporation Systems)
9. Electroporation cuvettes (Bio-Rad Laboratories, 0.4 cm gap)

## **Procedure**

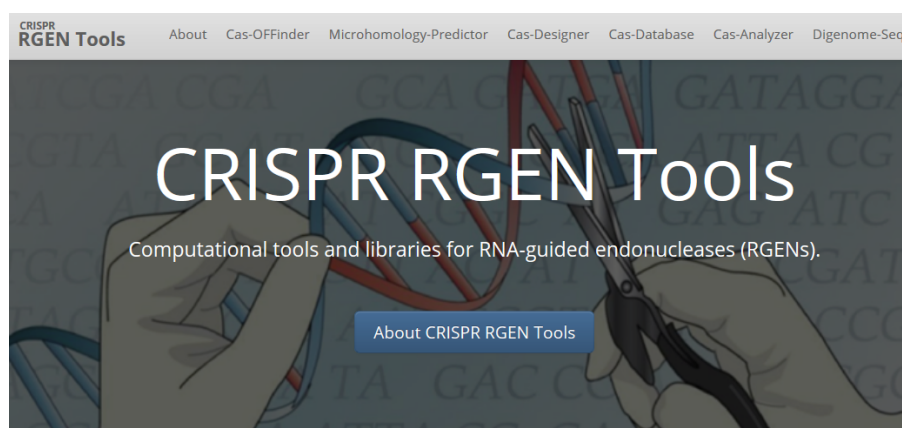
### A. CRISPR target selection for creating gene knock-outs of interest

1. Obtain the coding sequence (CDS) of the gene of interest from an appropriate database. We use Phytozome from JGI (<https://phytozome.jgi.doe.gov/pz/portal.html#>) (Figure 1).



**Figure 1. Main page of Phytozome: The plant genome database.** The coding sequence information of each gene can be obtained from Phytozome database.

2. For target design, we use CRISPR RGEN tools (<http://www.rgenome.net/>), a web-based set of tools for CRISPR target design and next-generation sequencing (NGS) data analysis (Figure 2).



**Figure 2. Main page of CRISPR RGEN Tools.** A web-based set of tools which provides all required tools from CRISPR target design to NGS data analysis.

3. In Cas-Designer, select the type of CRISPR endonuclease, such as SpCas9 from *Streptococcus pyogenes*, and the target genome, in this case that of the model organism *Chlamydomonas reinhardtii*. Cas-Designer now contains the versions 4 and 5 of it at 'Plants' organism type on our website. Then insert the CDS of the gene of interest in the 'Target Sequence' box. We recommend choosing target sites within the first half of the full CDS to increase the probability of generating complete knock-out mutations (Figure 3).

The screenshot shows the Cas-Designer web interface. It is divided into three main sections: PAM Type, Target Genome, and Target Sequence. The PAM Type section lists various CRISPR/Cas-derived RNA-guided Endonucleases (RGENs) with radio buttons for selection. The Target Genome section has a dropdown for Organism Type (set to 'Plants') and a list of Genomes, with *Chlamydomonas reinhardtii* (Chlre4) selected. The Target Sequence section has a text area for inserting target sequences (raw sequence or FASTA format, maximum 1000 chars) and a dropdown for crRNA length (set to 20). There is also an option to upload a FASTA-formatted file and an optional target searching range field.

**Figure 3. Overview of Cas-Designer.** Users can select various types of CRISPR endonucleases and the target genome including *Chlamydomonas reinhardtii* (versions 4 or 5).

- Choose several targets among the candidates from Cas-Designer. You should avoid targets that are associated with potential off-target sites bearing 1-2 mismatches compared to the on-target. Targets with GC content ranging from 30 to 70% and higher out-of-frame scores are recommended (Figure 4).

Cre01

RGEN Target (5' to 3')[?]	Position[?]	Cleavage Position (%) [?]	Direction[?]	GC Contents (% w/o PAM)[?]	Out-of-frame Score[?]	Mismatches[?]		
						0	1	2
GAAGCCGGGCAAGGAGGCGATGG	3	7.3	+	70.0	N/A	1	0	7
GCCGGGCAAGGAGGCGATGGAGG	6	8.5	+	75.0	N/A	1	0	5
TCCTCCATGCCCTCTTGCCCGG	7	4.6	-	65.0	N/A	1	0	2
CATGGAGGATTCACGCCAGG	21	14.2	+	65.0	54.8	1	0	0
AGGCGGCGGACCTGGGCTGG	33	14.6	-	85.0	53.6	1	0	4
CGCCAGGTCCGCGCCCTTCGG	37	20.4	+	80.0	54.7	1	0	0
GCCAGTCCGCGCCCTTCGGG	38	20.8	+	80.0	54.0	1	0	1
GCCCCAAGGCGGCGGACCTGG	39	16.9	-	85.0	64.5	1	0	2
GGTCCGCGCCCTTCGGGCTGG	42	22.3	+	85.0	53.5	1	0	1
GTCCGCGCCCTTCGGGCTGGG	43	22.7	+	80.0	54.3	1	0	0
CGCCAGCCCGAAGGCGGCGCGG	45	19.2	-	85.0	63.2	1	0	6

**Figure 4. An example data of an output table from Cas-Designer.** Cas-Designer shows possible CRISPR target sites from input sequences along with useful information such as GC content, out-of-frame score, and off-target information.

*Note: N/A means 'not applicable' due to the lack of flanking DNA nucleotides.*

## B. Purification of recombinant Cas9 protein

- Transform 50 µl competent BL21-Pro cells with a pET28 vector containing 6xHis-SpCas9.
- Spread the cells on an agar plate containing kanamycin. Incubate the plate overnight at 37 °C.
- Inoculate 10 ml LB medium containing 50 µg/ml kanamycin in a conical tube with a single colony from the plate. Grow to saturation overnight at 37 °C with shaking at 200 rpm.

4. The next morning, inoculate 200 ml of LB medium containing kanamycin in a flask with 2 ml saturated overnight culture.
5. Grow the cells at 37 °C with shaking at 200 rpm to mid-log phase (to an OD<sub>600</sub> of approximately 0.4-0.5).
6. Add 160 µl of 1 M IPTG (0.8 mM final concentration) and incubate the cells overnight at 18 °C with shaking at 200 rpm.
7. Transfer the culture to a 50 ml conical centrifuge tube and pellet the cells by centrifuging for 20 min at 3,000 x g and 4 °C. Discard the supernatant and repeat the harvest in the same tube.
8. Re-suspend the cell pellets in 10 ml lysis buffer (see Recipes).
9. Add 20 mg lysozyme and 100 µl of 100 mM PMSF (1 mM final concentration).
10. Incubate 1 h on ice.
11. Sonicate the cells in a conical tube on ice (amplitude 25%, 1 sec ON/ 1 sec OFF, pulse 30 times). Repeat the sonication process 5 times.
12. Transfer the sonicated cells to a 1.5 ml microtube using micropipette and pellet debris by centrifuging at 4 °C, 11,000 x g for 30 min.
13. Filter the supernatant through a 0.45-µm syringe filter and collect it in a 50 ml conical tube.
14. Add 2 ml Ni-NTA agarose to the lysate. Swirl agarose beads before adding. Incubate for 1 h with rotation at 4 °C.
15. Transfer the mixture to a chromatography column with pipette. Collect the flow-through in a conical tube.
16. Wash the column with 10 ml wash buffer (see Recipes). Collect this first batch of wash buffer in a conical tube.
17. Repeat the wash. Collect the second batch of wash buffer in a conical tube.
18. Elute the bound protein with 10 ml elution buffer (see Recipes). Collect the eluate in 1 ml sequential aliquots in 10 microtubes.
19. Using a Bradford assay, determine the protein concentration in each fraction.
20. Apply the eluate that contains the fusion protein to a protein concentrator (100K MWCO).
21. Centrifuge at 4 °C, 3,000 x g until the sample volume is reduced by 90%.
22. Apply 4 ml protein storage buffer (see Recipes) and centrifuge at 4 °C, 3,000 x g, until the volume is reduced by 90%.
23. Elute the protein on the filter with 300 µl protein storage buffer.
24. Measure the protein concentration through Bradford assay with standard BSA in the spectrophotometer (the final concentration of SpCas9 should be approximately ~10 mg/ml).

*Notes:*

- a. *Identify which samples from each step contain the fusion protein by SDS-PAGE.*
- b. *You should verify the activity of Cas9 protein using an in vitro cleavage assay.*

### C. *In vitro* transcription of sgRNA

#### 1. Template extension

Primer F (100 μM) (Table 3)	1 μl			
Primer R (100 μM) (Table 3)	1 μl	98 °C	10 sec	
5x HF buffer	10 μl	98 °C	10 sec	} 25~30cyc
10 mM dNTP	2.5 μl	54 °C	20 sec	
Phusion DNA polymerase	0.5 μl	72 °C	30 sec	
Distilled water	35 μl	72 °C	5 min	
<b>Total</b>	<b>50 μl</b>			

After template extension, purify DNA using a PCR purification kit.

*Note: Purified DNA template can be stored at -20 °C.*

**Table 3. Oligos for *in vitro* transcription**

Oligo F (target specific)	5'-GAAATTAATACGACTCACTATAG (target 20nt - 5' to 3' direction, without PAM) GTTTTAGAGCTAGAAATAGCAAG-3'
Oligo R (common)	5'-AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC TAGCCTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'

#### 2. *In vitro* transcription reaction

Template DNA	(1,560 ng)
100 mM MgCl <sub>2</sub>	28 μl
Each NTP (100 mM stock)	4 μl (x4)
10x T7 RNA buffer	10 μl
T7 RNA polymerase (50 U/μl)	5 μl
RNase inhibitor (40 U/μl)	2.5 μl
DEPC-treated water	up to 100 μl
<b>Total</b>	

4 h-overnight reaction, 37 °C water bath or incubator.

#### 3. Removal of DNA template

- Add 11 μl DNase I buffer
- Add 1 μl (2 U/μl) DNase I in 100 μl reaction
- Incubate at 37 °C, 30 min

#### 4. RNA purification

- Use an RNA cleanup kit following the manufacturer's manual.
- Elute RNA in 10~25 μl DEPC treated water or RNase-free water. The final RNA concentration should be 5-15 μg/μl.

*Note: RNA should be stored at -80 °C until the Chlami cells ready.*



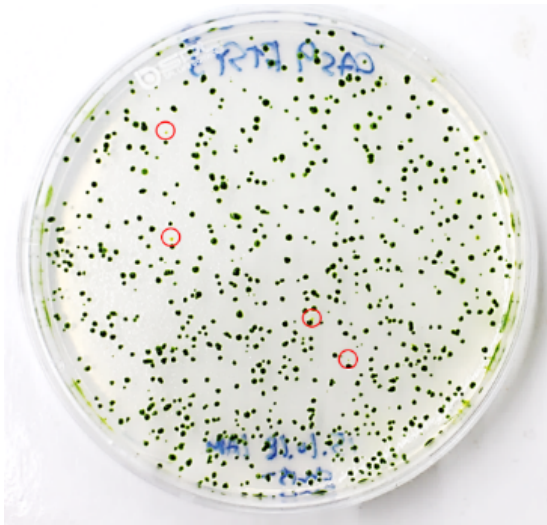
#### D. Transfection of ribonucleoproteins (RNPs) to *Chlamydomonas*

##### 1. Preparation of cells and RNP complex for transformation

- a. Cultivate the *Chlamydomonas* cells mixotrophically (in the light with acetate) in 50 ml liquid TAP medium at 25 °C under continuous light (50-70  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ ) in a 100 ml flask with shaking at 90 rpm until the cell density reaches an OD<sub>750</sub> of 0.3-0.5 as measured by a spectrophotometer.
- b. Estimate the cell density with a hemocytometer and microscope.
- c. Harvest  $5 \times 10^5$  cells by centrifugation in the 1.5 ml Eppendorf tubes (2,000  $\times g$ , 3 min, room temperature), discard the supernatant, and resuspend the cells in 250  $\mu\text{l}$  of TAP sucrose solution (see Recipes) by gently pipetting (prepare one aliquot of cells per transformation reaction).
- d. To prepare the RNP complex, premix purified Cas9 protein (200  $\mu\text{g}$ ) with *in vitro* transcribed sgRNA (140  $\mu\text{g}$ ) in the 1.5 ml Eppendorf tubes and incubate for 10 min at room temperature (prepare one aliquot of RNP complexes per transformation reaction).
- e. Add the RNP complex to the resuspended cells and gently tap the tube.
- f. Transfer 250  $\mu\text{l}$  (+ volume of the RNP complex) of the transformation mixture to an electroporation cuvette.
- g. Incubate at room temperature for 5 min.

##### 2. Electroporation of RNP complex

- a. Set parameters in the electroporation device (Bio-Rad Laboratories) (voltage: 600 V, capacity: 50  $\mu\text{F}$ , resistance: infinity).
- b. Gently tap the cuvette to mix the contents and place the cuvette in the cuvette chamber.
- c. Electroporate the cells and then resuspend them by gently pipetting in 750  $\mu\text{l}$  of TAP sucrose solution (total volume, 1 ml).
- d. Transfer all of the transformation mixture into a 15 ml conical tube.
- e. To obtain single mutant colonies, immediately transfer  $2-4 \times 10^3$  cells (4-6  $\mu\text{l}$ ) from the transformation mixture to a new 15 ml conical tube and then plate them on a 1.5% TAP agar plate (see Recipes) using top agar (see Recipes). After transformation, 400-600 colonies generally appear (Figure 5).



**Figure 5. Visual coloration examination to investigate CpFTSY gene knockout.** Red circles indicate the putative CpFTSY knockout mutant lines which have pale green colors grown on TAP agar medium.

- f. Incubate the rest of the cells for 12 h in dim light ( $5\text{-}10 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ ), after which they should be harvested for genomic DNA extraction for use in targeted deep-sequencing analysis.

#### E. Preparation of NGS library using PCR

##### 1. Deep sequencing library preparation by PCR amplification

- a. (Optional) Target gene ( $\sim$ cleavage site  $\pm$  250 bp) amplification

For successful library construction, we recommend amplifying the target gene, which should include the cleavage site  $\pm$  250 bp, first. We use Phusion DNA polymerase (Thermo).

- b. Pre-indexing PCR ( $\sim$ 25 cycles)

DNA template	(100 ng)
5x phusion HF buffer	4 $\mu$ l
10 mM dNTPs	0.4 $\mu$ l
Pre-index forward primer (10 $\mu$ M) (Table 1)	1 $\mu$ l
Pre-index reverse primer (10 $\mu$ M) (Table 1)	1 $\mu$ l
Phusion DNA polymerase (2 U/ $\mu$ l)	0.2 $\mu$ l
Distilled water	Up to 20 $\mu$ l
Total volume	20 $\mu$ l

For paired-end sequencing, the DNA library must have two adapters that include the i5 and i7 indices. Amplify the target DNA using Phusion polymerase with the pre-index tailed primers.

Note: We recommend that the length of the target DNA be ~250 bp for Miseq 300 cycles or ~450 bp for Miseq 500 cycles for optimal merging of the paired-end reads.

c. Indexing PCR (~25 cycles)

DNA template (step E1b sample dilution)	x
5x HF buffer	10 µl
10 mM dNTPs	1 µl
Index forward primer D50X (10 µM) (Table 2)	2.5 µl
Index reverse primer D7XX(10 µM) (Table 2)	2.5 µl
Phusion DNA polymerase (2 U/µl)	0.5 µl
Distilled water	Up to 50
Total volume	50 µl

After the pre-indexed amplification, amplify the PCR products with universal index primers. Purify the amplicons using a PCR DNA purification kit and measure the concentration of library using a spectrophotometer.

2. Targeted deep sequencing

We perform targeted deep sequencing using an Illumina Miseq Reagent Kit (v2).

## Data analysis

A. Analysis of targeted deep-sequencing data

1. We use Cas-Analyzer in RGEN tools (<http://www.rgenome.net/cas-analyzer/>) (Figure 6).

The screenshot displays the Cas-Analyzer web interface. It is divided into three main sections: 'Sequencing Data', 'Basic Information', and 'Analysis Parameters'.  
1. 'Sequencing Data': Includes a 'File Type' dropdown set to 'Paired-end reads', and two input fields for 'Read 1 (fastq or gzipped fastq):' and 'Read 2 (fastq or gzipped fastq):', each with a '찾아보기...' (Find) button.  
2. 'Basic Information': Contains a 'Full reference sequence (5' to 3\')' text area, a 'Nuclease Type' dropdown set to 'Single nuclease', a 'Select Nuclease' dropdown set to 'SpCas9 from Streptococcus pyogenes: 5'-NGG-3'', a 'Target DNA sequence (5' to 3', without PAM sequence):' text area, and an '(Optional) Donor DNA sequence for homology directed repair (HDR) (5' to 3\')' text area.  
3. 'Analysis Parameters': Includes a 'Comparison range (R) [7]' input field with a 'or use both ends' checkbox, a 'Minimum frequency (n) [7]' input field set to '1', and a checked '(Optional) WT marker (r) [7]' input field set to '5'. Below these fields is a schematic diagram of a DNA sequence with a 'Cleavage point' indicated by a green arrow. The diagram shows 'Left indicator sequence (12bp)', 'Right indicator sequence (12bp)', 'ZFNs, TALENs, Cas9, or Crf1 nucleases' at the cleavage site, a 'WT marker (r)' in the center, and a 'Comparison range (R)' on either side. A 'Submit' button is located at the bottom right of the diagram area.

Figure 6. Overview of Cas-Analyzer. Users analyze their targeted deep-sequencing data using Cas-Analyzer without uploading any data to the server or local tool installation.

2. Upload the NGS data files. Single-end reads, paired-end reads, and already merged sequencing data are allowed.
3. Input the reference sequence and target DNA sequence without the PAM (protospacer adjacent motif) sequences that are the recognition site of CRISPR endonucleases.
4. Submit the data.
5. The results contain information about mutation counts and frequencies in brief. Statistical data and detailed sequence alignment data are displayed further down on the page.

## B. Analysis of off-target mutations in an isolated mutant

1. Find more detailed information about potential off-targets associated with each selected target in the *Chlamydomonas* genome using Cas-OFFinder. Select a mismatch number (typically 4) and DNA/RNA bulge size (typically 1) (Figure 7).

**PAM Type**

CRISPR/Cas-derived RNA-guided Endonucleases (RGENs)

- SpCas9 from *Streptococcus pyogenes*: 5'-NGG-3'
- SpCas9 from *Streptococcus pyogenes*: 5'-NRG-3' (R = A or G)
- StCas9 from *Streptococcus thermophilus*: 5'-NNAGAAW-3' (W = A or T)
- NmCas9 from *Neisseria meningitidis*: 5'-NNNGMTT-3' (M = A or C)
- SaCas9 from *Staphylococcus aureus*: 5'-NNRRRT-3' (R = A or G)
- CjCas9 from *Campylobacter jejuni*: 5'-NNNRYAC-3' (V = G or C or A, R = A or G, Y = C or T)
- CjCas9 from *Campylobacter jejuni*: 5'-NNNRYAC-3' (R = A or G, Y = C or T)
- AsCpf1 from *Acidaminococcus* or *LbCpf1* from *Lachnospiraceae*: 5'-TTTN-3'
- FnCpf1 from *Francisella*: 5'-TTN-3'
- SpCas9 from *Streptococcus pasteurianus*: 5'-NNGTGA-3'

**Target Genome**

Organism Type

Plants

Genomes

- Arabidopsis thaliana* (TAIR10) - Thale cress
- Oryza sativa* (Os4v4) - Rice
- Solanum lycopersicum* (SL2.4) - Tomato
- Zea mays* (AGPv3) - Corn
- Chlamydomonas reinhardtii* (Chlr-e4)
- Solanum tuberosum* (PGSC v4.03) - Potato
- Glycine max* (v1.0) - Soybean
- Vitis vinifera* (IGGP\_12X/Enspant26) - European grapevine

**Query Sequences**

Query sequences (5' to 3'), one sequence per line.

Please write crRNA sequences **without PAM sequences** (e.g. without NGG for SpCas9). The length of each query sequence should be between 15 and 25 nt, and all be the same length!

CAGCACTCCAGGGGGCCGC  
AAGGAACCATTTGTGTAA

Mismatch Number (eq or less than) 0

DNA Bulge Size (eq or less than) 0

RNA Bulge Size (eq or less than) 0

Please note that large number of bulge size will significantly increase the calculation time!  
Mixed bases are allowed.

Submit

<DNA bulge> <RNA bulge>

**Figure 7. Overview of Cas-OFFinder.** Cas-OFFinder (<http://www.rgenome.net/cas-offinder/>) has a broad range of searching options on the number of mismatches (10 bp) and DNA or RNA bulges (2 bp).

2. Genomic DNA preparation from wild-type cells and isolated mutants.
3. For predicted off-targets, perform NGS library preparation, targeted deep sequencing (Procedure E), and NGS data analysis.

## Recipes

1. Cas9 protein purification
  - a. LB medium (for 1 L)
    - 10 g NaCl
    - 10 g tryptone
    - 5 g yeast extract

- Distilled water
- b. 1 M IPTG stock
  - 2.383 g of IPTG
  - 10 ml distilled water
  - Filtered by syringe filter
- c. Lysis buffer, pH 7.4 (10 ml for 200 ml *E.coli* culture)
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - 300 mM NaCl
  - 10 mM imidazole
  - Distilled water
  - Note: Adjust pH to 7.4 using NaOH, filtered.*
- d. Wash buffer, pH 7.4 (20 ml for 200 ml *E.coli* culture)
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - 300 mM NaCl
  - 20 mM imidazole
  - Distilled water
  - Note: Adjust pH to 7.4 using NaOH, filtered.*
- e. Elution buffer, pH 7.4 (10 ml for 200 ml *E.coli* culture)
  - 50 mM NaH<sub>2</sub>PO<sub>4</sub>
  - 300 mM NaCl
  - 250 mM imidazole
  - Distilled water
  - Note: Adjust pH to 7.4 using NaOH, filtered.*
- f. Cas9 storage buffer, pH 7.5 (10 ml for 200 ml *E.coli* culture)
  - 150 mM NaCl
  - 20 mM HEPES
  - 0.1 mM EDTA
  - 1 mM DTT
  - 2% sucrose
  - 20% glycerol
  - Distilled water
  - Note: Adjust pH to 7.5 using NaOH, filtered.*
- 2. Transfection
  - a. TAP sucrose solution
    - TAP medium with 40 mM sucrose
  - b. TAP agar plates
    - TAP medium with 1.5% agar
  - c. Top agar

TAP medium with 0.5% agar

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