Nuclear Transformation of *Chlamydomonas reinhardtii* by Electroporation

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[Abstract] The unicellular green alga *Chlamydomonas reinhardtii* is an important model organism for studying photosynthesis, acclimation to abiotic stress, cilia biology, and many other biological processes. Many molecular biology tools exist for interrogating gene function including the ability to easily transform the nuclear genome of *Chlamydomonas*. While technical advances such as TALENs, ZFNs and CRISPR are making it easier to precisely edit the nuclear genome, the efficiency of such methods in *Chlamydomonas* is at present very low. In contrast, random insertion by nuclear transformation tends to be a much more efficient process. This protocol describes a method for transformation of the *Chlamydomonas* nuclear genome by electroporation. The protocol requires at least 3 days of work and generally results in the appearance of small colonies within 1-2 weeks.

**Keywords:** Algae, Nuclear transformation, Electroporation, Fluorescent fusion protein, Venus, mCherry, FLAG tag, His tag, Paromomycin, Hygromycin

[Background] Numerous molecular, genetic and genomic resources make *Chlamydomonas reinhardtii* (*Chlamydomonas* hereafter) an excellent model organism for studies on diverse biological processes. Many techniques have been developed to transform the *Chlamydomonas* nucleus, chloroplast and mitochondria including particle bombardment (Boynton *et al.*, 1988), glass bead transformation (Kindle, 1990), and electroporation (Shimogawara *et al.*, 1998). Nuclear mutants may be generated by exposure of *Chlamydomonas* cells to physical or chemical mutagens (e.g., UV light or ethyl methanesulfonate), but are often obtained by random insertional mutagenesis of transgenic DNA. Since the efficiency of homologous recombination for nuclear transformation in *Chlamydomonas* is very low (Zorin *et al.*, 2009; Jinkerson and Jonikas, 2015), transformed DNA is generally integrated into the nuclear genome at random sites. A number of techniques exist for subsequently identifying the insertion sites of the ectopic DNA including classical genetic mapping (Rymarquis *et al.*, 2005), TAIL-PCR (Dent *et al.*, 2005), and next-generation sequencing of individual mutants (Dutcher *et al.*, 2012) or large mutant libraries (Zhang *et al.*, 2014; Li *et al.*, 2016). While recent technical advances have led to improvements in targeted genome editing in *Chlamydomonas* using CRISPR/Cas9 (Baek *et al.*, 2016; Shin *et al.*, 2016; Ferenczi *et al.*, 2017; Greiner *et al.*, 2017) and zinc-finger nucleases (Sizova *et al.*, 2013; Greiner *et al.*, 2017), random insertional mutagenesis is still a preferred method to generate mutant libraries for forward and reverse genetics.

This protocol describes a detailed method for nuclear transformation of *Chlamydomonas* by electroporation. It can be used to generate random insertion mutants using a plasmid fragment conferring antibiotic resistance (Jinkerson and Jonikas, 2015) or for the expression of fluorescent fusion
proteins using well-established, publically-available expression vectors. Once a suitable DNA fragment
has been obtained or generated, the transformation protocol takes two days and generally results in
visible, isolated colonies within 1-2 weeks.

**Materials and Reagents**

1. Pipette tips with filters
2. Sterile 0.6 ml microcentrifuge tubes
3. Sterile 15 ml centrifuge tubes
4. Sterile 50 ml centrifuge tubes
5. 0.4 cm gap electroporation cuvettes (Bio-Rad Laboratories, catalog number: 1652088)
6. Petri dishes
7. Blue Sharpie (permanent) marker pen
8. Sterile plastic inoculating loops (VWR, catalog number: 12000-810)
9. Parafilm
14. 70% ethanol
15. Canned air (Fisher Scientific, catalog number: 23-022-523)
16. Tris base (Biopioneer, catalog number: C0060)
17. Hydrochloric acid (HCl) (Fisher Scientific, catalog number: A144-212)
18. Potassium phosphate monobasic (KH₂PO₄) (Sigma-Aldrich, catalog number: P0662)
19. Potassium phosphate dibasic (K₂HPO₄) (Sigma-Aldrich, catalog number: P3786)
20. Ammonium chloride (NH₄Cl) (Sigma-Aldrich, catalog number: A4514)
21. Calcium chloride dihydrate (CaCl₂·2H₂O) (Sigma-Aldrich, catalog number: C3306)
22. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (Sigma-Aldrich, catalog number: 230391)
23. Ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA·Na₂·2H₂O) (Sigma-Aldrich, catalog number: E5134)
24. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 221473)
25. Ammonium molybdate tetrahydrate [(NH₄)₆Mo₇O₂₄·4H₂O] (Sigma-Aldrich, catalog number: A7302)
26. Sodium selenite (Na₂SeO₃) (Sigma-Aldrich, catalog number: S5261)
27. Zinc sulfate heptahydrate (ZnSO₄·7H₂O) (Sigma-Aldrich, catalog number: Z4750)
28. Manganese(II) chloride tetrahydrate (MnCl₂·4H₂O) (Sigma-Aldrich, catalog number: M3634)
29. Iron(III) chloride hexahydrate (FeCl₃·6H₂O) (Sigma-Aldrich, catalog number: F2877)
30. Copper(II) chloride dihydrate (CuCl₂·2H₂O) (Sigma-Aldrich, catalog number: C3279)
31. Glacial acetic acid (Fisher Scientific, catalog number: A38-212)
32. Sodium carbonate (Na₂CO₃) (Sigma-Aldrich, catalog number: S7795)
33. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
34. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L5750)
35. Agar (Caisson Laboratories, catalog number: A038)
36. Paromomycin sulfate salt (Paro) (Sigma-Aldrich, catalog number: P8692)
37. Hygromycin B (Hyg) 50 mg/ml solution (Clontech, catalog number: 631309)
38. Stock solutions (see Recipes)
   a. 1 M Tris Base (1 L, 50x)
   b. Solution A for TAP (500 ml, 100x)
   c. 125 mM EDTA·Na₂ pH 8.0 (300 ml)
   d. 285 µM (NH₄)₆Mo₇O₂₄ (250 ml)
   e. 1 mM Na₂SeO₃ (250 ml)
   f. 100 mg/ml paromomycin (1 ml)
39. Micronutrient stock solutions (see Recipes)
   a. 25 mM EDTA·Na₂ (250 ml)
   b. 28.5 µM (NH₄)₆Mo₇O₂₄ (250 ml)
   c. 0.1 mM Na₂SeO₃ (250 ml)
   d. Zn-EDTA (250 ml)
   e. Mn-EDTA (250 ml)
   f. Fe-EDTA (250 ml)
   g. Cu-EDTA (250 ml)
40. TAP liquid medium (see Recipes)
41. TAP 40 mM sucrose (see Recipes)
42. TAP + 20 µg/ml Paro selective agar medium (see Recipes)
43. TAP + 25 µg/ml Hyg selective agar medium (see Recipes)
   *Note: Materials used for generating plasmids of interest (polymerases, restriction enzymes, ligases, buffers, etc.) are not described here.

**Equipment**

1. Sterile 250 ml culture flasks (e.g., Corning, catalog number: 70980-250)
2. Sterile 1 or 2 L culture flasks (e.g., DWK Life Sciences, Kimble®, catalog numbers: 26500-1000 or 26500-2000)
3. Pipettes
4. Centrifuge (e.g., Eppendorf, model: 5810 R) and microcentrifuge (e.g., Eppendorf, model: 5424)
5. NanoDrop 2000 (or similar) spectrophotometer
6. Hemacytometer (e.g., Sigma-Aldrich, catalog number: Z359629) or automated cell counter (e.g., Bio-Rad Laboratories, model: TC20)
7. Water bath with thermometer
8. Biosafety cabinet (with optional UV light)
   Note: A laminar flow hood can also be used
9. Gene Pulser XCell Electroporator (Bio-Rad Laboratories, catalog number: 1652660)
10. Tube shaker (e.g., Thermo Fisher Scientific, catalog number: T415110Q)

**Procedure**

**A. On the days prior to the transformation**

1. Prepare and digest plasmids for transformation. Genes can be cloned into any suitable expression vector. The following vectors are useful for expressing genes as fluorescent fusion proteins:
   a. pLM005–Gene-of-interest (GOI) can be cloned to generate a C-terminal protein-of-interest (POI)-Venus-3xFLAG fusion protein, positive transformants selected on medium containing paromomycin (Yang et al., 2015; Mackinder et al., 2016).
   b. pLM006–GOI can be cloned to generate a C-terminal POI-mCherry-3xHis fusion protein, positive transformants selected on medium containing hygromycin (Mackinder et al., 2016).
   c. pMO449–GOI can be cloned to generate a C-terminal POI-Venus-3xFLAG fusion protein that is expressed along with a downstream selectable marker from a single bicistronic mRNA, positive transformants selected on medium containing paromomycin (Onishi and Pringle, 2016).

   *Note: In general, linearized plasmid DNA results in more efficient transformation than circular plasmid DNA. Digested pLM005 and pMO449 empty vectors can be used for expression of cytosolic Venus, while digested pLM006 empty vector can be used for expression of cytosolic mCherry, without the need to clone a GOI.*

2. Grow cells for transformation. In a sterile 250 ml culture flask, grow wild-type CC-1690 21gr+ and/or other desired *Chlamydomonas* strain(s) in 50 ml TAP liquid medium under moderate light (50-100 µmol photons m⁻² sec⁻¹) until cells reach exponential or early stationary phase (typically 3-4 days from TAP plates).
   a. For growth in TAP medium, a typical cell concentration for exponential or early stationary phase is 2-10 x 10⁶ cells/ml. Cells can be counted using a hemacytometer or automated cell counter.
   b. Dilute cells into a larger volume (~200 ml final volume in a sterile 1 L flask, or ~400 ml final volume in a 2 L flask) 1-2 days before transformation.
   c. Each transformation event requires 0.5 x 10⁸ cells. Therefore, it is highly recommended to count cells and dilute into an appropriate volume of TAP liquid medium at least 1-2 days
before transformation.

3. Determine the concentration of digested plasmid using a NanoDrop (or similar spectrophotometer) and aliquot 150 ng into a sterile 0.6 ml microcentrifuge tube. Add sterile water so that the final volume is 5 µl. These tubes can be frozen until the day of the transformation.

   Note: If digested plasmids are more dilute than 30 ng/µl, a final volume larger than 5 µl will be necessary. To minimize the chance of arcing during the electroporation and increase the efficiency of the transformation, avoid using volumes larger than 10 µl (see Step B10).

4. For each transformation, label a sterile 15 ml centrifuge tube and aliquot into it 10 ml of sterilized TAP 40 mM sucrose.

5. If reusing 0.4 cm electroporation cuvettes, dry in a biosafety cabinet (or a laminar flow hood) for several hours. If possible, treat with UV light in a biosafety cabinet for at least a few hours.

   Note: The drying and UV light treatment of the cuvettes can both be done overnight on the night before the transformation. After the transformation, the cuvettes can be cleaned, sterilized and reused many times. For cleaning, treat the used cuvettes and plastic caps with a mild bleach solution to kill any remaining cells and to degrade residual DNA. Then wash cuvettes and plastic caps several times with distilled or Milli-Q water. Finally, store cuvettes and plastic caps in 70% ethanol between uses.

B. On the day of the transformation

   Note: All steps performed in a sterile manner in a biosafety cabinet using sterile filter tips.

1. Prepare a water bath with a thermometer. Have a bucket of ice nearby to keep the temperature of the water bath at 16 °C at all times.

2. Label all cuvettes with a blue Sharpie (permanent) marker.

   Note: If reusing cuvettes, blue Sharpie ink is easier to remove with ethanol or methanol than red Sharpie ink.

3. Set out (or thaw) linearized plasmid fragments (Steps A1 and A3).

4. Move the Bio-Rad Gene Pulser Xcell electroporator near the biosafety cabinet, turn the power on, and select the following settings under ‘Exponential protocol’ at the home screen:

   - Voltage (V) 800
   - Capacitance (µF) 25
   - Resistance (Ω) ∞
   - Cuvette (mm) 4

5. Clean out the electroporator cuvette chamber with 70% ethanol and blow dry with canned air.

6. Collect 0.1-1 ml cells for counting. Count cells and determine the volume of culture needed for an appropriate number of transformations.

   Note: Each transformation requires 0.5 x 10⁸ cells, resuspended in 250 µl TAP 40 mM sucrose. (see Excel File for example calculations).

7. Collect the necessary volume of cells into 50 ml centrifuge tubes. Pellet the cells by
centrifugation (1,000 x g, 20 °C, 10 min).

**Note:** Multiple 50 ml centrifuge tubes may be required to collect the necessary volume of cells. Be sure to balance the tubes in the centrifuge.

8. Carefully discard the supernatant using a pipette and resuspend the cells in the appropriate volume of TAP 40 mM sucrose.

**Note:** Each transformation requires 0.5 x 10⁸ cells, resuspended in 250 µl TAP 40 mM sucrose. (see Excel File for example calculations).

9. Aliquot 250 µl cells into pre-labeled cuvette.

**Note:** Refer to Video 1 for Steps B9-B13.

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**Video 1. Chlamydomonas transformation demonstration.** This video demonstrates how to perform nuclear transformation of *Chlamydomonas reinhardtii* cells by electroporation and corresponds to Steps B9-B13 of the protocol. 250 µl cells are added to each sterile cuvette. 5 µl digested DNA is added to the cuvette and mixed. The mixture of cells and DNA are briefly incubated in a 16 °C water bath prior to electroporation. After electroporation, transformed cells are transferred from the cuvette to a 15 ml centrifuge tube containing 10 ml TAP 40 mM sucrose.

10. Add 5 µl linearized plasmid DNA fragment to the cuvette and mix briefly by pipetting.

**Note:** If necessary, more than 5 µl can be mixed with the 250 µl of concentrated cells (see Step A3). However, larger volumes may increase the chance of arcing during the electroporation and/or decrease the efficiency of the transformation.

11. Place the cuvette with cells and DNA (from Steps B8 and B9) into a water bath.

**Note:** Make sure the water bath is at 16 °C. To avoid long incubations and incorporation of ‘junk DNA’ (see Notes), set up no more than 10 transformation cuvettes at a time. Try not to incubate the cell/DNA mixture for more than 5 min.

12. Remove the cuvette from the water bath, briefly dry by blotting with a paper towel, and place the cuvette into electroporator cuvette chamber. Close the chamber lid and press the red ‘Pulse’ button.

**Note:** The time constants generally range from 7-12 msec. If longer than this, the transformation
might not work. In rare cases, arcing may occur and could be caused by air bubbles in the cuvette.

13. Transfer the electroporated cells to the corresponding pre-labeled 15 ml tube containing 10 ml TAP 40 mM sucrose (Step A4). Carefully, transfer back and forth 2-3 times to get as many cells out of the cuvette as possible.

   Note: It is important to transfer the cells as quickly as possible to minimize the potentially damaging effects of the electroporation (e.g., heat shock).

14. After all transformations are complete, place all 15 ml tubes on a tube shaker and gently shake overnight in low light (< 10 µmol photons m\(^{-2}\) sec\(^{-1}\)).

15. Prepare and label selective agar plates.

C. On the day after the transformation

1. Pellet transformed cells by centrifugation (1,000 x g, 20 °C, 10 min).
2. Discard supernatant by decanting. A small residual volume of supernatant will remain.
3. Resuspend the cells in the residual volume of supernatant (~200-500 µl) and plate out onto a pre-labeled selective agar plate.
4. Spread cells with sterile plastic inoculating loop and allow to dry (5-20 min).
5. Wrap the plate with Parafilm and grow under desired selection conditions (see Notes).
6. After 3-5 days, the plates should start to lose color (turn from green to almost white). This is the selection taking place (see Figure 1).

Figure 1. *Chlamydomonas* transformants. A. Lawn of green cells after plating transformants. Plate in panel A was incubated in the dark for 6 days. B. Loss of green lawn and appearance of small green colonies (*i.e.*, positive transformants). Plate in panel B was incubated in moderate light (~50 µmol photons m\(^{-2}\) sec\(^{-1}\)) for 6 days.
7. After 2-3 weeks, small colonies should start to appear (see Figure 2).

![Figure 2. Appearance of Chlamydomonas transformant colonies.](image)

**Figure 2. Appearance of Chlamydomonas transformant colonies.** Representative transformation plates with larger, more conspicuous colonies (i.e., positive transformants) after incubation in dim light (< 5 μmol photons m\(^{-2}\) sec\(^{-1}\)) for 13 days. A. Colonies selected on Paromomycin. B. Colonies selected on Hygromycin.

8. Pick colonies onto fresh selective agar medium.

9. Optional: Confirm insertions by PCR, Western blots, and/or fluorescence microscopy.

**Notes**

1. This protocol is optimized for Chlamydomonas strains containing cell walls. For mutants lacking (or having partial) cell walls (e.g., strain CC-425 cw15), special care must be taken to avoid damaging the cells. A starch embedding method for transformation of cell wall mutants was described previously (Shimogawara et al., 1998).

2. Cells can be synchronized by growing under a 12 h light:12 h dark regime. While this might not affect transformation efficiency, it can help to prevent cell clumping.

3. Long incubations of cells with transforming DNA can result in the integration of ‘junk DNA’ or Chlamydomonas DNA that has been endonucleolytically-digested (Zhang et al., 2014; Li et al., 2016).

4. In general, smaller plasmids (or GOIs) tend to result in higher transformation efficiencies and better expression in Chlamydomonas reinhardtii. GOIs larger than 5 kb may require optimization of the number of cells and/or concentration of DNA used in the transformation. GOIs larger than 10 kb may result in very low transformation efficiencies and/or no positive transformants. A DNA/cell ratio of 7.25 ng/kb of digested plasmid per 1 x 10\(^8\) cells has been suggested previously (Mackinder et al., 2017).
5. Selection of positive transformants can be done on medium containing antibiotic if the appropriate antibiotic resistance gene is transformed. Selection can also be performed in the absence of an antibiotic if an obvious phenotype can easily be scored (e.g., recovery of light-sensitive mutants under strong illumination or restored photoautotrophic growth).

6. It is strongly recommended that you perform a positive control by transforming cells with a DNA fragment that is known to efficiently generate transformants (e.g., linearized pMO449), as well as a negative control (e.g., water).


Recipes

1. Stock solutions (all prepared in Milli-Q water)
   a. 1 M Tris Base (1 L, 50x)
      121.14 g Tris Base
   b. Phosphate Buffer II for TAP (100 ml, 1,000x)
      10.8 g K2HPO4
      5.6 g KH2PO4
   c. Solution A for TAP (500 ml, 100x)
      20 g NH4Cl
      5 g MgSO4·7H2O
      2.5 g CaCl2·2H2O
   d. 125 mM EDTA·Na2 pH 8.0 (300 ml)
      13.959 g EDTA·Na2
      Dissolve in ~250 ml Milli-Q water, titrate pH to 8.0 with KOH, adjust volume to 300 ml
   e. 285 µM (NH4)6Mo7O24 (250 ml)
      0.088 g (NH4)6Mo7O24
   f. 1 mM Na2SeO3 (250 ml)
      0.043 g Na2SeO3
   g. 100 mg/ml paromomycin (1 ml)
      100 mg paromomycin sulfate salt

2. Micronutrient stock solutions (all prepared in Milli-Q water)
   Note: *Based on Kropat et al., 2011.
   a. 25 mM EDTA·Na2 (250 ml)
      50 ml 125 mM EDTA·Na2 pH 8.0
   b. 28.5 µM (NH4)6Mo7O24 (250 ml)
      25 ml 285 µM (NH4)6Mo7O24
   c. 0.1 mM Na2SeO3 (250 ml)
25 ml 1 mM Na₂SeO₃
d. Zn-EDTA (250 ml)
0.18 g ZnSO₄·7H₂O
5.5 ml 125 mM EDTA-Na₂ pH 8.0
e. Mn-EDTA (250 ml)
0.297 g MnCl₂·4H₂O
12 ml 125 mM EDTA-Na₂ pH 8.0
f. Fe-EDTA (250 ml)
1.35 g FeCl₃·6H₂O
2.05 g EDTA-Na₂
0.58 g Na₂CO₃
Combine EDTA-Na₂ and Na₂CO₃ in water and mix; add FeCl₃·6H₂O after first two components are fully dissolved (do not use 125 mM EDTA-Na₂ pH 8.0 stock)
g. Cu-EDTA (250 ml)
0.085 g CuCl₂·2H₂O
4 ml 125 mM EDTA-Na₂ pH 8.0

3. TAP medium (For 1 L)
Stock solution Volume Conc.
1 M Tris Base 20 ml 50x
Solution A 10 ml 100x
Phosphate Buffer II 1 ml 1,000x
Glacial acetic acid 1 ml 1,000x
25 mM EDTA-Na₂ 1 ml 1,000x
28.5 µM (NH₄)₆Mo₇O₂₄ 1 ml 1,000x
0.1 mM Na₂SeO₃ 1 ml 1,000x
Zn-EDTA 1 ml 1,000x
Mn-EDTA 1 ml 1,000x
Fe-EDTA 1 ml 1,000x
Cu-EDTA 1 ml 1,000x
Adjust pH to 7.3 with HCl
Sterilize by autoclaving

4. TAP 40 mM sucrose (1 L)
Same as TAP medium (see above), but add 13.69 g sucrose prior to autoclaving

5. TAP + 20 µg/ml Paro selective agar medium (1 L)
Same as TAP medium (see above), but with 15 g agar added prior to autoclaving, and 200 µl 100 mg/ml paromomycin sulfate stock solution added after autoclaving and prior to pouring plates (once the medium has cooled to around 50 °C)
6. TAP + 25 µg/ml Hyg selective agar medium (1 L)
   Same as TAP medium (see above), but with 15 g agar added prior to autoclaving, and 500 µl
   50 mg/ml hygromycin B solution added after autoclaving and prior to pouring plates (once the
   medium has cooled to around 50 °C)

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