Transfection of *Micromonas commoda* CCMP2709 V.2

Lisa Sudek[1], Alexandra Worden[1,2], Manny Ares[3]

[1]Monterey Bay Aquarium Research Institute, Moss Landing, USA, [2]Ocean EcoSystems Biology Unit, Marine Ecology Division, Helmholtz Centre for Ocean Research, Kiel, Germany, [3]University of California, Santa Cruz

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Worden Lab

Alexandra Worden

ABSTRACT

This protocol describes the transformation of the picoprasinophyte alga *Micromonas commoda*, a green alga that is found in tropical to temperate oceans. The strain used was genome-sequenced in (1). The original strain name was NOUM17, housed as RCC299. The latter was rendered axenic, genome-sequenced, and deposited at the CCMP (now NCMA) as CCMP2709 and also maintained in the Worden lab. The below protocol is an adaptation of transformation methods used for the choanoflagellate *Salpingoeca rosetta*. We are grateful to N. King and D. Booth for their invaluable support in initial stages of experiments, as well as help from H. Igel, C. Poirier, M. Hamilton, S. Sudek, K. Hoadley, C. Eckmann and especially C. Yung throughout the entire development.

GUIDELINES

All liquid handling steps should be performed in a laminar flow hood to maintain sterility. Aerosol resistant pipette tips or serological pipettes should be used at all times.

MATERIALS TEXT

REAGENT/PRODUCT	VENDOR	CATALOG NO		
ART 1000E Barrier Pipette Tip	Thermofisher Scientific	2079E		
ART 200 Barrier Pipette Tip	Thermofisher Scientific	2069-05		
ART 20 Barrier Pipette Tip	Thermofisher Scientific	2149E		
ART 20 Barrier Pipette Tip	Thermofisher Scientific	2140-05		
0.2 µm Polyethersulfone filter, 25 mm	Whatman	6780-2502		
Nunc™ Non-Treated 12-well culture plate	Fisher Scientific	12-566-81		
Disposable Pipette Basins	Fisher Scientific	13-681-502		
1.7 ml Microcentrifuge tubes	Axygen	2021-08-01		
50 ml conical centrifuge tubes	Corning	430828		
15 ml centrifuge tubes	Corning	430791		
Chemicals:				
D-Sorbitol	Acros Organics	50-70-4		
Ampicilling Sodium Salt	Fisher Scientific	69-52-3		

Poly(ethylene glycol)HEPES	Sigma-Aldrich	P2139-500G		
Adenosine triphosphate	Fisher Scientific	7365-45-9		
Plasmids/Preps:				
XL1-Blue Competent Cells	Agilent Technologies	200249		
QIAGEN Plasmid Maxi Kit (10)	Qiagen	12162		
Carrier Plasmid stock (pUC19, 20mg/ml in TrisHCl pH8)	Nature Technology Inc.			
Reporter Plasmid stock (RPS9proMco-eGFP, 10 µg/µl)	Integrated DNA Technologies, Inc.			
Nucleofection:				
4D-NucleofectorTM X Unit	Lonza	AAF-1002X		
4D-NucleofectorTM Core Unit	Lonza	AAF-1002B		
96-well ShuttleTM Device	Lonza	AAM-1001S		
SF Cell Line 96-well NucleofectorTM Kit	Lonza	V4SC-2096		
SG Cell Line 96-well NucleofectorTM Kit	Lonza	V4SC-3096		

BEFORE STARTING

Wipe the surface and sides of the laminar flow hood with 70% EtOH before wiping the following items and placing them in the hood:

- Pipettes and tips for transferring volumes of 1-1000 µl
- L1 medium
- Sterile 12-well culture plates
- Waste beaker
- 1.7 ml Eppendorf tubes
- Multichannel pipette for transferring 100 µl volumes
- 96-well nucleofection plate
- Disposable pipette basin

Plasmid Preparation

1 The Carrier DNA stock (*pUC19*) is purchased at a concentration of 20 µg μl^{-1} in 10 mM TrisHCl pH8, aliquoted in 100 µl volumes and stored at -20 °C.

2 The reporter plasmid stock (in this case of plasmid *RPS9proMco-eGFP*) is prepared by transforming the purchased plasmid into *Escherichia coli* (*E. coli*) XL-Blue Competent Cells according to the manufacturer's instructions.

3 Grow the transformed *E. coli* cells on LB-Ampicillin (LB-Amp) plates (final concentration 100 µg ml^{-1}) at 37°C overnight (16-18 hr).

4 Pick one colony and inoculate into 5 ml of liquid LB Amp medium (final concentration 100 µg ml^{-1}) at 37°C and ~250 rpm for ~16hr.

5 Centrifuge the culture at 6000 x g for 10 min. Remove the supernatant and resuspend the remaining pellet in 5 ml of LB medium. Use 2 ml of this cell suspension to inoculate 220 ml of LB-Amp medium (final concentration final concentration 100 µg ml^{-1}) and grow at 37°C and ~200 rpm for ~16 hr.

6 Purify the plasmid using the Qiagen Maxi Prep Kit according to the manufacturer's instructions.

7 Quantify the plasmid concentration on a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer. Aim for a plasmid concentration of 5-10 µg $µl^{-1}$ (ideally 10 µg $µl^{-1}$). Aliquot the plasmid into 1.7 ml microcentrifuge tubes and store at -20°C.

Culturing *M. commoda*

8 The growth chamber should be set to a 14:10-h light:dark cycle (~140 µmol photon $m^{-2}s^{-1}$ photosynthetically active radiation, PAR), 21°C.

9 Prepare L1 medium (2) in a natural seawater base adjusted to a salinity of 22 PSU (e.g. per liter: 550 ml of natural seawater from the North Eastern Pacific, 36 PSU) + 450 ml 18.2 MΩ·cm MilliQ H_2O). Other seawaters supporting the growth of this strain and details on how to prepare medium can be found at (https://www.mbari.org/wp-content/uploads/2015/11/KASW.pdf).

10 Transfer the culture approximately every 3 days (we monitor daily on a flow cytometer) to a density of 4 x 10^6 cells ml^{-1}. For transformation experiments maintain *M. commoda* in mid-exponential growth for ≥10 generations. The expected growth rate in these conditions is 0.56 ± 0.06 day^{-1}.

Transformation Instrument and Nucleofection preparation

11 For transformation use the Lonza 4D- Nucleofector™ System (X Unit AAF-1002X, Core Unit AAF-1002B, 96-well Shuttle AAM-1001S). Full details on instrumental setup can be found in the Lonza manual.

12 Briefly, before starting your experiment define a new experiment, by creating a template file in the Lonza program. Manually select the positions/wells in the Lonza 96 well nucleofection plate that will be pulsed and (for each well) select a "Cell type program" in the left part of the screen, choose a control (no pulse or no template) or a pre-defined Nucleofection program (pulse) from the following list:

CA-137	DS-150
CM-138	DS-120
CM-137	EH-100
CM-150	EO-100
DN-100	EN-138
DS-138	EN-150
DS-137	EW-113
DS-130	No pulse control

Here we only use pulse EW-113 and no program (i.e. no pulse; for controls).

13 Prepare the Recovery Buffer and the Adenosine triphosphate, pH 7.5 (ATP 250 mM) solution (see section: Appendix / Buffer recipes below)

Day-of transfection experiment set-up

14 Thaw Carrier DNA stock (pUC19, 20 µg µl^{-1}) and reporter plasmid stock (*RPS9proMco-eGFP*,10 µg µl^{-1}), recovery, ATP pH 7.5 (250 mM) and an aliquot of Lonza buffer SF amended with the provided supplement according to the manufacturer's instructions (Lonza V4-SC-2096) on ice.

15 Wipe the surface and sides of the laminar flow hood with 70% EtOH before wiping the following items and placing them in the hood:

- Pipettes and tips for transferring volumes of 1-1000 µl
- L1 medium
- Sterile 12-well culture plates
- Waste beaker
- 1.7 ml Eppendorf tubes
- Multichannel pipette for transferring 100 µl volumes
- 96-well nucleofection plate
- Disposable pipette basin

16 Determine the *Micromonas* cell concentration using a flow cytometer (a BD Accuri C6 was used here). Cell concentration should be between 15 and 22 x 10^6 cells ml^{-1}. For 22 transfection reactions harvest 46.2 ml of culture by centrifuging in 50 ml conical tubes at 5000 x g for 10 min at 4°C. Remove the supernatant and store at room temperature while preparing the mastermix (see below).

17 Resuspend the cell pellet in 46.2 µl of the SF buffer/supplement solution (Lonza) prepared above (step 14) and quickly add the cell suspension to the mastermix (see below) at 2 µl per transfection reaction. Carefully mix by gentle pipetting.

18 Add ice cold Recovery Buffer to the pipette basin just before moving the 96-well nucleofection plate containing the transfection reactions to the 96-well ShuttleTM System to apply the pulse (buffer needs to stay cold).

Nucleofection reaction

19 Create mastermix containing (per transfection reaction):

• 15 µl of Buffer SF (Lonza)
• 2 µl of 20 µg µl^{-1} *pUC19*
• 1µl of 250 mM ATP solution
• 2 µl of reporter DNA *RPS9proMco-eGFP* (10 µg µl^{-1}) and
• 2 µl of cell suspension in SF buffer (from step 17 above)

Add cell suspension last to ensure viability of the cells and gently mix into the mastermix by slow pipetting. Transfection reactions and no pulse controls are run in 4 biological replicates.

20 Add 22 µl of nucleofection mixture per well of a 96-well nucleofection plate (Lonza V4SP-1096) and insert (with lid) into the 96-well ShuttleTM System connected to a 4D-NucleofectorTM System. Apply pulse EW-113. The successful application of the pulse by the instrument to the solution is indicated on a per well basis using a colormetric approach. Green indicates a successful pulse while red indicates an unsuccessful pulse. Possible reasons for unsuccessful pulses can be bubbles in the well of the nucleofection plate. Note that pulses cannot be re-run and thus wells with a red color should be considered failed.

Post-Nucleofection treatment

21 Immediately after pulsing add 100 µl of ice cold Recovery Buffer to each well to re-seal the pores.

22 Gently mix by tapping on the sides of the nucleofection plate. Incubate this mixture at room temperature for 5 min.

23 Add 2 ml of L1 medium to each well of a 12- well culture plate. Transfer each transfection reaction to a separate well. Mix gently by pipetting before transferring. Allow cells to “rest” for 30 min. at room temperature. Subsample a 50 µl aliquot from each well and determine cell concentrations by flow cytometry. Cell concentrations average 3.27 x 10^6 cells ml^{-1} in each well.

24 Place 12-well culture plates at 21°C under a 14:10-h light:dark cycle. Increase light intensity as follows: Day 0-1 = 30 µmol photon m^{-2}s^{-1}, Day 1-2 = 40 µmol photon m^{-2}s^{-1}, Day 2-7 = 80 µmol photon m^{-2}s^{-1}.

25 Subsample for flow cytometry on day 2, 3, 4 and 6 to calculate transfection efficiencies and growth rates of transformed versus non-transformed cells.

Appendix / Buffer recipes

26 **Recovery Buffer:**

Final concentrations: 10 mM HEPES-KOH pH 7.5, 530 mM Sorbitol, 4.7% (w/v) PEG 8000 (note: salt concentrations are adjusted to yield salinity of 22 PSU)

- Per 100 ml: 1 ml of 1M HEPES pH7.5 + 99 ml 18.2 MΩ·cm MilliQ H_2O + 4.7g PEG 8000 + 10.2 g D-Sorbitol (560mM final conc.)
- Filter sterilize through 0.2 µm filter, aliquot into 2 x 50 ml in conical tubes, store at 4°C for up to 3 months.

27 **Adenosine triphosphate (ATP) solution:**

Final concentration: 250 mM, pH 7.5

- Per 100 ml: Add 15.13 g of ATP to 100 ml of 18.2 MΩ·cm MilliQ H_2O. Adjust to pH 7.5 (with NaOH). Completely dissolve, filter through a 0.2 µm filter, split up into 10 ml aliquots in 15 ml falcon tubes and store at 4°C for up to 6 months.

References

28 1. Worden AZ, *et al.* (2009) Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*. *Science* 324(5924):268-272.

2. Anderson R (2005) *Algal culturing techniques* (Elsevier Academic Press, San Francisco) p 578.