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Reporter titrations for transfection of *Micromonas commoda* CCMP2709 V.2

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ABSTRACT

This protocol describes the method used to test various amounts (0, 0.3, 3, 10, 20 and 30 µg) of reporter (*H3pro^{Mpo}-LUC*) DNA. The plasmid *H3pro^{Mpo}-LUC* carries a codon optimized Nanoluc coding region, whose expression is driven by the histone H3 promoter and 3' mRNA end formation and termination sequences. Lumen per total number of cells (including transfected and non-transfected cells within a culture) is used as a proxy for relative transfection efficiencies. It is determined in each culture based on luminescence measurements performed 72 hrs after transfection using the Nano-Glo® Luciferase Assay System. We would like to thank H. Igel, C. Poirier, M. Hamilton, S. Sudek, K. Hoadley, C. Eckmann and especially C. Yung for their support in the lab.

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 Eppendorf tubes	Axygen	2021-08-01	
50 ml conical centrifuge tubes	Corning™	430828	
15 ml centrifuge tubes	Corning™	430791	
96-Well Solid White Polystyrene Microplates		3917	
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		
Reporter Plasmid stock (H3proMpo-LUC)	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	
Nano-Glo Luciferase Assay System:			
Nano-Glo Luciferase Assay Substrate	Promega	N1110	
Nano-Glo Luciferase Assay Buffer	Promega	N1110	
Nano-Glo1000x Enzyme (Promega RealTime-Glo MT Cell Viability Assay)	Promega	G9711, Part # E499A, 1x 10 µl	

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 *H3proMpo-LUC*) 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 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 Prepare L1 medium (1) 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).

9 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.

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 predefined 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 Appendix / Buffer recipes)

Day-of transfection experiment set-up

14 Thaw Carrier DNA stock (*pUC19*, 20 µg µl^{-1}) and reporter plasmid stock (*H3proMpo-LUC*,10 µg µl^{-1}), recovery buffer, 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 28 transfection reactions harvest 58.8 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 mastermixes (see below).

17 Resuspend the cell pellet in 58.8 µl of the SF buffer/supplement solution (Lonza) prepared above (step 14) and quickly add the cell suspension to the mastermixes (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 6 different mastermixes for each reporter plasmid concentration (final conc.: 0, 0.3, 3, 10, 20 and 30 µg) containing (per transfection reaction):

• 15 µl of Buffer SF (Lonza)
• 2 µl of 20 µg/µl *pUC19*
• 1µl of 250 mM ATP solution
• Either 0, 0.3 (of a 1:10 dilution), 0.3, 1, 2 or 3 µl of reporter DNA (*H3proMpo-LUC*, 10 µg/µl) and
• 2 µl of cell suspension in SF buffer (from step 17).

No pulse controls are run with concentrations of 30 µg of reporter plasmid. Add cell suspension last to ensure viability of the cells and gently mix into the mastermixes by slow pipetting. Transfection reactions and no pulse controls are run in biological triplicates.

20 Add between 20 and 23 µl of nucleofection mixture (depending on reporter plasmid concentration: 20 µl for 0 µg; 20.3 µl for 0.3 and 3 µg; 21 µl for 10 µg; 22 µl for 20 µg and 23 µl for 30 µg) 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 while incubating the nucleofection 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.

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 $m^{-2}s^{-1}$, Day2-7= 80 µmol photon $m^{-2}s^{-1}$.

25 Subsample for flow cytometry on day 3 to calculate overall cell numbers in each of the culture prior to measuring luciferase (see below).

Nano-Glo® Luciferase Assay System

26 72 hrs after transfection determine cell numbers on a BD Accuri C6 flow cytometer.

27 Harvest 1 ml of each culture by spinning at 8000 x g for 10 min at room temperature.

28 Remove the supernatant and store pellets on ice along with luciferase buffer (see Appendix / Buffer recipes).

Standard curve

29 The standard curve for the luciferase assay consists of 9 dilutions of a Nano-Glo 1000x enzyme that are run in technical duplicates.

30 Set up the Running Buffer for a total of 45 reactions (22 nucleofection reactions, 20 standard reactions, 3 extra): mix 2.205 ml of Nano-Glo buffer and 45 µl of Nano-Glo substrate resulting in 50 µl of mastermix per reaction.

31 Set up 11 microcentrifuge tubes for serial dilutions of the Nano glo 1000x enzyme starting from a $1x10^{-2}$ stock (see step 32). Prior to making the stock add the following volumes of running buffer to the 11 tubes of serial dilutions:

1. 50 µl
2. 50 µl
3. 100 µl
4. 100 µl
5. 100 µl
6. 100 µl
7. 100 µl
8. 100 µl
9. 100 µl
10. 100 µl
11. 100 µl

32 Nano-Glo 1000x enzyme stock: mix 99 µl of luciferase buffer (see Appendix / Buffer recipes) with 1 µl Nano-Glo 1000x enzyme for a final concentration of $1x10^{-2}$. Mix well and store at 4 °C in the dark.

33 Add 25 µl of the $1x10^{-2}$ Nano-Glo 1000x enzyme stock to serial dilution reaction #1. Mix thoroughly and change the tip before adding 25 µl of reaction #1 to reaction #2. Add 50 µl of each previous dilution to the next one after that making sure to change tips in between.

34 Discharge serial dilution reactions # 1 and 2. Starting with serial dilution reaction # 3 split each Standard reaction into 2 by adding 50 µl each to one well of a white 96 well flat bottom microplate.- Store in the dark until Step 6 and 7 are completed.

35 Add 50 µl of Running Buffer to each cell pellet from the 22 nucleofection reactions and resuspend the pellets completely. Make sure to minimize foam formation or briefly spin in a tabletop centrifuge.

36 Add each Running Buffer/cell resuspension to one well of a white 96 well flat bottom microplate. Adapt in the dark for a minimum of 1 and a maximum of 10 min.

Luminometer Assay and Analysis

37 Measure luminescence on a Tecan plate reader using 20 kinetic cycles, 500 ms per sample, 0 settle time. Calculate mean and standard deviation of Standard readings from technical duplicates. Normalize readings to the mean of the no pulse controls and calculate lumen per total number of cells in each culture (including transfected and non-transfected cells) determined on the Accuri C6 flow cytometer that day.

Appendix / Buffer recipes

38 **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 2x 50 ml in conical tubes, store at 4°C for up to 3 months.

39 **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.

40 **Luciferase Buffer:**

Final concentration: 50mM HEPES pH 7.5, 200 mM NaCl, 20% Glycerol

- Per 100 ml: 80 ml 18.2 MΩ·cm MilliQ H_2O + 20 ml 50% glycerol + 1.1915 g HEPES
- Adjust to pH 7.5 with KOH pellet (~1.5 pellets)
- To 10 ml of HEPES/Glyc solution add 0.1168 g NaCl
- Filter sterilize through a 0.2 µm filter , split up into 15 ml aliquots and store at 4°C for up to 1 month.

References

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