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Cell sorting of marine heterotrophic flagellates for single-cell genome amplification

Camille Poirier¹, Raquel Rodríguez-Martínez^{2,3}, Emily Cook³, David S. Milner³, Alexandra Z. Worden¹, Thomas A. Richards³

¹GEOMAR Helmholtz Centre for Ocean Research Kiel, ²Universidad de Antofagasta, ³University of Exeter

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Richards Lab







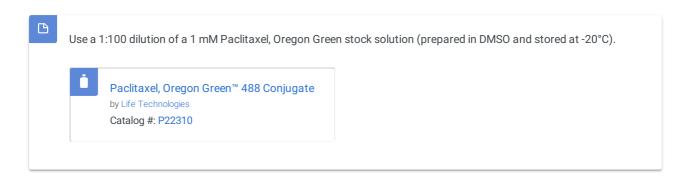
MATERIALS

NAME ~	CATALOG #	VENDOR ~
Phusion High-Fidelity DNA Polymerase - 100 units	M0530S	New England Biolabs
Qubit™ dsDNA BR Assay Kit	Q32853	Thermo Fisher Scientific
Paclitaxel Oregon Green™ 488 Conjugate	P22310	Invitrogen - Thermo Fisher
Hoechst 33342	H1399	Invitrogen - Thermo Fisher
Fluoresbrite® YG Microspheres 0.75µm	17153-10	Polysciences
PBS - Phosphate-Buffered Saline (10X) pH 7.4	AM9625	Invitrogen - Thermo Fisher
REPLI-g Single Cell Kit	150345	Qiagen
STEPS MATERIALS		
NAME ×	CATALOG #	VENDOR ~
Hoechst 33342	H1399	Invitrogen - Thermo Fisher
PBS - Phosphate-Buffered Saline (10X) pH 7.4	AM9625	Invitrogen - Thermo Fisher
REPLI-g Single Cell Kit	150345	Qiagen
Phusion High-Fidelity DNA Polymerase - 100 units	M0530S	New England Biolabs
Agencourt AMPure XP	A63880	Beckman Coulter

Water collection and preparation

Pre-filter the water sample through a 30 µm mesh, then concentrate by gravity filtration ~100x onto a 0.8 µm filter to approximately a 1-2 ml volume.

2 Gently resuspend the cells from the filter using 1-2 ml sterile artificial sea water (ASW) and stain a 500 μl volume of the cell concentrate with 10 μM Paclitaxel, Oregon Green® 488 Conjugate (to target cytoskeletal tubulin). Incubate for 1 hour at room temperature.

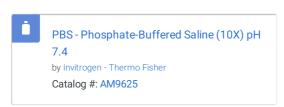


- Pellet cells by centrifugation (3 mins at 6,000 x g) and wash with 2 ml ASW to remove unbound dye. Repeat centrifugation and resuspend in 2 ml ASW.
- 4 Also stain with 2 μg/ml Hoechst 33342 (to target dsDNA) for 30 mins at room temperature.



Flow cytometry and sorting

- 5 Prior to sorting, illuminate 96-well plates by UV radiation inside the sort chamber (2 mins).
- 6 Perform cell sorting on a flow cytometer equipped with 488 nm and 355 nm lasers. Use sterile nuclease-free 1x PBS (pH 7.4) as sheath fluid.



7 Use sort window combinations to select cells that show both green and blue fluorescence (520/35 nm bandpass filter for Oregon Green [tubulin] and a 460/50 nm bandpass filter for Hoechst 33342 [dsDNA]) compared to unstained control samples, and that show baseline red fluorescence (692/40 nm bandpass filter) indicating the absence of chlorophyll. This will allow for the exclusion of the majority of photosynthetic cells.



Regularly check the sort quality by sorting a known number of fluorescent beads onto a slide, and counting them on an epifluorescence microscope. Do not add beads to actual sort samples (to limit possibility for contamination).

Please note that this protocol was performed using a BD Influx and it is possible that other cell-sorters would require different validation methods.

- Sort targeted cells into 96-well plates (one cell per well; Single-Cell sorting mode in BD FACS 'Sortware' software). Leave the outer column of wells empty for sheath-fluid only negative controls.
- 9 Cover the plates with foil and place at -80°C immediately after the sort.

DNA amplification for single cell genome or amplicon sequencing

- 10 UV-treat all materials (except cell samples) in a HL-2000 HybriLinker (UVP) for 30-90 mins prior to conducting multiple displacement amplification (MDA).
- 11 Negative controls include:
 - 4 cell-sort controls per 96-well plate (outer wells)
 - 2 buffer-only controls from the MDA kit (i.e. no sample added)
- Lyse samples (both sorted cells and negative controls) for 10 mins at 65°C using buffer D2 from the REPLI-g Single Cell Kit (Qiagen), according to the manufacturer's instructions for amplification of genomic DNA from single cells.



After neutralization, amplify samples using the REPLI-g kit in a final volume of 50 μ l. Perform MDA reactions in a thermal cycler for 8 h at 30°C with the lid temperature set to 65-70°C. After amplification, aliquot samples as follows:

a) 2 μ l diluted 100-fold for PCRs (see step 14; stored at -20°C) b) 20 μ l kept as a backup (stored at -80°C) c) 28 μ l (stored at -80°C). Use 25 μ l of this aliquot for sequencing (step 17) Dilute an aliquot (2 μl) of each MDA product 100-fold in nuclease-free water and use 2-5 μl of this dilution as the template for 25 μl PCR reactions. Carry out PCR amplification using the primers:

1389F (5'-TTGTACACACCGCCC-3')
1510R (5'-CCTTCYGCAGGTTCACCTAC-3')

Use Phusion High-Fidelity DNA Polymerase (NEB) in a 25 μ l reaction volume, with 0.35 μ M final concentration of each primer, 3% DMSO and 2X GC buffer.



Phusion High-Fidelity DNA Polymerase - 100 units

by New England Biolabs

Catalog #: M0530S

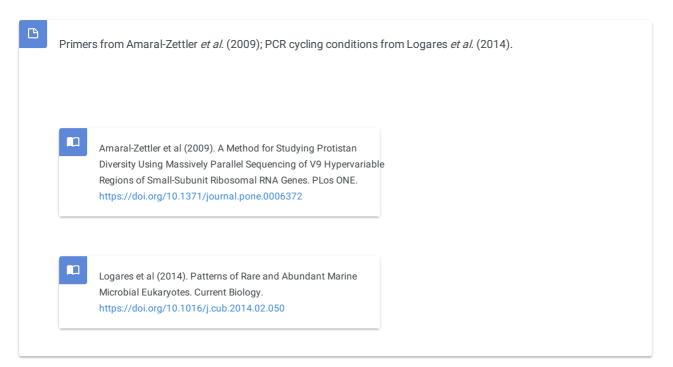
Initial denaturation step of 98°C for 30 s Followed by 25 cycles of:

98°C for 10 s

57°C for 30 s

72°C for 30s

Then a final elongation step of 72°C for 10 mins



- Run PCR products on a 1% agarose gel stained with GelGreen. Excise bands using a blue light transilluminator to prevent DNA damage.
- Purify amplicons using a GeneJet gel extraction kit (Thermo Scientific), quantify with a Qubit fluorometer using the dsDNA BR kit (Invitrogen) and send for Sanger sequencing (Eurofins Genomics).

Library preparation

Purify an aliquot (25 μl) of each chosen MDA sample (including 6 negative controls: 4 negative wells from sorting plate, plus 2 buffer controls from the kit) using AMPure XP beads following the manufacturer's instructions.



- Quantify using a Qubit fluorometer and dilute in 10mM TrisCl (pH 8.0) to 1 μ g in a final volume of 130 μ l. For the negative controls add the total amount of sample (~20 μ l) plus TrisCl until 130 μ L (~110 μ L).
- 19 Fragment DNA using focused acoustic waves (Covaris E220) and then concentrate the sample.
- 20 Prepare libraries using a Nextflex Rapid DNA library preparation kit and indexes (BIOO Scientific) without PCR amplification.
 - Method i. Pool 3 μl of each sample and concentrate, selecting 450-650 bp products using a Blue Pippin 1.5% agarose cassette (R2 marker). Average size of the recovered libraries is ~420 bp (with 295 bp inserts).
 - Method ii. Prepare libraries using bead-based size selection (420-620 bp), quantify by qPCR and pool equimolar amounts (2 nM).
- 21 Denature library pools, dilute and perform 250 bp paired-end sequencing across two lanes on a HiSeq 2500 using Rapid Run SBS v2 reagents (Illumina).

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