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

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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

1 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.

Use a 1:100 dilution of a 1 mM Paclitaxel, Oregon Green stock solution (prepared in DMSO and stored at -20°C).

Paclitaxel, Oregon Green™ 488 Conjugate
by Life Technologies
Catalog #: P22310

3 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.

Hoechst 33342
by Invitrogen - Thermo Fisher
Catalog #: H1399

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.

PBS - Phosphate-Buffered Saline (10X) pH 7.4
by Invitrogen - Thermo Fisher
Catalog #: AM9625

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.

8 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)

12 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.

REPLI-g Single Cell Kit
by Qiagen
Catalog #: 150345

13 After neutralization, amplify samples using the REPLI-g kit in a final volume of 50 µl. 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 µl diluted 100-fold for PCRs (see step 14; stored at -20°C)
b) 20 µl kept as a backup (stored at -80°C)
c) 28 µl (stored at -80ºC). Use 25 µl of this aliquot for sequencing (step 17)

Screening MDA products by amplification of the V9 region of the nSSU rRNA gene

14 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 µl reaction volume, with 0.35 µM 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

Primers from Amaral-Zettler *et al.* (2009); PCR cycling conditions from Logares *et al.* (2014).

Amaral-Zettler et al (2009). A Method for Studying Protistan Diversity Using Massively Parallel Sequencing of V9 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. PLos ONE.
https://doi.org/10.1371/journal.pone.0006372

Logares et al (2014). Patterns of Rare and Abundant Marine Microbial Eukaryotes. Current Biology.
https://doi.org/10.1016/j.cub.2014.02.050

15 Run PCR products on a 1% agarose gel stained with GelGreen. Excise bands using a blue light transilluminator to prevent DNA damage.

16 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

17 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.

Agencourt AMPure XP
by Beckman Coulter
Catalog #: A63880

18 Quantify using a Qubit fluorometer and dilute in 10mM TrisCl (pH 8.0) to 1 µg in a final volume of 130 µl. For the negative controls add the total amount of sample (~20µl) plus TrisCl until 130 µL (~110µL).

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).