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 We use this protocol and it's working

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

Nature Microbiology

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MATERIALS

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- Phusion High-Fidelity DNA Polymerase - 100 units [New England Biolabs Catalog](#) #M0530S
- Qubit™ dsDNA BR Assay Kit [Thermo Fisher Scientific Catalog](#) #Q32853
- Paclitaxel Oregon Green™ 488 Conjugate [Invitrogen - Thermo Fisher Catalog](#) #P22310
- Hoechst 33342 [Invitrogen - Thermo Fisher Catalog](#) #H1399
- Fluoresbrite® YG Microspheres 0.75µm [Polysciences Catalog](#) #17153-10
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 [Invitrogen - Thermo Fisher Catalog](#) #AM9625
- REPLI-g Single Cell Kit [Qiagen Catalog](#) #150345

STEP MATERIALS

- Hoechst 33342 [Invitrogen - Thermo Fisher Catalog](#) #H1399
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 [Invitrogen - Thermo Fisher Catalog](#) #AM9625
- REPLI-g Single Cell Kit [Qiagen Catalog](#) #150345
- Phusion High-Fidelity DNA Polymerase - 100 units [New England Biolabs Catalog](#) #M0530S
- Agencourt AMPure XP [Beckman Coulter Catalog](#) #A63880

Water collection and preparation

- 1 Pre-filter the water sample through a 30 μm mesh, then concentrate by gravity filtration $\sim 100\times$ 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.

Note

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 Life Technologies Catalog
#P22310

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

Hoechst 33342 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 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.

Note

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 'Software' 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 seque..

- 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 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 -80°C). Use 25 µl of this aliquot for sequencing (step 17)

Screening MDA products by amplification of the V9 region o..

- 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'-CCTTCYGCAGGTTACCTAC-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 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

Note

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

CITATION

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.

LINK

<https://doi.org/10.1371/journal.pone.0006372>

CITATION

Logares *et al.* (2014). Patterns of Rare and Abundant Marine Microbial Eukaryotes. Current Biology.

LINK

<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 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.

Note

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).

Note

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).