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Workflow for F₄₂₀-based fluorescence-activated cell sorting (FACS) of sedimentary microbes

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Jeemin Rhim¹, Alyson Santoro¹

¹University of California Santa Barbara



Jeemin Rhim

UCSB

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Protocol status: Working

We use this protocol and it's working



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Abstract

This protocol describes an overall workflow for genomic analysis of sedimentary microbes. This workflow applies the F₄₂₀ autofluorescence-based flow cytometric method, recently developed for the targeted sorting and sequencing of methanogenic archaea (Lambrecht et al., 2017), to investigate diverse lineages of (uncultivated) archaea in sedimentary environments. Pre-sorting steps (sample collection, cell extraction, and glycerol-TE cryopreservation) are optimized for the preservation of cell integrity and F₄₂₀ autofluorescence in archaeal cells for downstream sequencing applications. Post-sorting step can be modified with different whole genome amplification (WGA) methods. See Stepanauskas et al. (2017) and Bowers et al. (2024) for example applications of different WGA methods (e.g., multiple displacement amplification, WGA-X, primary template-directed amplification).

Citation

Lambrecht J, Cichocki N, Hübschmann T, Koch C, Harms H, Müller S (2017)
. Flow cytometric quantification, sorting and sequencing of methanogenic archaea based on F(420) autofluorescence.

<https://doi.org/10.1186/s12934-017-0793-7>

LINK

Citation

Stepanauskas R, Fergusson EA, Brown J, Poulton NJ, Tupper B, Labonté JM, Becraft ED, Brown JM, Pachiadaki MG, Povilaitis T, Thompson BP, Mascena CJ, Bellows WK, Lubys A (2017)
. Improved genome recovery and integrated cell-size analyses of individual uncultured microbial cells and viral particles.

<https://doi.org/10.1038/s41467-017-00128-z>

LINK



Citation

Bowers RM, Gonzalez-Pena V, Wardhani K, Goudeau D, Blow MJ, Uduary D, Klein D, Vill AC, Brito IL, Woyke T, Malmstrom RR, Gawad C
(2024). scMicrobe PTA: near complete genomes from single bacterial cells.

<https://doi.org/10.1093/ismeco/ycae085>

LINK

Materials

Sample collection

- Sterile sample containers to store sediment samples

Cell extraction

- Balance
- Plastic or metal spatula to weigh and transfer sample (clean and UV-crosslinked)
- 15-mL conical bottom centrifuge tubes
- 3X PBS (molecular biology grade) -- 1X PBS may be used for non-marine samples
- Tube rack
- Ice bath
- Ultrasonic homogenizer
- P1000 pipette
- P1000 pipette tips (PCR grade)
- Vortex
- 2-mL microcentrifuge tubes (sterile and UV-crosslinked)
- 22G needles (sterile)
- 1-mL syringes (sterile)
- 60% (w/v) Nycodenz solution
- Microcentrifuge
- 2-mL cryovials (sterile and UV-crosslinked)

Glycerol-TE cryopreservation

- Glycerol-TE (55%, filter-sterilized)
- Liquid nitrogen setup for flash-freezing

F420-based fluorescence-activated cell sorting (FACS)

- 70% ethanol
- 10% bleach
- Ultrapure water
- Optional: surface decontaminant (e.g., DNA AWAY™)
- Heating block for 2-mL cryovials
- Cryovials or 96-well or 384-well plate (clean and UV-crosslinked)
- Cell sorter equipped with blue (488 nm) and violet (405 nm) lasers

Whole genome amplification

- 70% ethanol
- 10% bleach
- Ultrapure water
- Optional: surface decontaminant (e.g., DNA AWAY™)
- Heating block for 2-mL cryovials
- Liquid nitrogen setup for freeze-thawing



- 2-mL microcentrifuge tubes (sterile and UV-crosslinked)
- PCR plate (sterile and UV-crosslinked)
- PCR plate seal
- Whole genome amplification kit (note: we used BioSkryb Genomics ResolveDNA® Whole Genome Amplification Kit v2.0)
- Optional: SYTO™ Green Fluorescent Nucleic Acid Stain (SYTO 9) and a real-time PCR machine



Sample collection

- 1 Collect sediment samples from the environment (e.g., marine sediment core samples). Do not freeze or add fixatives to the samples. Store samples at 4°C until cell extraction (next step).



Cell extraction

- 2 The cell extraction protocol was adapted from Lloyd et al. (2013) and Fazi et al. (2005).

Citation

Lloyd KG, Schreiber L, Petersen DG, Kjeldsen KU, Lever MA, Steen AD, Stepanauskas R, Richter M, Kleindienst S, Lenk S, Schramm A, Jørgensen BB (2013). Predominant archaea in marine sediments degrade detrital proteins.

<https://doi.org/10.1038/nature12033>

LINK

Citation

Fazi S, Amalfitano S, Pernthaler J, Puddu A (2005). Bacterial communities associated with benthic organic matter in headwater stream microhabitats.

<https://doi.org/>

LINK

- 3 Add ~1 g of sediment to a 15-mL conical bottom centrifuge tube.
- 4 Add 5 mL of sterile 3X PBS to each 15-mL tube. Gently invert tubes enough to mix the sediment and PBS, resulting in sediment slurries.
- 5 Place the 15-mL tubes containing sediment slurries in a rack in an ice bath. Ultrasonicate for 2 × 20-second cycles at 7-10 Watts.





- 6 Top off each 15-mL tube with additional 5 mL of 3X PBS. The slurries should contain 10 mL of 3X PBS and ~1 g of sediment at this point.
- 7 Vortex the slurries for 10 seconds.
- 8 Allow them to settle on ice for 10-15 minutes. 
- 9 For each sample, aliquot 750 μ L of the supernatant into each of four replicate 2-mL microcentrifuge tubes.
- 10 Using a 22G needle and a 1-mL syringe, add 750 μ L of 60% (w/v) Nycodenz to the bottom of each tube. As long as the tip of the needle releases Nycodenz at the bottom of the tube, the dense Nycodenz layer will stay below the sample.
- 11 Centrifuge the 2-mL tubes containing sample and Nycodenz at 14,000 g for 20 minutes at 4°C.  
- 12 Using a pipette, carefully collect the sample supernatant and the "cell layer" at the interface between the supernatant and the Nycodenz layer. Combine the supernatant and cell layers from two replicate tubes into one 2-mL tube. This will result in two 2-mL tubes per sample, each containing >1.4 mL of sample. 
- 13 Optional: the supernatant and cell layer collected from Step 11 may vary slightly across samples. You may transfer an exact amount of sample into a new 2-mL cryotube. 

Glycerol-TE cryopreservation

- 14 Add glycerol-TE to samples to a final concentration of ~5.5%.
- 15 Mix the cryotubes gently by inverting three times. Allow to sit at room temperature for 15-20 minutes. It is important to incubate the samples at room temperature prior to freezing. Shorter incubation times (1-5 minutes) will result in decreased cell recovery.  
- 16 After the 15-20 minute incubation, flash-freeze the cryotubes in liquid nitrogen.  
- 17 Keep the flash-frozen cryovials at -80°C until cell sorting (next step).  



F₄₂₀-based fluorescence-activated cell sorting (FACS)

- 18 Clean the sorting environment: wipe down the inside surfaces of the sorting chamber and other working surfaces with (in the following order) 70% ethanol, 10% bleach, and ultrapure water. Optionally, clean the surfaces with DNA AWAY™ surface decontaminant at the end.
- 19 Immediately before sorting, thaw cryotubes containing samples at 42°C.
- 20 Perform cytometric measurements of forward scatter (FSC), side scatter (SSC), and the F₄₂₀ fluorescence as described in Lambrecht et al. (2017). Briefly, the 488 nm laser is used for FSC (488/10) and SSC (trigger signal, 488/10), and the 405 nm laser is used for the F₄₂₀ fluorescence.

Citation

Lambrecht J, Cichocki N, Hübschmann T, Koch C, Harms H, Müller S (2017)
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- 21 Sort F₄₂₀-positive particles into a new (PCR-clean) 2-mL tube.

Note: you will need up to 3 µL per 12-µL reaction for the whole genome amplification protocol described below; sort enough particles for replicate reactions. If planning for single-cell sequencing, sort single F₄₂₀-positive particles into a 96-well or 384-well plate.

- 22 Keep sorted samples at -80°C until genome amplification (next step).

Whole genome amplification

- 23 Prepare a DNA contamination-free working environment: wipe down all working surfaces and pipettes with (in the following order) 70% ethanol, 10% bleach, and ultrapure water. Optionally, clean the surfaces and pipettes with DNA AWAY™ surface decontaminant at

the end. Use new PCR-grade pipette tips. UV-crosslink all 2-mL tubes used for amplification for at least 30-60 minutes.

- 24 Immediately before amplification, thaw sorted samples at 42°C.
- 25 Conduct primary template-directed amplification (PTA) DNA amplification on samples following the ResolveDNA® Whole Genome Amplification Kit Protocol. Below are modifications we recommend for environmental samples containing bacteria and archaea.
 - 25.1 Conduct 5 freeze-thaw cycles on samples prior to the lysis step of the protocol. This can be done by aliquoting just the samples in a PCR plate first, performing the freeze-thaw cycles, then finish preparing the plate with negative and positive controls before proceeding with the ResolveDNA® Whole Genome Amplification Kit Protocol.
 - 25.2 The thermal cycler program for DNA amplification in the Kit Protocol includes a hold at 30°C for 2.5 hours. For sorted environmental samples (e.g., microbial cells extracted from sediments and sorted as described above), we increased the duration of this isothermal amplification to ~4 hours.

It is important to include at least one (preferably two or more) negative control to ensure that the prolonged amplification did not result in amplification of negative controls. To do this, we added 0.12 µL of SYTO™ Green Fluorescent Nucleic Acid Stain (SYTO 9) to the Reaction Mix (final concentration of 5 µM) and monitored fluorescence of samples and controls on a real-time PCR machine. The 30°C hold was terminated at the first sign of fluorescence increase in negative controls.
- 26 Amplified products are stored at -20°C (or -80°C for long-term storage). Samples are ready for downstream sequencing applications.
- 27 Alternatively, whole genome amplification can be done using a different kit (e.g., QIAGEN® REPLI-g® Single Cell Kit).



Citations

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<https://doi.org/10.1093/ismeco/ycae085>

Step 2

Fazi S, Amalfitano S, Pernthaler J, Puddu A. Bacterial communities associated with benthic organic matter in headwater stream microhabitats.

<https://doi.org/>

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<https://doi.org/10.1038/nature12033>

Step 20

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