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Wet lab SOP of the deep-sea sponge microbiome project

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ABSTRACT

This protocol describes the wet lab standard operating procedures (SOPs) established for the Deep-sea Sponge Microbiome Project. It includes the field work procedures, as well as protocols for the isolation and multiplication of microbial genomic DNA for **16S rRNA amplicon sequencing**.

We developed this pipeline with the aim to process a maximum variety of sampletypes. The wet lab part of our amplicon pipeline is modified, but generally based on the guidelines established by the Earth Microbiome Project:

Thompson LR, Sanders JG, McDonald D, Amir A,…, Jansson JK, Gilbert JA, Knight R & The Earth Microbiome Project Consortium (2017) *A communal catalogue reveals Earth's multiscale microbial diversity.* Nature 551:457-463. doi:10.1038/nature24621.

Key characteristics in a nutshell:

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A	B
extract_type	DNA
library_source	genomic
library_strategy	Amplicon (ENA:0000049)
lib_layout	paired
lib_const_meth	PCR
lib_screen	gel electrophoresis
target_gene	16S rRNA
target_subfragment	V3V4
target_main_organism.domain	Bacteria (NEWT:2)
seq_meth.platform	Illumina
seq_meth.instrument_model	MiSeq
seq_chemistry_kitname	MiSeq Reagent Kit v3
read_length	2 × 300 bp
investigation_type	MIMARKS-survey
tissue_fixative	none
tissue_store_temp_degrees_c	-80
dna_store_temp_degrees_c	-80

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://github.com/KathrinBusch/16S-AmpliconCorePipeline/blob/master/ArticleCitation.md

KEYWORDS

molecular ecology, bacteria, amplicon sequencing, 16S, microbial diversity

IMAGE ATTRIBUTION

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MATERIALS TEXT

Working with deep-sea samples often comes along with large logistic efforts.
You can find our standard packing list for a 1-month research cruise here:

Example_packing_list.pdf

Note: Make sure to watch out for customs, dangerous goods + frozen sample shipping guidelines, and other legal regulations.

Please find a key resource table for Steps 2-4 here:

Key_resources_table.xlsx

*(the format of this table has been adopted from Cell's STAR*METHODS)*

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BEFORE STARTING

Consider the tab *Materials* for a full list of key resources needed to conduct this protocol.

According to the general principles of molecular work on microbes, we aim to minimise potential cross-contaminations by keeping workspace, devices, and gloves sterile throughout the whole sample handling process.

Field work

1

The deep-sea sponge microbiome project dataset has been collected during 21 **ship expeditions**.
For these cruises we established the following standard operating procedures:

A) Metadata collection:

- Gather as much metadata as possible
- Fill in this metadata table for all samples (and add anything else that is worth recording):

Example_metadata_sheet.xlsx

FYI:

- *We link every collected sample to cruise ids and event ids deposited in the Pangaea repository.*
- *Usage of the same sample partent ids allows linkage between researchers within the SponGES project.*
- *We store respective cruise reports in the OSIS portal.*

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B) Sponge collection:

- Collect four different specimens of the same sponge species per location. Our sampling focus for the deep-sea sponge microbiome project is on adult, healthy looking sponge individuals.

Note: After animal collection, proceed with the preparations as quickly as realistically possible.

- Take a photograph (with size standard and sample name) of every sponge individual.

- From each specimen, aseptically (wear gloves, use scalpel) remove four 5 cm^3 pieces of tissue. Our targeted body compartment for tissue sampling is the sponge mesohyl.

Note: Use a fresh scalpel each time between different individuals so that there is no cross-contamination between samples. If you do not have a sufficient amount, then swipe the scalpel with ethanol and a Kimwipe to reduce the carry-over of DNA from one sample to another.

- Rinse dissected pieces by transferring them 3x through sterile filtered seawater. Then gently dip sponge piece on Kimwipe to remove excess liquid and flash-freeze samples. Store samples at -80°C.

Note: Petri plates work well for rinsing of sponges. Technical replicates can be frozen together if tube size allows.

- Flash-freeze additional tissue individually (extra biomass) in a 50 mL falcon tube and store it at -80˚ C (alternatively -20°C).

All samples are shipped on dry-ice. To prevent a worse case scenario (i.e. loss of precious samples during transportation) we commonly store additional tissue pieces in RNA*later*™ Stabilization Solution (ThermoFisher) as backup.

C) Seawater collection:

- Collect 4x 2 L replicates of water (8 L total, 4 Niskins) at the same location where sponges are collected.

Note: Acid-cleaned graduated Nalgene bottles work well for sub-sampling from Niskin bottles.

- Filter each 2 L replicate of water on an individual PVDF filter membrane (Merck Millipore; 0.22 µm pore size, ⌀47 mm) using acid-cleaned filter towers.

Note: Rinse filter towers between different samples with MilliQ water.

- Using sterile forceps, remove filter from chimney base, roll up the filter biomass-side in, and place in 15mL falcon tubes. Store in -80˚ freezer and ship on dry-ice.

Note: To save space in the freezer, filters can also be folded twice in half (similar to a crêpe) and stored in cryo vials.

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- Extract and store all available measurements from the CTD cast. Usage of .cnv files facilitates analyses afterwards (.XMLCON +.HEX together work also).

D) Sediment collection:

- Collect 4 sediment push cores from the same location where sponges and water are collected.
- Using sterile spatula (wiped with 70% EtOH), slice off top of core to collect upper 2 cm of sediment.
- Store sediment in individual tubes, freeze in the -80˚ freezer and ship on dry-ice.

Curation of physical frozen sample collection (sponge tissue, sediment, seawater filters):
The deep-sea collection is situated in an own ½ -80 °C freezer in Prof. Ute Hentschel's lab at GEOMAR, Kiel, Germany. It is currently managed by Kathrin Busch. Storage boxes are labeled with cruise identifiers and tubes are labeled with abbreviated parent ids.

DNA extraction

2

LIMS (Laboratory Information Management System):
For products of the DNA extraction procedure we apply a consequtive labelling system (*WB-xxx* for sponges, *SW-xxx* for seawater, *SED-xxx* for sediment). Further we perform a standardised documentation for each sample processed:
Example_lab_documentation.xlsx .

DNA extraction is performed with the DNeasy Power Soil Kit (Qiagen, Cat.#: 12888-100).
(the following steps are closly linked to the manufacturers manual)

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A	B
Sample input	0.25 g of sponge tissue or sediment; ½ filter for seawater
Throughput	1 - 24 samples
Time per run or prep	3 - 4 h

2.1

- Add 60 µl of Solution C1 and invert several times or vortex briefly.

Note: If Solution C1 has precipitated, heat at 60 °C until precipitate dissolves.

2.2

- Add sponge tissue, sediment or half of a seawater filter to the PowerBead Tube included in the kit.
- Vortex PowerBead Tubes.
- Secure PowerBead Tubes for bead beating with PowerLyzer® 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, #13155) and run program. Settings: 3500 rpm, 2x 30 sec bead beating with 45 sec pause in between.

2.3

- Centrifuge tubes at 7500 x *g* for 2 min.

Note: All centrifugation steps in this protocol are performed at room temperature (15-25 °C).

- Transfer the supernatant to a clean 2 mL collection tube.

Note: Expect between 400–500 µL of supernatant.

2.4

- Add 250 µL of Solution C2 and vortex for 5 sec.

2.5

- Incubate at 2–8°C for 5 min.

2.6

- Centrifuge tubes at 10000 x *g* for 2 min.
- Avoiding the pellet, transfer up to 600 µL of supernatant to a clean 2 mL

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collection tube.

2.7

- Add 200 µL of Solution C3 and vortex briefly.

2.8

- Incubate at 2–8°C for 5 min.

2.9

- Centrifuge tubes at 10000 x *g* for 2 min.
- Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 mL customer tube.

Note: The total volume will not fit into the collection tubes provided in the kit, it will spill over when you close the lid.

2.10

- Shake to mix Solution C4 and add 1200 µL to the supernatant. Invert 8x.

2.11

- Load 640 µL onto a MB Spin Column.
- Centrifuge at 10000 x *g* for 1 min. Discard flow through.

Repeat step 2.11 twice, until the entire sample has been processed.

2.12

- Add 500 µL of Solution C5.

2.13

- Centrifuge at 10000 x *g* for 30 sec.
- Discard the flow through. Centrifuge again at 10,000 x *g* for 2 min.
- Carefully place the MB Spin Column into a clean 2 mL collection tube.

Note: Avoid splashing of Solution C5 onto the column.

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2.14

- Air dry filter with open lid under the clean bench for 5 min.

2.15

- Add 100 µL of prewarmed (37 °C) Solution C6 to the center of the white filter membrane.

Note: For processed seawater filters elute with 50 µL of Solution C6 instead of 100 µL.

2.16

- Centrifuge at 10000 x *g* for 1 min. Discard the MB Spin Column.

2.17

The DNA is now ready for downstream applications.

- Transfer 6 µL of the extract into an extra tube to be used for quality controls (see below).
- Store the rest of the extract in the freezer.

Note: Solution C6 is 10 mM Tris-HCl, pH 8.5 (it does not contain EDTA). We therefore recommend to store the extracted DNA frozen.

- We store the extracts at -20 °C until library preparation. Afterwards we use a rack system for organised long-term storage of extracts at -80 °C.

Remark: We also run kit blanks through our pipeline on a regular basis, aswell as two negative and two positive controls for every processed 96-well plate.

Quality control

2.18

Three steps are performed for quality check of the extracted DNA:

A
DNA concentration and purity measurements with a NanoDrop spectrophotometer
PCR with universal 16SrRNA primers
agarose gel electrophoresis

A) 16S rRNA PCR set up:

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A	B
PCR conditions	**Specifications**
27F Forward primer	5’-GAG TTT GAT CCT GGC TCA G-3’
1492R Reverse primer	5’-GGT TAC CTT GTT ACG ACT T-3’
Template	undiluted sample, 10 ng optimum
Template size	1500 bp

To set up parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and DreamTaq DNA polymerase in order. Prepare enough master mix for the number of reactions plus one or two extra. Aliquot the master mix into PCR-stripes/plate and then add template DNA and close with stripe cover/foil.

- Gently vortex and briefly centrifuge all solutions after thawing.
- Place PCR-strips/plate on a cold rack and add the components for a single 20 µL reaction:

A	B
dH2O	16.4 µL
10x Dream Taq Green Buffer*	2.0 µL
dNTP Mix 10 mM each	0.2 µL
Forward primer 10 mM	0.1 µL
Reverse primer 10 mM	0.1 µL
Dream Taq DNA Polymerase 5 U	0.2 µL
Template DNA 10 pg-1 µg	1.0 µL

- Gently vortex the sample stripes and spin down.
- Place the reactions in a thermal cycler. Perform a PCR using following thermal cycling conditions:

A	B	C	D
Step	**Temperature [°C]**	**Time [min]**	**Number of cycles**
Initial denaturation	95	3	1
Denaturation	95	0.5	34
Annealing	56	0.5	34
Extension	72	1.5	34
Final Extension	72	5	1

B) Agarose gel electrophoresis. Check your DNA on an 1% Agarose gel:

- Mix the agarose with 1xTAE buffer in a 300 mL Erlenmeyer flask.
- Heat up the solution in a microwave until the agarose is completely dissolved.

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- Add a magnetic stir bar let it mix and cool down till ~ 45 °C.
- Add Gelgreen (10000x) with a final concentration of 0,5x and mix.
- Carefully transfer the agarose solution into a gel tray with combs.
- After the gel is hardened it can be transferred into the electrophoresis chamber with 1xTAE running buffer.
- Pipette the DNA size (1kb) Ladder and your samples into the slots.
- Start the electrophoresis with a constant power supply of 180 V for 30 min.

We use this template for evaluation of agarose gel electrophoresis: **Example_gel_evaluation.xlsx** .

Library preparation

3

Amplification of the V3V4 variable regions of the 16SrRNA gene is done by a one-step PCR. Add the following components for a single 25 µL reaction:

A	B
dH2O	10.25 µL
5x Buffer	5 µL
dNTPs 10 µmol	0.5 µL
Forward primer 341F 0.28 µM	4 µL
Reverse primer 806R 0.28 µM	4 µL
Phusion Hot Start II High-Fidelity DNA Polymerase 0.5 U	0.25 µL
Template DNA	1 µL

We use the primer pair 341F-806R in a dual-barcoding approach. Check out the exact primer composition here: **PrimersV3V4.xlsx** .

Perform the PCR using the following thermal cycling conditions:

A	B	C	D
Step	**Temperature [°C]**	**Time**	**Number of Cycles**
Initial denaturation	98	30 sec	1
Denaturation	98	9 sec	30
Annealing	55	60 sec	30
Extension	72	90 sec	30
Final Extension	72	10 min	1
Hold	10	∞	-

The amplicon libraries are quality checked by gel electrophoresis, normalised with the SequalPrep Normalization Plate Kit (ThermoFisher Scientific) and pooled equimolarily.

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Sequencing

4

Sequencing is performed on a MiSeq platform (MiSeqFGx, Illumina) using v3 chemistry, resulting in 2x 300 bp.
We always run samples in a 384 pool (1 lane) and use a standardised sequencing layout for all runs: **Example_sequencing_layout.xlsx**.
A commercial mock community (Zymo Research) is added to every plate and a PhiX sequencing control (20 % spike-in) is utilised. For demultiplexing zero mismatches are allowed in the barcode sequence.

Bioinformatics

5

Our subsequent core bioinformatic pipeline can be found here: GitHub.

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