



VERSION 2

SEP 26, 2023

OPEN ACCESS



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**Protocol status:** In development  
We are still developing and optimizing this protocol

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**PROTOCOL integer ID:** 88358

18S V9 PCR V.2

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Better Biomolecular Ocean Practices (BeBOP)



Kathleen Pitz

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ABSTRACT

This protocol is aimed at amplifying the 18S rRNA hypervariable region 9 (18S V9) in eukaryotes with a focus on microbial eukaryotes. Amplicons generated using this protocol can then be sequenced using the Illumina platform. The primers (1391F, EukBr) utilized in this protocol are based on the primer utilized in Amaral-Zettler et al. (2009), Stoek et al. (2010), and the Earth Microbiome Project (EMP).

This work was supported by NASA grant NNX14AP62A 'National Marine Sanctuaries as Sentinel Sites for a Demonstration Marine Biodiversity Observation Network (MBON)' funded under the National Ocean Partnership Program (NOPP RFP NOAA-NOS-IOOS-2014-2003803 in partnership between NOAA, BOEM, and NASA), and the U.S. Integrated Ocean Observing System (IOOS) Program Office.

## Minimum Information about an Omics Protocol (MIOP)

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MIOP Term	Value
methodology category	omics analysis
project	Monterey Bay Time Series
purpose	time series design [OBI:0500020]
analyses	amplicon sequencing assay[OBI:0002767]
geographic location	Monterey Bay [GAZ:00002509]
broad-scale environmental context	marine biome [ENVO:00000447]
local environmental context	upwelling [ENVO:01000005]
environmental medium	sea water [ENVO:00002149]
target	18S Ribosomal RNA [NCIT:C48172]
creator	Kathleen Johnson Pitz
materials required	
skills required	laboratory technician with experience in PCR
time required	
personnel required	1
language	en

MIOP Term	Value
issued	
audience	scientists
publisher	Monterey Bay Aquarium Research Institute, Chavez Lab
hasVersion	
license	
maturity level	Demonstrated

## Authors

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PREPARED BY All authors known to have contributed to the preparation of this protocol, including those who filled in the template	AFFILIATION	ORCID (visit <a href="https://orcid.org/">https://orcid.org/</a> to register)
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Kobun Truelove	MBARI	
Kathleen Johnson Pitz	MBARI	0000-0002-4931-8592

## PROTOCOL REVISION RECORD

- 3 Version numbers start at “1.0.0” when the protocol is first completed and will increase when changes that impact the outcome of the procedure are made (patches: 1.0.1; minor changes: 1.1.0; major changes: 2.0.0). Please store all versions in the gDrive folder designated to your institute.

VERSION	RELEASE DATE This is the date when a given protocol version was finalised	DESCRIPTION OF REVISIONS Please include a brief description of what was changed relative to the previous version
1.0.0	2022-04-25	Initial release

## RELATED PROTOCOLS IN YOUR FOLDER

- 4 This is a list of other protocols deposited in your folder which should be known to users of this protocol. For example, if you create a derivative or altered protocol, you would link to the original protocol in the section below. Please include the link to each related protocol. Also include the version number of that protocol when you linked to it.

PROTOCOL NAME AND LINK	VERSION The version of the protocol you linked to	RELEASE DATE This is the date corresponding to the version listed to the left
protocol_18S_secondary_amplification.md		yyyy-mm-dd
protocol_18S_sequencing.md		yyyy-mm-dd

## RELATED EXTERNAL PROTOCOLS

- 5 This is a list of other protocols that are not in your folder which should be known to users of this protocol. These include, e.g., kit manuals. Please upload all relevant external protocols to Appendix A and link to them here.

EXTERNAL PROTOCOL NAME AND LINK	ISSUER / AUTHOR Please note who authored the protocol (this may also be a company name)	ACCESS DATE This is the date you downloaded the protocol
Environmental DNA (eDNA) 18S metabarcoding Illumina MiSeq NGS PCR Protocol V.2 <a href="https://dx.doi.org/10.17504/protocols.io.n2vdge6">https://dx.doi.org/10.17504/protocols.io.n2vdge6</a>	Collin Closek, Anni Djurhuus, Katie Pitz, Ryan Kelly, Reiko Michisaki, Kristine Walz, Hilary Starks, Francisco Chavez, Alexandria Boehm, Mya Breitbart	yyyy-mm-dd
18S Illumina Amplicon Protocol <a href="https://earthmicrobiome.org/protocols-and-standards/18s/">https://earthmicrobiome.org/protocols-and-standards/18s/</a> <a href="https://dx.doi.org/10.17504/protocols.io.nuvdew6">dx.doi.org/10.17504/protocols.io.nuvdew6</a>	Earth Microbiome Project	yyyy-mm-dd

## ACRONYMS AND ABBREVIATIONS

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ACRONYM / ABBREVIATION	DEFINITION
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ACRONYM / ABBREVIATION	DEFINITION
MBARI	Monterey Bay Aquarium Research Institute
PCR	polymerase chain reaction
NTC	no template control

## GLOSSARY

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SPECIALISED TERM	DEFINITION
amplicon	A piece of DNA or RNA that is the source and/or product of amplification or replication events ( <a href="https://en.wikipedia.org/wiki/Amplicon">https://en.wikipedia.org/wiki/Amplicon</a> )

## BACKGROUND

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This protocol is aimed at amplifying the 18S rRNA hypervariable region 9 (18S V9) in eukaryotes with a focus on microbial eukaryotes. Amplicons generated using this protocol can then be sequenced using the Illumina platform. The primers (1391F, EukBr) utilized in this protocol are based on the primer utilized in Amaral-Zettler et al. (2009), Stoek et al. (2010), and the Earth Microbiome Project (EMP).

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## Summary

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This method uses PCR to amplify the 18S V9 region using primers 1391F and EukBr from Amaral-Zettler et al 2009 and the Earth Microbiome Project (EMP).

## Method description and rationale

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This method is applied because of its ability to amplify the target region (18S V9) across many different groups of organisms, the target region's ability to discriminate between different taxa, and the common research application of this primer set allowing the data to be compared to a reference database and other published environmental datasets.

## Spatial coverage and environment(s) of relevance

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- ocean [ENVO:00000015]
- freshwater lake [ENVO:00000021]

## PERSONNEL REQUIRED

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1 technician

## Safety

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Note

Identify hazards associated with the procedure and specify protective equipment and safety training required to safely execute the procedure

## Training requirements

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#### Note

Specify technical training required for the good execution of the procedure.

### Time needed to execute the procedure

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#### Note

Specify how much time is necessary to execute the procedure.

### EQUIPMENT

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DESCRIPTION e.g. filter	PRODUCT NAME AND MODEL Provide the official name of the product	MANUFACTURER Provide the name of the manufacturer of the product	QUANTITY
<b>Durable equipment</b>			
ultraviolet light source [OBI:0002900]			
PCR instrument [OBI:0000989]			
electrophoresis system [OBI:0001053]			
fluorometer [OBI:0400143]	FMAX Fluorometer	Molecular Devices	
<b>Consumable equipment</b>			
Agarose gel			2
Agencourt AMPure XP bead system		Beckman Coulter, USA	
Quant-It Picogreen dsDNA Assay		Life Technologies	
<b>Chemicals</b>			
10% Bleach			
70% Ethanol			
RNase Away			
Amplitaq Gold Fast PCR mastermix			
molecular-biology grade water			
forward and reverse primers (5 µM)			

### Preparation

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1. Disinfect work surfaces with 10% bleach, followed by 70% ethanol.
2. RNase Away and pipets with RNase Away
3. UV pipets, molecular grade water, and tube racks for 20 minutes prior to starting protocol.

### PCR

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PCR reactions were run in single 75ul reactions for each sample using 12-basepair Golay barcoded reverse primers [Amaral-Zettler et al. (2009), Stoek et al. (2010), Earth Microbiome Project] with Fluidigm adapters CS1 & CS2. All primers listed in the 5' to 3' direction.

- 3 µl DNA extract template
- 37.5 µl Amplitaq Gold Fast PCR mastermix (Applied Biosystems)
- 3 µl each of forward and reverse primers (5 µM)
- 28.5 µl molecular-biology grade water

PCR Primer Name	Direction	Sequence (5' -> 3')
<b>Euk1391F</b> and Fluidigm CS1	forward	<b>ACACTGACGACATGGTTCTACAGTACA CACCGCCCGTC</b>
<b>EukBr</b> and Fluidigm CS2	reverse	<b>TACGGAGCAGAGACTTGGTCTTGATCC TTCTGCAGGTTCACTAC</b>

PCR reactions were run in 96-well plates with a NTC run in singleton for each plate

#### 18S thermal cycling parameters

- These parameters use a normal ramp speed

PCR step	Temperature	Duration	Repetition
denature	95° C	10 minutes	1
denature	94° C	45 seconds	35
anneal	57° C	30 seconds	35
extension	68 °C	90 seconds	35
extension	72° C	10 minutes	1
hold	4° C	infinity	1

### Quality control, PCR clean-up

- 19 After PCR amplification of the marker region, PCR products were run through an agarose gel to confirm the presence of target bands and absence of non-specific amplification across environmental samples as well as the absence of amplification in no-template controls (NTCs).
1. PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter, USA).
  2. A second agarose gel was run to confirm primer removal and retention of target amplicons after purification.
  3. Purified products were then quantified using Quant-It Picogreen dsDNA Assay (Life Technologies) on an fmax Molecular Devices Fluorometer with SoftMaxPro v1.3.1

### Basic troubleshooting guide

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#### Note

Identify known issues associated with the procedure, if any.  
Provide troubleshooting guidelines when available.

### REFERENCES

- 21 Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM (2009) A Method for Studying Protistan Diversity Using Massively Parallel Sequencing of V9 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. PLOS ONE 4(7): e6372. <https://doi.org/10.1371/journal.pone.0006372>
- Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D. M., Breiner, H.-W., & Richards, T. A. (2010). Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology*, 19 Suppl 1, 21–31. <https://doi.org/10.1111/j.1365-294X.2009.04480.x>
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6, 1621–1624. <http://doi.org/10.1038/ismej.2012.8>

### APPENDIX A: DATASHEETS

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