Sep 26, 2023 Version 2

18S V9 PCR V.2

DOI
dx.doi.org/10.17504/protocols.io.36wgq3d8olk5/v2

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Better Biomolecular Oce...

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Protocol Citation: Kathleen Pitz 2023. 18S V9 PCR. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq3d8olk5/v2Version created by Kathleen Pitz

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

Created: September 26, 2023

Last Modified: February 06, 2024

Protocol Integer ID: 88358

Keywords: earth microbiome project, demonstration marine biodiversity observation network, microbial eukaryote, 18s rrna hypervariable region, 18s rrna, national marine sanctuaries as sentinel site, eukaryote, national ocean partnership program, national marine sanctuary, v9 pcr this protocol, biodiversity, primer, mbon, nnx14ap62a, pcr

Disclaimer

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.

Troubleshooting

Minimum Information about an Omics Protocol (MIOP)

1

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

Authors

2

	PREPARED BY All authors known to have contributed to the preparation of this protocol, including those who filled in the template.	AFFILIATION	ORCID (visit https:/
	Jacoby Baker	MBARI	
	Kobun Truelove	MBARI	
	Kathleen Johnson Pitz	MBARI	0000-0002-4931-

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
	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 d
	Environmental DNA (eDNA) 18S metabarcoding Illumina MiSeq NGS PCR Protocol V.2 https://dx.doi.org/10.17504/protocols.io.n2vdge6 dx.doi.org/10.17504/protocols.io.n2vdge6	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 https://earthmicrobiome.org/protocols-and-standards/18s/ dx.doi.org/10.17504/protocols.io.nuvdew6	Earth Microbiome Project	yyyy-mm-dd

ACRONYMS AND ABBREVIATIONS

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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 (https://en.wikipedia.org/wiki/Amplicon)

BACKGROUND

8 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

9 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

10 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

11
- ocean [ENVO:00000015]
- freshwater lake [ENVO:00000021]

PERSONNEL REQUIRED

12 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

15

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 pro
	Durable equipment		
	ultraviolet light source [OBI:0002900]		
	PCR instrument [OBI:0000989]		
	electrophoresis system [OBI:0001053]		
	fluorometer [OBI:0400143]	FMAX Fluorometer	Molecular Devices
	Consumable equipment		
	Agarose gel		
	Agencourt AMPure XP bead system		Beckman Coulter, USA
	Quant-It Picogreen dsDNA Assay		Life Technologies
	Chemicals		
	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

18 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′)
	Euk1391F and Fluidigm CS1	forward	ACACTGACGACATGGTTCTACA**GTAC ACACCGCCCGTC**
	EukBr and Fluidigm CS2	reverse	TACGGAGCAGAGACTTGGTCT**TGAT CCTTCTGCAGGTTCACCTAC**

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 absense 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

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APPENDIX A: DATASHEETS

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