Contents

NMNH PCR Protocol 18S rRNA V4 (Stoeck-Piredda)

1 PROTOCOL INFORMATION

1.1 Minimum Information about an Omics Protocol (MIOP)

- MIOP terms are listed in the YAML frontmatter of this page.
- See MIOP_definition.md for list and definitions.

1.2 Making eDNA FAIR (FAIRe)

- FAIRe terms are listed in the YAML frontmatter of this page.
- See https://fair-edna.github.io/download.html for the FAIRe checklist and more information.
- See https://fair-edna.github.io/guidelines.html#missing-values for guidelines on missing values that can be used for missing FAIRe or MIOP terms.

1.3 Authors

PREPARED BY	AFFILIATION	ORCID	DATE
Steven Auscavitch	National Museum of Natural History, Smithsonian Institution	https://orcid.org/0000-0001-5777-4814	2025-09-03
Allen Collins	NOAA National Systematics Laboratory, Smithsonian Institution	https://orcid.org/0000-0002-3664-9691	2025-09-03

1.4 Related Protocols

- This section contains protocols that should be known to users of this protocol.
- Internal Protocols: Derivative or altered protocols, or other protocols in this workflow.
- External Protocols: Protcols from manufacturers or other groups.
- Include the link to each protocol.
- Include the version number (internal) or access date (external) of the protocol when it was accessed.

1.4.1 Internal Protocols

PROTOCOL NAME	LINK	VERSION	RELEASE DATE	INTERNAL/EXTERNAL
AOML 'Omics Protocols	https://github.com/aomlomics/protocols	ongoing		

1.4.2 External Protocols

PROTOCOL NAME	LINK	ISSUER / AUTHOR	ACCESS DATE
Qubit™ dsDNA HS Assay Kit	https://documents.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf	Thermo Fisher Scientific	2026-01-26
KAPA Pure Beads Manual	https://rochesequencingstore.com/wp-content/uploads/2022/07/KAPA-Pure-Beads-Technical-Data-Sheet.pdf	Roche	2026-01-26

1.5 Protocol Revision Record

VERSION	RELEASE DATE	DESCRIPTION OF REVISIONS
1.0.0	2025-09-03	Initial release
1.0.1	2025-12-15	Updated YAML front matter

1.6 Acronyms and Abbreviations

ACRONYM / ABBREVIATION	DEFINITION
eDNA	environmental DNA
NOAA	National Oceanic and Atmospheric Administration
NSL	National Systematics Laboratory
SI	Smithsonian Institution
NMNH	National Museum of Natural History
NTC	No template control
PCR	Polymerase chain reaction
EtOH	Ethanol

1.7 Glossary

SPECIALISED TERM	DEFINITION
None	None

2 BACKGROUND

Environmental DNA (eDNA) is rapidly becoming a "go-to" tool for monitoring and assess biodiversity in the marine environment. DNA extracted from an environmental sample contains molecular signatures in the environment. Therefore, these eDNA samples can be used to generate metabarcode libraries (amplifications of specific gene regions of interest) in order to characterize biological communities.

2.1 Summary

The 18S_V4 rRNA metabarcoding protocol detailed here is designed to prepare 18S_V4 rRNA fragments for sequencing on Illumina platforms using a two-step amplification process. The first step, described here, (which we call the amplification PCR) will amplify your region of interest and tail (Note: we opt for iTru tails), depending on your chosen primer design. The tail acts as the priming site for future indices and the Illumina-required sequencing adapter to your product. The resulting libraries are dual-indexed and can be sequenced on any Illumina sequencer. Indexing PCR protocols are not described but many options are available depending on if sequencing will be done with a commercial facility or in-house.

2.2 Method Description and Rationale

This procedure uses polymerase chain reaction to amplify the 18S_V4 rRNA region in an environmental sample of DNAs. This procedure is best suited for targeting eukaryotic taxa. This protocol was selected because it is reproducible, accurate, is easy to execute by individuals with basic wet lab bench skills.

2.3 Spatial Coverage and Environment(s) of Relevance

This protocol was designed for environmental DNA from marine environments. For the NOAA Okeanos Explorer program, samples typically are collected from oceanic mesopelagic, oceanic bathypelagic, and oceanic abyssopelagic zones.

3 PERSONNEL REQUIRED

One technician is sufficient to complete this procedure.

3.1 Safety

Basic laboratory safety measures and appropriate personal protective equipment should be used to avoid exposure to hazardous reagents and situations.

3.2 Training Requirements

Basic wet lab bench skills, including pipetting and aseptic technique, are required.

3.3 Time Needed to Execute the Procedure

This procedure can be conducted within 180 minutes, but is dependent on the number of samples processed and the skill of the user.

4 EQUIPMENT

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
Durable equipment				
Benchtop vortexer	Nonspecific	Nonspecific	1	N/A

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
PCR workstation with UV Lamp	AirClean600 PCR Workstation	BioExpress	1	UV lamp built into PCR workstation
P1000 Pipette	Pipet-Lite LTS Pipette L-1000XLS+	Mettler Toledo	1	Single or Multichannel model
P200 Pipette	Pipet-Lite LTS Pipette L-200XLS+	Mettler Toledo	1	Single or Multichannel model
P20 Pipette	Pipet-Lite LTS Pipette L-20XLS+	Mettler Toledo	1	Single or Multichannel model
P10 Pipette	Pipet-Lite LTS Pipette L-10XLS+	Mettler Toledo	1	Single or Multichannel model
PCR plate mat (Sterile/Autoclaved)	Unknown	Unknown	1 per reaction	Alternatively, heat-sealed sheets can be used to seal reactions
Thermocycler	Bio-Rad T100 Thermocycler	Bio-Rad	1	Or similar
Silicon PCR mats,reusable	Sealing Mats for 96-Well PCR Plates (#2239442)	Bio-Rad	1, or as needed	Can be substituted with sterile, consumable heat-sealed sheets
Gel Electrophoresis dock and power supply	Varies	Varies	1	NA
Microwave	Generic Microwave	Generic Brand	1	
Gel Combs	Gel Electrophoresis Combs	Generic Brand	8	NA
Magnetic Stand	DynaMag-96 Side Magnet (12331D)	ThermoFisher Scientific	1	NA
Nucleic Acid Quantification	Qubit or Quant-IT Assay Readers	ThermoFisher Scientific	1 kit(s)	Or other quantification assay
Consumable equipment				
Gloves	Nitrile Gloves, Exam Grade, Powder-free	Varies	1 box	Can be substituted with generic
Hype-Wipe	CurrentTechnologies 9103	Current Technologies	As needed	As needed
KimWipes	KimwipesTM 34155	Kimberly-Clark	1 box	As needed

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
0.2 mL 96-well PCR Plate	MSP 96-wWell Half-Skirt PCR Plate #15-3590	Med Supply Partners	As needed	Autoclave, if not sterilized
0.2 ml 8-well PCR strip tube	PCR Reaction Strips 8 x 0.2mL, attached dome cap (T320-3N)	Simport	As needed	Autoclave, if not sterilized
Eppendorf LoBind Tubes (1.5 mL)	EP022431021	Eppendorf	As needed	Autoclave, if not sterilized
1.5% Agarose Gels (pre-made)	Varies	Varies	Include appropriate DNA stain & loading dye when electing gel method. If pre-made gels are not available, prepare 1.5% agarose gels using genetic method.	
1000µL Filtered Tips	Filter Tips, 1000µL	Mettler Toledo	As needed	Can be substituted with generic
200µL Filtered Tips	Filter Tips, 200µL	Mettler Toledo	As needed	Can be substituted with generic
10 ul Filtered tips	Pipette Tips, 10 uL	Mettler Toledo	As needed	Can be substituted with generic
Clear Qubit Assay tubes	0.5 mL thin-walled, polypropylene tubes	Invitrogen	98	Must be correct tubes to allow for fluorometer to read concentration
Chemicals				
Surface Disinfectant	DNA Away	Thermo-Fisher	1 bottle	As needed. Can be substituted with 10% bleach
Ethanol (70%)	Varies	Varies	As needed	For surface and equipment cleaning
Ethanol (80%)	Varies	Varies	As needed	For bead cleaning
Absolute Ethanol (200 Proof)	Varies	Varies	As needed	
GoTaq Hot Start Polymerase	2X GoTaq Hot Start Master Mix (colorless) - M5132	Promega Corp.	Sample quantity dependent	
Bovine Serum Albumin (BSA) 20 mg/mL		Varies	Varies	Sample quantity dependent

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
18S_V4 primer F	NA	Integated DNA Technologies	Sample quantity dependent	NA
18S_V4 primer R	NA	Integated DNA Technologies	Sample quantity dependent	NA
Kapa Pure Beads	KK8000 - 5 mL	Roche	Sample quantity dependent	AmPure magnetic beads also work.
10 mM Tris, pH 8.0	Varies	Varies	Sample quantity dependent	Diluted 1X TE to 0.1X would also work.
Sterile, Nuclease-Free Water	NA	Sample quantity dependent	NA	
Unique Indexing primers (i5 & i7)				Nextera or iTru adapters and indexes are available. In this protocol, we used iTru.
DNA Quantification Assay Kit	Qubit™ dsDNA Quantification Assay Kit (High Sensitivity)	ThermoFisher Scientific	As needed	
Gel electrophoresis buffer	1X SB	Unknown	As needed	or any other run buffer substitute (e.g. TBE)
Nucleic acid stain	GelRed® Nucleic Acid Stain 10000X	Biotium	As needed	Can be substituted with other nucleic acid stains
Gel Loading Dye	Varies	Varies	As needed	NA
100bp DNA Ladder	Generuler 100 bp DNA Ladder	Thermo Scientific	As needed	Or similar reagent
Qubit Reagents	Qubit dsDNA Quantification Assay Kit	Invitrogen	1	(kit)

5 STANDARD OPERATING PROCEDURE

5.1 Preparation

Quantification & dilution of DNA 1. This protocol assumes a known DNA input quantity. If not known, quantify your DNA sample(s) using a QuBit or Quant-iT assay. 2. Normalize your DNA to desired concentration, if desired. 3. Set up a PCR plate map with triplcate reactions for each sample, along with 3 NTC reactions. NTC reactions will use 1 uL of sterile water instead of DNA template.

5.2 PCR

18S_V4 rRNA PCR Reaction 4. Retrieve reagents from the freezer and allow them to thaw at room temperature. Vortex well and spin down. 5. Perform the initial PCR in triplicate using 10 µL reactions. A master mix may be created from the master mix polymerase, sterile water, primer forward and reverse, and BSA. Follow the rection mixture below to make your master mix, scaling up accordingly. Allow for ~10%

overage calculation for pipetting error. Volumes are given in µL. Mix thoroughly, then spin briefly. Master mixes can be made in sterile, autoclaved 1.5 ml Eppendorf Lo-Bind tubes. 6. Aliquot 9.0 µL of master mix into each PCR tube, then add 1.0 µL of template DNA. Cover plate with a silicone mat or heat seal, then spin to collect liquid to the bottom of wells. 7. Place tubes/plate into a thermocycler and run the initial amplification PCR program (see PCR Cycling Program). 8. After the PCR finishes, proceed directly to Gel Verification, or samples may be stored at 4°C for up to 3 days (or at -20°C for longer periods).

Primers: PCR primer sequences

Note: Ensure that primer sequences come with the appropriate (e.g. Nextera or iTru) adapter sequences for your sequencing technology of choice. This should be decided upon beforehand, along with your sequencing facility and technology of choice. (ADD ADAPTER INFORMATION.)

PCR Primer Name	Direction	Sequence (5' -> 3')
18S-V4-TAReuk454FWD1	forward	CCAGCASCYGCGGTAATTCC
18S-V4-TAReuk454REV3.Piredda	ACTTTCGTTCTTGATYRATGA	

Reaction Mixture: PCR reagents, volumes, initial and final concentrations

Reagent	Volume	Initial Concentration	final concentration
sterile nuclease-free water	3.3 uL	NA	NA
2X GoTaq Hot Start Master Mix	5.0 uL	2X	1X
10 uM primer F 18S-V4-TAReuk454FWD1	0.3 uL	10 uM	0.3 uM
10 uM primer R 18S-V4-TAReuk454REV3.Piredda	0.3 uL	10 uM	0.3 uM
BSA	0.1 uL	20 mg/uL	0.2 mg/uL
Template eDNA	1.0 uL	NA	NA

PCR Cycling Program:

Initial PCR Amplification | PCR Step | Temperature | Duration | Repetition | | — | — | — | — | | Initial denaturation | 95°C | 5 min. | NA | | Denaturation | 95°C | 30s | 35 cycles | | Annealing | 55°C| 30s | 35 cycles | | Extension | 72°C | 30s | 35 cycles | | Final Extension | 72°C | 5 min. | NA | | Hold | 12°C | Infinite | NA |

5.3 Quality Control

Gel verification (Recommended) 1. Prepare a 1.5% agarose gel using 1X SB. Arrange combs to reflect the number of samples and replicates, including a space for ladder. 2. Mix 2 µl PCR product with 2 µl 2X loading dye/10X GelRed. Load 4 uL of PCR product + loading dye/10X GelRed into each respective well. Gel should be immersed with 1X SB. Run gels for a 5-7 minutes at 125V. 3. Product size should be ~430 bp for invertebrate metabarcoding COI primers, depending on your adapter and the presence of heterogeneity spacers, if included on the primer. 4. Following Gel Verification, pool PCR replicates for each sample, omitting any volumes that had incorrect bands present. A full volume of the remaining product should be 24 uL (8 uL X 3 replicates). 5. Samples may be stored at 4°C for up to 3 days (or at -20°C for longer periods).

5.3.1 Positive Control

As needed, but can be optional.

5.3.2 Negative Control

Non-template control (NTC) reaction(s) using sterile, nuclease-free water (or PCR grade water) in place of DNA template are used for each master mix prepared or PCR reaction plate.

5.4 PCR Clean-up

Clean up of initial amplification with Kapa Pure Beads (Recommended) 1. Remove KAPA Pure beads from the fridge and allow to come to room temperature. While you wait, make fresh 80% Ethanol (450 µl per sample). 2. Vortex the beads until fully resuspended (~30 seconds). 3. To 24 µL of pooled PCR product, add 28.8 µL of KAPA Pure beads (1.2X bead-to-sample ratio). 4. Mix thoroughly by pipetting up/down at least 10 times. 5. Incubate at room temperature for 5 minutes to bind the DNA to the beads. 6. Place the plate/tubes on a magnet for ~3 minutes, or until the supernatant is clear and beads are pelleted. 7. Carefully remove and discard the supernatant, making sure to not disturb the pellet. 8. With the plate/tubes still on the magnet, wash the beads by adding 200µL of 80% ethanol. Do not mix. 9. Incubate at room temp for 30 seconds. 10. Remove and discard the supernatant. 11. Repeat the ethanol wash. 12. Carefully remove any residual ethanol using a P20 pipette. 13. Allow beads to airdry for 3 to 5 minutes. Avoid overdrying to the point that the bead pellet starts to crack. 14. Remove the plate from the magnet. Add 22 µL of 10 mM Tris-HCl, pH 8.0 to each well and use a pipette to fully resuspend the beads. 15. Incubate the plate at room temperature for 5 minutes. 16. Place the plate back on the magnet for ~3 minutes, or until the supernatant clears. 17. Carefully transfer 20 µL of the clear supernatant to a new plate/tube, making sure to not disturb the bead pellet. 18. After the cleanup, go directly to Step 5, or samples may be stored at 4°C for up to 3 days (or at -20°C for longer periods).

5.5 Basic Troubleshooting Guide

Not applicable.

6 REFERENCES

Not applicable.

7 APPENDIX A: DATASHEETS

Not applicable.