Contents

NOAA/AOML PCR Protocol 12S rRNA MiFish (Miya-Wangensteen)

1 PROTOCOL INFORMATION

1.1 Minimum Information about an Omics Protocol (MIOP)

- MIOP terms are listed in the YAML frontmatter of this page.
- See https://github.com/BeBOP-OBON/miop/blob/main/model/schema/terms.yaml 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

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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
AOML 'Omics Protocols	https://github.com/aomlomics/protocols	1.2.2	ongoing
NOAA 'Omics Metabarcoding Assays	https://github.com/NOAA-Omics/noaa-omics-metabarcoding-assays	not applicable	ongoing

1.4.2 External Protocols

PROTOCOL NAME	LINK	ISSUER / AUTHOR	ACCESS DATE
AMPure XP Bead-Based Reagent Protocol for PCR Purification	https://www.beckman.com/reagents/genomic/cleanup-and-size-selection/pcr/ampure-xp-protocol	Beckman Coulter	2025-02-05
Invitrogen Qubit 1X dsDNA HS Assay Kits User Guide	https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017455_Qubit_1X_dsDNA_HS_Assay_Kit_UG.pdf	ThermoFisher Scientific	2025-02-05

1.5 Protocol Revision Record

VERSION	RELEASE DATE	DESCRIPTION OF REVISIONS
1.0.0	2021-08-22	Initial release
1.0.1	2024-10-23	Formatting edits
1.1.0	2024-11-16	Addition of FAIR eDNA terms in YAML front matter
1.1.1	2024-12-15	Updated YAML front matter

1.6 Acronyms and Abbreviations

ACRONYM / ABBREVIATION	DEFINITION
NOAA	National Oceanographic and Atmospheric Administration
AOML	Atlantic Oceanographic and Meteorological Laboratory
MSU	Mississippi State University
NGI	Northern Gulf Institute
PCR	Polymerase chain reaction
eDNA	environmental DNA
NTC	No template control
EtOH	Ethanol

1.7 Glossary

SPECIALISED TERM	DEFINITION
Extraction Blank	A type of negative control to confirm there is no contamination during DNA extractions. Normally an empty is filter extracted and PCR amplified alongside other samples.
No Template Control	A type of negative control during PCR to confirm there is no contamination during the PCR process. Normally nuclease-free water is run in place of DNA on a PCR.

2 BACKGROUND

2.1 Summary

This protocol describes steps for performing PCR for 12S rRNA V5-V6 marker gene regions using eDNA extracted from Sterivex at NOAA's AOML. The PCR protocol only includes a primary PCR step as the secondary PCR, library preparation and sequencing is completed by Michigan State University's RTSF Genomics Core. Steps related to preparing samples for sequencing and the Genomics Core's procedure are included. Some steps (e.g. PCR plate preparation) have been or can be optimized for use with the Opentrons OT2 robot. This protocol closely follows along with the following protocol: https://www.protocols.io/view/environmental-dna-edna-12s-metabarcoding-illumina-kqdg35kqzv25/v2.

2.2 Method description and rationale

This protocol is used for PCR amplifying the 12S MiFish marker gene regions of environmental DNA. Fluidigm adapters are already present on the primers described in the following protocol. It is highly reproducible and can easily be adapted for any number of samples (i.e. a full 96-well plate or a few samples).

2.3 Spatial coverage and environment(s) of relevance

This protocol can be used to amplify the 12S marker gene region of any eDNA sample.

3 PERSONNEL REQUIRED

One person with molecular biology experience.

3.1 Safety

There are no hazardous chemicals or materials involved in this protocol. Standard lab safety techniques should still be used such as wearing PPE to avoid skin or eye contact.

3.2 Training requirements

Basic molecular biology training is sufficient for this protocol including sterile technique, pipetting small volumes and programming/running PCR thermal cyclers.

3.3 Time needed to execute the procedure

Protocol takes about 4 hours (240 minutes) including thermal cycler run time.

4 EQUIPMENT

For 96-well Plate:

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
Durable equipment				
100-1000 ul Pipette	Eppendorf Research Plus Adjustable-Volume Pipette	Eppendorf	1	Can be substituted with any accurate pipette
10-100 ul Pipette	Eppendorf Research Plus Adjustable-Volume Pipette	Eppendorf	1	Can be substituted with any accurate pipette
0.1-2.5 ul Pipette	Eppendorf Research Plus Adjustable-Volume Pipette	Eppendorf	1	Can be substituted with any accurate pipette
10-100 ul 8-Channel Pipette	Eppendorf Research Plus 8 Channel Pipette	Eppendorf	1	Can be substituted with any accurate pipette
0.5-10 uL 8-Channel Pipette	Eppendorf Research Plus 8 Channel Pipette	Eppendorf	1	Can be substituted with any accurate pipette
Thermal cycler	Mastercycler Nexus Thermal Cycler	Eppendorf	1	Can be substituted with generic
Microwave	Generic Microwave	Generic Brand	1	
Flask	500 mL Flask	Generic Brand	1	Used for mixing agarose gel solution
1-L Glass Container	1 L Glass Container	Generic Brand	1	Used for storing 1x TBE buffer
Gel Tray & Box	Gel Electrophoresis Box and Tray	Generic Brand	1	Can be substituted with generic
Gel Combs	Gel Electrophoresis Combs	Generic Brand	2	Can be substituted with generic
Consumable equipment				
Gloves	Nitrile Gloves, Exam Grade, Powder-free	ULINE	1	(box) Can be substituted with generic
Kim Wipes	KimWipe Delicate Task Wipers	KimTech	1	(box) Can be substituted with generic

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
96-well PCR Plate		Armadillo PCR Plate, 96-well, clear, clear wells	ThermoFisher	3
PCR Plate Seal	AlumaSeal II Sealing Foils for PCR and Cold Storage	VWR	2	Can be substituted with generic, can use tightly-fitted strip caps in place of seal
1000µL Filter Tips	OT-2 Filter Tips, 1000µL	Opentrons	1	(box) Can be substituted with generic
200µL Filter Tips	OT-2 Filter Tips, 200µL	Opentrons	2	(boxes) Can be substituted with generic
10 ul Filter tips	TipOne Pipette Tips, 10 uL	TipOne	2	(boxes) Can be substituted with generic
AmpliTaq Gold PCR Mix	AmpliTaq Gold DNA Polymerase 5 mL	ThermoFisher	1.2	(mL)
Molecular water	Invitrogen RT-PCR Grade Water	Fisher Scientific	0.912	(mL)
Forward Primer - 12S MiFish F	12S MiFish_U F Fluidigm V2	IDT	105	(ul (10uM)) Primer must be diluted from 100uM stocks to 10uM
Reverse Primer - 12S MiFish R	12S MiFish_U R Fluidigm	IDT	105	(ul (10uM)) Primer must be diluted from 100uM stocks to 10uM
TBE Buffer (10x)	TBE Buffer 10X Solution, Molecular Biology Grade, UltraPure	Thermo Scientific	100	(uL)
Agarose	Agarose LE, Molecular Biology Grade, UltraPure	Thermo Scientific	4	(g)
SYBR Safe	SYBR Safe DNA Gel Stain	Invitrogen	20	(uL) Light sensitive - do not expose to light
Gel stain loading dye	DNA Gel Loading Dye (6x)	Thermo Scientific	480	(ul per plate)
100bp DNA Ladder	Generuler 100 bp DNA Ladder	Thermo Scientific	6	(ul per lane on gel)
Parafilm	Parafilm M Lab Film	Generic	1	(roll) Can substitute with generic brand

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
Chemicals				
RNase AWAY	RNase AWAY Surface Decontaminant	ThermoFisher Scientific	1	(bottle) Used to sterilize lab surfaces and equipment
EtOH	Ethanol	Generic Brand	1	(wash bottle) Must be molecular grade ethanol
DI water	Deionized water	Generic	900	(mL)
(OPTIONAL) Clean-Up Protocol				
AMPure XP Beads	AMPure XP Bead-Based Reagent	Beckman Coulter	1	(kit)
96-well magnetic plate	MagDTR 96-Well Magnetic Separator	Edge Biosystems Inc	1	Can be substituted with generic brand
(OPTIONAL) Qubit				
Qubit Reagents	Qubit dsDNA Quantification Assay Kit	Invitrogen	1	(kit)
Clear Qubit Assay tubes	0.5 mL thin-walled, polypropylene tubes	Invitrogen	98	Must be correct tubes to allow for fluorometer to read concentration

- 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: Provide quantities necessary for one application of the standard operating procedure (e.g., number of filters).
- Remark: For example, some of the consumable may need to be sterilized, some commercial solution may need to be diluted or shielded from light during the operating procedure.

5 STANDARD OPERATING PROCEDURE

5.1 Protocol

5.1.1 Preparation

1. Dilute primers from 100 uM to 10 uM if not already at 10uM.
2. Set up PCR under hood by wiping off all surfaces, pipettes, and racks with RNase AWAY and UV sterilizing for 5-10 mins.
3. Map out order of samples on 96-well PCR plate. Make sure to leave a space for a no template control (NTC) and positive control.

5.1.2 PCR

1. Make PCR master mix and add 24 ul to each well of PCR plate - possible use on Opentrons OT2 Pipetting Robot. Account for triplicate reactions.

- 12.5 ul AmpliTaq Gold 360 Master Mix
- 9.5 ul molecular water
- 1 ul Fwd primer (10 M) - 12S MiFish_U F Fluidigm V2
- 1 ul Rev primer (10 M) - 12S MiFish_U R Fluidigm

PCR Primer Name	Direction	Sequence (5' -> 3')	Sequence (5' -> 3') with Fluidigm Adapters	Fluidigm Adapter
12S MiFish F V2	forward	GCCGGTAAAACTCG	xxx GCCG-GTAAAACTCGT-GCCAGC	
12S MiFish R	reverse	CATAGTGGGGTATC	xxx CATAGTGGGGTATC-TAATC-CCAGTTTG	

2. Add 1 ul of sample DNA (or molecular water for NTC) to respective triplicate wells for a total reaction volume of 25 ul per well. Pipette up and down or vortex to fully distribute DNA into master mix.
3. Seal plate with PCR plate seal or strip caps.
4. Load plate onto thermal cycler and select "MBARI 12S Touchdown" program to run the following steps:

PCR step	Temperature	Duration	Repetition
Initial Denaturation	95°C	15min	1x
Touchdown Cycling			
Denaturation	94°C	30s	13x
Annealing	69.5°C	30s	13x (changes -1.5°C each cycle)
Extension	72°C	90s	13x
Normal Cycling			
Denaturation	94°C	30s	25x
Annealing	50°C	30s	25x
Extension	72°C	45s	25x
Final Extension	72°C	10min	1x
Hold	4°C	∞	

5.1.3 Quality Control and PCR Clean-Up 2% Agarose Gel Following PCR amplification, pool triplicate PCR products then run through 2% agarose gel to confirm presence of target bands: 1. Make stock solution of TBE buffer (1x) in a 1-L glass container by adding 100 ml of stock TBE buffer (10x) to 900 ml DI water. 2. For a 5.5in x 5.5in gel tray, mix 200 ml of TBE buffer (1x) and 4 g of agarose in a flask. Use scale to weigh agarose. 3. Microwave mixture for 1 minute, followed by 15-30 second intervals. Watch carefully after 1 minute - mixture can bubble out of flask! The agarose should be fully dissolved so that the solution is mostly clear. Wear a protective glove when handling flask as the mixture will be hot. 4. Allow for gel mixture to cool in flask for 5-10 min. While cooling, set up gel tray (5.5in x 5.5in) in gel box. Make sure the tray is oriented properly and sealed tight for gel pouring. Add two gel combs for 20 wells each lane - total of 40 wells. 5. Once cooled to 50°C, add 20 ul of SYBR safe to the mixture and swirl the flask gently to mix (don't create bubbles!). SYBR safe is light sensitive so after adding to mixture, immediately close the vial

and store in the dark. 6. Pour the gel mixture and remove any bubbles using a pipette tip. 7. Allow gel to set for 30-45 min. 8. Cut large strips of parafilm or use 8-strip tubes and label each sample as a position on the parafilm/tubes. 9. Pipette 1 ul of blue loading dye onto each sample position or into each tube. 10. Pipette 5 ul of DNA sample into loading dye and pipette to mix 2-3 times. 11. Once the gel is set, fill the gel box with enough TBE buffer (1x) to fully submerge the gel beneath ~1cm of buffer. 12. Carefully add samples (6 ul each) to gel and write down their positions. 13. Add 6 ul of ladder dye (green) to gel. 14. Run gel at 100 V for 40-50 min then visualize on gel reader machine.

(OPTIONAL) Purify PCR products using AMPure beads protocol (optimized for Opentrons) 1. Follow along with AMPure XP beads manufacturer protocol (begins on page 5 of manual - https://www.beckmancoulter.com/wsrportal/techdocs?docname=B37419). 2. Adjust bead ratio: 1.2x beads to sample volume for 12S. 3. Will need magnetic plate and fresh 70% ethanol. 4. End product will be ~40 ul of cleaned DNA eluted in molecular grade water.

(OPTIONAL) Run Qubit on final PCR Products 1. Follow manufacturer protocol for running Qubit: https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf.

(OPTIONAL) Run Second 2% Agarose Gel on Purified PCR Products 1. Follow along with previous gel instructions.

5.1.4 Sequencing Preparation

1. After performing a gel on the PCR products, pipette 10 uL of each sample into their respective wells of a new 96-well PCR plate.
   - Leave well H12 empty for Michigan State's sequencing negative control
2. Seal plate, label with ID and place in freezer till day of shipping.
3. Fill out Illumina Sample Submission form with sample information and 96-well plate format.
   - Can be found online at: LIMS Project Submission
4. Login to LabLink and create a project for the sequencing run.
5. Upload project and run information, sample submission form and gel images (annotated) to the project.
6. Prepare plates for shipping by obtaining dry ice (5-10lbs depending on quantity of plates), a styrofoam cooler and fitted cardboard box.
7. Place 1-2 inches of dry ice on bottom of styrofoam cooler followed by sequencing plates then the remainder of dry ice.
8. Place lid on cooler (do not tape shut) and place cooler into cardboard box.
9. Tape the cardboard box shut and attach a shipping label.

5.1.5 Sequencing Facility Protocol Information on sequencing is provided by Michigan State University's Genomics Core Facility:

The Genomics Core performs a secondary PCR using dual-indexed, Illumina compatible primers which target the Fluidigm CS1/CS2 oligomers at the ends of the primary PCR products. Amplicons are batch normalized using the Invitrogen SequalPrep DNA Normalization plates and the recovered product is pooled. The pool is QC'd and quantified using a combination of Qubit dsDNA HS and Agilent 4200 TapeStation HS DNA1000 assays.

The amplicon pool often has two target peaks (plus a likely primer dimer peak) in which the smaller of the two, a eukaryotic derived amplicon is the focus. This peak is isolated using the Sage Science BluePippin instrument. The pool is QC'd and quantified using a combination of Qubit dsDNA HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Library Quantification qPCR assays.

Each pool is loaded onto one (1) Illumina MiSeq v2 Standard flow cell. Sequencing is carried out in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent cartridge. Custom sequencing and index primers complementary to the Fluidigm CS1 and CS2 oligomers are added to appropriate wells of the reagent cartridge. Base calling is done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA is demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. A summary of the run output is provided by MSU and basic QC information about sequence data is provided by the accompanying

FastQC reports. For information regarding interpretation of these reports, please see the FastQC Tutorial and FAQ from MSU's website.

Data is downloaded using an account on the Genomics FTP server. See the Genomics FAQ for general instructions. Sequence data typically remains available on the FTP server for 60 days. It is the responsibility of the researcher to download and store data long term. The RTSF Genomics Core only guarantees retention of sequence data for one year from the date of availability.

5.2 Basic troubleshooting guide

Low Volume Post-PCR

- If using strip-caps, ensure they are tightly fitting on wells. Any gap in the lid will allow for some volume to evaporate during the PCR process on the thermal cycler. If using PCR plate seals, spin down the plate after taking it off the thermal cycler to ensure all condensation is drawn back into the well.

Contamination

- If there are contamination bands appearing on the gel, run another PCR ensuring full sterilization of work spaces and equipment under the hood and use new vials of AmpliTaq Gold and molecular water. If diluted primers are contaminated, use freshly-made aliquot of primers.

Weak Amplification

- If there are weak amplification bands on the gel, ensure the master mix and DNA is being fully mixed. You can also increase the concentration of primers or tweak the PCR process on the thermal cycler (increasing # of cycles of PCR or optimize annealing temperature). The addition of Bovine Serum Albumin (BSA) to master mix is also useful in some cases.

6 REFERENCES

Not applicable.

7 APPENDIX A: DATASHEETS

Not applicable.