Contents

NMNH PCR Protocol 12S rRNA MiFish (Miya)

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

PREPARED BY	AFFILIATION	ORCID	DATE
Katie Murphy	Smithsonian NMNH	not provided	2023-02-10
Allen Collins	Smithsonian NMNH	https://orcid.org/0000-0002-3664-9691	not provided
Steven Auscavitch	Smithsonian NMNH	https://orcid.org/0000-0001-5777-4814	not provided
Luke Thompson	NOAA/AOML, MSU/NGI	https://orcid.org/0000-0002-3911-1280	2025-12-19

PREPARED BY	AFFILIATION	ORCID	DATE
Sammy Harding	NOAA/AOML, MSU/NGI	https://orcid.org/0009-0008-8885-6140	2025-12-19

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/aomomics/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	2025-12-19	Initial release
1.1.0	2026-01-05	Updated protocol with NMNH PCR protocol details

1.6 Acronyms and Abbreviations

ACRONYM / ABBREVIATION	DEFINITION
NMNH	National Museum of Natural History
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

The 12S metabarcoding protocol detailed here is designed to prepare 12S fragments for sequencing on Illumina platforms using a two-step amplification process. This protocol was created and is used by the Smithsonian Institution, National Museum of Natural History.

2.2 Method description and rationale

The first step (amplification PCR) will amplify your region of interest and add an iTru tail. This tail acts as a priming site for the second step (indexing PCR) that will add indices and the Illumina-required adapter to your product. The resulting libraries are dual-indexed and can be sequenced on any Illumina sequencer.

2.3 Spatial coverage and environment(s) of relevance

This protocol is designed such that it can be modified for any organism or gene by using custom primers in place of the 12S primers for the amplification PCR, as long as the primers also contain the appropriate iTru Tail. To modify, replace the 12S forward and 12S reverse portions of the primer (in the case below, MiFish_U_F and MiFish_U_R, respectively) with your specific forward and reverse primers. The indexing primers will remain the same regardless of your study, so you will not need to modify these.

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 8 hours 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
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
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

DESCRIPTION	PRODUCT NAME AND MODEL	MANUFACTURER	QUANTITY	REMARK
10 ul Filter tips	TipOne Pipette Tips, 10 uL	TipOne	2	(boxes) Can be substituted with generic
2X KAPA HiFi Master Mix	2X KAPA HiFi Master Mix	Roche	1.2	(mL)
Molecular water	Invitrogen RT-PCR Grade Water	Fisher Scientific	0.912	(mL)
Forward Primer - 12S MiFish-U-F	12S MiFish-U-F	IDT	105	(ul (10uM)) Primer must be diluted from 100uM stocks to 10uM
Reverse Primer - 12S MiFish-U-R	12S MiFish-U-R	IDT	105	(ul (10uM)) Primer must be diluted from 100uM stocks to 10uM
iTru-tailed locus-specific primers Indexing primers (i5 & i7)	iTru-tailed locus-specific primers Indexing primers (i5 & i7)			
KAPA Pure Beads	KAPA Pure Beads	Roche		
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
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)

- 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 Step 1 – Quantification of DNA Step 1 is not always mandatory, especially if you are amplifying a large number of samples for barcoding (instead of metabarcoding). However, not quantifying may affect amplification success and product concentration.

1. Quantify your DNA sample(s) using QuBit or Quant-iT.

5.1.2 Step 2 – 12S PCR This protocol describes the use of triplicate reactions. Please note that your project may require more replicates than this; we strongly suggest you review recent literature when planning your project.

1. Retrieve reagents from the freezer and allow them to thaw at room temperature. Vortex and spin down.
2. Perform the initial PCR in triplicate using 10 µL reactions. Follow the recipe below to make your master mix (scale up accordingly). Volumes are given in µL. Mix thoroughly, then spin briefly.

Component	Per Reaction (uL)	Final Concentration
Nuclease-free water	3.4	not applicable
2X KAPA HiFi master mix	5.0	1X
12S MiFish-U-F with tail (10 M)	0.3	0.3 M
12S MiFish-U-R with tail (10 M)	0.3	0.3 M
Template DNA	1.0	varies

PCR Primer Name	Direction	Sequence (5' -> 3')	Sequence with iTru tails
12S MiFish-U-F_iTru_HS	forward	GTCGGTAAAACTCGTGCCAGC	ACACTCTTTCCCTACACGACGCTCTTCCGATC xxx GTCGGTAAAACTCGTGCCAGC
12S MiFish-U-R_iTru_HS	reverse	CATAGTGGGGTATCTAATCCCAGTTTG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGA xxx CATAGTGGGGTATCTAATCCCAGTTTG

3. 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, then spin to collect liquid to the bottom of wells.
4. Place tubes/plate into a thermocycler and run the following program:

PCR step	Temperature	Duration	Repetition
Initial Denaturation	95°C	3min	1x
Denaturation	98°C	20s	35x
Annealing	64°C	15s	35x
Extension	72°C	30s	35x
Final Extension	72°C	1min	1x
Hold	12°C	∞	

5. After the PCR finishes, go directly to Step 3, or samples may be stored at 4°C for up to 3 days (or at 20°C for longer periods).

5.1.3 Step 3 – Gel verification & Pooling

1. Prepare a 1.5% agarose gel.
2. Mix 2 µl PCR product from Step 2 with 2 µl 2X loading dye/10X GelRed. Run gels for 6 minutes at 125V.
3. Product size should be 275–321 bp for MiFish 12S primers with heterogeneity spacers.
4. Pool PCR replicates for each sample, omitting any that had incorrect bands present.
5. Go directly to Step 4, or samples may be stored at 4°C for up to 3 days (or at -20°C for longer periods).

5.1.4 Step 4 – PCR Cleanup with KAPA Pure Beads Clean up your 12S PCR products using either 1.5X KAPA Pure beads If you have less than 24 uL per sample, please scale the amount of beads accordingly. If you are using a different locus, you may need to adjust the bead ratio based on your amplicon size.

1. Remove KAPA Pure beads from the fridge and allow to come to room temperature. While you wait, make fresh 80% Ethanol (you need 450 µl per sample).
2. Vortex the beads until fully resuspended (~30 seconds).
3. To 24 µL of PCR product, add 36 µL of KAPA Pure beads (1.5X 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 (steps 8-10).
12. Carefully remove any residual ethanol using a P20 pipette.
13. Allow beads to air dry for 3 to 5 minutes (not longer!).
14. Remove the plate from the magnet. Add 22 µL of 10 mM Tris-HCl (pH 8.0) (or TLE: 10 mM Tris-HCl, 0.1 mM EDTA) 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).

5.1.5 Step 5 – Indexing PCR

1. Determine which indices will be used for which samples; make sure to record these exactly. Each sample needs an i7 and an i5. a. Make sure you use the indices that match the tails on your locus-specific primers (eg. iTru indices w/ iTru-tailed locus primers).

2. Thaw the 2X KAPA HiFi master mix and indexing primers. Once thawed, briefly vortex the master mix. Invert the indexing primers ~10 times to mix. Spin briefly.
3. Set up the following 25 µL PCR reactions.
    a. Remember, your indices are sample-specific, so do NOT include them in your master mix if you choose to make one.

Component	Per Reaction (uL)	Final Concentration
Nuclease-free water	9.0	not applicable
2X KAPA HiFi master mix	12.5	1X
i7 index (10 M)	0.75	0.3 M
i5 index (10 M)	0.75	0.3 M
Template (12S tailored product)	2.0	varies

4. If using a reaction mix (that includes everything except indices and DNA), pipette 21.5 µL of master mix into each well of your plate (or tubes).
5. Add 0.75 µL of the required i5 primer (S5xx or i5xx) to each well. Next, add 0.75 µL of the required i7 primer (N7xx or i7xx) to each well.
6. Lastly, add 2 µL of your template DNA (12S PCR product). a. Your total reaction volume in each well should be 25 µL.
7. Using a P20 or P200 pipette, set to ~15 µL, mix up/down 5 times. Avoid making bubbles.
8. Seal the plate with a silicone mat or plate seal and spin briefly to collect liquid at the bottom of the wells.
9. Place the plate (or tubes) into a thermocycler and run the following indexing program with 5-8 cycles:

PCR step	Temperature	Duration	Repetition
Initial Denaturation	95°C	3min	1x
Denaturation	98°C	30s	5-8x
Annealing	65°C	15s	5-8x
Extension	72°C	30s	5-8x
Final Extension	72°C	1min	1x
Hold	12°C	∞	

10. After the PCR finishes, go directly to Step 6, or samples may be stored at 4°C for up to 3 days (or at 20°C for longer periods).

5.1.6 Step 6 – Gel verification

1. Prepare a 1.5% agarose gel.
2. Mix 2 µl PCR product from Step 6 with 2 µl 2X loading dye/10X GelRed. Run gels for 6 minutes at 125V.
3. Product size should be 344-384 bp for correctly indexed libraries. A second band at ~275-305 bp indicates that not all of your 12S PCR product was indexed.

5.1.7 Step 7 – PCR Cleanup Clean up your PCR products using KAPA Pure beads. If you have less than 23 uL per sample, please scale the amount of beads accordingly.

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. In your indexing PCR plate, add 18 µl of KAPA Pure beads to the ~23 µL of PCR product. This represents a ~0.8X bead-to-sample ratio. a. This ratio is dependent upon the size of the product. You can use less for larger products (refer to KAPA Pure manual).
4. Mix thoroughly by pipetting at least 10 times, until the mixture is homogenous.
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 200 µL 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 (steps 8-10).
12. Carefully remove any residual ethanol using a P20 pipette. Let the plate dry on the magnet for 3-5 minutes.
13. Remove the plate from the magnet. Add 22 µL of 10 mM Tris-HCl (pH 8.0) (or TLE: 10 mM Tris-HCl, 0.1 mM EDTA) to each well and use a pipette to fully resuspend the beads.
14. Incubate the plate at room temperature for 5 minutes.
15. Place the plate back on the magnet for ~3 minutes, or until the supernatant clears.
16. Carefully transfer 20 µL of the clear supernatant to a new plate/tube.
17. After the cleanup, go directly to Step 8, or samples may be stored at 4°C for up to 3 days (or at -20°C for longer periods).

5.1.8 Step 8 – Library Quantification & Pooling

1. Quantify your libraries using the Qubit or Quant-iT HS dsDNA assay kit, following manufacturer's instructions.
2. Pool each library to be sequenced in equal ng or equimolar amounts in a new 1.5 mL tube: a. If you are using a single gene and all samples will be approximately the same size (such as with 12S or COI), you can pool using ng. b. If you are using multiple amplicons that vary in size, you should instead pool in equimolar amounts.

5.1.9 Step 9 – Final Pool QC

1. Use HS dsDNA Qubit or Quant-iT to measure the concentration (ng/µL) of your library pool.
2. Determine the molarity (nM) of your pool using the equation given in the "Additional Information" section of this guide.
3. Run a 1:10 dilution of your pool on a HS D1000 tape or on the Bioanalyzer, to ensure the product is the correct size and no adapter-dimer is present. a. Alternatively, you can run 2 uL of your undiluted pool on a 1.5% agarose gel with an appropriate ladder. However, this will not be as sensitive as the Tapestation or Bioanalyzer.
4. Your pool should be stored at -20°C until submitted for sequencing.

5.1.10 Additional Information If you need to convert from ng/ul to nM, use the following equation:

$$\frac{\text{concentration (ng/µL)}}{660\,\text{g/mol} \times \text{average library size (bp)}} \times 10^6 = \text{concentration (nM)}$$

5.1.11 Quality Control An initial quantification of DNA is performed to ensure product concentration and amplification success. PCR triplicates are used to reduce PCR bias and increase reliability. Initial PCR products are verified using gel electrophoresis followed by a cleanup step. PCR products are indexed and once again verified using gel electrophoresis followed by another cleanup step. Libraries are quantified and pooled. The final pool is quantified and stored at -20°C.

5.2 Basic troubleshooting guide

Please note, if you use alternative locus-specific primers that include inosines, you must use Taq (such as GoTaq) instead of a high fidelity, proof-reading DNA polymerase. If your primers do not contain inosine, then we recommend using a high fidelity enzyme. Based on our experience, we now recommend using KAPA HiFi over NEB Q5, as KAPA HiFi has shown more consistent amplification. Please make sure to adjust thermocycler conditions accordingly.

For running these libraries on the MiSeq, we recommend spiking 20% PhiX into your pool due to the low complexity of amplicons, assuming your primers did not include heterogeneity spacers. If your locus-specific primers include heterogeneity spacers, then the PhiX spike-in can be decreased to 1%.

6 REFERENCES

Glenn, T. C., R. Nilsen, T. J. Kieran, J. W. Finger Jr., T. W. Pierson, K. E. Bentley, S. L. Hoffberg, S. Louha, F. J. Garcia-De Leon, M. A. D. R. Portilla, K. D. Reed, J. L. Anderson, J. K. Meece, S. E. Aggrey, R. Rekaya, M. Alabady, M. Belanger, K. Winker, and B. C. Faircloth. Adapterama I: Universal stubs and primers for thousands of dual-indexed Illumina libraries (iTru & iNext). Preprint at http://biorxiv.org/content/early/2016/06/15/049114

Glenn TC, Pierson TW, Bayona-Vásquez NJ, Kieran TJ, Hoffberg SL, Thomas IV JC, Lefever DE, Finger JW, Gao B, Bian X, Louha S, Kolli RT, Bentley KE, Rushmore J, Wong K, Shaw TI, Rothrock Jr MJ, McKee AM, Guo TL, Mauricio R, Molina M, Cummings BS, Lash LH, Lu K, Gilbert GS, Hubbell SP, Faircloth BC. 2019. Adapterama II: universal amplicon sequencing on Illumina plat- forms (TaggiMatrix). PeerJ 7:e7786 http://doi.org/10.7717/peerj.7786.

Miya, M., Y. Sato, T. Fukunaga, T. Sado, J.Y. Poulson, K. Sato, T. Minamoto, S. Yamamoto, H. Yamanaka, H. Araki, M. Kondoh, W. Iwasaki. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society Open Science, 2: 150088.

Bell, J. (2011). Overview of tailed amplicon sequencing approach with MiSeq. Illumina.

KAPA Pure Beads technical data sheet, KR1245 – v3.16. July 2016. 6. Herbold, C.W. et al. (2015). A flexible and economical barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. Front Microbiol. https://doi.org/10.3389/fmicb.2015.00731

16S Metagenomic sequencing library preparation. Illumina part # 15044223 Rev. A.

Smithsonian Global ARMS Program – 18S Metabarcoding protocol.

7 APPENDIX A: DATASHEETS

Not applicable