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12S rRNA-Gene Metabarcoding Library Prep: Dual-PCR Method

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Andreas Novotny¹

¹University of British Columbia

Pelagic Ecosystems Lab

Tech. support email: a.novotny@oceans.ubc.ca



Andreas Novotny

University of British Columbia, Hakai Institute, Stockholm U...

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Protocol status: Working

We use this protocol and it's working

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Keywords: mitochondrial 12s rRNA gene, level resolution of fish diversity, fish diversity, rRNA gene, end illumina miseq sequencing, part of the urban ocean biodiversity project, urban ocean biodiversity project, fishery, PCR method this protocol, PCR method, eDNA metabarcoding, fish, species, PCR, institute for the ocean, sequencing

Abstract

This protocol is used for **eDNA metabarcoding of the mitochondrial 12S rRNA gene (Miya et al 2015)** using Pair-End Illumina Miseq Sequencing. This protocol was implemented as part of the Urban Ocean Biodiversity project, a collaboration between the University of British Columbia's Institute for the Oceans and Fisheries, and Tsleil-Waututh First Nation. This protocol is developed to give a **species-level resolution of fish diversity**.

Guidelines

MIOP: Minimum Information about an Omics Protocol

MIOP Term	Value
analyses	Amplicon Sequencing, 12S
audience	scientists
broad-scale environmental context	marine biome ENVO_00000447
creator	Andreas Novotny
environmental medium	sea water [ENVO:00002149]
geographic location	North Pacific Ocean [GAZ:00002410]
hasVersion	1
issued	2024
language	en
license	CC BY 4.0
local environmental context	coastal sea water [ENVO: 00002150]
materials required	Sterile workbench, Thermo Cykler, MiSeq, Gel Electrophoresis syste, Qbit, Bioanalyzer
maturity level	Mature
methodology category	Omics Analysis
personnel required	1
project	Urban Ocean Biodiversity
publisher	University of British Columbia, Pelagic Ecosystems Lab.
purpose	DNA metabarcoding
skills required	sterile technique pipetting skills
target	Fish mitochondrial DNA
time required	3-5 days

AUTHORS

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://orcid.org)
Rute Carvalho	Hakai Institute	https://orcid.org/0000-0001-
Colleen Kellogg	Hakai Institute	https://orcid.org/0000-0001-
Matt Lemay	Hakai Institute	https://orcid.org/0000-0001-
Andreas Novotny	University of British Columbia	https://orcid.org/0000-0001-

RELATED PROTOCOLS

PROTOCOL NAME AND LINK	ISSUER / AUTHOR	RELEASE DATE This is the date corresponding to the version listed to the left
Content Cell	Content Cell	yyyy-mm-dd
Content Cell	Content Cell	yyyy-mm-dd

This is a list of other protocols which should be known to users of this protocol. Please include the link to each related protocol.

ACRONYMS AND ABBREVIATIONS

	ACRONYM / ABBREVIATION	DEFINITION
	Content Cell	Content Cell

GLOSSARY

	SPECIALISED TERM	DEFINITION
	Content Cell	Content Cell
	Content Cell	Content Cell

BACKGROUND

This protocol is used for **eDNA metabarcoding of the mitochondrial 12S rRNA gene (Miya et al 2015)** using Pair-End Illumina MiseqSequencing.

Citation

M. Miya , Y. Sato , T. Fukunaga , T. Sado , J. Y. Poulsen , K. Sato , T. Minamoto , S. Yamamoto , H. Yamanaka , H. Araki , M. Kondoh and W. Iwasaki (2015) . MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R. Soc. Open Sci..
<https://doi.org/10.1098/rsos.150088>

LINK

The protocol builds on the Hakai Institutes protocol for 12S metabarcoding, but the bead cleanup protocol have been updated for a more efficient cleanup, for the smaller 12S fragment. More specifically, a sample-to-bead ratio of 1.2 or 1.5 instead of 0.8. See the parent protocol here:

Protocol

NAME

12S rRNA-Gene Metabarcoding Library Prep: Dual-PCR Method

CREATED BY

Andreas Novotny

Preview

Method description and rationale

Assuming extracted DNA as starting material, this protocol includes the following steps:

1. First PCR: Triplicate locus-specific amplification of the mitochondrial 12S rRNA gene (Miya et al 2015) .
2. First PCR product purification (using magnetic beads)
3. Second PCR: Sample indexing using Nextera V2 indexing primers
4. Second PCR product purification (using magnetic beads)
5. Quantification and Pooling
6. Library concentration (using magnetic beads)
7. Gel purification with size selection
8. Quality control
9. Pair End Sequencing on Illumina MiSeq V3 2*300 bp

Due to the risk of cross contamination, it is pivotal to separate work with amplified PCR products from pre-PCR steps. We preform pre-PCR steps (including DNA extractions) in separate clean rooms on surfaces steralized with hydrogen peroxide (PreEmpt) and UV.

Spatial coverage and environment(s) of relevance

This protocol was implemented as part of the Urban Ocean Biodiversity project, a collaboration between University of British Columbias Institute for the Oceans and Fisheries, and Tsleil-Waututh First Nation. In 2023 we sambled multiple stations in the Burrard Inlet BC, Canada (from Point Grey to Port Moody Arm, Including the Port of Vancouver). This protocol is developed to give a **species-level resolution of fish diversity**.

Personnel Required

1 Technician

Safety



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

Training requirements

Serile work technique, pipetting skills, PCR, gel electrophoresis.

Time needed to execute the procedure

This protocol will take several days to complete depending on sample size.

Materials

Equipment

- pre-PCR and post-PCR separated workspaces
- Thermocycler (1 or 3)
- Gel electrophoresis equipment
- Qubit or plate reader
- Magnetic plate
- BioAnalyzer
- Real-Time PCR
- Illumina MiSeq

Protocol materials

- ☒ Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo Fisher Catalog #12368050**
- ☒ 100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog #G2101**
- ☒ Gel Red Nucleic Acid Gel Stain **Biotium Catalog ##41003**
- ☒ Qubit dsDNA Broad Range assay kit (500 assays) **Invitrogen - Thermo Fisher Catalog #Q32853**
- ☒ Quant-iT dsDNA Pico Green assay kit (Invitrogen) **Life Technologies Catalog #P7589**
- ☒ Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**
- ☒ Bioanalyzer chips and reagents (DNA 1000) **Agilent Technologies Catalog #5067-1504**
- ☒ MiSeq v3 (150 cycle) Kit **Illumina, Inc. Catalog #MS-102-3001**
- ☒ PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**
- ☒ TBE Buffer, 10X Molecular Biology Grade, 1000ml **Promega Catalog #V4251**
- ☒ RedSafe Nucleic Acid Staining Solution **Froggabio Catalog #21141**
- ☒ Wizard® SV Gel and PCR Clean-Up System **Promega Catalog #A9281**
- ☒ NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog #E7630S**

Before start

Read Minimum Information about an Omics Protocol (MIOP) and other recommendations under the "Guidelines" tab.

Preparations

- 1 Ensure that the laboratory is appropriately configured and that staff has appropriate training. See "Guidelines" for more information. Pay attention to the separation of pre and post-PCR spaces and equipment.
- 2 Ensure that all reagents are aliquoted in appropriate amounts, and stored according to manufacturers' recommendations. Never pipet directly from reagent stocks.
- 3 Prepare the SPRI beads' working solution, and test their efficiency following this protocol.



Protocol

 NAME
Serapure Beads Preparation and Testing

CREATED BY
 Andreas Novotny

Preview

- 4 Prepare primer working stocks (10µM) for both the first and second PCR steps. Here we use Nextera V2 Kit Sets A, B, C, and D. We advise preparing the indexing primers on 96-well plates according to this configuration:

 Indexes_plate.xlsx 38.5KB

- 5 We advise adding aliquots of the extracted DNA to a 96-Well PCR plate to facilitate the setup of the PCR reaction. This metadata template will help keep track of the samples, and if indexes are configured as described above, also the identity of sample indexes.

Triplicate PCR Amplification (1st PCR)

6 Preparations



Note

1. **Prepare PCR reactions in a clean working space (such as a biosafety cabinet) dedicated to pre-PCR tasks only.**
2. Do not need to Qubit DNA samples before starting, only do it if the reaction does not work.
3. **Use UNDILUTED DNA**
4. Test at least 8 samples before doing a batch/plate.
5. Include a negative control, an extraction blank (if you have it), and a positive control.
6. After testing, **perform the PCR for all of the samples in triplicates.**

Reagents:

☒ Molecular Biology Grade Water **Corning** Catalog #46-000-CV (Or equal)

☒ Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo**
Fisher Catalog #12368050

☒ Froggarose LE **Froggabio** Catalog #A87-500G (Or equal)

☒ 100bp DNA Ladder, 250ul (50 lanes) **Promega** Catalog #G2101 (Or equal)

☒ Gel Red Nucleic Acid Gel Stain **Biotium** Catalog ##41003 (Or equal)

- Custom-designed primers (*Miya et al 2015*) including:

PCR Primer Name	Direction	Sequence (5' → 3')
MiFish-U-F_overhang	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GGTCGGTAAACTCGTGCCAGC

PCR Primer Name	Direction	Sequence (5' → 3')
MiFish-U-R_overhang	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGCATAGTGGGGTATCTAATCCCAGTTG

7 **UV for 30 minutes the following:**

- 96-well PCR plates (or 8-strip tubes)
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water

Thaw Platinum, Primers, and nuclease-free water. Keep them in a cooling microcentrifuge tube rack.

8 PCR reactions are carried out in triplicate 12.5 µl reactions:

Reagent	Volume (µl)
Sterile Nuclease-Free water	3.65
Forward primer (10µM)	0.3
Reverse Primer (10µM)	0.3
Platinum SuperFi	6.25
DNA (1-10 ng)	2
TOTAL	12.5

30m



9 Seal the 96-well plates and transfer them to thermocyclers.

2h



Note

Amplified PCR products should never come in contact with equipment used for non-amplified DNA.
From this point, no samples will reenter the pre-PCR working space.

PCR step	Temperature	Duration	Repetition
denaturation	94°C	3 minutes	
denaturation	94°C	30 seconds	
annealing	63°C	30 seconds	
extension	68°C	30 seconds	
GO TO step 2			39 times
final extension	68°C	10 minutes	
HOLD	12°C	HOLD	

10 Pool the three replicates.

11 Run sample pools on a 2% gel (90V, ~40-60 min) to check the size of the amplicons and the success of the PCR (5µl/sample).

1h



**Expected result**

You may see 2 amplified fragments: one ~ 300 bp, and other one ~400 bp. The target band is ~300 bp, do not worry if you see two bands, because we will eliminate the larger one at the end of the library prep.

Purification of first PCR product using SPRI beads**12 Preparations****Note**

Prepare the purification in the post-PCR working space.

Size selection can be achieved using different ratios of magnetic beads to sample. A ratio of bead to a sample of 1.2 will efficiently purify the amplicons away from primer dimers and allow the selection of fragments larger than 200 bp. (Note, this is a higher ratio than for 18S and COI, as the fragment is smaller).

Materials

- Serapure SPRI beads. If not already prepared: ➡
- Magnetic 96-well plate stand
- Anhydrous Ethanol to make a fresh 80% ethanol solution
- Molecular grade water

UV for 30 minutes the following:

- 96-well PCR plates (or 8-strip tubes)
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water

Remove the magnetic beads from the fridge (allow 30 min to reach room temperature).

- 13
- Vortex the beads before use.
 - As you now have about 32.5µl reaction (pooled samples - 5µl used on a gel/Qiaxcel), add 39 µl beads to the product to obtain a ratio of 1.2.
 - Pipette up and down about ten times (or until the solution is well mixed – you will see that the color changes).
 - Spin tubes down to remove drops from the walls.
- 14
- Incubate a room temperature without shaking for 5 min. For samples that you know have a low DNA concentration, you can increase this incubation time to 30 min.
 - Place the plate on the magnetic stand until the supernatant has cleared (~ 3 min).
- 15
- Remove the supernatant with a multichannel pipette, ensuring to not disturb the beads.
- 16
- With the samples on the magnetic rack, wash the beads by adding 180 µl of freshly prepared 80% ethanol and incubate for 30s. Carefully remove the supernatant without disturbing the beads.
- 17
- Repeat the washing step ➡ [go to step #16](#)
- 18
- Remove all residual ethanol using a pipette and air dry, leaving the samples on the magnetic stand (~ 5 min*).

8m

5m

10m

10m

5m





Note

*This depends on the type of the magnetic rack – the O-ring magnet dries faster than the side magnet. Keep an eye on the beads and do not over-dry. Otherwise, you will not get an efficient DNA recovery.

- 19 Remove the plate from the magnetic stand and add 32 μ l of nuclease-free water for elution. Gently pipet up and down ten times to resuspend the beads. Incubate the plate at room temperature for 5 min. 5m
- 20 Place the plate back on the magnetic rack for at least 5 min or until the supernatant is cleared. 5m
- 21 Carefully transfer 30 μ l of the clear supernatant to a new plate. Seal the plate.
- 22 Name the plate: Project, [Gene_name], PCR 1, Post-Purification Plate #, Date, Initials. Samples can be stored at -20°C for up to 7 days. II

Indexing PCR amplification (2nd PCR)

23 Preparations

Reagents:

- Platinum™ SuperFi II PCR Master Mix **Invitrogen - Thermo**
Fisher Catalog #12368050
- Molecular Biology Grade Water **Corning** Catalog #46-000-CV
- Froggarose LE **Froggabio** Catalog #A87-500G
- 100bp DNA Ladder, 250ul (50 lanes) **Promega** Catalog #G2101
- Gel Red Nucleic Acid Gel Stain **Biotium** Catalog ##41003

- i5 and i7 index plates (10 μ M) – If not already prepared: ➞ [go to step #4](#)

PCR Primer Name	Direction	Sequence (5' → 3')
Nextera V2 Index1	forward	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTC GTGGGCTCGG
Nextera V2 Index 2	reverse	AATGATACGGCGACCACCGAGATCTACAC[i5]T CGTCGGCAGCGTC

UV for 30 minutes the following:

- 96-well PCR plates (or 8-strip tubes)
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water

Thaw Taq, i5 and i7 indexes, and nuclease-free water. Keep them in the IsoFreeze microcentrifuge tube rack.

24 Prepare PCR reaction in 25 μ l reactions:

Reagent	Volume (μ l)
Sterile Nuclease-Free water	5
Forward primer (10 μ M)	2.5
Reverse Primer (10 μ M)	2.5
2XTaq	12.5



	Reagent	Volume (μl)
	DNA (1-10 ng)	2.5
	TOTAL	25

- 25 Seal the 96-well plates and transfer them to thermocyclers.



	PCR step	Temperature	Duration	Repetition
	denaturation	94°C	3 minutes	
	denaturation	94°C	30 seconds	
	annealing	55°C	30 seconds	
	extension	68°C	30 seconds	
	GO TO step 2			7X
	final extension	68°C	5 minutes	
	HOLD	12°C	HOLD	

- 26 Run the product on a 2% agarose gel to check the size of the amplicons and success of the PCR (5μl).



Note

Again, you may see two bands (~350bp and ~450bp).

Purification of indexed libraries (Second bead cleanup)

27 Preparations

Materials

- Prepared serapure SPRI beads.
- Magnetic 96-well plate stand
- Anhydrous Ethanol to make a fresh 80% ethanol solution
- Molecular grade water

UV for 30 minutes the following:

- 96-well PCR plates (or 8-strip tubes)
- Sharpie
- Pipette tips
- Multichannel pipettes
- Pipettes
- Sterile Nuclease-Free Water

Remove the magnetic beads from the fridge (allow 30 min to reach room temperature).

- 28
- Vortex the beads before use.
 - Add 24 μl beads to 20 μl of PCR product to obtain a ratio of 1.2.
 - Pipette up and down ten times (or until the solution is well mixed – you will see that the color changes).
 - Spin tubes down to remove drops from the walls.
- 29
- Incubate a room temperature without shaking for 5 min. For samples that you know have a low DNA concentration, you can increase this incubation time to 30 min.
 - Place the plate on the magnetic stand until the supernatant has cleared (~ 3 min).
- 30
- Remove the supernatant with a multichannel pipette, making sure not to disturb the beads.
- 31
- With the samples on the magnetic rack, wash the beads by adding 180 μl of freshly prepared 80% ethanol.



- Incubate for 30 s.
- Carefully remove the supernatant without disturbing the beads.

32 Repeat the washing step. ➞ [go to step #31](#)

33 Remove all residual ethanol using a pipette and air dry, leaving the samples on the magnetic stand (~ 5 min*). Keep an eye on the beads and do not over-dry, otherwise, you will not get an efficient DNA recovery.

Note

*the length of time depends on the type of the magnetic rack – the O-ring magnet dries faster than the side magnet.

34

- Remove the plate from the magnetic stand and add 28 µl of nuclease-free water for elution.
- Gently pipette up and down ten times to resuspend the beads.
- Incubate the plate at room temperature for 5 min.

35 Place the plate back on the magnetic rack for at least 5 min or until the supernatant has cleared.

36 Carefully transfer 25 µl of the clear supernatant to a new plate. Seal the plate.

37 Name the plate: Project, [Gene_name], PCR 1, Post-Purification Plate #, Date, Initials.
Samples can be stored at -20°C for up to 7 days.



Quantification and pooling, and quality control

38 Use a fluorometric quantification method that uses dsDNA dyes to measure the concentration of your libraries (Qubit or plate reader). If using Qubit, give preference to the broad range kit if you visualize a strong band in the gel:

☒ Qubit dsDNA Broad Range assay kit (500 assays) **Invitrogen - Thermo**
Fisher Catalog #Q32853

OR

☒ Quant-iT dsDNA Pico Green assay kit (Invitrogen) **Life**
Technologies Catalog #P7589

Expected result

Samples will have approximately similar concentrations (usually). Re-check samples that showed very high or low concentrations on Qubit/plate reader quantification.

39 Calculate sample volume to have a final amount of 10-40 ng. This amount may vary depending on the overall quantification. For example, if on average the concentration of your samples is about 3 ng/µl and you have 20 µl of product, you can calculate the volume to make up to 60 ng per sample.

Note

Check the final volume that you will get after pooling – sometimes you will end up with 2 mL or more. Then use the proper Eppendorf tube for pooling (1.5, 2.0, or 5 mL).

40 Measure the final library pool concentration on Qubit using

☒ Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**



- 41 Label tube: [Gene_name], [Project_Name], Pooled Amplicons. Date, Initials, pool concentration.

Concentrating library pool with magnetic beads

42

Note

You may need to perform an extra beads purification step to decrease the volume of your pool. For example, if you ended up with 2 mL pool you won't be able to load this volume in the gel in the next step of the library prep. So, decreasing the volume to 40-50 μ l is necessary at this point.

Since the purpose of this protocol is to concentrate the library, not selecting the size, we increase the bead-to-sample ratio to 1.5 to get maximum DNA recovery.

Materials

- Magnetic beads* (SPRI beads or AMPure XP, find aliquots in fridge)
- Magnetic rack for 1.5-2 mL tubes
- Anhydrous Ethanol to make a fresh 80% ethanol solution
- Sterile Nuclease-Free water

UV for 30 minutes the following:

- 1.5 mL Io-Bind tubes
- Sharpie
- Pipette tips
- Pipettes
- Sterile Nuclease-Free Water

Remove the magnetic beads from the fridge (allow 30 min to reach room temperature).

- 43 Aliquot your pool into 1.5 mL tubes (try to keep similar volumes between aliquots, a maximum of 350 μ l per tube). If you have a 2 mL pool, you can aliquot 350 μ l pool in 5 tubes, and have the 6th tube with 250 μ l.
- 44 Calculate the volume of beads to add into each tube (1.5 x beads). For the tubes with 300 μ l – add 450 μ l beads, and for the tube with 200 μ l, add 300 μ l beads. You will obtain a ratio of 1.5. Pipette up and down ten times (or until the solution is well mixed – you will see that the color changes). Spin tubes down to remove drops from the walls. Incubate a room temperature without shaking for 5 min.
- 45 Place the tubes on the magnetic rack until the supernatant has cleared (~ 3 min).
- 46 Remove the supernatant with a P1000 pipette, making sure not to disturb the beads.
- 47 With the samples on the magnetic rack, wash the beads by adding 500 μ l of freshly prepared 80% ethanol and incubate for 30 s. Carefully remove the supernatant without disturbing the beads.
- 48 Repeat washing step ➡ [go to step #47](#)
- 49 Remove all residual ethanol using a pipette and air dry, leaving the samples on the magnetic rack (~ 5 min*). Keep an eye on the beads and do not over dry, otherwise you will not get an efficient DNA recovery.

Note

This length of time may vary. It is important to keep an eye on the beads (they must look opaque).



tubes at room temperature for 5 min.

- 51 Place the tubes back on the magnetic rack at least 5 min or until the supernatant is cleared.
- 52 Carefully transfer 30 μ l of the clear supernatant to a single new tube (pooling the volumes of the 6 tubes).
- 53 Repeat the bead cleanup to reduce the volume even more. You may now have 180 μ l in your pool, so you must add 270 μ l of beads to have a rate of 1.5. [➡ go to step #44](#)
Make final elution in 50 μ l of water, transferring 45 μ l of the clear supernatant to a new tube.
- 54 Name the tube: [Gene_name], Project, Concentrated Pool, Date, Initials.

Gel purification with size selection

55

Note

This preparation may take 2 days. Allow enough time for autoclaving the buffer, preparing, and purifying the gel. There are some safe stopping points if you do not have two days in a row available to perform this procedure.

Materials:

- ☒ TBE Buffer, 10X Molecular Biology Grade, 1000ml **Promega Catalog #V4251** (or similar)
- ☒ Froggarose LE **Froggabio Catalog #A87-500G**
- ☒ RedSafe Nucleic Acid Staining Solution **Froggabio Catalog #21141**
- ☒ Wizard® SV Gel and PCR Clean-Up System **Promega Catalog #A9281**
- ☒ 100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog #G2101**

- 55.1 Prepare 1.5-2 L of 1x TBE buffer and autoclave it. Wait for it cool down to room temperature. *safe stopping point.
- 56 Prepare a 3% agarose gel. It may polymerize faster than the gels that you are used to prepare. You may need about 120 mL of buffer and 3.6 g of agarose if using the small electrophoresis system. Add 6 μ l of RedSafe. Use a comb with large teeth – it may accommodate a higher volume of product.
- 57 If you have 45 μ l of product, add 8 μ l of loading dye to the tube and mix well. Load the entire tube's content in the well. You may need more than one well to load the entire content.
- 58 Load the Ladder 100 bp in the first and last well (~3 μ l).
- 59 Run the gel for about 1h at 90V. In this mean time, weigh one 1.5 mL Eppendorf tube and record the weight. Place on your counter: sterile blade for scalpel, a scalpel, and the LED transilluminator.
- 60 When the gel run is done, transfer the gel to the transilluminator, and excise the 350 bp band. Place it in the 1.5 mL tube that you have weighed. * Sometimes the stronger band is the non-target one. That's why it is important to have the ladder running beside your samples to help you to find the right band. See Figure 1.

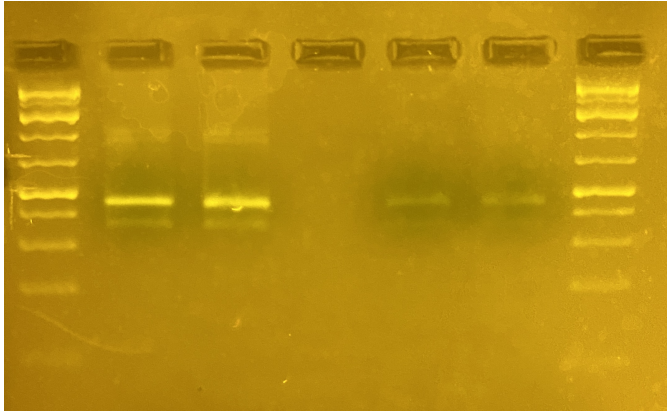


Figure 1. 12S rRNA libraries loaded in the 3% gel. Ladder (100 bp) was loaded in the first and last wells. Second and third well has one library loaded (~20 μ l in each well), fourth well is empty, and the fifth and sixth wells have other 12S library loaded (~20 μ l in each well). Note that the strong band has ~450 bp, but this is not our target!

- 61 Weigh the tube with the gel slice. Record the weight.
- 62 Use the Promega Wizard SV gel and PCR clean-up to perform the gel purification (follow instructions in the manual - <https://www.promega.ca/products/nucleic-acid-extraction/clean-up-and-concentration/wizard-sv-gel-and-pcr-clean-up-system/?catNum=A9281#protocols>).
- 63 Add 10 μ l Membrane Binding Solution per 10 mg of gel slice (for example, if you weight 102 mg of gel slice in the tube, add 102 μ l of Membrane Binding Solution).
- 64 Vortex and incubate at 50-65°C until gel slice is completely dissolved (~10 min). Vortex again to be sure that you do not have any gel in the tube. Spin down.
- 65 Insert SV minicolumn into a collection tube.
- 66 Transfer dissolved gel mixture to the minicolumn assembly. Incubate at room temperature for 1 min.
- 67 Centrifuge at 16,000 x g for 1 min. Discard flowthrough, and reinsert the minicolumn into the collection tube. *dry the collection tube edges using Kim wipes if necessary.
- 68 Add 700 μ l Membrane Wash Solution (ethanol added). Centrifuge at 16,000 x g for 1 min. Discard flowthrough, and reinsert the minicolumn into collection tube.
- 69 Repeat the step 15 with 500 μ l Membrane Wash Solution. Centrifuge at 16,000 x g for 5 min.
- 70 Empty the collection tube and recentrifuged the column assembly for 1 min to allow evaporation of any residual ethanol.
- 71 Carefully transfer minicolumn to a clean 1.5 ml tube.
- 72 Add 35-50 μ l of elution buffer (or Nuclease-Free water) to the minicolumn. Incubate at room temperature for 1 min. Centrifuge at 16,000 x g for 1 min. Perform a second elution if you consider it to be necessary.
- 73 Quantify the purified product on Qubit using the ds DNA BR kit.



74 Label tube: [Gene_name], [Project_Name], Final Library. Date, Initials, concentration.

Sequencing parameters

75 Library fragment size (BP) is determined using

 Bioanalyzer chips and reagents (DNA 1000) **Agilent Technologies** Catalog #5067-1504

76 Molarity of the final pool is assessed using

 NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs** Catalog #E7630S

77 COI libraries are sequenced on a MiSeq instrument using:

 MiSeq v3 (150 cycle) Kit **Illumina, Inc.** Catalog #MS-102-3001 with pair-end

setup (2*300 bp), spiked with 10%

 PhiX Control v3 **Illumina, Inc.** Catalog #FC-110-3001 .

Citations

M. Miya , Y. Sato , T. Fukunaga , T. Sado , J. Y. Poulsen , K. Sato , T. Minamoto , S. Yamamoto , H. Yamanaka , H. Araki , M. Kondoh and W. Iwasaki. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species

<https://doi.org/10.1098/rsos.150088>