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COI Metabarcoding Library Prep: Dual-PCR Method

Forked from PCR Protocol Template

This protocol is a draft, published without a DOI.

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

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Protocol Integer ID: 99147

Disclaimer

Draft!

Abstract

Draft!

Guidelines

MIOP: Minimum Information about an Omics Protocol

MIOP Term	Value
methodology category	Environmental DNA - dual PCR illumina library preparation for the sequencing of cytochrme oxidase I gene.
project	Hakai Genomics
purpose	Metazooan community and diversity assessment from environmental DNA.
analyses	Polymerase chain reaction. Amplicon sequencing.
geographic location	Coastal Noth Pacific Ocean
broad-scale environmental context	Pelagic ecosystem
local environmental context	Coastal monitoring station in Northern Strait of Georgia
environmental medium	Sea water
target	Metazoan invertebrate metacommunity
creator	Hakai Institute
materials required	Illumina Sequencing
skills required	laboratory technician with experience in PCR
time required	
personnel required	
language	English
issued	
audience	
publisher	
hasVersion	
license	
maturity level	

See https://github.com/BeBOP-OBON/miop/blob/main/model/schema/terms.yaml for list and definitions.

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/ to register)	DAT
Content Cell	Content Cell	Content Cell	yyyy-mm-dd
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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
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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 document describes the required protocol to conduct insert name of the method/protocol.

Summary

Insert a short description of the background for the method/protocol (e.g. why and for which purpose do you perform water sampling).
Please provide a brief summary of your method including, as appropriate, a brief description of what techniques your best practice is about, which ocean environments or regions it targets, the primary sensors covered, what type of data/measurements/observing platform it covers, limits to its applicability.

Method description and rationale

Insert a short description of the functioning principal of the methodology used in the protocol (i.e. how does the method work?). Please note that this is different from the step-by-step description of the protocol procedure.
Insert a short statement explaining why the specific methodology used in the protocol has been selected (e.g. it is highly reproducible, highly accurate, procedures are easy to execute etc.…).

Spatial coverage and environment(s) of relevance

If applicable, please specify the region where the protocol is applied. For regional term guidance see here. If applicable, please indicate here the environment(s) of relevance for the protocol, e.g. Abyssal plain. Select from the ENVO terminology.

Personnel Required

Insert the number of technicians, data managers, and scientists required for the good execution of the procedure

Safety

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

Training requirements

Specify technical training required for the good execution of the procedure.

Time needed to execute the procedure

Specify how much time is necessary to execute the procedure.

Materials

EQUIPMENT

	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 qua
	Durable equipment			
	Content Cell	Content Cell	Content Cell	Content Cell
	Content Cell	Content Cell	Content Cell	Content Cell
	Consumable equipment			
	Content Cell	Content Cell	Content Cell	Content Cell
	Content Cell	Content Cell	Content Cell	Content Cell
	Chemicals			
	Content Cell	Content Cell	Content Cell	Content Cell
	Content Cell	Content Cell	Content Cell	Content Cell

Protocol materials

Molecular Biology Grade Water **Corning Catalog #**46-000-CV In 3 steps

Taq FroggaMix **Froggabio Catalog #**FBTAQM96 In 2 steps

BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S Step 6

Froggarose LE **Froggabio Catalog #**A87-500G In 2 steps

100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog #**G2101 In 2 steps

Before start

Read background information, MIOP and BePOP-OBON information 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:

4 Prepare primer working stocks (10µM) for both the first and second PCR steps.
We advise preparing the indexing primers according to this configuration:
Indexes_plate.xlsx 38KB

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 samples diluted 1:10 (1 µl DNA in 9 µl Nuclease-Free Water)
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)
- Taq FroggaMix **Froggabio Catalog #**FBTAQM96
- BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S
- Froggarose LE **Froggabio Catalog #**A87-500G
- 100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog #**G2101

	PCR Primer Name	Direction	Sequence (5' -> 3')
	mlCOIintF_overhang	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC
	dgHCO2198R_overhang	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAAACTTCAGGGTGACCAAARAAYCA

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

7 PCR reactions are carried out in triplicate 25µl reactions: 30m

	Reagent	Volume (µl)
	Sterile Nuclease-Free water	7.3
	Forward primer (10µM)	0.6

	Reagent	Volume (µl)
	Reverse Primer (10µM)	0.6
	BSA (10mg/ml)	2
	2XTaq	12.5
	DNA (1-10 ng)	2
	TOTAL	25

8 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	95°C	5 minutes	
	denaturation	95°C	30 seconds	
	annealing	50°C	30 seconds	
	extension	72°C	45 seconds	
	GO TO step 2			39 times
	final extension	72°C	5 minutes	
	HOLD	12°C	HOLD	

9 Run a subset of the PCR product (5µl) on a 1.5% agarose gel to check the size of the amplicons and the success of the amplification.

1h

Expected result

If any additional bands appear that are not the desired product's size, increase the PCR's annealing temperature or perform additional purification steps.

Purification of first PCR product using SPRI beads

10 **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 rate of bead to a sample of 0.8-1.5 will efficiently purify the amplicons away from primer dimers and allow the selection of fragments larger than 200 bp.

Materials

- Magnetic beads working solution. If not already prepared: go to step #3
- Magnetic 96-well plate stand
- Anhydrous Ethanol to make a fresh 80% ethanol solution

Molecular Biology Grade Water **Corning Catalog #**46-000-CV

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).

11 Vortex the beads before use. 15m
- Add 16 µl beads to 20 µl of PCR product to obtain a ratio of 0.8.
- 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.

12 Incubate at room temperature without shaking for 5 min. 8m
Then, place the plate on the magnetic stand until the supernatant has cleared (~ 3 min).

13 Remove the supernatant with a multichannel pipette, ensuring to not disturb the beads. 5m

14 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. 10m

15 Repeat the washing step go to step #14 10m

16 Remove all residual ethanol using a pipette and air dry, leaving the samples on the magnetic stand (~ 5 min*). 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.

17 Remove the plate from the magnetic stand and add 40 µl 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

18 Place the plate back on the magnetic rack for at least 5 min or until the supernatant is cleared. 5m

19 Carefully transfer 30 µl of the clear supernatant to a new plate. Seal the plate.

20 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.

(**IF** this is the cleanup of the second PCR product go to step #27)

Indexing PCR amplification (2nd PCR)

21 **Preparations**

Reagents:

Taq FroggaMix **Froggabio Catalog #**FBTAQM96

Molecular Biology Grade Water **Corning Catalog #**46-000-CV

Froggarose LE **Froggabio Catalog #**A87-500G

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

- i5 and i7 index plates (10 µM) – If not already prepared: go to step #4

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.

22 Dilute the cleaned-up PCR (1:10) with sterile nuclease-free water

23 Prepare PCR reaction in 25µl reactions:

Reagent	Volume (µl)
Sterile Nuclease-Free water	5
Forward primer (10µM)	2.5
Reverse Primer (10µM)	2.5
2XTaq	12.5
DNA (1-10 ng)	2.5
TOTAL	25

24 Seal the

PCR step	Temperature	Duration	Repetition
denaturation	95°C	3 minutes	
denaturation	95°C	30 seconds	
annealing	55°C	30 seconds	
extension	72°C	30 seconds	
GO TO step 2			7X
final extension	72°C	5 minutes	
HOLD	12°C	HOLD	

25 Run a subset of the PCR product (5µl) on a 1.5% agarose gel to check the size of the amplicons and the success of the amplification.

Note

If any additional bands appear that are not the size of the desired product, additional purification steps need to be carried out.

Purification of indexed libraries (Second bead cleanup)

26 Repeat the Ampure XP bead cleanup for all the indexed libraries.

go to step #10 Purification

Quantification and pooling, and quality control

27 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 theBroad Range kit (BR) if you visualize a strong band in the gel.

Expected result

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

28 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).

29 Measure the final library pool concentration on Qubit using the High Sensitivity (HS) kit.

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

31

32

BASIC TROUBLESHOOTING GUIDE

33 Identify known issues associated with the procedure, if any.

Provide troubleshooting guidelines when available.

Protocol references

Insert all references cited in the document.
Please insert full DOI address when available, e.g. http://doi.dx.org/10.1007/s11258-014-0404-1