Jun 01, 2024

COI-Gene Metabarcoding Library Prep: Dual-PCR Method

In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.261ge5d7yg47/v1

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Hakai Genomics

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DOI: https://dx.doi.org/10.17504/protocols.io.261ge5d7yg47/v1

External link: https://hakai.org

Protocol Citation: rute.carvalho Carvalho, Colleen Kellogg, Matt Lemay 2024. COI-Gene Metabarcoding Library Prep: Dual-PCR Method. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.261ge5d7yg47/v1

Manuscript citation:
Andreas Novotny, Caterina Rodrigues, Loïc Jacquemot, Rute B G Clemente-Carvalho, Rebecca S Piercey, Evan Morien, Moira Galbraith, Colleen T E Kellogg, Matthew A Lemay, Brian P V Hunt, DNA metabarcoding captures temporal and vertical dynamics of mesozooplankton communities, ICES Journal of Marine Science, Volume 82, Issue 2, February 2025, fsaf007,
https://doi.org/10.1093/icesjms/fsaf007

Protocol status: Working
We use this protocol and it's working

Created: May 28, 2024

Last Modified: June 01, 2024

Protocol Integer ID: 100678

Keywords: edna metabarcoding of the cytochrome oxidase, gene metabarcoding library prep, level resolution of marine invertebrate, plankton communities in the northern salish sea, biomolecular sample, marine invertebrate, plankton community, part of the hakai institute ocean observing program, cytochrome oxidase, hakai institute ocean observing program, edna metabarcoding, pcr method this protocol, pcr method, northern salish sea, metabarcoding

Abstract

This protocol is used for **eDNA metabarcoding of the Cytochrome Oxidase I (COI) "Leray fragment"**, using Pair-End Illumina MiseqSequencing. As part of the Hakai Institute Ocean Observing Program, biomolecular samples have been collected weekly, from 0 m to near bottom (260 m), to genetically characterize plankton communities in the Northern Salish Sea since 2015. This protocol is developed to give a species-level resolution of **marine invertebrates**.

Guidelines

MIOP: Minimum Information about an Omics Protocol

	MIOP Term	Value
	analyses	Amplicon Sequencing, COI
	audience	scientists
	broad-scale environmental context	marine biome ENVO_00000447
	creator	Rute Carvalho
	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	Biomolecular surveys of marine biodiversity in the Northern Salish Sea, BC
	publisher	Hakai Institute, Ocean Observing Program
	purpose	DNA metabarcoding
	skills required	sterile technique | pipetting skills
	target	Marine Invertebrates
	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/
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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
	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 Cytochrome Oxidase I (COI) "Leray fragment" 320 bp** using Pair-End Illumina MiseqSequencing. (Leray et al 2013).

Citation

Leray, M., Yang, J.Y., Meyer, C.P. et al. (Invalid date)
. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents..
Front. Zool.

https://doi.org/10.1186/1742-9994-10-34 LINK

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 320 bp ling "Leray fragment" of the COI gene.
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. Quality control
7. 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
As part of the Hakai Institute Ocean Observing Program, biomolecular samples have been collected weekly, from 0 to near bottom (260 m), to genetically characterize plankton communities in the Northern Salish Sea since 2015, developing a climatology from which we can begin uncover the physical, chemical and biological drivers of community and functional change in the dynamic coastal waters of coastal British Columbia. This protocol is developed to give a species-level resolution of **marine invertebrates.**

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 may 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

- NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog** #E7630S
- Bioanalyzer chips and reagents (DNA 1000) **Agilent Technologies Catalog** #5067-1504
- PhiX Control v3 **Illumina, Inc. Catalog** #FC-110-3001
- MiSeq v3 (150 cycle) Kit **Illumina, Inc. Catalog** #MS-102-3001
- 100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog** #G2101
- Gel Red Nucleic Acid Gel Stain **Biotium Catalog** ##41003
- Taq FroggaMix **Froggabio Catalog** #FBTAQM96
- Molecular Biology Grade Water **Corning Catalog** #46-000-CV
- Froggarose LE **Froggabio Catalog** #A87-500G
- 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
- 100bp DNA Ladder, 250ul (50 lanes) **Promega Catalog** #G2101
- Gel Red Nucleic Acid Gel Stain **Biotium Catalog** ##41003
- Molecular Biology Grade Water **Corning Catalog** #46-000-CV
- Taq FroggaMix **Froggabio Catalog** #FBTAQM96
- BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog** #B9000S
- Froggarose LE **Froggabio Catalog** #A87-500G

Troubleshooting

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 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

Gel Red Nucleic Acid Gel Stain **Biotium Catalog** ##41003

- Custom-designed primers (*Leray et al 2013*) including:

PCR Primer Name	Direction	Sequence (5′ → 3′)
mICOlintF_overhang	forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC
dgHCO2198R_overhang	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAAACTTCAGGGTGACCAAARAAYCA

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

8 PCR reactions are carried out in triplicate 25µl reactions:

30m

Reagent	Volume (µl)
Sterile Nuclease-Free water	7.3
Forward primer (10µM)	0.6
Reverse Primer (10µM)	0.6
BSA (10mg/ml)	2
2XTaq	12.5
DNA (1-10 ng)	2
TOTAL	25

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	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	

10 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

11 **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

- Serapure SPRI beads. If not already prepared: go to step #3
- 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).

12 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.

13 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).

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

15 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

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

17 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.

18 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

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

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

21 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 #28)

Indexing PCR amplification (2nd PCR)

22 **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
- Gel Red Nucleic Acid Gel Stain **Biotium Catalog** ##41003
- i5 and i7 index plates (10 µM) – If not already prepared: go to step #4

PCR Primer Name	Direction	Sequence (5′ → 3′)
Nextera V2 Index1	forward	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG
Nextera V2 Index 2	reverse	AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

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.

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

24 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

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

PCR step	Temperature	Duration	Repetition
denaturation	95°C	3 minutes	

	PCR step	Temperature	Duration	Repetition
	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	

26 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)

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

go to step #11 Purification

Quantification and pooling, and quality control

28 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.

29 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).

30 Measure the final library pool concentration on Qubit using

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

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

Sequencing parameters

32 Library fragment size (BP) is determined using

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

Molarity of thefinal pool is assessed using

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

33 COI libraries are sequenced an 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

Leray, M., Yang, J.Y., Meyer, C.P. et al.. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents.
https://doi.org/10.1186/1742-9994-10-34