

Cytochrome c oxidase subunit I (COI) metabarcoding

This protocol is designed to amplify the mini COI barcode (313bp) as described in [Leray et al \(2014\)](#). The primer set (mlCOLintF and jgHCO2198) is successfully amplifying a wide range of marine invertebrate phyla. Multiplexing is made possible by a variation of the initial primers tailed on the 5' end with a 6 bp "tag".

The protocol described below is tailored for the [Illumina MiSeq platform](#) and uses the [Illumina Truseq kit](#). We use single indexing and a hierarchal tagging approach that can be modified depending on your number of samples. The number of samples in each illumina run will depend on the minimum number of sequences needed per sample. This will depend on the expected diversity. As a safety net, we suggest that at least 100,000 sequences be obtained for each sample.

1- Primers

The primer sequences are always listed in the 5' -> 3' orientation:

mlCOLintF: GGWACWGGWTGAACWGTWTAYCCYCC ([Leray et al, 2014](#))
jgHCO2198: TAIACYTCIGGRTGICCRAARAAYCA ([Geller et al, 2014](#))

It is also possible to multiplex the samples by using the following tailed primer sets ([Table S4 of Leray et al \(2015\)](#)) where the first 6 bp represents the tag.

Primer Label	Primer Sequence (5' – 3')
mlCOLint_Tag1	AGACGCGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag2	AGTGTAGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag3	ACTAGCGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag4	ACAGTCGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag5	ATCGACGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag6	ATGTCGGGWACWGGWTGAACWGTWTAYCCYCC
mlCOLint_Tag7	ATAGCAGGWACWGGWTGAACWGTWTAYCCYCC
jgHCO_Tag1	AGACGCTAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag2	AGTGATAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag3	ACTAGCTAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag4	ACAGTCTAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag5	ATCGACTAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag6	ATGTCGTAIACYTCIGGRTGICCRAARAAYCA
jgHCO_Tag7	ATAGCATAIACYTCIGGRTGICCRAARAAYCA

2- Amplicon library preparation

For better PCR success, we recommend diluting the gDNA to 10-ng/ μ l. Each sample will be amplified in triplicates (3 x 20 μ l reactions per sample). We will prepare a negative control for each tailed primer set used.

A hierarchical tagging approach will be used with a combination of tailed PCR primers and Illumina Indexes. For example: if you are analyzing 9 ARMS and 3 fractions per ARMS, you will have 27 samples. You can use 9 Illumina Indexes and 3 tagged primer pairs per index. In that case you will prepare three PCR master mixes for primer set Tag1, Tag2 and Tag3.

Materials:

- Bulk fractions purified gDNA
- TaKaRa [Advantage® 2 PCR Kit](#)
- [Qubit® dsDNA HS Assay Kit](#) or [Quant-iT PicoGreen dsDNA Assay Kit](#) (ThermoFisher/Invitrogen)
- [AMPure XP beads](#) (Beckman Coulter)
- Thermal cycler
- Dedicated pipettors and pipette tips
- PCR reaction tubes and/or plates
- PCR grade water
- agarose/EtBr gel and loading buffer

Protocol:

1. Prepare the appropriate number of PCR master mixes depending on the number of tagged primer sets used. If using three as in the above example, prepare three master mixes, one for each tagged primer set. Calculate the number of samples per primer set and multiply by three (each sample will be run in triplicates). Add enough reagents to also run a negative control for each tagged primer set. For the negative control, replace the DNA with PCR grade water.
2. Dilute each primer set to 10 μ M.

3. Prepare PCR master Mixes:

Reagent	Volume (μL)
PCR grade water	12.2
10X Advantage 2 PCR Buffer	2
mICOLint_Tagx (10 μM)	1
lgHCO_Tagx (10 μM)	1
50X dNTP Mix (10 mM ea.)	1.4
50X Advantage 2 Polymerase Mix	0.4
Sample gDNA or water for neg. control	2
Final volume	20

4. Run thermocycler with the following, touch-down, PCR profile:

Temperature (°C)	Time	Cycles #
95	1 mn	
95	10 s	16
62	30 s	
72	60 s	
95	10 s	20
46	30 s	
72	60 s	
72	7 mn	
4	∞	

1. Run each PCR on an agarose gel to check PCR success. The band observed should be ~365-380 bp.
2. Combine successful triplicate PCR reactions for each sample into a single tube (do not yet pool samples together at this point).
3. Quantify amplicons using [Qubit® dsDNA HS Assay Kit](#) or [Quant-iT PicoGreen dsDNA Assay Kit](#) (ThermoFisher/Invitrogen) following manufacturer's instructions.
4. For each Illumina Index you will use downstream, pool 1 μg of each tagged PCR sample into a single clean tube (or an equal amount to totalize >1 μg for each index). Be careful that each single Illumina Index (ligated below) contains only samples tagged with different PCR primers. For example, for Index AD002 we will pool libraries tagged with PCR primers tag 1, 2 and 3. We can use those same tags for each Index used but within an index those tags must be different in order to bioinformatically separate the different libraries.
5. Clean the amplicon's pool using [AMPure XP beads](#), following manufacturer's instructions.

6. Quantify each amplicon's pool with [Qubit® dsDNA HS Assay Kit](#) or [Quant-iT PicoGreen dsDNA Assay Kit](#).
7. Transfer 1 µg in a 0.5ml tube or PCR plate.

// Safe stopping point // You can freeze the pool of amplicons at -20°C //

3- Illumina TruSeq DNA Sample Preparation

For this step, we are following the [Illumina TruSeq DNA LT Sample Preparation protocol](#). If you are not using all 12 Illumina Indexes in a single run, make sure to choose the indexes according to the Low Plex Pooling guidelines described on page 45 of the [protocol](#).

Because we are starting with pooled PCR products, we will start the [protocol](#) at the Perform End Repair step (page 63).

Follow carefully the instructions provided in the [Illumina TruSeq DNA LT Sample Preparation protocol](#) as the protocol below only contains headlines of the process.

*Note: If, for the bead cleanup, you will use a magnetic rack and not a magnetic plate, you will have to transfer the samples to 1.5-mL tubes before each cleanup.

Materials:

- 1 µg of each pool of amplicons
- [Illumina TruSeqDNA LT Sample Prep Kit](#)
- [Qubit® dsDNA HS Assay Kit](#) or [Quant-iT PicoGreen dsDNA Assay Kit](#) (ThermoFisher/Invitrogen)
- [MiSeq Reagent kit v3 \(600 cycles\)](#)
- Thermal cycler
- Dedicated pipettors and pipette tips
- PCR reaction tubes and/or plates
- 80% Ethanol
- PCR grade water

Protocol:

1. Perform End Repair (Page 63):
Bring the volume of each pooled amplicon to 60 µL with provided Resuspension Buffer.
2. Add 40 µl of End Repair Mix 2 to each tube and mix thoroughly.

3. Incubate the samples in the thermocycler at 30°C for 30 minutes.
4. Proceed to Cleanup using the AMPure XP beads as described on Page 64. The final volume after cleanup step is 15 µl.

// safe stopping point // You can freeze the sample at -20°C //

5. Adenylate 3' Ends (Page 67):
Add 2.5 µl of Resuspension Buffer to each pooled amplicon.
6. Add 12.5µl of A-Tailing Mix to each sample and mix thoroughly.
7. Incubate the samples in the thermocycler at 37°C for 30 minutes.
8. Ligate Adapters (page 69):
Choose the adapters using the guidelines provided page 45 of the [Illumina TruSeq DNA LT Sample Preparation protocol](#).
9. Add 2.5µl of Resuspension Buffer to each sample.
10. Add 2.5µl of Ligation Mix (directly from the freezer and put back immediately).
11. Carefully add 2.5µl of corresponding adapter to each sample and mix thoroughly.
12. Incubate the samples in the thermocycler at 30°C for 30 minutes.
13. Remove the samples and add 5µl of Stop Ligation Buffer. Mix thoroughly.
14. Proceed to Cleanup using the AMPure XP beads as described on Page 73-75. The final volume after cleanup step is 20 µl.

// safe stopping point // You can freeze the sample at -20°C //

15. Quantify the libraries using [Qubit® dsDNA HS Assay Kit](#) or [Quant-iT PicoGreen dsDNA Assay Kit](#) following manufacturer's instructions.
16. Pool 50ng of each library into a single tube and mix thoroughly.
17. Quantify the mixed library pool as in step 15.
18. Validate the library with qPCR. We use [KAPA Library Quantification Kit](#) for Illumina platforms.
19. Prepare DNA template for sequencing. Use the [MiSeq Reagent kit v3 \(600 cycles\)](#).