CMarZ mtCOI Barcoding Protocol

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1. DNA Extraction

DNA is extracted with the Qiagen DNeasy Tissue Kit using the Animal Tissue Protocol included with the kits manual. DNA is eluted in final step with 50µl for specimens < 1mm, 100uL for 1-3 mm and 200µL for >3 mm.

[69504 DNeasy Tissue Kit, Qiagen Inc. (Valencia, CA)]

2. PCR Reaction Conditions

50 uL reaction	Stock concentration
10 µL	5X GoTaq Flexi Buffer
4 µL	25 mM $MgCl_2$
5 µL	2 mM each dNTP mix
0.5 µL	10 µM forward primer
0.5 µL	10 µM reverse primer
0.25 µL	5 U/µL GoTaq Flexi DNA pol
2 µL	DNA (typical volume)*
27.75 µL	dH_2O (typical volume)*

* Note: More or less DNA may be used, depending on concentration after extraction. Adjust dH_2O accordingly.

Primer sequences from Folmer et al. (1994)

LCO1490: 5'- GGTCAACAAATCATAAAGATATTGG -3'

HCO2198: 5'- TAAACTTCAGGGTGACCAAAAAATCA -3'

[M8295 GoTaq Flexi DNA Polymerase, Promega Corporation (Madison, WI)]

3. Thermal Cycling Conditions

Our standard protocol is: 94° (1 min); 45° (2 min); 72° (3 min) for 40 cycles.

Results will vary among PCR machines; conditions should be optimized for each machine. For mismatched primers, our best results have been obtained using a Perkin Elmer 480 DNA Thermal Cycler, an older – and more slowly-ramping – PCR machine.

Check PCR with agarose gel electrophoresis. PCR should yield a 710 bp fragment.

4. PCR reaction cleanup

PCR reactions are cleaned using the QIAquick PCR Purification Kit to remove excess primer, dNTP, and Taq.

[28106 Qiagen QIAquick PCR purification Kit, Qiagen Inc. (Valencia, CA)]

5. Cycle Sequencing Reaction
Sequencing is performed in 96 well reaction plate.

Reagents:
- 4 µL Ready Reaction Premix
- 2 µL BigDye Sequencing Buffer
- 0.32 µL 1490 or 2198 10 µM Primer
- 5-20 ng PCR template
- to 20 µL reaction volume with Deionized water

[4337455 BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems (Foster City, CA)]

6. Cycle Sequencing
Our protocol follows the cycle sequencing conditions for Applied Biosystems 9700 in 9600 emulation mode: 96° (1min); 96° (10 sec); 50° (5 sec); 60° (4 min) for 25 cycles; 4° hold.

7. Ethanol/EDTA precipitation
Protocol steps:
- Add 5 µL of 125 mM EDTA.
- Add 60 µL of 100% ethanol.
- Seal plate with aluminum tape and mix by inverting 4 times.
- Incubate at room temperature (27°C) for 15 min.
- Centrifuge at 3000 x *g* for 30 min.
- Invert plate and spin up to 185 x *g*.
- Add 60 µL of 70% ethanol.
- Centrifuge at 1650 x *g* for 15 min at 4°C.
- Invert plate and spin up to 185 x g for 1 min.
- Make sure wells are dry.
- Add 10 µL Hi Di Formamide.
- Denature at 95°C for 5 min
- Place on ice for 2 min and store at 4°C until ready to run.

8. DNA Sequencing
Our samples are sequenced by capillary electrophoresis on an Applied Biosystems 3130 Genetic Analyzer using a 50cm array and POP-7 polymer.

[Hi Di Formamide, Applied Biosystems (Foster City, CA)]
[POP-7 polymer, Applied Biosystems (Foster City, CA)]

9. Reference
Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3(5): 294-299.