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Chagos eDNA metabarcoding protocol

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We use this protocol and it's working

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Abstract

Full eDNA metabarcoding protocol for Chagos eDNA metabarcoding project, funded by the Bertarelli Marine Science Program.

Extraction from frozen encapsulated filters frozen, simplified from Spens et al, 2017.

1 Before extraction: Carefully wipe all surfaces with 5% bleach using clean tissue paper. Dry and wipe with 70% Ethanol using tissue paper. Syringes, tubes and pipettes should be sterilised in UV box for 30 mins prior to each set of extractions.

2 Label all filters using lab marker. Turn on incubators. Turn on heating block to 70°C to heat ddH2O for elution.

3 Make a premix of Lysis working solution by adding 720 µL ATL buffer and 80 µL proteinase K per sample provided by the kit.

4 Using a sterile filter tip, load 800 µL of lysis working solution into a sterile 3ml syringe with luer adapter.

5 Keeping the outlet end loosely closed with the outlet cap. Carefully add the 800 µL Lysis working solution to the filter using a 3ml sterile syringe with luer adapter. If bubbles/lysis solution can be seen from the outlet, tighten the cap. Close with an new inlet cap and handshake vigorously for a few seconds.

6 Incubate samples, while rotating, at 56°C for 24 hours.

7 Transfer: Remove ALL the liquid from outlet of capsule by using a Luer Lock syringe to pull the lysis buffer out of the filter capsule and add to 5 mL LoBind tube.

8 From this point on, this follows Qiagen B&T protocol. Add Buffer AL and ice-cold molecular grade 99% ethanol to the sample in equal volumes. Sample:Buffer:Ethanol = 1:1:1. Note: AL and ethanol can be premixed.

9 Vortex vigorously.

10 Pipet the mixture (max 650 µL at a time) into a DNeasy Mini Spin column in a 2 mL collection tube provided in the kit.

11 Spin in micro-centrifuge at 6000 ∗ g (8000 rpm) for 1 min.

12 Discard flow through.

13 Repeat steps 10-12 until all sample is filtered through DNeasy Mini spin column (most likely 4 times)

14 Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 ∗ g (8,000 rpm). Discard flow-through and collection tube.

15 Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 ∗ g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

16 Place spin column in a new collection tube, centrifuge 1 min at 17,000 ∗ g (13,000 rpm).

17 Transfer spin column to a new 1.5 or 2 mL DNA LoBind tube with caps removed.

18 Place tubes with spin columns, four at a time, on a 70°C heating plate, add 100 µl 70°C ddH2O to the membrane, immediately transfer spin column with filter to room temperature.

19 Incubate at room temperature for 10 min.

20 Centrifuge for 1 min at 6,000 ∗ g (8,000 rpm)

21 Re-elute DNA from DNA LoBind tube. (Apply eluate back on spin column on heating plate).

22 Incubate at room temperature for 10 min.

23 Centrifuge for 1 min at 6,000 ∗ g (8,000 rpm)

24 Discard the spin column.

25 Transfer DNA to pre-marked DNA LoBind tube with lid intact.

26 Qubit 2 µL for DNA measurement.

27 Store at -20°C or at -80°C.

Extraction from Durapore filters, rolled and stored either frozen or in RNAlater in 5ml Cryovials. Also simplified from Spens et al, 2017.

28

29 Before extraction: Carefully wipe all surfaces with 5% bleach using clean tissue paper. Dry and wipe with 70% Ethanol using tissue paper. Syringes, tubes and pipettes should be sterilised in UV box for 30 mins prior to each set of extractions.

30 Label all filters using lab marker. Turn on incubators. Turn on heating block to 70°C to heat ddH2O for elution.

31 Make a premix of Lysis working solution by adding 720 µL ATL buffer and 80 µL proteinase K per sample provided by the kit.

32 If stored in RNAlater, remove the buffer using a sterile pipette tip and add to a labelled tube.

33 Add 800 µL Lysis working solution to the filter in the cyrovial. Close the cap and handshake vigorously for a few seconds.

34 Incubate all samples, while rotating, at 56°C for 24 hours.

35 Using a pipette, transfer ALL the liquid from the cryovial to a 5 mL LoBind tube. Add the lysis buffer from the pellet incubation.

36 From this point on, this follows Qiagen B&T protocol. Add Buffer AL and ice-cold molecular grade 99% ethanol to the sample in equal volumes. Sample:Buffer:Ethanol = 1:1:1. Note: AL and ethanol can be premixed.

37 Vortex vigorously.

38 Pipet the mixture (max 650 µL at a time) into a DNeasy Mini Spin column in a 2 mL collection tube provided in the kit.

39 Spin in micro-centrifuge at 6000 ∗ g (8000 rpm) for 1 min.

40 Discard flow through.

41 Repeat steps 10-12 until all sample is filtered through DNeasy Mini spin column (most likely 4 times)

42 Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 ∗ g (8,000 rpm). Discard flow-through and collection tube.

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52 Discard the spin column.

53 Transfer DNA to pre-marked DNA LoBind tube with lid intact.

54 Qubit 2 µL for DNA measurement.

55 Store at -20°C or at -80°C.

Qubit DNA Quantification

56 The Qubit kit consists of the Qubit sample buffer, Qubit reagent and two standards (or if 1X qubit kits are bought, the buffer and reagent are pre-mixed). The Qubit sample buffer is simply the solution used to dilute your samples, the Qubit reagent is the fluorescent dye that, in this case, binds to dsDNA. It is advisable to prepare standards each time you use the Qubit. The Qubit requires specific 0.5ml thin wall tubes. The final volume in the assay tubes need to be 200ul, but you can vary the volume of DNA you add, 1-20ul, to allow concentration to be maintained within detection range. Standards are always added at a volume of 10ul.
If there is lots of samples, there is a qubit flex which allows for running strips of tubes rather than one at a time. The chemistry is exactly the same but the tubes are smaller, and must be thin walled as above.

57 Remove Qubit reagents from the fridge 30 minutes before use and vortex just before use.

58 If not using 1X buffer, make enough master mix for duplicates of each sample + 2 for standards

58.1 Per sample: 199ul Qubit reaction buffer and 1ul of Qubit reagent

59 Label Qubit tubes for the standards and the duplicate sample tubes.

60 To each standard tube add 190ul of master mix and 10ul of the relevant standard.

61 To each sample tube add 198ul of master mix and 2ul of library.

62 Turn on the Qubit and select DNA - dsDNA HS assay.

63 Select to read new standards.

64 Follow on screen instructions to read standards and insert the first assay tube.

65 After reading the first assay tube select “calculate stock concentration”.

66 Change the sample volume to 2µl and ensure that the units are ng/µl - record the concentrations.

1st round PCR with metabarcoding assays

67 **18S (AmarZett)**
Triplicate 10 µL PCRs should be performed for each sample to be sequenced.

67.1 The PCR reaction should be made up of 2 µL of 5x HOT FIREPol® Blend Master Mix Ready to Load, 0.4 µL of forward and reverse primers (10nM), 5.7 µL of ddH2O and 1.5 µL of DNA template.

67.2 The PCR protocol comprised 95°C denaturation for 15 minutes, followed by 30 cycles of 95°C for 10 seconds, 57°C for 30 seconds and 72°C for 30 seconds, with a final elongation step of 72°C for 7 minutes.

67.3 Pool the three replicates for each sample and run 5 µL on 1% agarose gel with SYBR safe stain to check for successful amplification (TLUM light and UV32 filter). COI (Leray)

68 **COI (Leray)**
Triplicate 25 µL PCRs should be performed for each sample to be sequenced.

68.1 The PCR reaction should be made up of 5 µL HOT FIREPol 5X MasterMix, 0.5 µL of each primer (10µM), 2 µL extracted DNA and 17 µL H2O for a final reaction volume of 25 µL.

68.2 The PCR protocol comprised 95°C denaturation for 95°C for 15 minutes followed by 35 cycles of 95°C for 1 60s, 55°C for 60s and 72°C for 60s, with a final extension of 72°C for 5 minutes.

68.3 Pool the three replicates for each sample and run 5 µL on 1% agarose gel with SYBR safe stain to check for successful amplification (TLUM light and UV32 filter). 12S (MiFish-U)

69 **12S (MiFish-U/E)**
Triplicate 10 µL PCRs should be performed for each sample to be sequenced.

69.1 The PCR reaction should be made up of 3 µL HOT FIREPol 5X MasterMix, 0.7 µL of each primer (MiFish-U-F & -R (10µM) and MiFish-E-F & -R (5µM)), 2.5 µL extracted DNA and 6.7 µL H2O for a total volume of 15 µL. Thermal conditions consisted of an initial denaturation step of

69.2 The PCR protocol comprised 95°C denaturation for 95°C for 2 minutes followed by 40 cycles of 95°C for 15s, 65°C for 30s and 72°C for 30s, with a final extension of 72°C for 5 minutes.

69.3 Pool the three replicates for each sample and run 5 µL on 1% agarose gel with SYBR safe stain to check for successful amplification (TLUM light and UV32 filter). If not using the green mastermix, each sample will need to be mixed with loading dye and gel red for visualisation (no gel stain). 16S (EMP)

70 **16S (EMP)**
Triplicate 25 µL PCRs should be performed for each sample to be sequenced.

70.1 The PCR reaction should be made up of 5 µL HOT FIREPol 5X MasterMix, 0.5 µL of each primer (10µM), 1 µL extracted DNA and 18 µL H2O to make the mixture up to 25 µL.

70.2 The PCR protocol comprised 94°C for 12 minutes followed by 35 cycles of 94°C for 45s, 50°C for 60s and 72°C for 90s, with a final extension of 72°C for 10 minutes.

70.3 Pool the three replicates for each sample and run 5 µL on 1% agarose gel with SYBR safe stain to check for successful amplification (TLUM light and UV32 filter). If not using the green mastermix, each sample will need to be mixed with loading dye and gel red for visualisation (no gel stain).

Primer sequences (inlcuding illumina adapters)

71

	A	B
	Primer	Primer sequence in bold (5′ – 3′)
	18S AmarZett (Amaral-Zettler et al., 2009)	
	1380F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCCTGCCHTTTGTACACAC
	1510R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTCYGCAGGTTCACCTAC
	COI (Leray et al., 2013)	
	mlCOIintF	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC
	jgHCO2198	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAIACYTCIGGRTGICCRAARAAYCA
	12S MiFish (Miya et al, 201)	
	MiFish-U-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTCGGTAAAACTCGTGCCAGC
	MiFish-U-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATAGTGGGGTATCTAATCCCAGTTTG
	MiFish-E-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTTGGTAAATCTCGTGCCAGC
	MiFish-E-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATAGTGGGGTATCTAATCCTAGTTTG
	V4 16S (Earth Microbiome Project)	
	515-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA
	806-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT

First round PCR bead clean up (protocol the same for each marker from this point)

72 **Before you start:** The volume of each sample should be about 70µl (70 µL total PCR product with 5 µL run on gel). Bring the Ampure XP beads to room temperature (~30 minutes) and thoroughly mix. Make up 400µl of 80% ethanol for each sample to be cleaned. Prepare an empty tip box filled with tissue to discard liquid waste into. Add the beads to a reagent reservoir or strip of tubes if cleaning a plate of samples.

73 Vortex clean-up beads for 30s.

74 Add 60 µL of beads to each sample and pipette mix 10 times - incubate the plate at room temperature for 5 minutes.

75 Put the plate on the magnetic stand for 2 minutes. There are two magnetic plate stands, one in Kev's drawer and one in the drawer under the MiSeq, you can use either. They pull the beads in slightly different ways so choose whichever you find easiest to not disturb the bead pellets.

76 Remove 120 µl of supernatant and discard but avoid disturbing the beads.

77 If beads are also being aspirated, it might be necessary to remove less initially and remove the rest of the volume with a 10µl tip afterwards.

78 With the plate still on the magnet add 200µl of 80% ethanol without disturbing the beads - Incubate for 30 seconds.

79 Remove and discard the supernatant.

80 Repeat steps 5-6 for a total of two washes.

81 Incubate the plate for 5 minutes at room temperature to dry the beads.

82 Using a 10µl pipette remove and discard any remaining ethanol from each sample.

83 Remove the plate from the magnet, add 52.5µl of RSB or PCR grade water to each well and pipette up and down 10 times to re-suspend the beads - Incubate at room temperature for 2 minutes.

84 Put the plate back onto the magnet and incubate for 2 minutes or until the solution has cleared.

85 Transfer 50µl of the clean product to a new PCR tube / plate.

Index PCR

86 Individual samples are identified post sequencing by the indexes that are added during this PCR. The libraries are dual-indexed, the combination of indexes is unique for each sample. Before starting in the lab decide which indexes are to be used – we have previously used Nextera XT index kits B or C, which come with enough indexes to dual index 96 samples, four times (each box can has 96 indexes and can index two plates (192 samples). The Illumina guidance on index choice and low-plexity pooling is included in Appendix-1.

87 Make the PCR master mix, using 10 µL of 5X hot fire pol and 25 µL of water per sample.

88 Aliquot 35 µL of master mix into the bottom of each well, try to avoid the edges of the wells so you can keep tips clean when pipetting the indexes.

89 Arrange index 1 and 2 primers in the “Index Fixture Plate” (available in the post PCR genomic lab). Take a picture of them in order so you can be sure of the index combinations.

90 Set a fresh PCR plate on the “index Fixture Plate” and add 5ul of the corresponding index primer 1 to each well. To reduce the need for a new pipette tip for each well, pipette the indexes onto opposite sides of well walls to avoid contamination.

91 Add 5ul of the corresponding index primer 2 to each well.

92 Add 5ul of cleaned first round PCR product to the corresponding well and pipette mix.

93 Seal the plate and briefly centrifuge.

94 Run a PCR protocol comprising 95°C denaturation for 15 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final elongation step of 72°C for 10 minutes.

Index PCR clean up

95 **Before you start:** Bring the Ampure XP beads to room temperature (~30 minutes) and thoroughly mix. Make up 400µl of 80% ethanol for each sample to be cleaned. Prepare an empty tip box filled with tissue to discard liquid waste into. Add the beads to a reagent reservoir or strip of tubes if cleaning a plate of samples.

96 Vortex clean-up beads for 30s.

97 Add 56 µL of beads to each sample and pipette mix 10 times- Incubate the plate at room temperature for 5 minutes.

98 Put the plate on the magnetic stand for 2 minutes.

99 Remove 42 µL of supernatant and discard, avoid disturbing the beads.

100 If beads are also being aspirated, it might be necessary to remove less initially and remove the rest of the volume with a 10µl tip afterwards.

101 With the plate still on the magnet add 200 µL of 80% ethanol without disturbing the beads - Incubate for 30 seconds.

102 Remove and discard the supernatant.

103 Repeat steps 5-6 for a total of two washes.

104 Incubate the plate for 5 minutes at room temperature to dry the beads.

105 Using a 10µl pipette remove and discard any remaining ethanol from each sample.

106 Remove the plate from the magnet, add 27.5 µL of PCR grade water to each well and pipette up and down 10 times to re-suspend the beads - Incubate at room temperature for 2 minutes.

107 Put the plate back onto the magnet and incubate for 2 minutes or until the solution has cleared.

108 Transfer 25 µL of the clean product to a new PCR plate.

PCR Check on TapeStation (D1000 HS)

109 Label 0.2ml tubes, the first for the ladder, then one for each sample.

110 Add 3µl of sample buffer to each tube.

111 To the first tube add 1µl of ladder.

112 Add 1ul of PCR product to the remaining tubes.

113 Seal the tubes and put onto the plate vortex for 60 seconds.

114 Briefly spin down.

115 Remove the lids (cut hinged lids so they come off totally), check liquid is still in the very bottom of the tube and put into the tube holder in the TapeStation.

116 Insert a tape, and make sure the loading tip rack is full.

117 In the TapeStation controller software select the relevant tube loading positions and name the samples in the sample table.

118 Start the run (save the run stating it is the 1st round PCR, your name and the date).

119 Once complete: In TapeStation analysis click on “Electropherogram” and check each sample for amplicon sizing (target gene +100BP) and signs of primer dimer.

Sample QC: Qubit

120

121 Vortex and centrifuge Qubit reagents

122 In a 1.5ml tube prepare 5 reactions of master mix by adding 5µl of Qubit reagent to 995µl of sample buffer, vortex and briefly centrifuge

123 Add the following to 5 Qubit tubes -
Tube-1 190µl of master mix, 10µl of standard 1
Tube-2 190µl of master mix, 10µl of standard 2
Tube-3 198µl of master mix, 2µl of pooled sample
Tube-4 198µl of master mix, 2µl of pooled sample
Tube-5 198µl of master mix, 2µl of pooled sample

124 Turn on the Qubit and select DNA - dsDNA HS assay

125 Select to read new standards

126 Follow on screen instructions to read standards and sample tubes

127 After reading the first sample tube select “calculate stock concentration”, change the sample volume to 2µl and change the units to ng/µl

128 Record the concentrations for the three sample replicates

129 Calculate nM values based on amplicon size from previous TapeStation values

MiSeq Loading

130 **Denature and Dilute Sample and PhiX**
Prepare a fresh dilution of 0.2N NaOH - 400µl H2O + 100µl 1N NaOH (in fridge)

131 To a 1.5ml tube add 10µl of sample pool (or 1:10 sample pool if necessary) and 10µl of 0.2N NaOH

132 To a different 1.5ml tube add 2µl of 10nM PhiX, 3µl of H2O and 5µl of 0.2N NaOH

133 Vortex the library and PhiX tubes for 5 seconds and spin for 1 minute at 400rcf

134 Incubate the tubes for 5 minutes at room temperature

135 To the denatured library tube add 980µl of HT1

136 To the denatured PhiX tube add 990µl of HT1, now 20pM (20pM dilution can be stored at -20 for three weeks)

137 Vortex library and PhiX and centrifuge for 1 minute

138 Dilute the sample to 3.5pM using the volumes from the Library Dilution Template sheet

139 Dilute the PhiX to 3.5pM by adding 175µl of 20pM PhiX to 825µl of HT1

140 Invert library and PhiX tube to mix and pulse centrifuge

141 Put 950µl of 3.5pM library in a 1.5ml tube and add 50µl of 3.5pM PhiX, put this sample on ice

142 **Prepare Reagent Cartridge**
Remove the cartridge from the water bath and dry, tap on paper towel to remove as much water as possible from the base

143 Invert cartridge 10 times to mix reagents and inspect the reservoirs to make sure they are fully defrosted

144 Tap on the bench to make sure there are no bubbles at the bottom of the tubes

145 Set aside the MiSeq cartridge until the library is ready to Load

146 **Load Flow Cell**
On the MiSeq press sequence

147 Remove the flow cell and PR2 reagent box from fridge

148 Carefully remove flow cell from the buffer and rinse thoroughly with dd H2O, dry using Kimwipes paying particular attention to the edges of the glass

149 Wet another Kimwipe with absolute ethanol and clean the glass slide on both sides, avoid contact with the rubber gasket

150 Check the flow cell has no smudges or fibres from the wipes

151 Follow the instructions on the MiSeq to load the flow cell and PR2 buffer bottle, empty and replace the waste bottle

152 **Heat Denature Sample**
Using the heat block incubate the combined Library and PhiX tube for 2 minutes at 96°C

153 After the incubation invert the tube 2 times to mix and place in ice water bath for 5 minutes

154 **Load MiSeq Cartridge**
Using a 1000µl pipette tip pierce well 17 (it might be 16 – its labelled sample) on the reagent cartridge

155 Load 600µl of the heat denatured Library / PhiX pool into the cartridge

156 Load the cartridge into the MiSeq and wait for it to read the RFID

157 Follow on screen instructions, check the cycle numbers for the sequencing reads and the indexes

158 Wait for the MiSeq to complete its pre-run checks and then press start