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DNA Barcoding Protocol for Darwin Tree of Life at The Natural History Museum of London V.1

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Darwin Tree of Life



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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol outlines the DNA barcoding process at the Natural History Museum of London (NHM) for the Darwin Tree of Life project (DToL). DNA barcoding is used as part of the species identification process and sample tracking to check that the genome sequence corresponds to the material that was sent.

Materials

Reagents

- **KAPA Express Extract kit**
- Molecular grade water
- **KAPA Plant PCR kit**
- 1X TAE buffer
- Agarose powder
- Gel red
- Loading dye
- 1KB hyperladder
- Qubit HS kit
- **Ligation sequencing library prep kit V14 (SQK-LSK114)**
- **NEB nanopore companion module**
- Indexed Folmer primers (containing sample identifying indexes for multiplexing)

Labware/Equipment

- Clear PCR seals
- 96 well PCR plates
- 1.5ml Eppendorf tubes
- Gel electrophoresis tank, tray and combs
- Masking tape
- Duran bottle
- **Flongle v10.4.1 (FLO-FLG114)**
- **Nanopore sequencing expansion pack (EXP-FSE002)**
- Thermocycler
- Qubit
- Gridion/Minion
- Flongle adaptor
- Magnet for 1.5ml eppendorfs

Before start

The DTOL sample team prepare submissions for barcoding by removing a small piece of tissue from specimens and plating them out into 96 well plates in 80% Ethanol (refer to taxon specific SOPs). This is the format the samples are provided in for the following barcoding protocols.



DNA Extraction

- 1 Spin down the sample plate and inspect to check no tissue/sample is stuck in the lids of the sealing caps.
- 2 Remove the caps and remove the ethanol. Use a single channel pipette for very small samples and take care to ensure the sample is not aspirated into the tip and removed with the ethanol.
- 3 Place the plate on a thermocycler with no lid or seal and with the thermocycler lid open to allow the remaining ethanol to evaporate. Set the thermocycler to run at 70 °C for 10 minutes or longer if required until remaining ethanol has evaporated off. Take care when moving the plate onto and off of the thermocycler without lids- static can cause dry samples to pop out of the wells.
- 4 Prepare a lysis mastermix using the KAPA Express Extract kit (Table 1).

	Reagent	Volume μ l (1 rxn)
	Molecular grade water	44
	1U/ μ l KAPA Express Extract Enzyme	1
	10X KAPA Express Extract Buffer	5

Table 1: KAPA Express Extract lysis mastermix reagents and volumes

- 5 Aliquot 50 μ l lysis master mix into each well and seal with a clear PCR seal. (Note: lysis master mix volume can be reduced for very small samples)
- 6 Spin down the plate and incubate on a thermocycler using the following program:

	Temperature (°C)	Time (minutes)
	30	30
	60	30
	95	5
	4	∞

Table 2: Thermocycling regime for lysis incubation.

Lysate dilution

- 7 Make 1:10 dilution of lysate, adding 2.5µl lysate to 22.5µl PCR grade water
NOTE: Lysate can be stored at -20°C until needed.

PCR with indexed primers

- 8 Prepare the PCR mastermix using the KAPA Plant PCR kit as follows:

Component	Volume (1 × 20µl rxn)
KAPA Plant PCR buffer (contains dNTPs)	10.1
PCR grade water	2.9
Polymerase	0.2
MgCl ₂ (25mM)	0.4

KAPA Plant PCR master mix volumes.

(NOTE: This PCR amplifies a 750bp COI amplicon using primers containing indexes for ONT sequencing. The plant PCR kit is used because it was found result in a higher success rate for samples containing inhibitors.)

Primer sequences can be found here:

 Nanopore Tagged Folmer Barcodin...

- 9 Aliquot 13.6µl PCR mastermix into each well.
- 10 Add 1.6µl indexed primer mix to each well.
- 11 Add 4.8µl 1:10 diluted lysate to each well
- 12 Seal the plate with PCR seal and mix and spin the plate.



13 Place the plate on a thermocycler and run the following program:

1 cycle:

95°C for 3 mins

5 cycles:

95°C for 40s

45°C for 40s

72°C for 1 min

40 cycles:

95°C for 40s

65°C for 40s

72°C for 1 min

1 cycle:

72°C for 5 mins

4°C ∞

NOTE: Plate can be stored at -20°C until needed.

Gel QC

14 Pour 500ml 1x TAE into a glass duran bottle. (This makes enough gel solution to make two large gels with 4 rows of combs with 50 teeth each to take 2 plates of samples each).

15 Weigh out 5g of agarose powder and add to the duran bottle.

16 Close the lid tightly and mix the agarose powder into the TAE by swirling the bottle.

17 Loosen the lid so that air can escape the bottle (so it doesn't explode) and heat the bottle in a microwave in short bursts checking between heating until the liquid starts to boil and the agarose has gone into solution and the liquid is completely clear.

18 Allow the agarose to cool so that it is still molten but not hot. Tape off the ends of a large gel tray with masking/autoclave tape and place combs in the tray.

19 Pour half of the agarose (~250ml) into the tray, making sure there are no bubbles present and allow to set firm.

- 20 When completely cooled and set, remove the plastic combs and tape, place in a tank filled with enough 1x TAE buffer to cover the gel.
- 21 Add Gel Red to loading dye in a 1:1000 ratio. Load 2µl PCR product mixed with 3µl loading dye/Gel Red mix to the gel and run alongside 1Kb hyper ladder at 80 volts for ~45 mins. Check bands of the expected size are present by visualising the gel in a UV transilluminator.

Pooling and quantification

- 22 Pool 5µl PCR product for each sample in your pool (taking care that no two samples in the pool have the same indexes).
- 23 Quantify the pool using Qubit, taking 3 × 1µl replicates from the pool and take the average ng/µl from the three replicates as the concentration.

 Qubit_HSDNA_Protocol.pdf

Library Preparation and sequencing

- 24 Prepare library for sequencing using the Nanopore Ligation Sequencing kit V14 as described in the attached protocol.  Flongle Library Prep Protocol (ligati...
The library prep takes 500ng DNA in 24.5µl volume as input.
The resulting library is sequenced on a flongle (v10.4.1) for 24 hours using the super-accurate basecalling setting.

Analysis

- 25 Currently, ONTBarcode software is used to extract consensus barcodes from the raw nanopore data. First fastq files from the run are concatenated into one fastq file containing all of the reads from the run.
This file is loaded into ONTBarcode as input along with a demultiplexing file containing the indexing information for each sample (see manual below for details).
 ONTBarcode_manual.pdf
ONTBarcode can be downloaded here: <https://github.com/asrivathsan/ONTbarcode>
- 26 The barcodes from ONTBarcode output are then searched in BOLD for species identification. In this pipeline, a BOLD search tool called BOLDigger is used to query multiple barcodes and return the top 20 hits in BOLD. Please see BOLDigger documentation here: <https://github.com/DominikBuchner/BOLDigger>