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Working

DNA Barcoding in a Field Setting

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ABSTRACT

This protocol was used in the 2018 Genomics in the Jungle field course held at the Inkaterra Green Lab in the Madre de Dios Department of Peru. We were able to use it to take 112 amplicons and multiplex them using a 96 barcode kit on an Oxford Nanopore Technologies MinION sequencer. This protocol can be used to multiplex much larger numbers of samples onto a single flowcell if the amplicons are from differing taxonomic groups.

We multiplexed the following taxonomic groups:
1. Invertebrates
2. Mammals
3. Plants
4. Environmental DNA

We isolated amplicons using markers for:
1. rDNA (see Krehenwinkel, H., Pomerantz, A., Henderson, J.B., Kennedy, S.R., Lim, J.Y., Swamy, V., Shoobridge, J.D., Patel, N.H., Gillespie, R.G. and Prost, S., 2018. Nanopore sequencing of long ribosomal DNA amplicons enables portable and simple biodiversity assessments with high phylogenetic resolution across broad taxonomic scale. *bioRxiv*, p.358572.)
2. COI using a mammal cocktail as recommended in Kress, W.J. and Erickson, D.L., 2012. DNA barcodes: methods and protocols. In *DNA Barcodes* (pp. 3-8). Humana Press, Totowa, NJ.
3. rBCL and matK for plant samples

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME	CATALOG #	VENDOR
GoTaq(R) G2 Hot Start Polymerase, Sample	M7402	Promega
Q5 High-Fidelity 2X Master Mix - 500 rxns	M0492L	New England Biolabs
Agencourt Ampure XP	A63880	Beckman Coulter

BEFORE STARTING

We assume that this protocol begins with extracted DNA, and thus we do not detail DNA extraction protocols at this time.

PCRMixes

1 1We used a master mix (See below) to set up PCR reactions

GoTaq(R) G2 Hot Start Polymerase, Sample
by Promega
Catalog #: M7402

Q5 High-Fidelity 2X Master Mix - 500 rxns
by New England Biolabs
Catalog #: M0492L

Butterfly rDNA (Total vol = 25 µl)

1.25 µl each of the forward and reverse primer set

9.5 µl l water

3 µl **template** DNA

10 µl q5 MasterMix

Mammal rDNA (Total vol = 13.5 µl)

1 µl each of the forward and reverse primer set

2 µl l water

3 µl **template** DNA

6.5 µl q5 MasterMix

Plant rDNA (Total vol = 13.5 µl)

1 µl each of the forward and reverse primer set

2 µl l water

3 µl **template** DNA

6.5 µl q5 MasterMix

Butterfly COI (Total vol = 25 µl)

1.25 µl each of the forward and reverse primer set

7 µl l water

3 µl **template** DNA

12.5 µl q5 MasterMix

Plant rBCL and matK (Total vol = 25 µl)

1.25 µl each of the forward and reverse primer set

7 µl l water

3 µl **template** DNA

12.5 µl q5 MasterMix

Ectoparasites, eDNA, dung beetles, snails COI (Total vol = 12.5 µl)

2.5 µl Taq buffer

1.25 µl MGCl2

0.0625 µl dNTPs (10mM)

0.125 µl each of the forward and reverse primer set

6.3775 µl l water

2 µl **template** DNA

0.06 µl **Hotstart GoTaq**

Dung beetles, ectoparasites, butterflies **(Total vol =** 12.5 µl **)**

2.5 µl Taq buffer

2 µl MGCl2

0.0625 µl dNTPs (10mM)

0.125 µl each of the forward and reverse primer set

5.63 µl l water

2 µl **template** DNA

0.06 µl **Hotstart GoTaq**

PCR conditions

2 rDNA PCR conditions

- **Initial denaturation for** 00:00:30 **at** 98 °C
- **Denaturation for** 00:00:10 **at** 98 °C
- **Annealing for** 00:00:30 **at** 68 °C
- **Extension for** 00:02:40 **at** 72 °C
- **Repeat cycles 35 times**
- **Final extention for** 00:02:00 **at** 72 °C

COI (2 sets of conditions, but run one after the other since our program could not handle 2 sets at once.)

- **Initial denaturation for** 00:02:00 **at** 94 °C
- **Denaturation for** 00:00:30 **at** 94 °C
- **Annealing for** 00:00:40 **at** 50 °C
- **Extension for** 00:01:00 **at** 72 °C
- **Repeat cycles 5 times**
- **Final extention for** 00:01:00 **at** 94 °C
- **Initial denaturation for** 00:00:01 **at** 94 °C
- **Denaturation for** 00:00:30 **at** 94 °C
- **Annealing for** 00:00:40 **at** 52 °C
- **Extension for** 00:01:00 **at** 72 °C
- **Repeat this set for 35 cycles**
- **Final extention for** 00:10:00 **at** 72 °C

Gel electrophoresis

3 **Equipment**

- BlueGel system
- MiniOne system

Create .8 - 1.0% agarose 1 gel with 13 combs

- Measure 1 g of agarose
- Mix agraose with 100 mL of 1xTBE
- Microwave the mixture until agarose is completely dissolved (1-3 min)

- Pour the agarose gel into the tray with the comb in place.
- Allow the agarose gel to harden (20-30 min)

Insert the agarose gel into electrophoresis equipment and add 1xTBE buffer until the agarose gel is submerged

Spot check with 2 µl of each sample

Mix 1 µl of loading dye to 2 µl of each sample and load the gel. (If Green Taq buffer with built in loading dye was used, skip this step).

Load 5 µl of 100bp ladder into the agarose gel.

Turn on the electrode and let the DNA run until the band is identifiable

Barcoding PCR

4
- A barcoding PCR was run to attach barcodes from the 96-barcode kit for the MinION to each sample
- We did not use special PCR mastermix at this stage, using instead a mix similar to that of the PCRs above
- We used 1 µl of each barcode primer and 2 µl of every postive PCR amplicon in a total volume of 25 uL

We ran the PCR for amplicons ~ 500bp in length at the following conditions:

- Initil denaturation of hotstart taq at 95 °C for 00:02:00
- Denaturation at 95 °C for 00:00:30
- Annealing at 62 °C for 00:00:30
- Extension at 72 °C for 00:00:40 **extend this to 85s for amplicons 1000bp long**
- Total number of cycles - 18
- Final Extension at 72 °C for 00:05:00

Normalisation and Quantification

5 At this point, our field course was running very short on time, so we omitted a quantus fluorometer check of each successful PCR amplicon. We simply added 5 uL of each amplicon directly into a library pool. We would NOT recommend this system, since it resulted in some highlyg concentrated samples overshadowing others. We recommend normalsing to 50 nM and then pooling so each sample is balanced.

SPRI cleanup

6 Run a SPRI cleanup of the library using your choice of bead purification systems/kits in a 1:1 ratio. Resuspend in the same volume.

Agencourt Ampure XP
by Beckman Coulter
Catalog #: A63880