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We use this protocol and it's working

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DNA Barcoding in a Field Setting

In 1 collection

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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

MATERIALS

STEP MATERIALS

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

BEFORE START INSTRUCTIONS

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 **Promega Catalog #M7402**

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

Butterfly rDNA (Total vol = 25 μ L)

1.25 μ L each of the forward and reverse primer set

9.5 μ L water

3 μ L template DNA

10 μ L q5 MasterMix

Mammal rDNA (Total vol = 13.5 μ L)

1.00 μ L each of the forward and reverse primer set

2.0 μ L water

3 μ L template DNA

6.5 μ L q5 MasterMix

Plant rDNA (Total vol = 13.5 μ L)

1.00 μ L each of the forward and reverse primer set

2.0 μ L water

3 μ L template DNA

6.5 μ L q5 MasterMix

Butterfly COI (Total vol = 25 μ L)

1.25 μ L each of the forward and reverse primer set

7 μ L water

3 μ L template DNA

12.5 μ L q5 MasterMix

Plant rBCL and matK (Total vol = 25 μ L)

1.25 μ L each of the forward and reverse primer set

7 μ L water

3 μ L template DNA

12.5 μ L q5 MasterMix

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

2.5 μ L Taq buffer

1.25 μ L MGCl2

0.0625 μ L dNTPs (10mM)

🧪 0.125 µL each of the forward and reverse primer set

🧪 6.3775 µL 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 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 extension 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 extension 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 extension 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 agarose 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

- 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 positive PCR amplicon in a total volume of 25 µL

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

- Initial 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 highly concentrated samples overshadowing others. We recommend normalising 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 **Beckman Coulter Catalog #A63880**