Protocol ID: NEON

Primer sets to use for metabarcoding analyses

Target gene	F primer (5'-3')	R primer (5'-3')	Approx length	Reference
CO1, F230 fragment	GGTCAACAAATCATA AAGATATTGG	CTTATRTTRTTTATICGIGG RAAIGC	314	F: Folmer et al.; R: Gibson et al. 2015
CO1, BE fragment	CCIGAYATRGCITTYCCICG	GTRATIGCICCIGCIARIAC	224	F&R: Hajibabaei et al., BMC Bioinformatics, 2012

Updated: Jan 12, 2018

Parent protocol: none
Update notes: none

General Precautions:

Gloves must be worn at all times and changed frequently. Surfaces must be cleaned with Rnase Away followed by 70% Ethanol prior to preforming any work and again after completing work.

Sample Prep:

1. Surber samples are 6 were blended with a hand immersion blender. Pulsed each sample for 2 seconds for a total of 30 seconds. Steel blender was cleaned via dipping/mixing in Rneasy followed by 80% ethanol between samples. Gloves were changed between samples.
2. Tow samples were allowed to sit unbothered for 4 hours. Using a 25ml transfer pipette, most of the ethanol was decanted until ~50ml of liquid was remaining.
3. 50ml of each of the 1-12 samples were transferred to sterile 50ml conical vials and spun down for 30 minutes at 4000 RPM.
4. Supernatants were poured off and pellet was frozen at -20 °C.

QC – Samples were visually inspected to ensure sample was homogenized prior to freezing.

Extraction Protocol:

Pelleted samples were thawed at room temp. A sterile swab was used to collect homogenized material and placed into a well of the extraction plate. Genomic DNA from samples was extracted using the MoBio PowerSoil htp-96 well Isolation Kit (Carlsbad, CA) according to the manufacturer's protocol.

QC-gDNA was visually inspected. gDNA should (and was) clear in color, indicating samples were mostly free of PCR inhibitors and debris. Yellow or brown colored samples need to be extracted again.

PCR Protocol:
PCR plate set up was performed in a biosafety cabinet. Two portions of CO1 gene were PCR amplified from each genomic DNA sample using the primer pairs listed above. Both primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega catalog # M5133, Madison, WI) which included 0.4 µM of each primer and 1 µl of gDNA. DNA was PCR amplified using the following conditions: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 40 seconds at 94 °C, 1 minute at 46 °C, and 30 seconds at 72 °C, and a final elongation at 72 C for 10 minutes. (Protocol was from Hajibabaei 2012 and Gibson 2015 papers listed above)

QC- 5ul of each PCR product was run on a 2% agarose gel. DNA bands of the desired size (either ~200 or ~300 bp) were scored on their intensity value on a scale of 0-3 with 3 being the brightest and thus containing the most PCR product.

Post-PCR protocol:
Amplicons were then cleaned by incubating amplicons with Exo1/SAP for 30 minutes at 37 °C following by inactivation at 95 °C for 5 minutes. A second round of PCR was performed to give each sample a unique 12-nucleotide index sequence. The indexing PCR included Promega Master mix, 0.5 µM of each primer and 2 µl of template DNA (cleaned amplicon from the first PCR reaction) and consisted of an initial denaturation of 95 °C for 3 minutes followed by 8 cycles of 95 °C for 30 sec, 55 °C for 30 seconds and 72 °C for 30 seconds.

QC- 5µl of PCR products of each sample were visualized on a 2% agarose gel and scored on the same 0-3 scale as above.

Normalization and Pooling
Final indexed amplicons from each sample were cleaned and normalized using SequalPrep Normalization Plates (Life Technologies, Carlsbad, CA). 25 µl of PCR amplicon is purified and normalize using the Life Technologies SequalPrep Normalization kit (cat#A10510-01) according to the manufacturer's protocol. 5ul of each normalized samples was then pooled together.

Sequencing QC
Pooled libraries were quantified via Qubit using a high sensitivity assay reagents. Amplicon size of the pooled library was sized by running on a TapeStation using 1000bp high sensitivity reagents. Size and concentration were used to determine the molarity of the library.

QA-Library should be free of 200bp amplicons. All 200bp fragments are purify out using AmpPure beads at a 0.7X ratio.

Sequencing protocol:

10pM of library with a 15% spike in of PhiX was Sequenced on an Illumina MiSeq (San Diego, CA) in the CU Boulder BioFrontiers Sequencing Center using the v2 500-cycle kit (cat# MS-102-2002).