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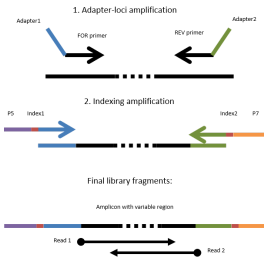
Library Preparation of Bee 18S and 28S rRNA amplicons for High-Throughput Illumina Sequencing



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We use this protocol and it's working

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Abstract

The objective of this protocol is to prepare amplicon sequencing libraries for high-throughput sequencing (via Illumina MiSeq) of the 18S SSU rRNA and 28 LSU rRNA loci for molecular identification of bee communities.

Guidelines

For best results, process samples as soon as possible after they are collected from the field (with not storage or freezing time if possible).

Materials

MATERIALS

- DNeasy 96 Blood & Tissue Kit **Qiagen Catalog #**69581
- Molecular Biology Grade Water **Fisher Scientific Catalog #**10154604
- DreamTaq™ Hot Start Green PCR Master Mix **Thermo Fisher Catalog #**K9021
- 2.0-ml flat bottom microcentrifgue tubes
- ceramic beads
- 96-well PCR plates
- 96-well plate adhesive sealing film
- Zymo 96-well cleanup kit

Sample preparation and gDNA extration

1 Place one mesothoracic leg from each specimen (of a sample) into a 2.0 ml microcentrifuge tube, and repeat this with a new microcentrifuge tube for all samples that are to be separately barcoded.

2 Add five ceramic beads and 500 ul genomic lysis buffer from the desired DNA extraction kit (e.g. Qiagen 96-well Tissue and Cells) to each tube. Optional: and two mealworm beetle (*Tenebrio molitar*) legs as inter-sample controls.

3 Lyse tissue at room temperature overnight, then pulverize for 10 min in a TissueLyser prior to proceeding with the desired genomic DNA extraction kit instructions (e.g. Qiagen 96-well Tissue and Cells kit). Elute DNA into 60 ul elution buffer, pH 8.0.

First Round PCR Amplifcation

4 Prepare Master Mix 1 for each locus separately (18S and 28S) for first-round PCR amplification:

	Volume (per reaction)	Working stock concentration	Reagent
	10 ul	2X	DreamTaq MasterMix
	0.4 ul	10 uM	18S FOR primer BD0150*
	0.4 ul	10 uM	18S REV primer BD0151*

	8.2 ul		Molecular grade water
	19 ul Total MasterMix1		

*For the 28S locus, use FOR primer BD0152 and REV primer BD0153.
BD0150 = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GTGCGGTTAAAAAGCTCGTAGTTG 3'
BD0151 = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GAACCATACTTCCCCCGGAAC 3'
BD0152 = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GTGAAACCGTTCAGGGGTAAACC 3'
BD0153 = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGTGTTTCAAGACGGGTCCTG 3'
(underlined portions anneal to gDNA targets)

4.1 For each locus separately, add 19 ul of Master Mix 1 and 1 ul of gDNA (10 ng/ul) to a suitable PCR reaction tube (or well in a 96-well plate).

4.2 Amplify first round PCR reactions in a thermocycler with 30 cycles of 95 °C for 0:30, 55 °C for 0:30, and 72 °C for 0:30, followed by a final extension at 72 °C for 5 minutes.

5 Keeping each sample separate, pool 10 ul of PCR product from each locus (e.g. 18S and 28S), and purify with a PCR clean-up kit (e.g. Zymo 96-well PCR CLeanup kit).

Second Round PCR Amplification

6 For each sample, add reagents to an appropriate PCR reaction tube (or well of a 96-well plate):

	Volume (per reaction)	Working stock concentration	Reagent

	10 ul	2X	DreamTaq MasterMix
	2 ul	2 uM	FOR Nextera barcoded primer*
	2 ul	2 uM	REV Nextera barcoded primer*
	5 ul		Molecular grad water
	2 ul		purified 1st-round PCR product
	20 ul Total reaction volume		

*FOR Nextera barcoded primer = 5' AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN TCGTCGGCAGCGTC 3'
*REV Nextera barcoded primer = 5' CAAGCAGAAGACGGCATACGAGAT NNNNNNNN GTCTCGTGGGCTCGG 3'
where "NNNNNNNN" is a unique 8-nt barcode sequence.

6.1 Amplify second round PCR reactions in a thermocycler with 8 cycles of 95 °C for 0:30, 55 °C for 0:30, and 72 °C for 0:30, followed by a final extension at 72 °C for 5 minutes.

7 Pool 5 ul of second-round PCR product from each sample and clean half of this using a PCR cleanup kit (e.g. Zymo PCR Cleanup kit). Elute in 30 ul elution buffer (EB), pH 8.0, and this eluted product is ready to submit for cluster formation on the Illumina MiSeq (using 2×300 bp paired end reads).

7.1 For best results, gel purify a large portion of the cleaned second-round PCR products to target the >500 bp bands and minimize <200 bp dimers (which can occupy a disproportionately large number of flowcell colonies if they are present).