Treseder Lab Pyrosequencing Protocol

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Abstract

1. **DNA Extraction**

- Extract DNA from sample using the phenol/chloroform procedure or your kit of choice. We typically use the Mo Bio Power Soil DNA extraction kit for extracting DNA from soil and plant litter samples.
- Use the Nanodrop in the Martiny lab to estimate the concentration and purity of your DNA extraction.
- Dilute DNA to 10 ng/ul prior to PCR.

2. **PCR Amplification**

Pre-amplification checklist:

- All pipetting should be done with aerosol barrier, PCR certified pipet tips in a PCR work station
- On ice thaw primers, DNA and PCR master mix
- Decontaminate pipettors and interior surfaces of a PCR workstation with DNA/RNA Away or 10% bleach solution.
- UV irradiate PCR work station interior, pipettors and consumables for minimum 15 min
- All samples should be amplified in triplicate.
- Include negative (no template) and positive (DNA isolated from mushroom) controls.

Primer information:

Forward primer:

454 primer B + 'AG' linker + SSU817f

GCCTTGCCAGCCCGCTCAGAGTTAGCATGGAATAATRRAATAGGA

Reverse Primer:

454 primer A + 12-base bar code + 'AC' linker + ssu1196r

GCCTCCCTCGCGCCATCAG-12 base bar code-ACTCTGGACCTGGTGAGTTTCC

Example barcode: ACACACTATGGC

Example primer sequence:
GCCTCCCTCGCGCCATCAGACACACTATGGCACTCTGGACCTGGTGAGTTTCC

PCR Cocktail:

22.5 ul PCR master mix (we use the Invitrogen Platinum PCR SuperMix)

1 ul BSA

0.75 ul 10 uM forward primer (all samples get the same forward primer)

0.75 ul 10 uM reverse primer (all samples get a unique barcoded reverse primer)

1 ul template DNA

PCR cycle:

Samples are initially denatured at 94°C for 10 min, then amplified using 30 cycles of 94°C for 45 sec, 52°C for 30 sec, and 72°C for 90 sec. A final extension of 10 min at 72°C is added at the end of the program to ensure complete amplification of the target region.

3. **Check for PCR product on a gel**

4. **Amplicon Cleaning**

- Combine the triplicate PCR reactions into a single volume
- Clean PCR reactions with a PCR clean up kit. The MoBio UltraClean PCR clean up kit and the Invitrogen Purelink PCR purification kit have both worked well.

5. **Amplicon Quantification**

- Use the qubit system for quantifying DNA, with a few modifications.
- Instead of using the qubit assay tubes, prepare the samples in black microplates.
- Perform assay with 1 ul of PCR product
- Read the plates on the microplate reader in the Allison lab set at 485ex /530em. Estimate amplicon concentration using the standard curve.

6. **Amplicon Pooling**

- Combine equal amounts of DNA per sample into single vessel (usually a 15 mL tube).
- Concentrate pooled amplicons using the Invitorgen Purelink PCR purification kit
- Run a gel to visualize pooled samples
- Depending on how your pooled amplicons look on the gel, you may need to gel purify the pooled amplicons. Pyrosequencing is biased towards shorter reads. If you have a messy band on your gel, it is important to purify your samples to improve the quality of the DNA you will receive.
    - We used the Qiagen QIAquick Gel Extraction Kit. We ran samples out on a 1.4 % gel.
    - The main drawback to performing a gel extraction is that you will almost certainly lose some of your sample. You may need to pool additional PCR product and perform a second gel extraction to obtain enough sample for sequencing.
    - Remove contaminants from gel purified samples using an Invitrogen Purelink PCR purification kit.

7. **Estimate final sample concentration and purity**

- Use the qubit system for estimating sample concentration. The sequencing facility requires 2-3 ug of DNA for sequencing.
- Use the Nanodrop in the Martiny lab for estimating sample purity. Samples with an A260/A280 ratio of ~1.8 typically provide optimal sequencing results.

8. **Send sample for sequencing**

- Send samples to the Engencore sequencing facility at the University of South Carolina. Check their website for more specific details about what else to include in the shipment.
- Send samples overnight on dry ice

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Materials

- MoBio UltraClean PCR by Contributed by users
- qubit system by Contributed by users
- Qiagen QIAquick Gel Extraction Kit by Contributed by users
- nanodrop by Contributed by users

Protocol