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McKenzie Lab 16S/ITS Sample Processing Protocol

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Fungal Dev Data

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ABSTRACT

This is the protocol used in the McKenzie Lab at CU Boulder for 16S and ITS1 sample processing for eventual amplicon sequencing on a MiSeq.

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ABSTRACT

This is the protocol used in the McKenzie Lab at CU Boulder for 16S and ITS1 sample processing for eventual amplicon sequencing on a MiSeq.

McKenzie Lab 16S/ITS Sample Processing Protocol

I. PCR

Primers for 16S are for paired-end sequencing and amplify the V4 region at 515F-806R (we use Earth Microbiome Project primers: <http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/>).

Primers for ITS are also for paired-end sequencing and amplify the ITS1f-ITS2 region (again, we use Earth Microbiome Project primers: <http://www.earthmicrobiome.org/protocols-and-standards/its/>).

Make sure that you record what primer plate is used and DO NOT use the same primer combinations for any samples that are going onto the same sequencing run. Also, do a test PCR plate with a subset of the samples to first make sure that they will amplify. Always amplify samples in triplicate.

Reagents used:

Master mix	Promega GoTaq G2 colorless master mix
Water	Nuclease-free water that comes with master mix OR Sigma sterile-filtered, BioReagent, suitable for cell culture water
Primers	Elim bio

PCR mix (1x):

Master mix 12.5 µl

Water 10.5 µL

Primer mix 1 µL

DNA template 1 µL (2 µL if you have low biomass samples that didn't amplify with a test PCR – subtract 1 µL from water)

PCR mix for 96 well plates (110x):

Master mix 1375 µL

Water 1155 µL

-----23 µL mix in each well, plus 1 µL of primer mix and 1 µL of DNA template-----

In the lab, the protocol in the PCR machine is under the folder "HOLLY" and is called "515/806 Universal Bac." The thermocycler conditions are as follows:

94°C 3 min

95°C 45 sec

50°C 1 min

72°C 1:30

-----35 cycles-----

72°C 10 min

14°C ∞

II. Gel Electrophoresis

Check each plate of samples on a 2% agarose gel (with SybrSafe gel stain), which we run at 130V for 30 min. Combine triplicates of samples into one plate.

III. Quantify with Quant-iT PicoGreen dsDNA Assay Kit

Calculate how much of reagents go into assay mixture:

110 samples per plate (96-well plate + extra included for pipetting error) * # of plates = Y

1x TE: $Y * 150 = Z$

20x TE: $Z / 20 = X$

Water: $Z - X = W$

-----Combine X mL of 20X TE and W mL of Water into a conical-----

PicoGreen: 5 µL (stock) * Z = P µL of stock PicoGreen added into conical above

In black PicoGreen assay plate:

Samples: 2 μL amplicon + 150 μL assay solution

Standards: In first column of plate,

-Add 300 μL assay solution into first well and 150 μL into wells B1-H1

-Add 2 μL of DNA standard in well A1

-Pipette up and down to mix, then transfer 150 μL of well A1 into well B1, mix, repeat down the column

Read the plate and check that your standards worked. Find the slope of the standards line and use this to convert fluorescence into ng/ μL of DNA. Put this information into a spreadsheet, then calculate how much volume you need to put into the pool so that you have 300 ng of DNA per sample ($300 \text{ ng} / X \text{ ng } \mu\text{L}^{-1}$). Put this new info into another spreadsheet and print for use in pooling. For a more detailed technical protocol, see "Amplicon Quantification Protocol."

IV. Pooling and Clean-up

Using your pooling values from the previous step, pool any samples that need $\leq 50 \mu\text{L}$ of liquid in the total pool (55 μL absolute max if certain samples are critical). Do one microcentrifuge tube per plate, then use 300 μL of sample from each tube in the MoBio UltraClean PCR Clean-Up Kit to clean up the pooled samples.

Next, do another PicoAssay for each tube. Use this data to figure out how much of each tube needs to be added into the final pool if you wanted each tube to represent the same percentage of the final pool and for the samples to be at 5000 ng of DNA. To do this, use the pooling spreadsheet in the lab files.

