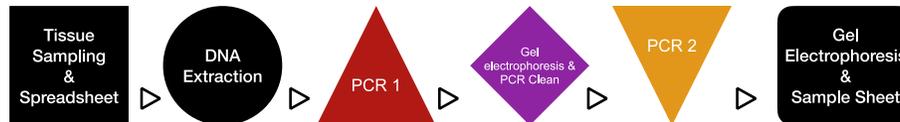


High Throughput DNA Barcoding – Methods

These protocols cover part of the laboratory steps described in Olds et al. (1).

This methodology is designed for sequences to be run through University of Idaho's IIDS Genomics and Bioinformatics Resource Core (GBRC). Please contact Dan New (dnew@uidaho.edu) to open an account or to consult if you wish to apply these sequencing protocols at your facility.

Workflow



The stages of the HT DNA barcoding process for the Wilson Lab are encapsulated in the colored (not black) icons of the workflow above. A version of this icon will indicate which part of the workflow that section describes.

DNA Extraction

Samples from 25mg of hymenophore tissue are extracted using a simple extraction method such as Extract-n-amp (Millipore Sigma) or the “[simple extraction](#)” method on the Colorado Macrofunga Project website under protocols. This is done 24-48 hours prior to PCR 1 step and stored at -20 °C until ready for PCR.

PCR1 Step Description



This step amplifies the ITS 2 region of the nuclear ribosomal internal transcribed spacer region. This region is targeted by using primer regions fITS7 (2) and ITS4 (3). The primers are appended with sequence tags labeled CS1 and CS2 respectively and are each 22 nucleotides long. The unique tag sequences produce primer binding sites for Illumina MiSeq paired end index adapters in the PCR2 step. Between the ITS primers and the tags is the spacer region that may vary from 0-6 bp in the forward primer (fITS7+ variable spacer + CS1=**CS1**) and 0-4 bp in the reverse primer (ITS4+ variable spacer + Shokralla barcode +CS2=**CS2**).

The novel feature to this HT DNA Barcoding method is the insertion of a 5pb barcode in the reverse primer between the ITS4 primer and the spacer region. There are 8 primers initially produced for this process. These eight primers correspond to novel barcodes developed by Shokralla et al. (4) for 454-Sequencing. This gives the CS2 primer eight unique barcodes that are labeled A-H.

CS1 primers, for the purposes of this project, are labeled, CS1-fITS7.(1-7)-for. The part in parentheses indicate the seven unique spacer combinations. The combinations of spacers used must be documented for downstream demultiplexing of sequences. The eight CS2 barcoded primers are labeled according to the unique Shokralla barcodes as CS2-Shok1(A-H).(0-4)-ITS4, with the letters in parentheses indicating the specific barcode. (See Table X) CS1 (AKA CS1-fITS7-for) primers with unique spacers are ordered individually and combined in equal amounts. CS2 primers (CS2-Shok1-ITS4) with unique spacers are ordered individually for each unique barcode. Primers with different spacers are only combined with the same barcode.

For PCR preparation, there should have one forward direction primer mix CS1-fITS7-for (mix with equal parts different spacers 1-7) and eight different reverse primers: CS2-Shok1A-ITS4, CS2-Shok1B-ITS4, CS2-Shok1C-ITS4, CS2-Shok1D-ITS4, CS2-Shok1E-ITS4, CS2-Shok1F-ITS4, CS2-Shok1G-ITS4, CS2-Shok1H-ITS4. Each reverse primer is a mix of spacers 0-4.

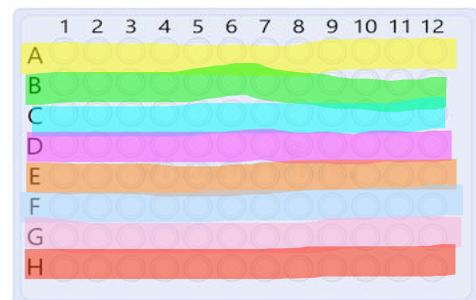
PCR1 Method

PCR are performed in 25 μL reactions consisting of:

- 12.5 μL of *MyTaq* mix ([Bioline](#)),
- 1.25 μL forward primer CS1-fITS7-for
- 1.25 μL one version of reverse barcode barcode CS2-Shok1(A-H)-ITS4.
- 9 μL PCR pure water.
- 1 μL of DNA Template from simple extraction process.

Setup

- Organize PCR1 specimens in the order you want them on your plate. ***Insure you are keeping good records of which samples have been assigned to which PCR1 barcode. This will be critical for downstream demultiplexing of samples.***
 - Make sure you use the sample template spreadsheet provided. Also make sure you are using the correct specimen catalog number that can be referenced using GBIF. This will facilitate downstream submission of the data.
 - Ideally you should organize your specimens so that they alter taxonomic groups regularly, if possible. Having specimens all of the similar taxonomic group bunched together will prevent recognition of possible error downstream. I.e., you will not know if labels were switched, or cross contamination took place between two unidentified *Lactarius* specimens vs. a *Lactarius* and *Mycena* specimens that are next to each other on the plate.
- Create 8 individual PCR master mixes, one for each barcode A-H.
- Calculate $N_{\text{mix}} + 1$.
 - In this case just say $N = 13$.
- Label eight 1.5 mL eppi tubes for PCR mixes A-H.
- Using $N = 13$ add the following to each of the PCR mix tubes A-H.
 - 117 μL of PCR H_2O .
 - 162.5 μL of Taq mix.
 - 16.25 μL of CS1 primer mix.
 - For individual tubes A-H, add the 16.25 μL of the CS2 primer mix whose letter corresponds to the letter on the PCR mix tube.
- Add volumes of 144 μL in columns 1 and 7 from PCR mixes whose letter corresponds to the lettered row.
- Use a 5-50 μL multichannel pipettor to distribute 24 μL of PCR master mixes from column 1, to columns 2-6. Repeat by dispersing column 7 through columns 8-12.
-



- Add 1 μL of DNA template to the corresponding well.
- Add a silicon plate sealer to the plate.
- Label the plate appropriately using a sharpee.
- Put in the thermalcycler and run the program labeled PCR1 in the “Metabarcoding” folder.

Thermocycler program PCR1: initial denature @ 94°C for 2 min; 35 cycles of: denature @ 94°C for 30 sec, anneal @ 57°C for 30 sec, extension @ 72°C for 30 sec; final extension @ 72°C for 5 min; stasis @ 4°C forever.

Gel Electrophoresis and PCR Clean



Follow the [PCR cleaning protocol](#) to remove excess reagents and primers before you run the gel. This will indicate how much *cleaned* product is available for pooling.

Preparing the large gel in the DBG lab. (Gene Mate 50-well combs E-4112-150MC)

- Set up the large blue gel rig and the gel cast. Orient so the black terminal is on the left and the red terminal is on the right.
 - Remember that DNA “Runs to red”.
- Use the white nobs and the bubble-level in the rig to make sure the gel cast is level to produce an even-thickness gel.
- Large gels use 2.5g of agarose in 250-260 mL of 1x TE buffer. (See cheat sheet for gels in the lab.)
- Mix above ingredients in an Erlenmeyer flask and microwave for 2 minutes. Keep an eye on when it’s boiling to avoid boiling-over.
 - Using the “lobster claw”, hold flask up to the light while swirling to see if agarose crystals have melted. Microwave for another minute if there are still unmelted agarose.
 - Once agarose has melted, add diH₂O to bring volume back to ~260 mL volume and swirl to mix in the water.
 - Set aside to let it cool to the touch. Swirl occasionally.
- When the flask is cooled but still warm and liquid, swirl gently and pour in the gel cast starting at one corner, pouring evenly and moving diagonally to the other corner to produce an even layer. Avoid splashing and creating bubbles.
- Add the clear plastic combs with black rubber ends for the first three rows of wells. These combs are custom and spaced apart to match the width of the multi-channel pipettors. For the last row use the comb with 24 wells.
- Using the blue sample plate, add 26 μL of EZ Vision Three loading dye (in refrigerator door) to all the wells in column 1. Then using a 1-10 μL multichannel pipettor, dispense 2 μL of loading dye from column 1 wells to wells in columns 2-12.
- To add samples from the PCR plate, keep samples organized based on columns, e.g., load column 1 samples A-H, then column 2... etc., using the multichannel pipettor to transfer 5 μL of sample to the blue collection plate with EZ Vision Three loading dye.

- Once dye and PCR product is mixed, transfer 5-7 μ L of product to the gel using the multichannel pipettor. By the third row of wells on the gel you will need to transfer to the single pipettor to load the remaining samples.

A successfully amplified product will produce a thick, somewhat fuzzy, band due to the incorporation of spacers of different lengths.

Pool Samples



Combine columns of samples with one sample from each row A-H.

- From your gel image qualify the success of PCR amplification into 3 categories. Highlight the sample according to the category. Suggested highlight colors are in parentheses.
 - Strong (No highlight): Nice clear bright bands or simply clear bands.
 - Weak (yellow): Bands visible, but faint. Not strong or clear.
 - No amplification (red): No PCR amplification is apparent. Maybe if you squint you can imagine a band, otherwise nothing.
- Pool sets of eight PCR product, each with one of A-H PCR1 barcodes into a single tube with the volume to be added based on the categories defined above.
 - Strong = 2 μ L
 - Weak = 5 μ L
 - No amplification = 10 μ L
- Label tubes appropriately, based on plate number and column number. Arrange tubes in a new plate configuration based on PCR2 tab in the spreadsheet.

PCR2 Step Description



PCR2 will use primers supplied by GRBC and/or homemade primers. For homemade primers, use the PCR2 primer spreadsheet. You will need to supply the GRBC with a “barcode lookup table”, and possibly the full sequences for the homemade primers. Examples for our lab are available as “DBGWilson_CS1_CS2_Sequences.txt” and “DBGWilsonBarcodeLookupTable.txt” respectively. PCR reactions include the following:

PCR2 Method

PCR2 reactions are performed in volumes 25 μ L consisting of:

- 12.5 μ L of *MyTaq* mix ([Bioline](#)),
- 11.5 μ L PCR pure water.
- 5 μ L of pooled PCR1 samples as the DNA template.

Use the same formula identified above for N number of reactions to calculate the volume of reagents to add to you master mix.

Do not dispense your Master Mix into the wells as you did in PCR1. Each well in the PCR2 plate contains a unique primer combination which you would screw up if you shared the contents between wells. You should set up your mastermix on a separate clean plate, or in 8-strip PCR tubes prior to dispensing the master mix between wells. Next add 1 μ L of your pooled PCR1 template to a previously designated well. Once all the template has been added, seal with a silicon mat and run PCR2 program on the thermalcycler.

Thermocycler program PCR2: initial denature @ 95°C for 1 min; 15 cycles of: denature @ 95°C for 30 sec, anneal @ 60°C for 30 sec, extension @ 68°C for 1 min; final extension @ 68°C for 5 min; stasis @ 4°C forever.

Next: Submitting sequences to University of Idaho's IIDS Genomics and Bioinformatics Resources Core (GBRC)

References

1. C. G. Olds, J. W. Berta-Thompson, J. J. Loucks, R. A. Levy, A. W. Wilson (In Review) Applying a Modified Metabarcoding Approach for the Sequencing of Macrofungal Specimens. in *Applications in Plant Sciences* (Submitted Dec 2021).
2. K. Ihrmark *et al.*, New primers to amplify the fungal ITS2 region—evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol. Ecol.* **82**, 666-677 (2012).
3. T. J. White, T. D. Bruns, S. Lee, J. W. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies." in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, 1990), pp. 315-322.
4. S. Shokralla *et al.*, Next-generation DNA barcoding: using next-generation sequencing to enhance and accelerate DNA barcode capture from single specimens. *Molecular ecology resources* **14**, 892-901 (2014).