16S Library PCR Protocol with PNA Blockers

This protocol is a modified version of the Earth Microbiome Project's PCR protocol and the JGI's PNA PCR Protocol.

Notes: There are 25 each of forward and reverse primer barcodes that can be combined to make 625 total unique barcode combinations. The primers are at 10μM each and are mixed in equal parts to create the 10μM combinations.

We use the bacteria/archaeal primers 515F/806R with an inhouse barcode system designed by Aaron Darling.

More about the primers can be found here:

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J.

1X Recipe:
22.5 μL of Invitrogen Platinum SuperMix
1.25 μL of Primer Mix (10μM)
0.62 μL of PNA chloroplast blocker ($\sim$ 50μM)
0.62 μL of PNA mitochondria blocker ($\sim$ 50μM)
1.0-5.0 μL of template DNA

Total: 26-30μL

Protocol:

PCR

1. Amplify samples in triplicate, meaning each sample will be amplified in 3 replicate 25 μL PCR reactions.

2. Combine the triplicate PCR reactions for each sample into a single volume. Combination will result in a total of 75 μL of amplicon for each sample. Do NOT combine amplicons from different samples at this point.

3. Run 5 μl of amplicons for each sample on an agarose gel to verify amplification. Expected band size for roughly 300-400 bp

Clean-up

4. Clean PCR reactions with magnetic beads. See "Magnetic-bead PCR Cleaning Protocol" for an example protocol.

5. Quantify amplicons with Qubit.

Pooling

6. Combine an equal amount of amplicon from each sample into a single, sterile tube. Generally 240 ng of DNA per sample are pooled. However, higher amounts can be used if the final pool will be gel-isolated or when working with low biomass samples. *Note:* When working with multiple plates of samples, it is typical to produce a single tube of amplicons for each plate of samples.

7. If spurious bands were present on gel (in step 3), half of the final pool can be run on a gel and then gel-extracted to select only the target bands.*

   *We routinely see high molecular-weight bands or smears in the gel. We use the Pippin prep gel from Sage Science (http://www.sagescience.com/products/pippin-prep/) to select the correct band size.

8. Measure concentration and 260/280 of final pool that has been cleaned. For best results the 260/280 should be between 1.8-2.0.

9. Send an aliquot for sequencing along with sequencing primers.

Thermocycler Conditions:

For 96 well thermocyclers:

1. 94°C 3 minutes
2. 94°C 45 seconds
3. 50°C 60 seconds
4. 72°C 90 seconds
5. Repeat steps 2-4 35 times
6. 72°C 10 minutes
7. 4°C HOLD

For 384 well thermocyclers:

1. 94°C 3 minutes
2. 94°C 60 seconds
3. 50°C 60 seconds
4. 72°C 105 seconds
5. Repeat steps 2-4 35 times
6. 72°C 10 minutes
7. 4°C HOLD