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Library preparation of bacterial and fungal amplicons and sequencing

Bacterial 16S rRNA gene amplicons were generated in 20 µL PCR reactions using 20 ng of fecal DNA with 20 cycles using high-fidelity Platinum™ SuperFi Polymerase (Life Technologies, Carlsbad, CA) at 52.7°C annealing using with degenerate 8F (AGAGTTTGATCMTGGCTCAG) and 357R (CTGCTGCCTYCCGTA) primers. Fungal ITS1 amplicons were generated in 20 µL PCR reactions using 20 ng DNA with 30 cycles using Platinum™ SuperFi Polymerase at 56.1°C annealing using ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers yielded sufficient amplification of ITS targets. All PCR reactions were purified using Agencourt AmPure Magnetic Beads (Beckman), resuspended in 20 µL of nuclease-free water and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA). Amplicons were further qualified using the DNA 1000 assay on the Agilent Bioanalyzer (Agilent Technologies).

Illumina paired-end adapters with unique Nextera XT v2 indexes were ligated to 100 ng of 16S and ITS1 amplicons using Nextera XT DNA Sample Preparation (Illumina, San Diego, CA). Library enrichment was performed with 10 cycles of PCR and purified using Agencourt Ampure Magnetic Beads (Beckman). All libraries were subjected to quality control using qPCR, DNA 1000 Bioanalyzer (Agilent), and Qubit (Life Technologies, Carlsbad, CA) to validate and quantitate library construction then pooled at equimolar concentrations. Pooled libraries were assayed on Agilent Bioanalyzer (Santa Clara, CA) to check final sizing as well as on KAPA Biosciences qPCR for quantitation. Samples were multiplexed and sequenced on an Illumina MiSeq platform (Illumina) with paired-end 300bp sequencing chemistry. Raw data processing and run demultiplexing was performed using on-instrument analytics as per manufacture recommendations.

Data analysis

Raw FASTQ data were merged with overlap into single reads using SeqPrep v1.0 wrapped by QIIME v1.9 (Caporaso JG et al., 2010) with default setting. A custom script was used to remove any reads that do not contain the proximal primer sequence or any reads containing a single N (unknown base) to enrich for high quality reads. For bacterial samples, the remaining high-quality reads were aligned to the Greengenes reference database (May 2013 release) using BLAST v2.2.22 in QIIME v1.9 wrapper with an identity percentage ≥97% to select the operational taxonomic units (OTUs). Similarly, for fungal samples, the filtered reads were aligned with the Targeted Host Fungi (THF) custom fungal ITS database (version 1.5) (Tang et al., 2015) with an identity percentage ≥97% for OUT picking.

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Reference:

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335-6.

Tang J*, Iliev ID*, Brown J, Underhill DM*, Funari VA*. Mycobiome: Approaches to analysis of intestinal fungi. J Immunol Methods. 2015 Jun;421:112-21.