Methods

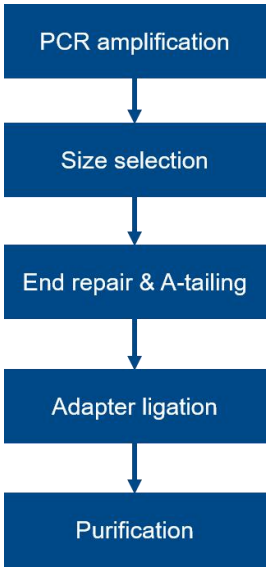
Sequencing

1. Sample Quality Control

Methods of sample quality control refer to QC report.

2. Library Construction, Quality Control and Sequencing

PCR amplification of targeted regions was performed by using specific primers connecting with barcodes. The PCR products with proper size were selected by 2% agarose gel electrophoresis. Same amount of PCR products from each sample was pooled, end-repaired, A-tailed and further ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to generate 250bp paired-end raw reads. The experimental procedures of DNA library preparation are shown:



Workflow of library construction

The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.Quantified libraries will be pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

Data analysis

1. Paired-end reads merged and quality control

1.1 Data Split

Paired-end reads were assigned to samples based on their unique barcodes and were truncated by cutting off the barcodes and primer sequences.

1.2 Paired-end reads merged

Paired-end reads were merged using FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/)[1], a very fast and accurate analysis tool designed to merge paired-end reads when at least some of the reads overlap with the reads generated from the opposite end of the same DNA fragment, and the splicing sequences were called Raw Tags.

1.3 Data Filtration

Quality filtering on the raw tags were performed using the fastp (Version 0.20.0) software to obtain high-quality Clean Tags.

1.4 Chimera Removal

The Clean Tags were compared with the reference database (Silva database https://www.arb-silva.de/ for 16S/18S,Unite database https://unite.ut.ee/ for ITS) using Vsearch (Version 2.15.0)to detect the chimera sequences, and then the chimera sequences were removed to obtain the Effective Tags[2].

2. ASVs Denoise and Species annotation

2.1 ASVsDenoise

For the Effective Tags obtained previously, denoise was performed with DADA2 or deblur module in the QIIME2 software (Version QIIME2-202006) to obtain initial ASVs (Amplicon Sequence Variants) (default: DADA2), and then ASVs with abundance less than 5 were filtered out[3].

2.2 Species Annotation

Species annotation was performed using QIIME2 software. For 16S/18S, the annotation database is Silva Database, while for ITS, it is Unite Database.

2.3 Phylogenetic Relationship Construction

In order to study phylogenetic relationship of each ASV and the differences of the dominant species among different samples(groups), multiple sequence alignment was performed using QIIME2 software.

2.4 Data Normalization

The absolute abundance of ASVs was normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on the output normalized data.

3.AlphaDiversity

In order to analyze the diversity, richness and uniformity of the communities in the sample,alpha diversity was calculated from 7 indices in QIIME2, including Observed_otus, Chao1, Shannon, Simpson, Dominance, Good's coverage and Pielou_e.

Three indices were selected to identify community richness:

Observed_otus - the number of observed species (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.observed_otus.html);

Chao - the Chao1 estimator (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.chao1.html#skbio.diversity.alpha.chao1);

Dominance - the Dominance index (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.dominance.html#skbio.diversity.alpha.dominance);

Two indices were used to identify community diversity:

Shannon - the Shannon index (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.shannon.html#skbio.diversity.alpha.shannon);

Simpson - the Simpson index (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.simpson.html#skbio.diversity.alpha.simpson);

One indice was used to calculate sequencing depth:

Coverage - the Good's coverage (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.goods_coverage.html#skbio.diversity.alpha.goods_coverage);

One indice was used to calculate species evenness:

Pielou_e - Pielou's evenness index (http://scikit-bio.org/docs/latest/generated/skbio.diversity.alpha.pielou_e.html#skbio.diversity.alpha.pielou_e).

4. Beta Diversity

In order to evaluate the complexity of the community composition and compare the differences between samples(groups), beta diversity was calculated based on weighted and unweighted unifrac distances in QIIME2.

Cluster analysis was performed with principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the ade4 package and ggplot2 package in R software (Version 3.5.3).

Principal Coordinate Analysis (PCoA) was performed to obtain principal coordinates and visualize differences of samples in complex multi-dimensional data. A matrix of weighted or unweighted unifrac distances among samples obtained previously was transformed into a new set of orthogonal axes, where the maximum variation factor was demonstrated by the first principal coordinate, and the second maximum variation factor was demonstrated by the second principal coordinate, and so on. The three-dimensional PCoA results were displayed using QIIME2 package, while the two-dimensional PCoA results were displayed using ade4 package and ggplot2package in R software (Version 2.15.3).

To study the significance of the differences in community structure between groups, the adonis and anosim functions in the QIIME2 software were used to do analysis. To find out the significantly different species at each taxonomic level (Phylum, Class, Order, Family, Genus, Species), the R software (Version 3.5.3) was used to doMetaStat and T-test analysis. The LEfSe software (Version 1.0) was used to do LEfSe analysis (LDA score threshold: 4) so as to find out the biomarkers.

Further, to study the functions of the communities in the samples and find out the different functions of the communities in the different groups, the PICRUSt2 software (Version 2.1.2-b)was used for function annotation analysis.

Reference

[1]Magoč T,Salzberg S L. FLASH: fast length adjustment of short reads to improvegenome assemblies. Bioinformatics 27.21 (2011): 2957-2963.

[2]Haas, Brian J., et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons.Genome research 21.3 (2011): 494-504.

[3]LiMinjuan,Shao Dantong,Zhou Jiachen et al. Signatures within esophageal microbiota with progression of esophageal squamous cell carcinoma.[J] .Chin J Cancer Res, 2020, 32: 755-767.