# Analysis of Gut Microbiota in Rheumatoid Arthritis Patients: Disease-Related Dysbiosis and Modifications Induced by Etanercept

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**4. Materials and Methods**

**4.3.** Next-Generation Sequencing of Bacterial 16S Ribosomal RNA Gene
The Illumina 16S Metagenomic Sequencing Library Preparation instructions were followed for high-throughput sequencing. Firstly, 12.5 ng of each DNA extract was employed for the amplification of the V3–V4 hypervariable regions of the bacterial 16S ribosomal RNA (rRNA) gene, using the following primers with Illumina adapters (underlined): forward primer: 50-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, reverse primer: 50-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, selected from Klindworth et al. [43]. The amplification reaction was carried out in the presence of the 2_ KAPA HiFi HotStart Ready Mix (Roche, Milan, Italy) under the following conditions: initial denaturation at 95 _C for 3 min, followed by 25 cycles of denaturation at 95 _C for 30 s, primer annealing at 55 _C for 30 s, extension at 72 _C for 30 s, with a final elongation at 72 _C for 5 min. PCR amplicons were then purified by means of Agencourt AMPure XP beads (Beckman Coulter, Milan, Italy). The purified DNA products were then subjected to a further PCR to attach dual Illumina indices (Nextera XT Index Kit, Illumina Inc., San Diego, CA, USA) necessary for multiplexing. The reaction was performed under the following conditions: initial denaturation at 95 _C for 3 min, followed by eight cycles of denaturation at 95 _C for 30 s, primer annealing at 55 _C for 30 s, extension at 72 _C for 30 s, with a final elongation at 72 _C for 5 min. Following a further PCR purification, the eluted DNA products were quantified using the Qubit dsDNA BR Kit assay, diluted to a concentration of 4 nM, and pooled in equal proportion into a single library. Paired-end sequencing (2 _ 300 cycles) was carried out on an Illumina MiSeq device (Illumina Inc.) according to the manufacturer's instructions. Sequences were demultiplexed based on index sequences, and FASTQ files were generated.

# A powerful machine learning approach to identify interactions of differentially abundant gut microbial subsets in patients with metastatic and non-metastatic pancreatic cancer

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**Materials and methods**

***Next-generation sequencing and analysis of bacterial 16S rRNA gene***
For each sample, 12.5 ng of DNA obtained through the above procedure was used to amplify the V3-V4 region of the 16S rRNA gene using KAPA HiFi HotStart Ready Mix (Roche Diagnostics, Milan, Italy, Cat. N° 07958935001) and the following primers selected by Klindworth[20] with Illumina adapters added: forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG reverse

primer: 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. Samples were then barcoded with the Nextera XT Index Kit (Illumina, Milan, Italy, Cat. N° FC-131-1002), as previously described.[21] Next, the libraries were purified, quantified using a Qubit™ Flex Fluorometer (Thermo Scientific, Milan, Italy), pooled and sequenced in pairs (2 × 300 cycles) on an Illumina MiSeq platform (San Diego, CA, USA). FASTQ files generated by MiSeq were deposited in ArrayExpress under the code E-MTAB-12513 and de-multiplexed and analyzed using the 16S Metagenomics GAIA 2.0 software, Sequentia Biotech, Barcelona, Spain (2019). Read pairs were quality-controlled (i.e., trimming, clipping and adapter removal) based on FastQC and BBDuk and, to obtain the taxonomic profile of each sample, they were mapped with BWA-MEM against the 16S reference database available in NCBI GenBank. Specifically, all full-length sequences of the 16S rRNA gene from all prokaryotes are included except those longer than 3000 bp which are filtered out, since the gene is expected to be shorter. A prediction of the microbial functions was carried out using the Tax4Fun2 package in R. The Mann–Whitney test was used to analyze the differences of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways between the two groups, which were then depicted using STAMP software.

# 16S Metagenomic Sequencing Library Preparation

*Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*

## Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached. For more information on primer sequences, see Amplicon Primers, on page 3.

### Consumables

**NOTE**
For more information on consumables and equipment for this protocol see Consumables and Equipment, on page 21.

| Item | Quantity | Storage |
|---|---|---|
| Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5) | 2.5 µl per sample | -15° to -25°C |
| Amplicon PCR Reverse Primer (1 µM) | 5 µl per sample | -15° to -25°C |
| Amplicon PCR Forward Primer (1 µM) | 5 µl per sample | -15° to -25°C |
| 2x KAPA HiFi HotStart ReadyMix | 12.5 µl per sample | -15° to -25°C |
| Microseal 'A' film | | |
| 96-well 0.2 ml PCR plate | 1 plate | |
| [Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504) | | |

### Procedure

1 Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:

| | Volume |
|---|---|
| Microbial DNA (5 ng/µl) | 2.5 µl |
| Amplicon PCR Forward Primer 1 µM | 5 µl |
| Amplicon PCR Reverse Primer 1 µM | 5 µl |
| 2x KAPA HiFi HotStart ReadyMix | 12.5 µl |
| Total | 25 µl |