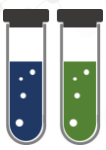
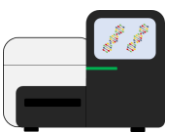
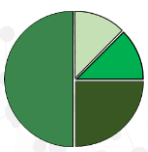
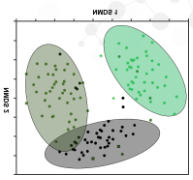
16S rRNA Analysis – A Quick Guide

Sampling and Wet Lab

Step	Look out for:
Sampling 	▪ Preserve samples to maintain original sample community ▪ Include sampling controls ▪ Include biological replicates if possible
DNA Extraction 	▪ Select (and test) sample appropriate extraction method ▪ Work in sterile environment ▪ Include extraction controls ▪ Include technical replicates if possible ▪ Assess DNA quality and quantity
Amplification (PCR)  	▪ Select Primers that target region of interest, e.g. V3/V4 region ▪ Use barcoded primers when working with more than one sample ▪ Select correct temperature settings (e.g. primer specific annealing temperature) ▪ 30 - 35 cycles are commonly used for 16S rRNA PCR ▪ Include negative and positive controls ▪ Include technical replicates ▪ Check PCR product visually (Gel, tape station) and assess quantity
Sequencing 	▪ Clean up and pool (if more than one sample) ▪ Select correct sequencing platform and chemistry depending on length of PCR product (e.g. Illumina 2x150bp) ▪ Attach sequencing adapters

Bioinformatics

Step	What to watch for
QC and pre-processing 	▪ Remove reads of poor quality or length ▪ Trim sequencing adapters ▪ Common tools: FastQC, Cutadapt ▪ Demultiplex samples based on used barcodes
ASV/OTU picking 	▪ Merge reads (not necessary when single end sequencing was used) ▪ Identify amplicon sequence variants (ASVs) or operational taxonomic units (OTUs) to identify which sequences are similar to each other ▪ Set an appropriate threshold for sequence similarity (OTUs)
Taxonomic assignment 	▪ Assign taxonomic identities to ASVs/OTUs using reference databases such as SILVA or RDP ▪ Use negative control data to remove contamination ASVs/OTUs
Diversity Analysis 	▪ Calculate alpha diversity metrics (e.g., Shannon) to measure diversity within individual samples. ▪ Evaluate beta diversity using distance metrics (e.g., Bray-Curtis) to assess differences in community composition between samples. ▪ Perform statistical tests (e.g., ANOSIM, PERMANOVA) to determine the significance of observed differences.
Visualization	▪ Visualize data using bar charts, bubble plots, heatmaps, PCoA plots or trees

Need more information?
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