MRC microbiome analysis toolbox-A singularity container version

Prepared by MRC bioinformatics team **Dr Xiao-Tao Jiang**, **Dr Emily McGovern** and **Dr Fan Zhang**

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Due to complex dependency and version conflicts, bioinformaticians are strugglying about installation of different tools and solving their dependence relationship and debuging while version conflicts happens. To solve this problem and establish a comprehensive, mobile and easy to use framwork for microbiome analysis to beginners, we build this MRC microbiome singularity image. This document describe the interface for different aspects of analysis using a demo example and the mrcmicrobiome.sif singularity image.

16S amplicon data analysis

In this tutorial, we use singularity version of the pipeline ***mrcmircobiome.sif*** to run a high fat diet mouse model stool microbiome analysis. The demo dataset includes stools samples from mice on divergent diets: high fat diet v normal chow. Using this demo dataset, users will learn:

1. How to prepare data for analysis
2. Quality control of amplicon sequences
3. Taxonomic annotation of 16S rRNA sequences
4. Alpha and beta diversity analysis
5. Differential abundant analysis using Linear discriminant analysis Effect Size LEfSe

> ## Prerequisites

> You'll need a laptop with a web browser and a terminal. See also the Setup page.
>
> You'll need a katana account for the training/have the the singularity file ***mrcmicrobiome.sif*** (This file is already on katana)
>
> You might want to brush up on the fundamentals of the Linux Shell for HPC.

Structure

This tutorial has been designed in a modular way. Modules are categorised as follows:

- Basic linux and setup prerequest environment
- 16S microbiome analysis: taxonomy, alpha, beta diversity analysis and basic statistical analysis
- Microbial signatures identification with LEfSe

Once you've gone through this tutorial, you will be able to analyze 16S amplicon sequences microbiome data You can refer to QIIME2, DADA2, Mothur, UPARSE and Phyloseq for more information

Schedule

	Setup	
10:00 - 10:30 am	1. Linux and HPC setup and data transfer	
10:40 - 12:00 am	2. Introduce of demo data set and quality control	
13:30 - 14:30 Pm	3. Taxonomy, phylogenetic tree, alpha and beta diversity analysis	
14:50 - 15:30 Pm	4. LEfSe to identify microbial signatues	

The actual schedule may vary slightly depending on the topics and exercises chosen by the instructor.

1. Linux and HPC setup

> ### Overview
>
> **Teaching:** 30 min **Exercises:** 0 min

Objectives

- Learn basic linux commands and HPC
- Learn how to transfer data from HPC to local computers

Katana | UNSW Research

Katana is a shared computational cluster located on campus at UNSW that has been designed to provide easy access to computational resources for groups working with non-sensitive data. Katana will be used for your next generation sequencing analysis.

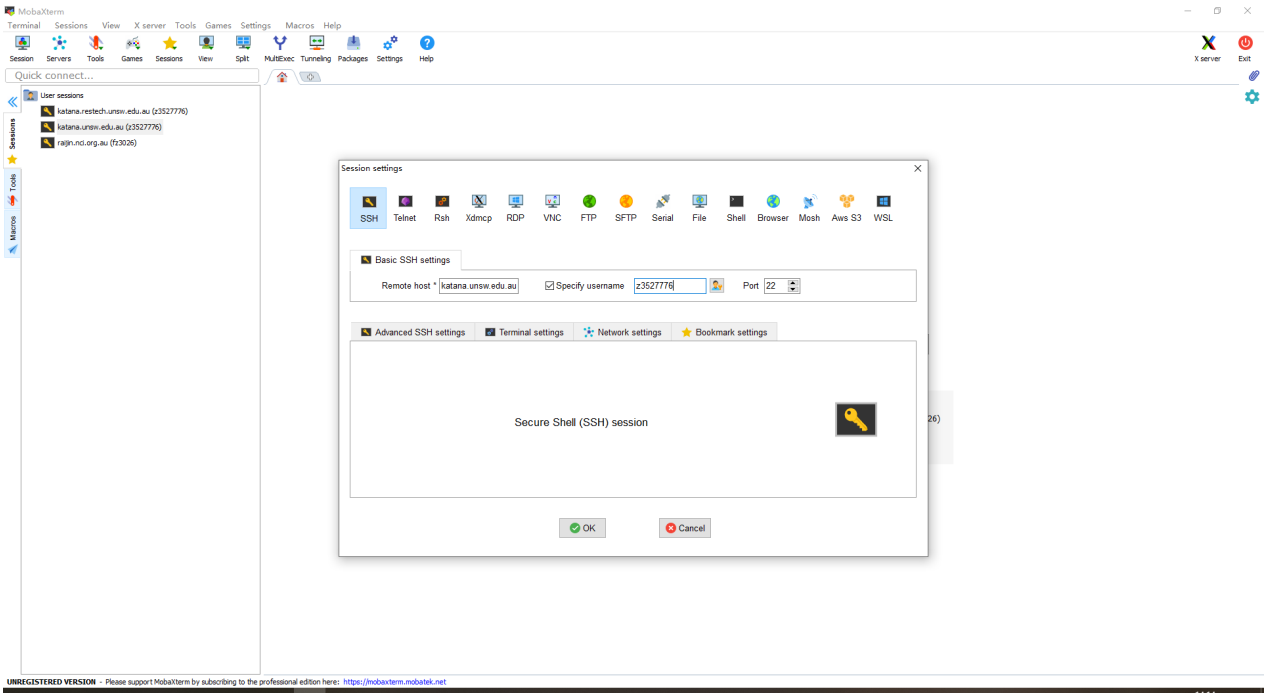
Log on to Katana

Log on from Iterm (Mac)

Download a terminal in order to log on to a remote terminal e.g. Iterm (Mac) or mobaxterm (Windows)

```
ssh zID@katana.restech.unsw.edu.au
```

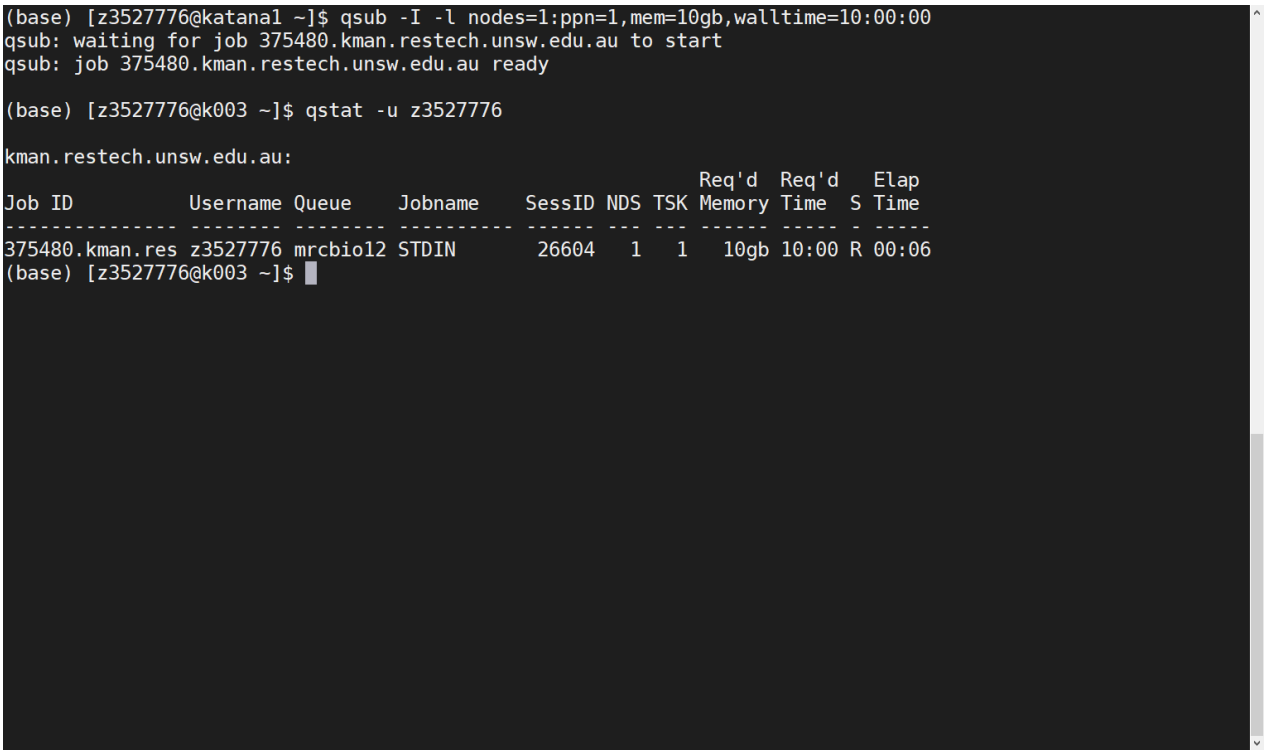
Log on from Mobaxterm (Windows)



Working on Katana

Always create an interactive job to work on Katana - Do not run jobs on head node

```
qsub -I -q R717942 -l nodes=1:ppn=1,mem=10gb,walltime=6:00:00
```



Navigating Katana

Check your filepath

```
pwd
```

Move into your personal scratch directory. Always create an interactive job to work on Katana - Do not run jobs on head node

```
cd /srv/scratch/zID
```

List what is in your filepath

```
ls
```

Make a new directory called `bio_tutorial`

```
mkdir bio_tutorial
```

Move into the `bio_tutorial` directory

```
cd bio_tutorial
```

Move back a directory

```
cd ..
```

Data upload

On your desktop and open notepad and create a file with the following text and save to your desktop as `test_file.txt`

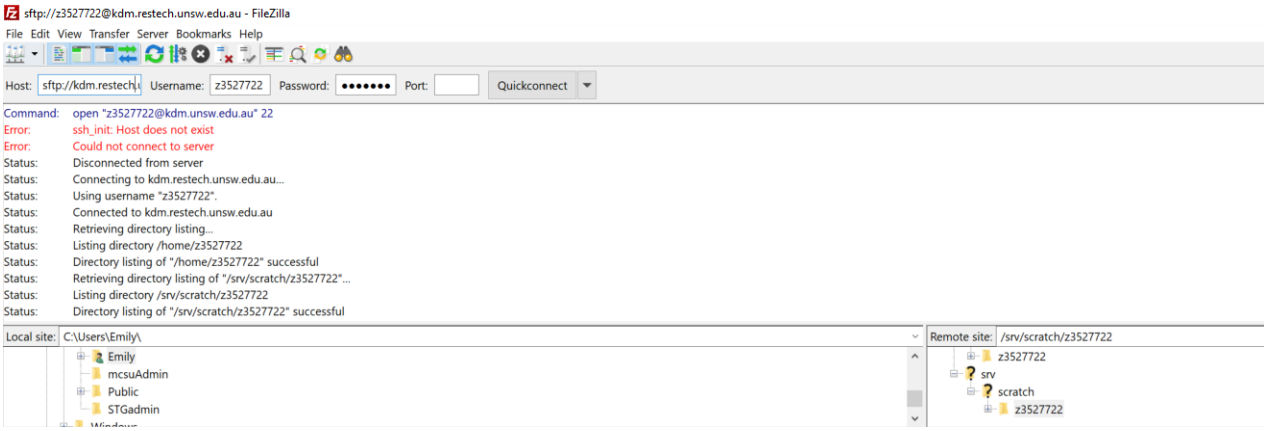
```
-8ACCGGB@=8=6BCFFCDEFGFGGGGGGGGGGGGGFFGF@C@CFGGGFCCBFGGGFGCFGGGFGDCFGGFGGGGGF
FCGFFGDFCGGCGGCC<EBFFGFBBE<EFFGEEFGG?<F9?E7++8+,,:<?
<,=++@:FEGGD9F@7F7>=F;8FFG@7FFCFCFFCCBCF8>DFCFEBFGGFGGDC:F:*
<F7<F,4<B9C*CCCACEFFEGC:EE:CE**A89CFCFCC=C5**<+55;C*CCE=EC>C58?F7:@7<:
<>+37:C7C99@E77C:<C*;++8:735B))8@36>>BC?)
@M01153:567:000000000-CNVPV:1:1101:23398:1773 1:N:0:GCTCATGA+GCGTAAGA
CCTACGGGGGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGT
TTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAG
ACAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCCAGCGTTGTCCGGTTTTATTGGGCGTAAA
GCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGTT
```

Next let's upload this file to your scratch drive on Katana using **Katana data Mover**

Open FileZilla

1. Enter the following details in the top tool bar

- Hostname = `ssh zID@kdm.restech.unsw.edu.au`
- Username = zID
- Password = zID password
- Port = 22



Enter remote site filepath

2. Once your logged on add `/srv/scratch/zID` to the bar saying `remote site`

Uploading file

Copy `test_file.txt` into your `/srv/scratch/zID`

Futher Navigation of Katana

```
cp test_file.txt bio_tutorial/
```

Move into `bio_tutorial` directory

```
cd bio_tutorial/
```

Now lets list whats in the `bio_tutorial` directory

```
ls
```

Duplicate the file

```
cp test_file.txt test_file2.txt
```

List all files with the suffix `.txt`

```
ls *.txt
```

Remove test_file2.txt

```
rm test_file2.txt
```

Look inside test_file.txt

```
less test_file.txt
```

Move back into scratch directory

```
cd ..
```

Delete `bio_tutorial`

```
rm -r bio_tutorial/
```

Further Training

UNSW research technology offer a range of training courses, that as of 2020 have been moved online. These are a great introduction to general programming and will help you improve the efficiency of your work. Explore them here

Introduce yourself to basic linux command using an online course

Reference Linux commands

Use this table as a reference during the workshop

Command	Meaning	Example
`ls`	list files in current directory, with -l, it also displays file permissions, sizes and last updated date/time	`ls -l`, `ls`
`pwd`	displays your current location in the file system	`pwd`
`env`	displays your user environment settings (e.g. search path, history size and home directory)	`env`
`cd`	change directory	`cd /file/path`
`mv`	move file, change file name - **Careful with this, no undo!**	`mv /path/file/ /newpath/file` , `mv oldname newname`
`cp`	copy files, -R copy directory	`cp /path/file/ /newpath/file`, `cp -R ./directory ./newdirectorylocation`
`head`	print top lines in file	`head filename` , `head -10 filename`
`tail`	print last lines in file	`tail filename` , `tail -10 filename`
`less`	view lines in file, use spacebar to go down file	`less filename`
`grep`	search file for pattern or string	`cat filename |grep "aaatttcc"`
`*`	wildcard, use with other commands	`ls *.fastq` : list all files with suffix ".fastq"
`wc`	word count, with -l = linecount	`wc filename`, `wc -l filename`
`rm`	remove filename , with -R directory **Again careful**	`rm filenmae`
`exit`	exit remote server	`exit`
`.`	This represents the current directory	`pwd .`, `cd ./path/to/subdirectory/from/currentdir`
`..`	This represents the parent directory	`pwd ..`, `cd ../path/to/subdirectory/from/parentdir`

Table:Useful everyday commands

Text editing from the command line

When learning bioinformatics, you will most likely need to create or edit text files, shell scripts or Python scripts from the command line. Using a Unix-based text editor good practice for getting used to the environment if you are new to the command line.

There are multiple text editors available. Which one you use is based on user preference, beginners usually go for nano or vim.

Text Editors

- nano
- vim
- emacs

User Guides

- Simple guide from a beginners perspective
- More comprehensive guide to vim

Useful Tip

If using `vim` and you are stuck in a wrong mode etc, you can always escape using by pressing `ESC` followed by `:q!` and this will allow you exit without saving any changes.

2. Quality control

Understand the demo data set

> ### Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Download the demo data set
- Understand the meta data file and learn how to prepare it

Using the FTP software to upload the demo data set and the relevant meta data file to your HPC account

Singularity on katana

login to compute nodes to run jobs.

```
qsub -I -q R717942 -l nodes=1:ppn=1,mem=10gb,walltime=6:00:00
```

mrcmicrobiome singularity sif have conda, qiime2-2020.8 and lefse installed, all the relevant dependence are resolved. Copy the demo data set to your own work directory

Check the singularity image file /data/bio/workshop/mrcmicrobiome.sif

Using the following command to create the wrok directory and copy the demo data set to your directory **Remember to replace the yourzID with your zID**

```
#create the hfdiet working directory
mkdir /srv/scratch/yourzID/hfdiet

#enter into the directory
cd /srv/scratch/yourzID/hfdiet

#copy the demo data set into your work directory
cp -r /data/bio/workshop/demo/  /srv/scratch/yourzID/hfdiet
```

Check the demo and database db directory

There are in total 16 samples in the demo, this demo compare mice stool microbiome with normal and high fat diet chow check the **meta_data.txt** file, you can find the samples information

```
[z3524677@katana2 hfdiet]$ tree demo/

#output
demo/
|-- all_r1.fq
|-- all_r2.fq
|-- meta_data.txt
`-- rawfq
    |-- 5821_S50_L001_R1.fastq.gz
    |-- 5821_S50_L001_R2.fastq.gz
    |-- 5822_S34_L001_R1.fastq.gz
    |-- 5822_S34_L001_R2.fastq.gz
    |-- 5824_S96_L001_R1.fastq.gz
    |-- 5824_S96_L001_R2.fastq.gz
    |-- 5825_S7_L001_R1.fastq.gz
    |-- 5825_S7_L001_R2.fastq.gz
    |-- 5832_S8_L001_R1.fastq.gz
    |-- 5832_S8_L001_R2.fastq.gz
    |-- 5833_S78_L001_R1.fastq.gz
    |-- 5833_S78_L001_R2.fastq.gz
    |-- 5859_S27_L001_R1.fastq.gz
    |-- 5859_S27_L001_R2.fastq.gz
    |-- 5860_S122_L001_R1.fastq.gz
    |-- 5860_S122_L001_R2.fastq.gz
    |-- 5862_S51_L001_R1.fastq.gz
    |-- 5862_S51_L001_R2.fastq.gz
    |-- 5863_S30_L001_R1.fastq.gz
    |-- 5863_S30_L001_R2.fastq.gz
    |-- 6319_S128_L001_R1.fastq.gz
    |-- 6319_S128_L001_R2.fastq.gz
    |-- 6320_S67_L001_R1.fastq.gz
    |-- 6320_S67_L001_R2.fastq.gz
    |-- 6321_S20_L001_R1.fastq.gz
    |-- 6321_S20_L001_R2.fastq.gz
    |-- 6322_S131_L001_R1.fastq.gz
    |-- 6322_S131_L001_R2.fastq.gz
    |-- 6328_S93_L001_R1.fastq.gz
    |-- 6328_S93_L001_R2.fastq.gz
    |-- 6329_S37_L001_R1.fastq.gz
    `-- 6329_S37_L001_R2.fastq.gz
```

Check meta data file

```
#enter into your own directory
cd demo/

#show the contents of the meta data file
[z3524677@katana2 demo]$ cat meta_data.txt
#SampleID LANID Diet
6319_S128_L001  fvb-a Normal
6320_S67_L001 fvb-a Normal
6321_S20_L001 fvb-b Normal
6322_S131_L001  fvb-b Normal
6328_S93_L001 fvb-c Normal
6329_S37_L001 fvb-c Normal
5821_S50_L001 181-1 HFD
5822_S34_L001 181-1 HFD
5824_S96_L001 181-2 HFD
5825_S7_L001  181-2 HFD
5832_S8_L001  181-3 HFD
5833_S78_L001 181-3 HFD
5859_S27_L001 181-4 HFD
5860_S122_L001  181-4 HFD
5862_S51_L001 181-5 HFD
5863_S30_L001 181-5 HFD
```

Check the database folder

```
tree /data/bio/workshop/db/

#output
/data/bio/workshop/db
|-- gg-v3v4-classifier.qza
|-- gg-v4-classifier.qza
|-- mouse.genome.bowtie2.1.bt2
|-- mouse.genome.bowtie2.2.bt2
|-- mouse.genome.bowtie2.3.bt2
|-- mouse.genome.bowtie2.4.bt2
|-- mouse.genome.bowtie2.rev.1.bt2
`-- mouse.genome.bowtie2.rev.2.bt2
```

QIIME2 manifest file preparation, example below

```
sample-id,absolute-filepath,direction
5821_S50_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5821_S50_L001_R1.fastq.
gz,forward
5821_S50_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5821_S50_L001_R2.fastq.
gz,reverse
5822_S34_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5822_S34_L001_R1.fastq.
gz,forward
5822_S34_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5822_S34_L001_R2.fastq.
gz,reverse
5824_S96_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5824_S96_L001_R1.fastq.
gz,forward
5824_S96_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5824_S96_L001_R2.fastq.
gz,reverse
5825_S7_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5825_S7_L001_R1.fastq.gz
,forward
5825_S7_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5825_S7_L001_R2.fastq.gz
,reverse
5832_S8_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5832_S8_L001_R1.fastq.gz
,forward
5832_S8_L001,/srv/scratch/z3524677/hfdiet/demo/rawfq//5832_S8_L001_R2.fastq.gz
,reverse
```

Prepare manifest and run dada2

> ## Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Generate quality control shell scripts with ***mima_prepare_manifest_and_qc.pl***
- Understand how to perform quality control with dada2

```
[z3524677@katana2 demo]$ singularity exec --cleanenv
/data/bio/workshop/mrcmicrobiome.sif bash -c 'perl
/home/applications/mima/mima_prepare_manifest_and_qc.pl'

perl /home/applications/mima/mima_prepare_manifest_and_qc.pl <Absolute path to
store fastq files> <output.manifest> <total_1.fq> <total_2.fq> <outputdir> <#
of threads> <Singularity_sif>
  <inputdir_abs_path>  The absolute directory to store the pair-end fastq
files
  <output.manifest>    The manifest file that qiime needed when running dada2
  <Merged_1.fq>        The overall fastq file pair 1
  <Merged_2.fq>        The overall fastq file pair 2
  <Output_dir>         Output directory store all the QC files and dada2
output
  <#ofthreads>         Deafault 1
  <Singularity sif img> the singualrity image to use
```

Run the one comman pipeline to generate all the necessary scripts. This one command will generate all the necessary scripts for the QC analysis. You can submit this as a job or run each command individually in an interactive job. As QC can take time, it's advisable to submit all the commands as a job `qsub qc.pbs`. For learning purposes today we will go through each command and it's output in an interactive model as the demo small

Remember to change the zID to your own zID

```
#run the command to generate commands for quality control
#Absolute path should be used input and output directory
[z3524677@katana2 demo]$ singularity exec --cleanenv
/data/bio/workshop/mrcmicrobiome.sif bash -c 'perl
/home/applications/mima/mima_prepare_manifest_and_qc.pl
/srv/scratch/z3524677/hfdiet/demo/rawfq/
/srv/scratch/z3524677/hfdiet/demo/hf_d.manifest all_r1.fq all_r2.fq
/srv/scratch/z3524677/hfdiet/demo/hf_qc 1
/data/bio/workshop/mrcmicrobiome.sif'
```

```
[z3524677@katana2 demo]$ tree hf_qc/
hf_qc/
|-- hf_d.manifest
`-- qc.pbs
```

Check the commands generated

```
[z3524677@katana2 demo]$ cat hf_qc/qc.pbs
#!/bin/bash
#PBS -l nodes=1:ppn=1
#PBS -l mem=80gb
```

```
#PBS -l walltime=100:00:00
export LC_ALL=en_AU.utf8
export LANG=en_AU.utf8
cd /srv/scratch/z3524677/hfdiet/demo/hf_qc

singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c ' fastp -i
all_r1.fq -I all_r2.fq -o /srv/scratch/z3524677/hfdiet/demo/hf_qc/ALL_R1.fq -O
/srv/scratch/z3524677/hfdiet/demo/hf_qc/ALL_R2.fq -h
/srv/scratch/z3524677/hfdiet/demo/hf_qc/fastp.outreport.html'

singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && qiime tools import --type
'SampleData[PairedEndSequencesWithQuality]' --input-path
/srv/scratch/z3524677/hfdiet/demo/hf_d.manifest --output-path demux.qza --
input-format PairedEndFastqManifestPhred33'

singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && qiime dada2 denoise-paired  --i-demultiplexed-seqs demux.qza
--p-trunc-len-f 295 --p-trunc-len-r 220 --p-trim-left-f 17  --p-trim-left-r 21
--p-n-threads 1 --o-representative-sequences rep-seqs.qza --o-table table.qza
--o-denoising-stats stats-dada2.qza'
```

Files in the folder after running the ***mima_prepare_manifest_and_qc.pl*** script. Now a file 'hf_d.manifest' and a folder 'hf_qc' are newly created.

```
(base) [z3527776@k212 demo]$ ll
total 52740
-rwx------ 1 z3527776 MRCBIO 27015199 Sep 10 18:45 all_r1.fq
-rwx------ 1 z3527776 MRCBIO 26974109 Sep 10 18:45 all_r2.fq
-rw-r--r-- 1 z3527776 MRCBIO     2890 Sep 14 12:43 hf_d.manifest
drwxr-xr-x 2 z3527776 MRCBIO       53 Sep 14 12:43 hf_qc
-rwx------ 1 z3527776 MRCBIO      322 Sep 10 18:45 meta_data.txt
drwx------ 2 z3527776 MRCBIO     4096 Sep 10 18:45 rawfq
(base) [z3527776@k212 demo]$
```

Overall assessment of quality of sequences with fastp

Prior to importing data into QIIME, the data will be assessed with Fastp. This step functions to assess overall sequence quality. Output information will be in a html format and will include: Run Fastp to assess the overall quality of the sequences.

```
[z3524677@katana2 demo]$ singularity exec /data/bio/workshop/mrcmicrobiome.sif
 bash -c 'fastp -i all_r1.fq -I all_r2.fq -o hf_qc/ALL_R1.fq -O
hf_qc/ALL_R2.fq -h hf_qc/fastp.outreport.html'
bash: warning: setlocale: LC_ALL: cannot change locale (en_AU.utf8)
Read1 before filtering:
total reads: 40000
```

```
total bases: 12022031
Q20 bases: 10726471(89.2235%)
Q30 bases: 9355072(77.8161%)

Read2 before filtering:
total reads: 40000
total bases: 12001486
Q20 bases: 9746062(81.2071%)
Q30 bases: 8306044(69.2085%)

Read1 after filtering:
total reads: 38912
total bases: 11695532
Q20 bases: 10522964(89.9742%)
Q30 bases: 9202096(78.6804%)

Read2 aftering filtering:
total reads: 38912
total bases: 11674221
Q20 bases: 9568399(81.9618%)
Q30 bases: 8182633(70.0915%)

Filtering result:
reads passed filter: 77824
reads failed due to low quality: 1952
reads failed due to too many N: 224
reads failed due to too short: 0
reads with adapter trimmed: 24
bases trimmed due to adapters: 1575

Duplication rate: 22.3048%

Insert size peak (evaluated by paired-end reads): 460

JSON report: fastp.json
HTML report: /srv/scratch/z3524677/hfdiet/demo/hf_qc/fastp.outreport.html
```

Check the output directory

```
[z3524677@katana2 demo]$ ls -lh hf_qc/
total 51M
-rw-r--r--. 1 z3524677 unsw  26M Sep  9 12:09 ALL_R1.fq
-rw-r--r--. 1 z3524677 unsw  26M Sep  9 12:09 ALL_R2.fq
-rw-r--r--. 1 z3524677 unsw 436K Sep  9 12:09 fastp.outreport.html
-rw-r--r--. 1 z3524677 unsw 2.9K Sep  9 12:02 hf_d.manifest
-rw-r--r--. 1 z3524677 unsw 1.1K Sep  9 12:02 qc.pbs
```

Fastp Output explained:

- Insert size is the length of DNA that you want to sequence and that is inserted between the adapters (adapters excluded).
- Total reads refer to the total number of reads generated by the sequencing run
- Total bases refer to the total number of nucleotide bases sequenced during the sequencing run
- Quality Scores for Next-Generation Sequencing (NGS) assessing sequencing accuracy using Phred quality scoring (Q score). Low Q scores can increase false-positive variant calls, which can result in inaccurate conclusions and higher costs for validation experiments.

Phred Quality	Score Probability of incorrect bases call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%

Table: Phred Quality table

Check the quality of the overall sequences in the ***fastp.outreport.html*** after downloading to local computer

fastp.outreport.html open on local browser

fastp report

Summary

General

fastp version:	0.20.1 (https://github.com/OpenGene/fastp)
sequencing:	paired end (301 cycles + 301 cycles)
mean length before filtering:	300bp, 300bp
mean length after filtering:	300bp, 300bp
duplication rate:	22.304833%
Insert size peak:	460

Before filtering

total reads:	80.000000 K
total bases:	24.023517 M
Q20 bases:	20.472533 M (85.218717%)
Q30 bases:	17.661116 M (73.515947%)
GC content:	55.948049%

After filtering

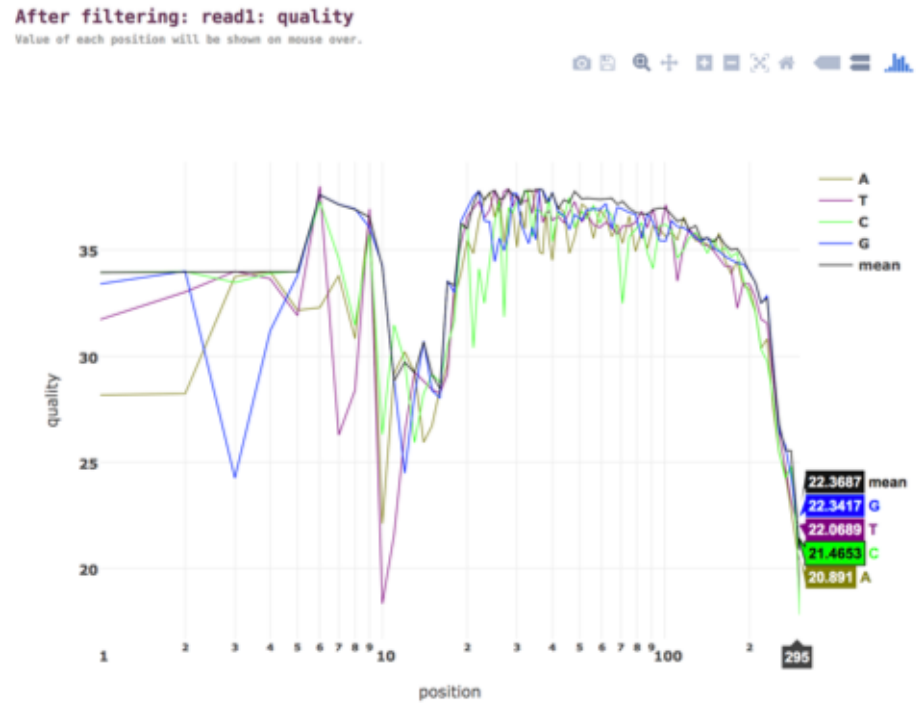
total reads:	77.824000 K
total bases:	23.369753 M
Q20 bases:	20.091363 M (85.971653%)
Q30 bases:	17.384729 M (74.389871%)
GC content:	55.963411%

Filtering result

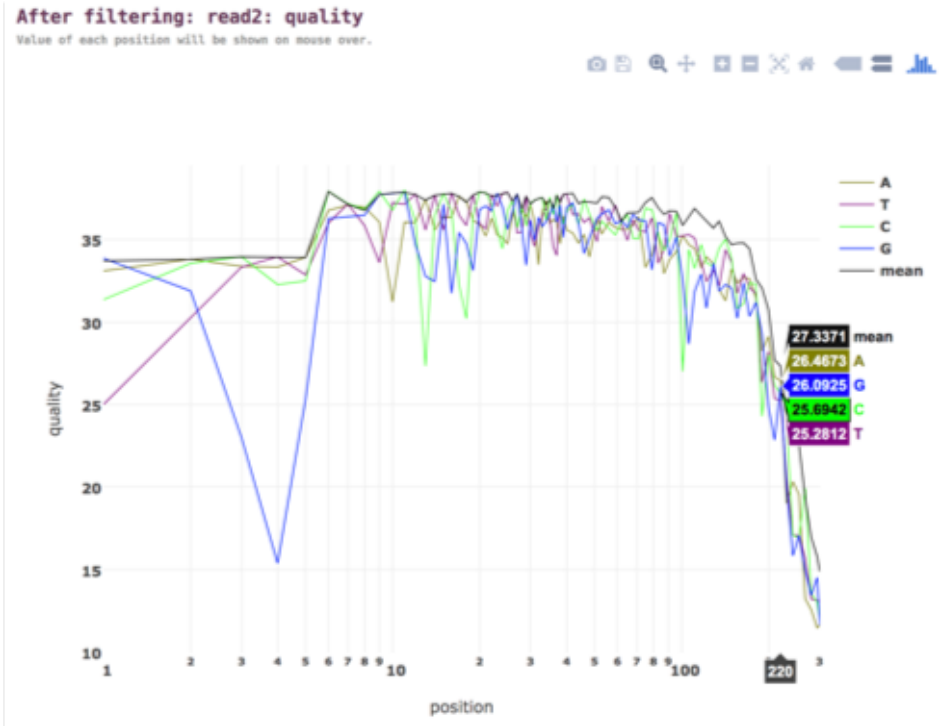
reads passed filters:	77.824000 K (97.280000%)
reads with low quality:	1.952000 K (2.440000%)
reads with too many N:	224 (0.280000%)
reads too short:	0 (0.000000%)

Determine the trim location at read1 and read2 For Miseq reads, usually the quality of bases decrease dramatically while towarding to the end of the reads, in order to loss less sequences during merge of pair end reads, it is better operation to trim the end of reads.

Read1, to keep higher quality bases, the trim location is set to 295



Read2, to keep higher quality bases, the trim locaiton is set to 220



Import fasta sequences into demux.qza

```
[z3524677@katana2 demo]$ singularity exec /data/bio/workshop/mrcmicrobiome.sif
 bash -c '. activate qiime2-2020.8 && \
qiime tools import \
 --type 'SampleData[PairedEndSequencesWithQuality]' \
 --input-path hf_d.manifest \
 --output-path demux.qza \
 --input-format PairedEndFastqManifestPhred33'

Imported /srv/scratch/z3524677/hfdiet/demo/hf_d.manifest as
PairedEndFastqManifestPhred33 to demux.qza
```

Pay attention to the PATH of the input and output files in the command, make sure it is your PATH, e.g., /srv/scratch/'yourzID'/hfdiet/demo/hf_d.manifest. A file 'demux.qza' is newly generated.

```
(base) [z3527776@k212 demo]$ ll
total 65484
-rwx------ 1 z3527776 MRCBIO 27015199 Sep 10 18:45 all_r1.fq
-rwx------ 1 z3527776 MRCBIO 26974109 Sep 10 18:45 all_r2.fq
-rw-r--r-- 1 z3527776 MRCBIO 13049747 Sep 14 12:53 demux.qza
-rw-r--r-- 1 z3527776 MRCBIO     2890 Sep 14 12:43 hf_d.manifest
drwxr-xr-x 2 z3527776 MRCBIO       53 Sep 14 12:43 hf_qc
-rwx------ 1 z3527776 MRCBIO      322 Sep 10 18:45 meta_data.txt
drwx------ 2 z3527776 MRCBIO     4096 Sep 10 18:45 rawfq
(base) [z3527776@k212 demo]$
```

Running dada2 within qiime2 to perform quality control, this takes about 1 minute

Run dada2 to trim end low quality bases, remove chimera sequencing and merge pair-end sequences, generate feature tables and statistics output of the results

```
  [z3524677@katana2 demo]$ singularity exec
/data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate qiime2-2020.8 && \
  qiime dada2 denoise-paired \
    --i-demultiplexed-seqs demux.qza \
    --p-trunc-len-f 295 \
    --p-trunc-len-r 220 \
    --p-trim-left-f 17 \
    --p-trim-left-r 21 \
    --p-n-threads 1 \
    --o-representative-sequences rep-seqs.qza \
    --o-table table.qza --o-denoising-stats stats-dada2.qza'

  Saved FeatureTable[Frequency] to: table.qza
  Saved FeatureData[Sequence] to: rep-seqs.qza
  Saved SampleData[DADA2Stats] to: stats-dada2.qza
```

Three files: 'table.qza', 'rep-seqs.qza' and 'stats-dada2.qza' are newly generated.

```
(base) [z3527776@k212 demo]$ ll
total 65552
-rwx------ 1 z3527776 MRCBIO 27015199 Sep 10 18:45 all_r1.fq
-rwx------ 1 z3527776 MRCBIO 26974109 Sep 10 18:45 all_r2.fq
-rw-r--r-- 1 z3527776 MRCBIO 13049747 Sep 14 12:53 demux.qza
-rw-r--r-- 1 z3527776 MRCBIO     2890 Sep 14 12:43 hf_d.manifest
drwxr-xr-x 2 z3527776 MRCBIO       53 Sep 14 12:43 hf_qc
-rwx------ 1 z3527776 MRCBIO      322 Sep 10 18:45 meta_data.txt
drwx------ 2 z3527776 MRCBIO     4096 Sep 10 18:45 rawfq
-rw-r--r-- 1 z3527776 MRCBIO    28860 Sep 14 13:03 rep-seqs.qza
-rw-r--r-- 1 z3527776 MRCBIO    10706 Sep 14 13:03 stats-dada2.qza
-rw-r--r-- 1 z3527776 MRCBIO    22922 Sep 14 13:03 table.qza
(base) [z3527776@k212 demo]$
```

Decompress **stats-dada2.qza** and check the quality details:

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path  stats-dada2.qza \
 --output-path stats-dada2'
```

Dada2 stats

- The **input column** refers to the sequence reads imported into QIIME2 from the initial QC filtration.
- Sequence reads are then filtered/denoised using parameters set by user. These are determined based on sequence 16SrRNA gene region amplified and quality assessment from the fastp report.
- The **merge column** (only present for paired end samples) refers to the number of the forward and reverse reads which were successfully merged together to obtain the full denoised sequences. Merging is performed by aligning the denoised forward reads with the reverse-complement of the corresponding denoised reverse reads, and then constructing the merged “contig” sequences. By default, merged sequences are only output if the forward and reverse reads overlap by at least 12 bases, and are identical to each other in the overlap region.
- The **non-chimeric column** refers to the number of sequences remaining after chimera removal. Chimeras are artifactual PCR product/amplicon generated erroneously from more than one DNA template. It is well-known that chimeras are inevitable when preparing amplicon sequencing libraries for NGS. It is therefore important to detect and filter them out before any types of microbiome analyses.

Look into the quality details:

```
cat stats-dada2/stats.tsv
```

```
#output:

sample-id input filtered  percentage of input passed filter denoised  merged
percentage of input merged  non-chimeric  percentage of input non-chimeric
#q2:types numeric numeric numeric numeric numeric numeric numeric numeric
5821_S50_L001 2500  1337  53.48 1215  1089  43.56 1073  42.92
5822_S34_L001 2500  1467  58.68 1364  1256  50.24 1243  49.72
5824_S96_L001 2500  1312  52.48 1203  1100  44  1098  43.92
5825_S7_L001  2500  1365  54.6  1268  1131  45.24 1116  44.64
5832_S8_L001  2500  1350  54  1229  1069  42.76 1058  42.32
5833_S78_L001 2500  1218  48.72 1092  881 35.24 877 35.08
5859_S27_L001 2500  1279  51.16 1138  994 39.76 987 39.48
5860_S122_L001  2500  1449  57.96 1344  1229  49.16 1225  49
5862_S51_L001 2500  1232  49.28 1121  1000  40  998 39.92
5863_S30_L001 2500  1371  54.84 1263  1140  45.6  1133  45.32
6319_S128_L001  2500  1248  49.92 1176  1025  41  987 39.48
6320_S67_L001 2500  1283  51.32 1180  985 39.4  951 38.04
6321_S20_L001 2500  1339  53.56 1190  973 38.92 932 37.28
6322_S131_L001  2500  1466  58.64 1339  1116  44.64 1045  41.8
6328_S93_L001 2500  1531  61.24 1420  1256  50.24 1146  45.84
6329_S37_L001 2500  1327  53.08 1217  1012  40.48 1000  40
```

**Determine the depth to normalize for alpha and beta diversity comparison By analyzing the depth of clean sequences of all the samples, 800 is decided as the cut off for normalization to keep all samples.

Contents of two important files **rep-seqs.qza** and **table.qza**

rep-seqs.qza

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path  rep-seqs.qza \
 --output-path rep-seqs'

#Exported rep-seqs.qza as DNASequencesDirectoryFormat to directory rep-seqs
```

Look the feature sequences

```
head -4 rep-seqs/dna-sequences.fasta

#output:
>dfa833b266bd2993b86feab3617b34c3
TCGAGAATCATTCACAATGGGGGAAACCCTGATGGTGCGACGCCGCGTGGGGGAATGAAGGTCTTCGGATTGTAAACC
CCTGTCATGTGGGAGCAAATTAAAAAGATAGTACCACAAGAGGAAGAGACGGCTAACTCTGTGCCAGCAGCCGCGGTA
ATACAGAGGTCTCAAGCGTTGTTCGGAATCACTGGGCGTAAAGCGTGCGTAGGCTGTTTCGTAAGTCGTGTGTGAAAG
GCGCGGGCTCAACCCGCGGACGGCACATGATACTGCGAGACTAGAGTAATGGAGGGGGAACCGGAATTCTCGGTGTAG
CAGTGAAATGCGTAGATATCGAGAGGAACACTCGTGGCGAAGGCGGGTTCCTGGACATTAACTGACGCTGAGGCACGA
AGGCCAGGGGAGCGAAAG
>f67a6d5da8c0b71206955aeae1505a74
TGAGGAATATTGGTCAATGGTCGGGAGACTGAACCAGCCAAGCCGCGTGAGGGAAGAAGGTACAGCGTATCGTAAACC
TCTTTTGCCGGGGAACAAGAGCTTCCACGAGTGGGAGTGTGAGCGTACCCGGAGAAAAAGCATCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCGAGTTAA
GTCAGCGGTAAAAGCCCGGGGCTCAACCCCGGCCCGCCGTTGAAACTGGCTGGCTTGAGTTGGGGAAAGGCAGGCGGA
ATGCGCGGTGTAGCGGTGAAATGCATAGATATCGCGCAGAACCCCGATTGCGAAGGCAGCCTGCCGGCCCCACACTGA
CGCTGAGGCACGAAAGCGTGGGTATCGAACA
```

table.qza

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path  table.qza \
 --output-path table'
#Exported table.qza as BIOMV210DirFmt to directory table

#convert biom file to tsv file
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
biom convert -i table/feature-table.biom -o table/feature-table.txt --to-tsv'
```

Look into the table matrix file, the numbers in the table are abundance of that feature sequences in different samples

```
head -4 table/feature-table.txt

#output:

# Constructed from biom file
#OTU ID 5821_S50_L001 5822_S34_L001 5824_S96_L001 5825_S7_L001  5832_S8_L001
5833_S78_L001 5859_S27_L001 5860_S122_L001  5862_S51_L001 5863_S30_L001
6319_S128_L001  6320_S67_L001 6321_S20_L001 6322_S131_L001  6328_S93_L001
6329_S37_L001
dfa833b266bd2993b86feab3617b34c3  193.0 186.0 271.0 290.0 208.0 176.0 210.0
322.0 223.0 230.0 140.0 63.0  54.0  117.0 0.0 0.0
f67a6d5da8c0b71206955aeae1505a74  55.0  126.0 72.0  85.0  57.0  39.0  46.0
51.0  48.0  36.0  131.0 35.0  38.0  0.0 109.0 14.0
```

Host contamination removal if necessary when processing mucosal samples

> ## Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Understand why there are contamination in the amplicons data
- Finish the commands to decontamination

Although the sequencing target is the 16S hyper variable region PCR products, the data generated by Ramaciotti sometimes includes host contaminations. Hence, we need to remove the host sequences in the **table.qza** and **rep-seq.qza**, and update the stats-dada2.qza again to check the percentage of host contamination.

Check the help information for **mima_decontaminate_host.pl**

```
[z3524677@katana2 demo]$ singularity exec /data/bio/workshop/mrcmicrobiome.sif
 bash -c '. activate qiime2-2020.8 && perl
/home/applications/mima/mima_decontaminate_host.pl '
perl /home/applications/mima/mima_decontaminate_host.pl <rep_seqs.qza>
<table.qza> <outputdir> <host_genome.fa_bowtie2_index> <threads>

  <rep_seqs.qza> qiime format represntative sequences output from dada2
  <table.qza>    qimme format features table
  <outputdir>    output directory to store the decontaminated updated
rep_seqs.qza and table.qza
  <host_genome.fa_bowtie2_index> bowtie2 index of the host genome
  <threads>      number of threads used in bowtie2 mapping

  This script use bowtie2 to maping the rep_seqs.qza aganist host genome to
identify the contaminated sequences and update the sequences and table with
qiime2. The updated files can be used for downstream analysis.
```

Run the decontamination script to generate the bash file and run the analysis with singularity

```
[z3524677@katana2 demo]$ singularity exec /data/bio/workshop/mrcmicrobiome.sif
 bash -c '. activate qiime2-2020.8 && perl
/home/applications/mima/mima_decontaminate_host.pl rep-seqs.qza table.qza
hf_host_decontamination /data/bio/workshop/db/mouse.genome 1'

[z3524677@katana2 demo]$ ls hf_host_decontamination/
decontaminationhost.sh
```

A folder 'hf_host_decontamination/' is newly creatd, with one file 'decontaminationhost.sh' in it.

```
(base) [z3527776@k212 demo]$ ll
total 65552
-rwx------ 1 z3527776 MRCBIO 27015199 Sep 10 18:45 all_r1.fq
-rwx------ 1 z3527776 MRCBIO 26974109 Sep 10 18:45 all_r2.fq
-rw-r--r-- 1 z3527776 MRCBIO 13049747 Sep 14 12:53 demux.qza
-rw-r--r-- 1 z3527776 MRCBIO     2890 Sep 14 12:43 hf_d.manifest
drwxr-xr-x 2 z3527776 MRCBIO       44 Sep 14 13:29 hf_host_decontamination
drwxr-xr-x 2 z3527776 MRCBIO       53 Sep 14 12:43 hf_qc
-rwx------ 1 z3527776 MRCBIO      322 Sep 10 18:45 meta_data.txt
drwx------ 2 z3527776 MRCBIO     4096 Sep 10 18:45 rawfq
-rw-r--r-- 1 z3527776 MRCBIO    28860 Sep 14 13:03 rep-seqs.qza
drwxr-xr-x 2 z3527776 MRCBIO       31 Sep 14 13:05 stats-dada2
-rw-r--r-- 1 z3527776 MRCBIO    10706 Sep 14 13:03 stats-dada2.qza
-rw-r--r-- 1 z3527776 MRCBIO    22922 Sep 14 13:03 table.qza
(base) [z3527776@k212 demo]$ tree hf_host_decontamination
hf_host_decontamination
└── decontaminationhost.sh

0 directories, 1 file
(base) [z3527776@k212 demo]$
```

Execute the shell commands

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && sh hf_host_decontamination/decontaminationhost.sh'
```

Aftering running the decontamination pipeline, we get an updated **rep-seqs.qza** and **table.qza**

Explain of the output folder

- The file 'align-hostgenome.sam.txt' in folder 'rep-seqsold' holds the mapping info of the fasta sequences in 'dna-sequences.fasta'. Due to the small size of the demo data, there is no host reads detected therefore no record in 'align-hostgenome.sam.txt'.
- The file 'metadata.table.txt' holds the IDs of the fasta sequences that are mapped to host reference, therefore there is no record in this file, either.
- The file 'filtered_dnaseqs.fasta' has the fasta sequences that are not mapped to host reference, therefore we shall focus on this file in the downstream analysis.

```
(base) [z3527776@k212 demo]$ tree hf_host_decontamination
hf_host_decontamination
├── decontaminationhost.sh
├── filtered_dnaseqs.fasta
├── metadata.table.txt
├── rep-seqsold
│   ├── align-hostgenome.sam.txt
│   └── dna-sequences.fasta
└── rep-seqs.qza

1 directory, 6 files
(base) [z3527776@k212 demo]$
```

3. Taxonomy annotation and diversity analysis

> ### Overview
>
> **Teaching:** 60 min **Exercises:** 0 min

Objectives

- Running diversity analysis pipeline and taxonomy annotation, alpha and beta diversity analysis
- Understand how to interpret the output

Taxonomy annotation of **rep-seqs.qza** with naive bayesian classifier, for this part analysis, we have a comprehensive pipeline script, it can generate a series of commands that can finish different analysis, we firstly run this pipeline and then go to details of each part of the flow.

Create a directory **hf_diver** under the demo directory and copy the updated rep-seqs.qza and table.qza into this folder

```
#make the diversity output directory
mkdir hf_diver

#move the two files feature table and featues sequences into the hf_diver
directory
cp rep-seqs.qza hf_diver/
cp table.qza hf_diver/
```

Execute taxonomy annotation and diversity analysis pipeline command

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
perl /home/applications/mima/mima_diversity-basic-statistic.pl \
 -m meta_data.txt \
 -c /data/bio/workshop/db/gg-v3v4-classifier.qza \
 -n 1 \
 -d 800 \
 -o hf_diver'
```

The **mama_diversity-basic-statistic.pl** pipeline will generate a shell file *beta-diversity-commands.sh*, which includes all the taxonomical, alpha, beta diversity analysis . A file *beta-diversity-commands.sh* and a folder **hf_diver** are newly generated.

```
(base) [z3527776@k212 demo]$ ll
total 65560
-rwx------ 1 z3527776 MRCBIO 27015199 Sep 10 18:45 all_r1.fq
-rwx------ 1 z3527776 MRCBIO 26974109 Sep 10 18:45 all_r2.fq
-rw-r--r-- 1 z3527776 MRCBIO     4103 Sep 14 13:44 beta-diversity-commands.sh
-rw-r--r-- 1 z3527776 MRCBIO 13049747 Sep 14 12:53 demux.qza
drwx------ 4 z3527776 MRCBIO      100 Sep 14 13:44 hf_diver
-rw-r--r-- 1 z3527776 MRCBIO     2890 Sep 14 12:43 hf_d.manifest
drwxr-xr-x 3 z3527776 MRCBIO      155 Sep 14 13:31 hf_host_decontamination
drwxr-xr-x 2 z3527776 MRCBIO       53 Sep 14 12:43 hf_qc
-rwx------ 1 z3527776 MRCBIO      322 Sep 10 18:45 meta_data.txt
drwx------ 2 z3527776 MRCBIO       10 Sep 14 13:43 mkdir
drwx------ 2 z3527776 MRCBIO     4096 Sep 10 18:45 rawfq
-rw-r--r-- 1 z3527776 MRCBIO    28860 Sep 14 13:03 rep-seqs.qza
drwxr-xr-x 2 z3527776 MRCBIO       31 Sep 14 13:05 stats-dada2
-rw-r--r-- 1 z3527776 MRCBIO    10706 Sep 14 13:03 stats-dada2.qza
-rw-r--r-- 1 z3527776 MRCBIO    22922 Sep 14 13:03 table.qza
(base) [z3527776@k212 demo]$ tree hf_diver
hf_diver
├── alpha-group
├── beta-group
├── rep-seqs.qza
└── table.qza

2 directories, 2 files
(base) [z3527776@k212 demo]$
```

Run the above diversity analysis in batch model (This command run the pipeline)

```
#Run the analysis in batch model, the /scratch should be bind for this
function, the database directory should be bind as well to get access to
classifier database
singularity exec -B /data/bio/workshop/db/,/scratch
/data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate qiime2-2020.8 && sh
beta-diversity-commands.sh '
```

Taxonomy assignment of feature sequences

> ## Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Understand what is a phylogenetic tree and the purpuse of why we need this
- Finish the commands to generate rooted tree

```
#taxonomy assignment of feature representative sequences
singularity exec -B /data/bio/workshop/db/
/data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate qiime2-2020.8 && \
qiime feature-classifier classify-sklearn \
 --i-classifier /data/bio/workshop/db/gg-v3v4-classifier.qza \
 --i-reads hf_diver/rep-seqs.qza \
 --o-classification hf_diver/taxonomy.qza'
```

Check the contents of the taxonomy.qza by decompress it and view it

Decompress *hf_diver/taxonomy.qza*

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/taxonomy.qza \
 --output-path hf_diver/taxonomy'

#output meassage
#Exported hf_diver/taxonomy.qza as TSVTaxonomyDirectoryFormat to directory
hf_diver/taxonomy
```

Check the taxonomic annotaion of represent sequences

```
head -4 hf_diver/taxonomy/taxonomy.tsv

#output
Feature ID  Taxon Confidence
dfa833b266bd2993b86feab3617b34c3  k__Bacteria; p__Verrucomicrobia;
c__Verrucomicrobiae; o__Verrucomicrobiales; f__Verrucomicrobiaceae;
g__Akkermansia; s__muciniphila 0.9999999999999716
f67a6d5da8c0b71206955aeae1505a74  k__Bacteria; p__Bacteroidetes;
c__Bacteroidia; o__Bacteroidales; f__S24-7; g__; s__ 0.9999839589828744
d272bf25781448dde9031a24679a9012  k__Bacteria; p__Bacteroidetes;
c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides;
s__acidifaciens 0.9995083080422182
```

Normalize the feature table to a uniform depth

Note: The decision of how deep should be selected is based on **stats-dada2/stats.tsv** clean reads in all samples, you should keep as much samples as possible, and should keep a good enough depth, usually for stool samples over 10000 sequences should be fine. Here, as this is a demo, the raw sequences are 2500 and the final clean reads are all over 800, we chose 800 reads as the cut off.

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime feature-table rarefy \
 --i-table hf_diver/table.qza \
 --p-sampling-depth 800 \
 --o-rarefied-table hf_diver/rarefy.table.qza'
```

Generate stack barchart

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime taxa barplot \
 --i-table hf_diver/rarefy.table.qza \
 --i-taxonomy hf_diver/taxonomy.qza \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/normalized.taxa-bar-plots.qzv'
```

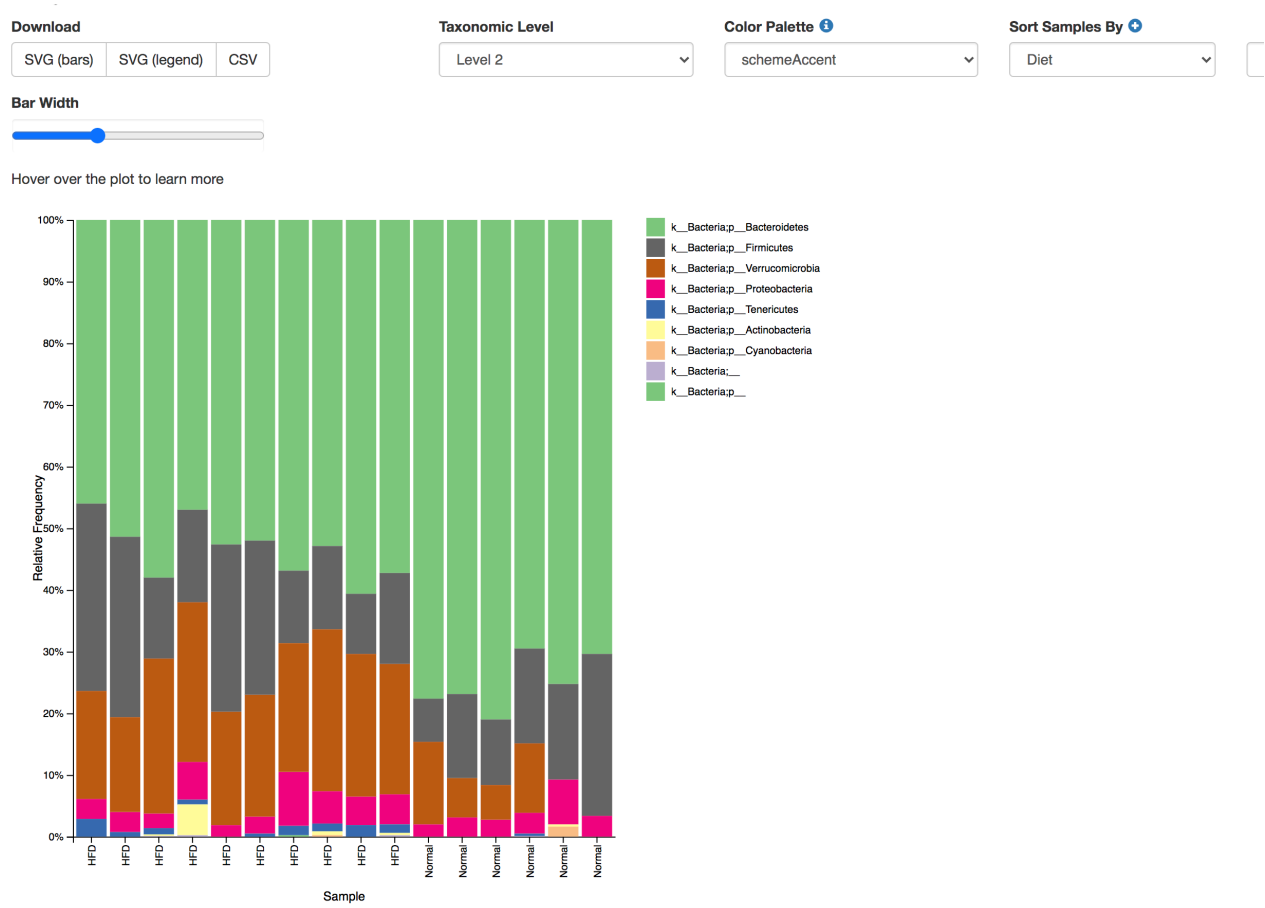
Decompress qzv file and download to local to browse the file

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/normalized.taxa-bar-plots.qzv \
 --output-path hf_diver/normalized.taxa-bar-plots'
```

Three files 'taxonomy.qza', 'rarefy.table.qza', 'normalized.taxa-bar-plots.qzv' and one filder 'normalized.taxa-bar-plots' are generated sequentially.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll hf_diver/
total 884
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 alpha-group
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 beta-group
drwxr-xr-x 4 z3527776 MRCBIO   4096 Sep 14 13:59 normalized.taxa-bar-plots
-rw-r--r-- 1 z3527776 MRCBIO 348445 Sep 14 13:58 normalized.taxa-bar-plots.qzv
-rw-r--r-- 1 z3527776 MRCBIO  27779 Sep 14 13:58 rarefy.table.qza
-rw------- 1 z3527776 MRCBIO  28860 Sep 14 13:43 rep-seqs.qza
-rw------- 1 z3527776 MRCBIO  22922 Sep 14 13:43 table.qza
-rw------- 1 z3527776 MRCBIO 420230 Sep 14 13:48 table-summarize.qzv
-rw-r--r-- 1 z3527776 MRCBIO  40406 Sep 14 13:49 taxonomy.qza
```

If you download file 'normalized.taxa-bar-plots.qzv' locally then upload to qiime2 view, the visualization should be look like this. 'Taxonomic Level', 'Color Palette' and 'Sort Samples By' all can be manully modified.



Phylogenetic tree construction

> ## Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Understand what is a phylogenetic tree and the purpuse of why we need this
- Finish the commands to generate rooted tree

In order to generate the UniFrac distance, a phylogenetic tree should be build for all the representative feature sequences

```
#constructing phylogenetic tree
singularity exec -B /scratch /data/bio/workshop/mrcmicrobiome.sif  bash -c '.
activate qiime2-2020.8 && \
qiime alignment mafft \
 --i-sequences hf_diver/rep-seqs.qza \
 --o-alignment hf_diver/aligned-rep-seqs.qza'

singularity exec -B /scratch /data/bio/workshop/mrcmicrobiome.sif  bash -c '.
activate qiime2-2020.8 && \
qiime alignment mask \
 --i-alignment hf_diver/aligned-rep-seqs.qza \
 --o-masked-alignment hf_diver/masked-aligned-rep-seqs.qza'

singularity exec -B /scratch /data/bio/workshop/mrcmicrobiome.sif  bash -c '.
activate qiime2-2020.8 && \
qiime phylogeny fasttree \
 --i-alignment hf_diver/masked-aligned-rep-seqs.qza \
 --o-tree hf_diver/unrooted-tree.qza --p-n-threads 1'

singularity exec -B /scratch /data/bio/workshop/mrcmicrobiome.sif  bash -c '.
activate qiime2-2020.8 && \
qiime phylogeny midpoint-root \
 --i-tree  hf_diver/unrooted-tree.qza \
 --o-rooted-tree hf_diver/rooted-tree.qza'
```

Four files 'aligned-rep-seqs.qza', 'masked-aligned-rep-seqs.qza', 'unrooted-tree.qza' and 'rooted-tree.qza' are generated sequentially. The **rooted-tree.qza** will be used in the core diversity analysis

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/
total 1036
-rw-r--r-- 1 z3527776 MRCBIO  37356 Sep 14 14:09 rooted-tree.qza
-rw-r--r-- 1 z3527776 MRCBIO  32900 Sep 14 14:08 unrooted-tree.qza
-rw-r--r-- 1 z3527776 MRCBIO  38095 Sep 14 14:07 masked-aligned-rep-seqs.qza
-rw-r--r-- 1 z3527776 MRCBIO  34835 Sep 14 14:07 aligned-rep-seqs.qza
drwxr-xr-x 4 z3527776 MRCBIO   4096 Sep 14 13:59 normalized.taxa-bar-plots
-rw-r--r-- 1 z3527776 MRCBIO 348445 Sep 14 13:58 normalized.taxa-bar-plots.qzv
-rw-r--r-- 1 z3527776 MRCBIO  27779 Sep 14 13:58 rarefy.table.qza
-rw-r--r-- 1 z3527776 MRCBIO  40406 Sep 14 13:49 taxonomy.qza
-rw------- 1 z3527776 MRCBIO 420230 Sep 14 13:48 table-summarize.qzv
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 beta-group
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 alpha-group
-rw------- 1 z3527776 MRCBIO  22922 Sep 14 13:43 table.qza
-rw------- 1 z3527776 MRCBIO  28860 Sep 14 13:43 rep-seqs.qza
(qiime2-2020.2) [z3527776@k212 demo]$
```

Run the core diversity analysis

> ### Overview

> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Understand the output for the core diversity analysis
- Checking the results for PcoA with different beta diversity distance

Sometimes we need to filter samples by the meta data to select part of the samples for analysis

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime feature-table filter-samples \
 --i-table hf_diver/table.qza \
 --m-metadata-file meta_data.txt \
 --o-filtered-table hf_diver/core.table.qza'
```

core diversity with phylogenetic information

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity core-metrics-phylogenetic \
 --i-phylogeny  hf_diver/rooted-tree.qza \
 --i-table hf_diver/core.table.qza \
 --p-sampling-depth 800 \
 --m-metadata-file meta_data.txt \
 --output-dir hf_diver/core-metrics-results'
```

One files 'core.table.qza' and one filder 'core-metrics-results', which has the core diversity analysis outputs, are generated sequentially. This step generate alpha diversity indexes such a Shannon, observed features, jaccard, evenness, and faith phylogenetic distance data and plot. The beta diversity of PcoA such as bray-curtis distance, weighted and unweighted UniFrac distance.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/
total 1068
drwxr-xr-x 2 z3527776 MRCBIO   4096 Sep 14 14:11 core-metrics-results
-rw-r--r-- 1 z3527776 MRCBIO  28304 Sep 14 14:11 core.table.qza
-rw-r--r-- 1 z3527776 MRCBIO  37356 Sep 14 14:09 rooted-tree.qza
-rw-r--r-- 1 z3527776 MRCBIO  32900 Sep 14 14:08 unrooted-tree.qza
-rw-r--r-- 1 z3527776 MRCBIO  38095 Sep 14 14:07 masked-aligned-rep-seqs.qza
-rw-r--r-- 1 z3527776 MRCBIO  34835 Sep 14 14:07 aligned-rep-seqs.qza
drwxr-xr-x 4 z3527776 MRCBIO   4096 Sep 14 13:59 normalized.taxa-bar-plots
-rw-r--r-- 1 z3527776 MRCBIO 348445 Sep 14 13:58 normalized.taxa-bar-plots.qzv
-rw-r--r-- 1 z3527776 MRCBIO  27779 Sep 14 13:58 rarefy.table.qza
-rw-r--r-- 1 z3527776 MRCBIO  40406 Sep 14 13:49 taxonomy.qza
-rw------- 1 z3527776 MRCBIO 420230 Sep 14 13:48 table-summarize.qzv
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 beta-group
drwxr-xr-x 2 z3527776 MRCBIO     10 Sep 14 13:44 alpha-group
-rw------- 1 z3527776 MRCBIO  22922 Sep 14 13:43 table.qza
-rw------- 1 z3527776 MRCBIO  28860 Sep 14 13:43 rep-seqs.qza
(qiime2-2020.2) [z3527776@k212 demo]$ tree hf_diver/core-metrics-results
hf_diver/core-metrics-results
├── bray_curtis_distance_matrix.qza
├── bray_curtis_emperor.qzv
├── bray_curtis_pcoa_results.qza
├── evenness_vector.qza
├── faith_pd_vector.qza
├── jaccard_distance_matrix.qza
├── jaccard_emperor.qzv
├── jaccard_pcoa_results.qza
├── observed_features_vector.qza
├── rarefied_table.qza
├── shannon_vector.qza
├── unweighted_unifrac_distance_matrix.qza
├── unweighted_unifrac_emperor.qzv
├── unweighted_unifrac_pcoa_results.qza
├── weighted_unifrac_distance_matrix.qza
├── weighted_unifrac_emperor.qzv
└── weighted_unifrac_pcoa_results.qza

0 directories, 17 files
(qiime2-2020.2) [z3527776@k212 demo]$
```

d

Check alpha diversity contents, shannon vector for example

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/core-metrics-results/shannon_vector.qza \
 --output-path hf_diver/core-metrics-results/shannon_vector'
```

Look into the output file

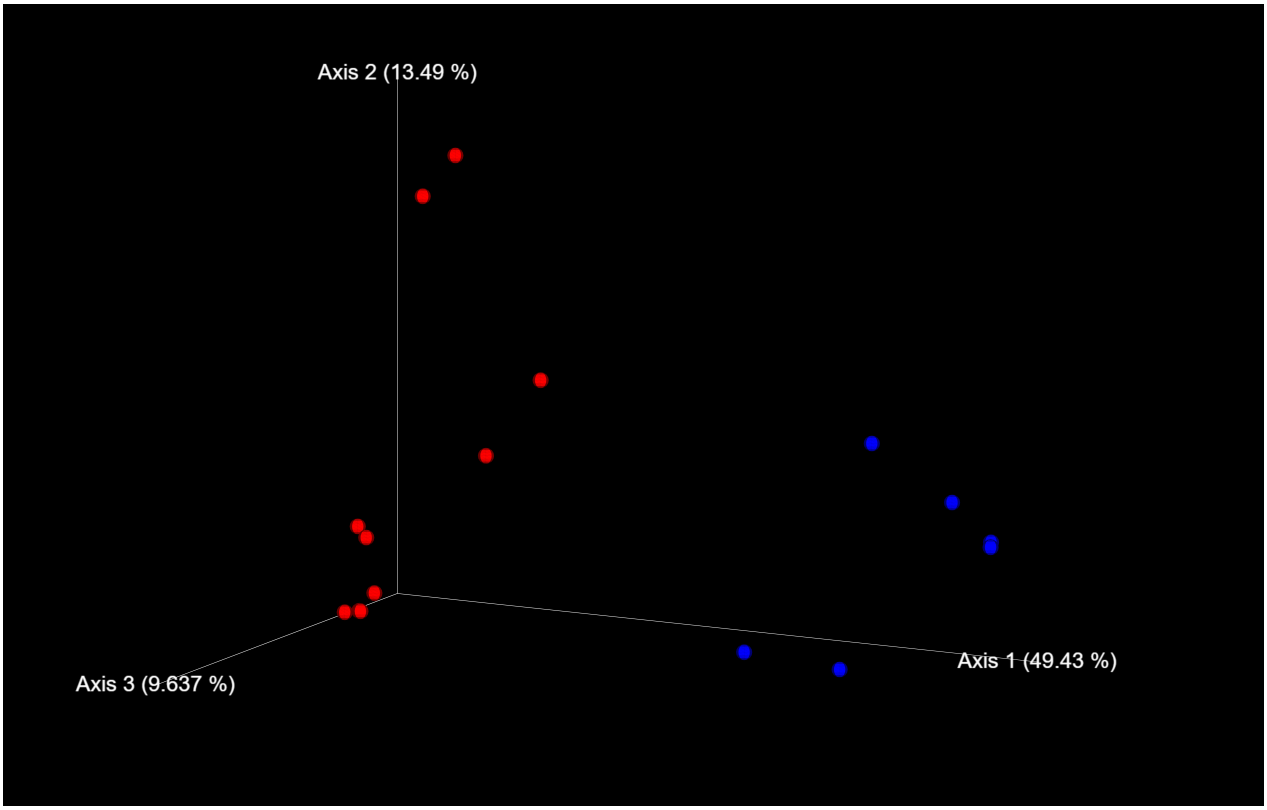
```
head -4 hf_diver/core-metrics-results/shannon_vector/alpha-diversity.tsv

#output:
  shannon_entropy
5821_S50_L001 4.284775238361043
5822_S34_L001 4.492180841533424
5824_S96_L001 4.474363582402979
```

Check PcoA plot for bray-curtis distance and weighted unifrac distance

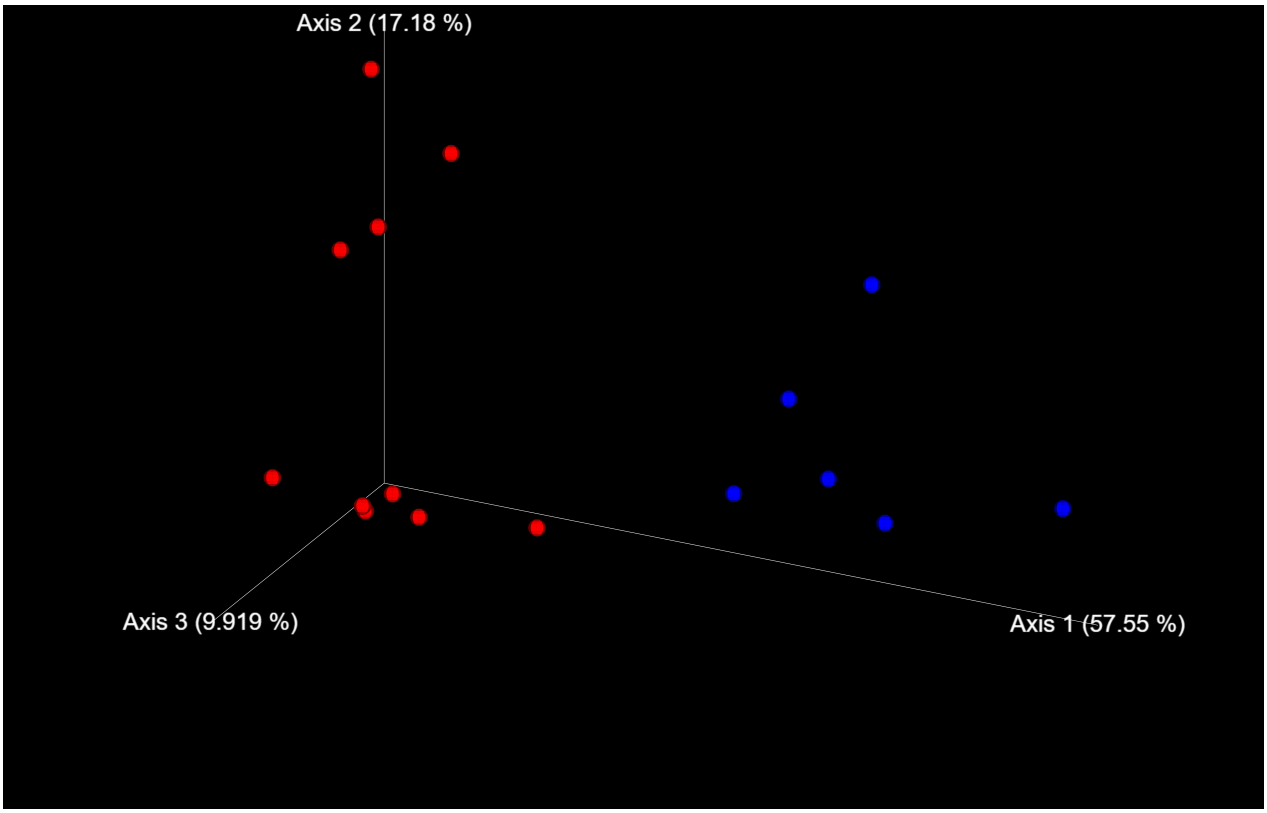
Decompress the qzv file and visulize at local computer or donwload qzv file and upload to qiime2 view for visulization

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/core-metrics-results/weighted_unifrac_emperor.qzv \
 --output-path hf_diver/core-metrics-results/weighted_unifrac_emperor'
```

If you download file 'bray_curtis_emperor.qzv' locally then upload to qiime2view, the visualization should be look like this. (Red is high fat diet samples and blue are healthy)



If you download file 'weighted_unifrac_emperor.qzv' locally then upload to qiime2 view, the visualization should be look like this.



Now check all the other alpha diversity index and beta diveristy PcoA results 5 minutes given

Alpha diversity rarefaction curve

> ## Overview
>
> **Teaching:** 5 min **Exercises:** 0 min

Objectives

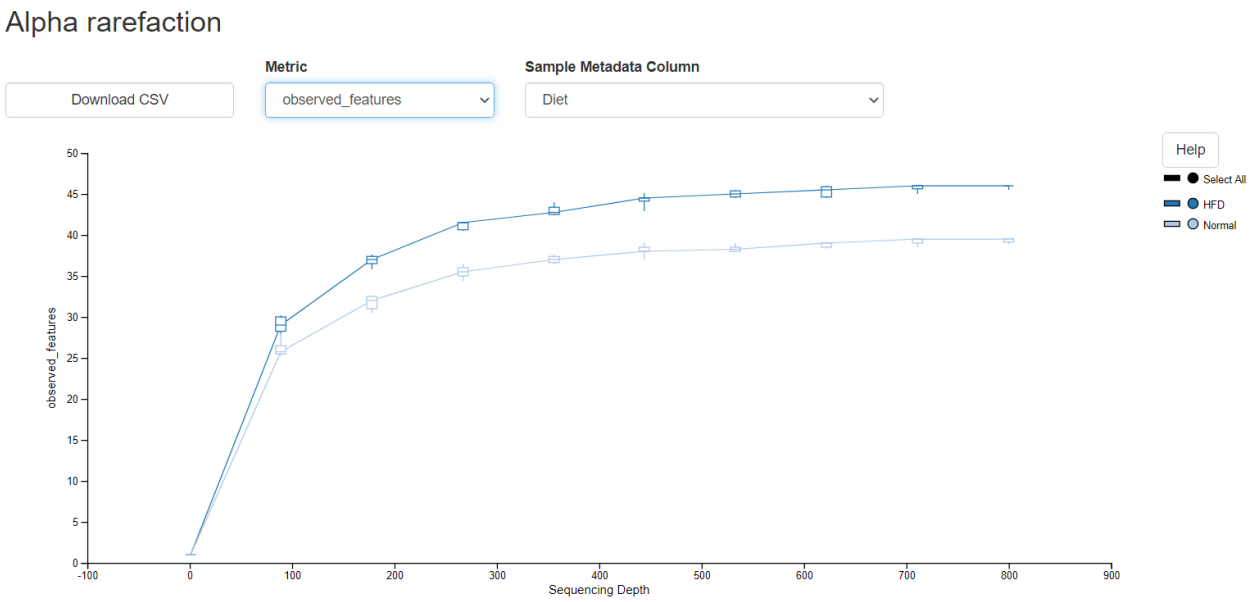
- Understand the meaning of rarefaction curve
- Generating rarefaction curve with qiime diversity plugin

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif bash -c '. activate
qiime2-2020.8 && \
qiime diversity alpha-rarefaction \
 --i-table hf_diver/table.qza \
 --i-phylogeny hf_diver/rooted-tree.qza \
 --p-max-depth 800 \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/alpha-rarefaction.qzv'
```

A file 'alpha-rarefaction.qzv' is created.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/
total 1396
-rw-r--r-- 1 z3527776 MRCBIO 332060 Sep 14 14:24 alpha-rarefaction.qzv
drwxr-xr-x 2 z3527776 MRCBIO   4096 Sep 14 14:11 core-metrics-results
-rw-r--r-- 1 z3527776 MRCBIO  28304 Sep 14 14:11 core.table.qza
-rw-r--r-- 1 z3527776 MRCBIO  37356 Sep 14 14:09 rooted-tree.qza
-rw-r--r-- 1 z3527776 MRCBIO  32900 Sep 14 14:08 unrooted-tree.qza
```

If you download file 'alpha-rarefaction.qzv' locally then upload to qiime2 view, the visualization should be look like this. 'Metric' has options (1) observed_features, (2) shannon and (3) faith_pd.



PERMANOVA statistical testing of alpha and beta diversity with meta data

> ## Overview
>
> **Teaching:** 10 min **Exercises:** 0 min

Objectives

- Understand PERMANONA testing for group significance
- Identifying the most significant influential meta data that associated with microbial alpha and beta diversity

PERMANOVA statistical testing of alpha diversity for groups

Faith_ph

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity alpha-group-significance \
 --i-alpha-diversity hf_diver/core-metrics-results/faith_pd_vector.qza \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/alpha-group/faith-pd-group-significance.qzv'
```

A file 'faith-pd-group-significance.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/
total 324
-rw-r--r-- 1 z3527776 MRCBIO 328428 Sep 14 14:28 faith-pd-group-significance.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'faith-pd-group-significance.qzv' locally then upload to qiime2 view, the visualization should be look like this.

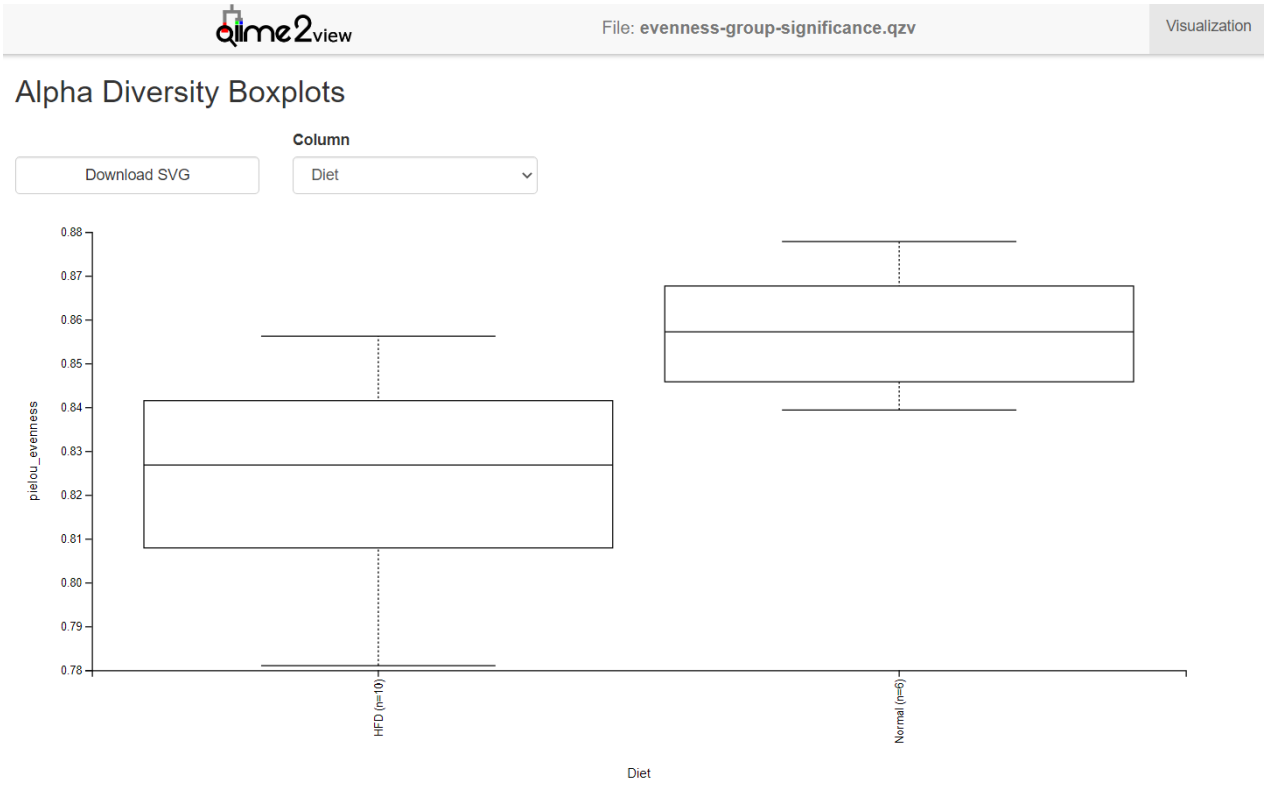
evenness

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity alpha-group-significance \
 --i-alpha-diversity hf_diver/core-metrics-results/evenness_vector.qza \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/alpha-group/evenness-group-significance.qzv'
```

A file 'evenness-group-significance.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/
total 648
-rw-r--r-- 1 z3527776 MRCBIO 328463 Sep 14 14:33 evenness-group-significance.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328428 Sep 14 14:28 faith-pd-group-significance.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'evenness-group-significance.qzv' locally then upload to qiime2 view, the visualization should be look like this.



observed features

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity alpha-group-significance \
 --i-alpha-diversity hf_diver/core-metrics-
results/observed_features_vector.qza \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/alpha-group/observed_features_vector.qzv'
```

A file 'observed_features_vector.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/
total 972
-rw-r--r-- 1 z3527776 MRCBIO 328061 Sep 14 14:35 observed_features_vector.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328463 Sep 14 14:33 evenness-group-significance.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328428 Sep 14 14:28 faith-pd-group-significance.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'observed_features_vector.qzv' locally then upload to qiime2 view, the visualization should be look like this.

shannon index

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity alpha-group-significance \
 --i-alpha-diversity hf_diver/core-metrics-results/shannon_vector.qza \
 --m-metadata-file meta_data.txt \
 --o-visualization hf_diver/alpha-group/shannon_vector.qzv'
```

A file 'shannon_vector.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/
total 1296
-rw-r--r-- 1 z3527776 MRCBIO 328483 Sep 14 14:36 shannon_vector.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328061 Sep 14 14:35 observed_features_vector.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328463 Sep 14 14:33 evenness-group-significance.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328428 Sep 14 14:28 faith-pd-group-significance.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'shannon_vector.qzv' locally then upload to qiime2 view, the visualization should be look like this.

Output alpha diversity values from qiime2 output

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/alpha-group/shannon_vector.qzv \
 --output-path hf_diver/alpha-group/shannon_vector'
```

A folder 'shannon_vector' is newly generated. You can download the whole directory to your local pc or laptap and open the file 'index.html' to visualize.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/
total 1296
drwxr-xr-x 4 z3527776 MRCBIO    173 Sep 14 14:37 shannon_vector
-rw-r--r-- 1 z3527776 MRCBIO 328483 Sep 14 14:36 shannon_vector.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328061 Sep 14 14:35 observed_features_vector.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328463 Sep 14 14:33 evenness-group-significance.qzv
-rw-r--r-- 1 z3527776 MRCBIO 328428 Sep 14 14:28 faith-pd-group-significance.qzv
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/alpha-group/shannon_vector
total 16
drwxr-xr-x 2 z3527776 MRCBIO   85 Sep 14 14:37 dist
drwxr-xr-x 6 z3527776 MRCBIO   71 Sep 14 14:37 q2templateassets
-rw-r--r-- 1 z3527776 MRCBIO  924 Sep 14 14:37 column-Diet.jsonp
-rw-r--r-- 1 z3527776 MRCBIO 2502 Sep 14 14:37 index.html
-rw-r--r-- 1 z3527776 MRCBIO  117 Sep 14 14:37 kruskal-wallis-pairwise-Diet.csv
-rw-r--r-- 1 z3527776 MRCBIO  647 Sep 14 14:37 metadata.tsv
(qiime2-2020.2) [z3527776@k212 demo]$
```

PERMANOVA statistical testing of beta diversity for groups

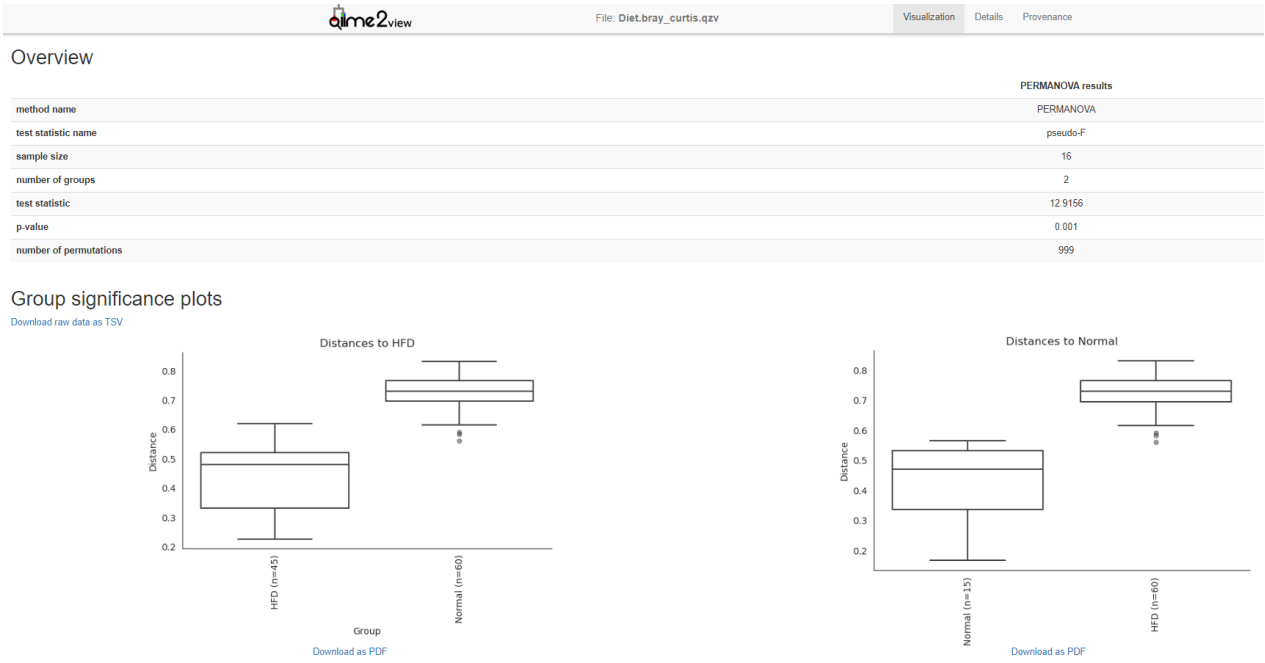
Bray curtis distance on diet

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity beta-group-significance \
 --i-distance-matrix hf_diver/core-metrics-
results/bray_curtis_distance_matrix.qza \
 --m-metadata-file meta_data.txt \
 --m-metadata-column "Diet" \
 --o-visualization hf_diver/beta-group/Diet.bray_curtis.qzv --p-pairwise'
```

A file 'Diet.bray_curtis.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/beta-group/
total 288
-rw-r--r-- 1 z3527776 MRCBIO 294459 Sep 14 14:40 Diet.bray_curtis.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'Diet.bray_curtis.qzv' locally then upload to qiime2 view, the visualization should be look like this.



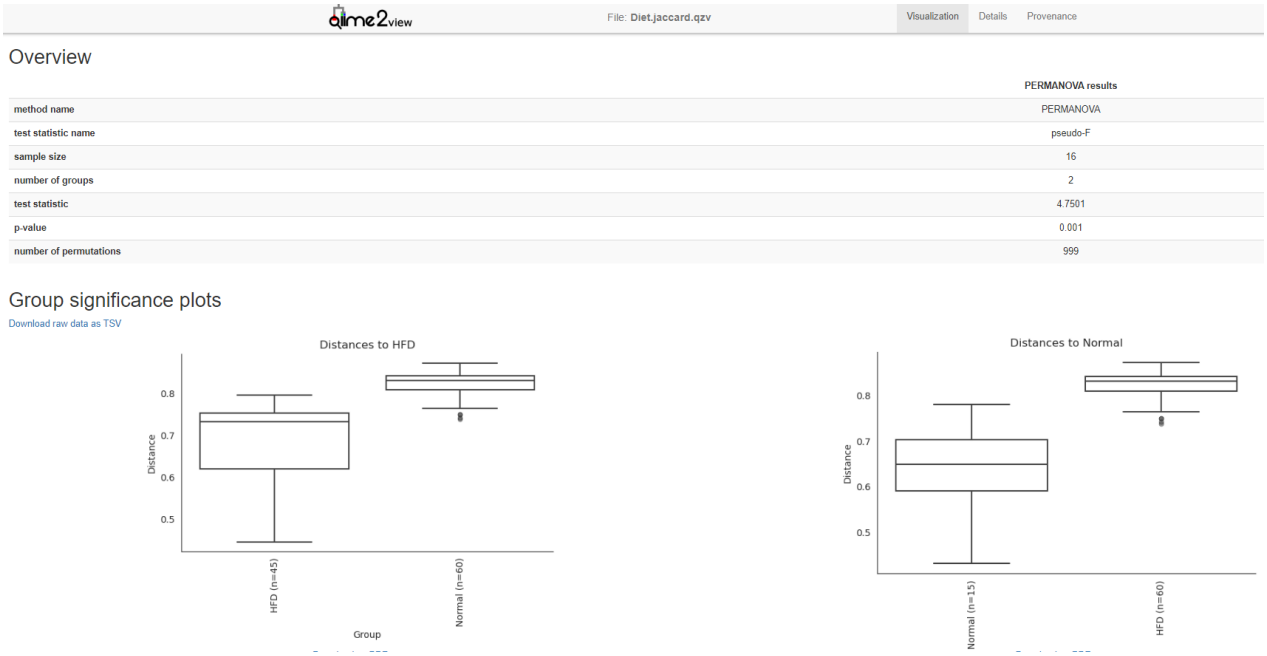
Jaccard distance

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity beta-group-significance \
 --i-distance-matrix hf_diver/core-metrics-results/jaccard_distance_matrix.qza
\
 --m-metadata-file meta_data.txt \
 --m-metadata-column "Diet" \
 --o-visualization hf_diver/beta-group/Diet.jaccard.qzv --p-pairwise'
```

A file 'Diet.jaccard.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/beta-group/
total 576
-rw-r--r-- 1 z3527776 MRCBIO 291627 Sep 14 14:42 Diet.jaccard.qzv
-rw-r--r-- 1 z3527776 MRCBIO 294459 Sep 14 14:40 Diet.bray_curtis.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'Diet.jaccard.qzv' locally then upload to qiime2 view, the visualization should be look like this.



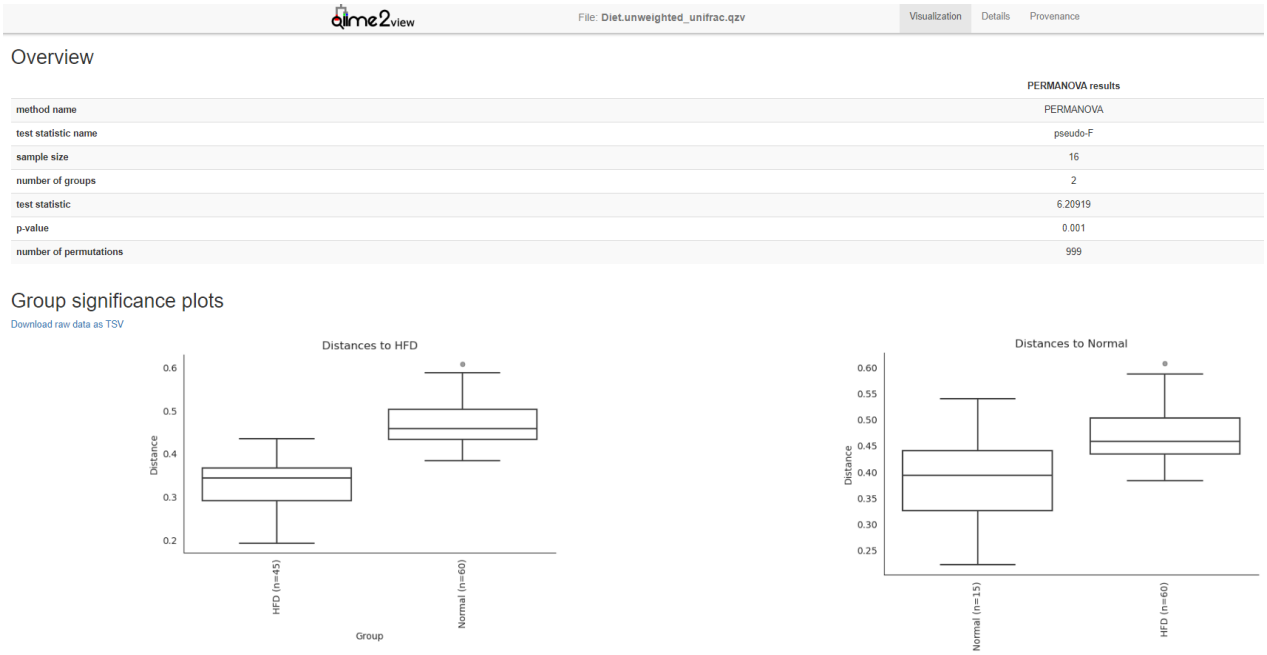
Unweighted UniFrac distacne

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity beta-group-significance \
 --i-distance-matrix hf_diver/core-metrics-
results/unweighted_unifrac_distance_matrix.qza \
 --m-metadata-file meta_data.txt \
 --m-metadata-column "Diet" \
 --o-visualization hf_diver/beta-group/Diet.unweighted_unifrac.qzv --p-
pairwise'
```

A file 'Diet.unweighted_unifrac.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/beta-group/
total 868
-rw-r--r-- 1 z3527776 MRCBIO 296326 Sep 14 14:45 Diet.unweighted_unifrac.qzv
-rw-r--r-- 1 z3527776 MRCBIO 291627 Sep 14 14:42 Diet.jaccard.qzv
-rw-r--r-- 1 z3527776 MRCBIO 294459 Sep 14 14:40 Diet.bray_curtis.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'Diet.unweighted_unifrac.qzv' locally then upload to qiime2 view, the visualization should be look like this.



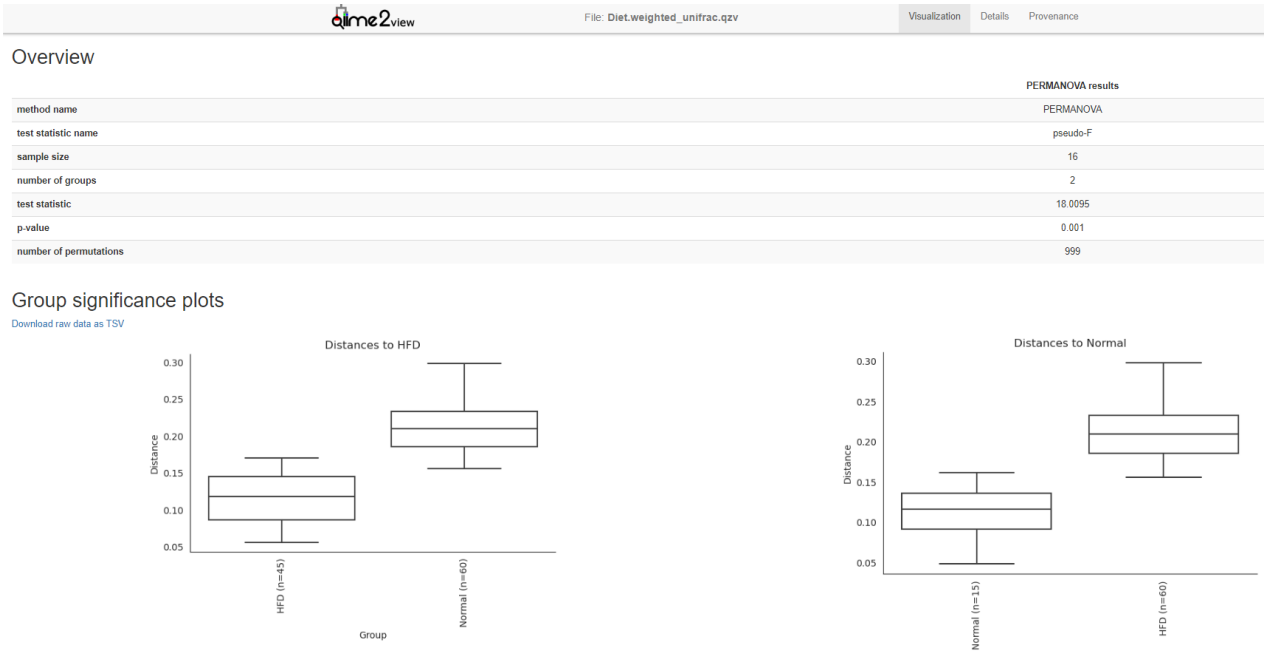
Weighted UniFrac distacne

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime diversity beta-group-significance \
 --i-distance-matrix hf_diver/core-metrics-
results/weighted_unifrac_distance_matrix.qza \
 --m-metadata-file meta_data.txt \
 --m-metadata-column "Diet" \
 --o-visualization hf_diver/beta-group/Diet.weighted_unifrac.qzv --p-pairwise'
```

A file 'Diet.weighted_unifrac.qzv' is newly generated.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/beta-group/
total 1156
-rw-r--r-- 1 z3527776 MRCBIO 293949 Sep 14 14:46 Diet.weighted_unifrac.qzv
-rw-r--r-- 1 z3527776 MRCBIO 296326 Sep 14 14:45 Diet.unweighted_unifrac.qzv
-rw-r--r-- 1 z3527776 MRCBIO 291627 Sep 14 14:42 Diet.jaccard.qzv
-rw-r--r-- 1 z3527776 MRCBIO 294459 Sep 14 14:40 Diet.bray_curtis.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

If you download file 'Diet.weighted_unifrac.qzv' locally then upload to qiime2 view, the visualization should be look like this.



Decompress visulization qzv file and download to local computer to check the results

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif  bash -c '. activate
qiime2-2020.8 && \
qiime tools export \
 --input-path hf_diver/beta-group/Diet.weighted_unifrac.qzv \
 --output-path hf_diver/beta-group/Diet.weighted_unifrac'
```

A folder 'Diet.weighted_unifrac' is newly generated. You can download the whole directory to your local pc or laptap and open the file 'index.html' to visualize.

```
(qiime2-2020.2) [z3527776@k212 demo]$ ll -t hf_diver/beta-group/
total 1156
drwxr-xr-x 3 z3527776 MRCBIO    236 Sep 14 14:46 Diet.weighted_unifrac
-rw-r--r-- 1 z3527776 MRCBIO 293949 Sep 14 14:46 Diet.weighted_unifrac.qzv
-rw-r--r-- 1 z3527776 MRCBIO 296326 Sep 14 14:45 Diet.unweighted_unifrac.qzv
-rw-r--r-- 1 z3527776 MRCBIO 291627 Sep 14 14:42 Diet.jaccard.qzv
-rw-r--r-- 1 z3527776 MRCBIO 294459 Sep 14 14:40 Diet.bray_curtis.qzv
(qiime2-2020.2) [z3527776@k212 demo]$
```

4. Using LEfSe to identify differential abundant taxa

> ## Overview
>
> **Teaching:** 20 min **Exercises:** 0 min

Objectives

- Understand how LEfSe identify microbial signatures
- Identify differential abundant taxa in the high fat diet mouse

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif bash -c '. activate
qiime2-2020.8  && perl /home/applications/mima/mima_lefsetable_16s.pl
hf_diver/rarefy.table.qza hf_diver/taxonomy.qza Diet meta_data.txt
hf_lefse_tables '
```

LEfSe outputs are in directory 'hf_lefse_tables', this step will generate input file of differnt ranks and merge all 7 ranks taxa into one file to be used in the following step to draw clade plot and bar plot of LDA scores.

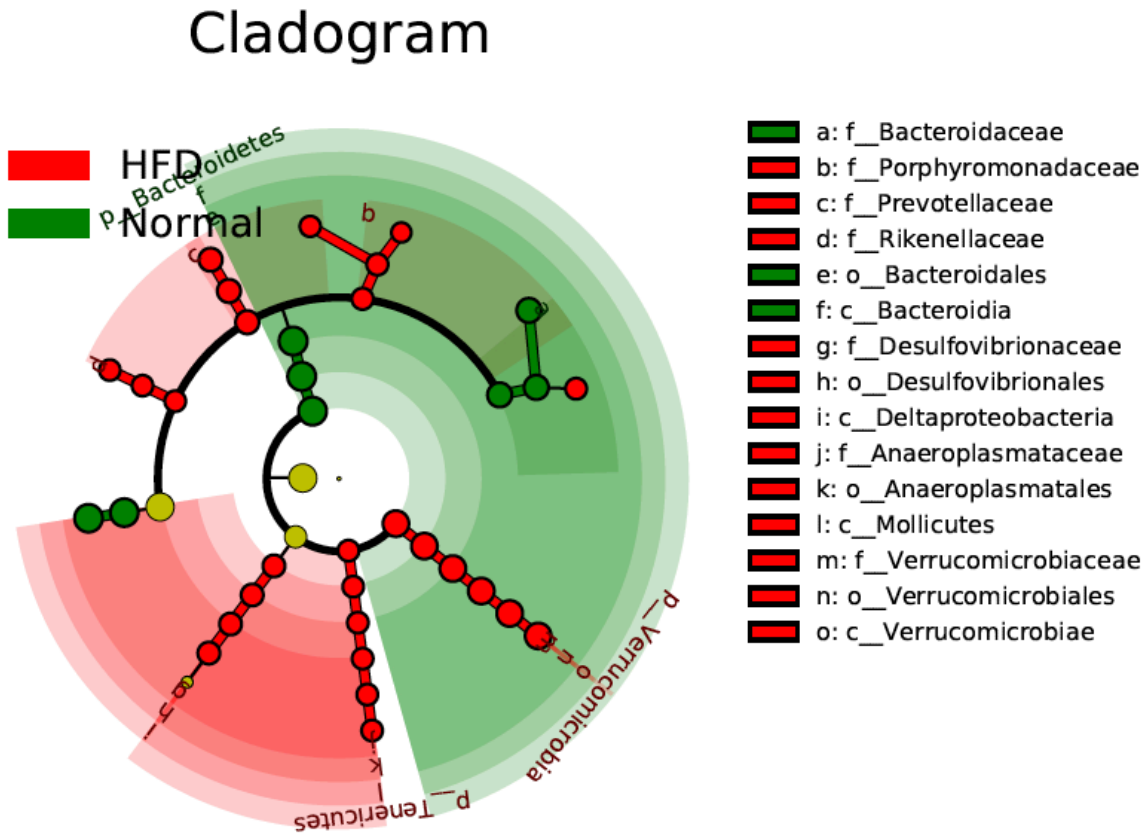
For a regular tab seperated table to do LEfSe analysis, the following pipeline can be used Check the help information for the pipeline

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif bash -c '. activate
lefse-conda && perl /home/applications/mima/mima_lefse_pipeline.pl '

  -i table.tsv, the rarefied feature table used to do LEfSe
  -m meta datafile, a table with rows as sample and column as differnt meta
data
  -g group vector should be column_name:group1,group2,group3 #the detail
groups in one column to compare
  -o output dir/ string
  -f prefix to the output file,  string format
  -d LDA schore forcut off default 2
  -x width of figure  default 8 inch
  -y height of figure default 8 inch
  -h print this help information
```

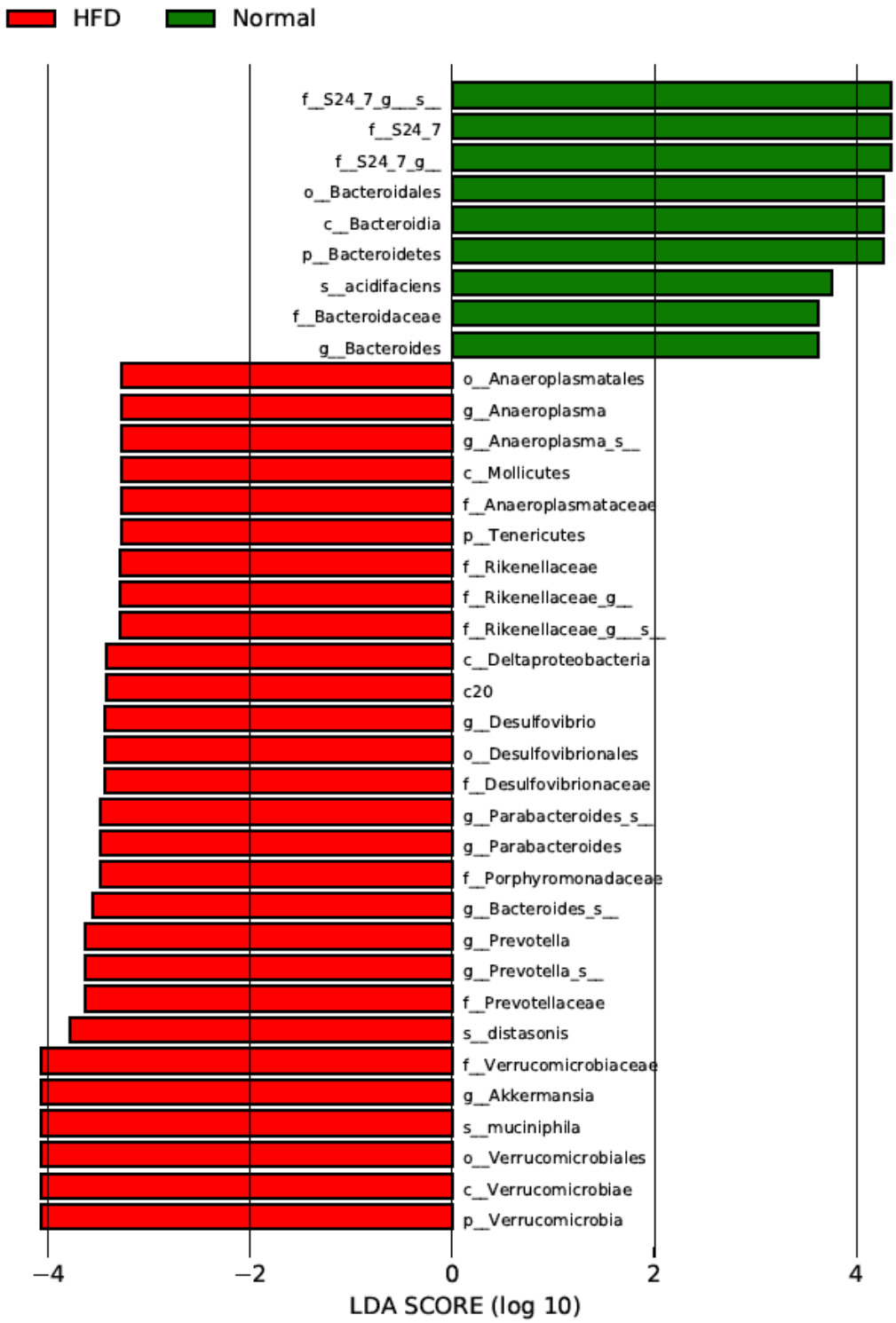
Here in this example, we would like to identify the differential abundant taxa that enriched in high fat diet mouse.

```
singularity exec /data/bio/workshop/mrcmicrobiome.sif bash -c '. activate
lefse-conda && perl  /home/applications/mima/mima_lefse_pipeline.pl -i
hf_lefse_tables/lefse_allrank/merge_taxonomy.csv -m meta_data.txt -g
Diet:Normal,HFD -o hf_lefsenew -f dietlefse -d 3'
```

cladoplot presenting of signatures



LEfSe signatures barchart



Congratulations!
