TAXONOMY COMPOSITION AND DIVERSITY OF BACTERIA AND ARCHAEA WITH QIIME 2

Pipeline steps:

A. Preparing the data
B. Demultiplexing of paired-end reads
C. Denoising of poor quality reads
D. Merging data (optional)
E. Taxonomy classification
F. Excluding samples (optional)
G. Diversity analysis

Considerations:

- This pipeline is made for processing multiplexed paired-end reads from V4 16S rRNA.
- This pipeline assumes you have at least a basic knowledge about bioinformatics or programming.
- QIIME 2 needs to be installed and activated from Conda on your computer. Use the following steps to guide you (this pipeline was made with QIIME 2 version 2020.11): https://docs.qiime2.org/2020.8/install/native/
- I invite you to look over some interesting information from the QIIME 2 page to understand concepts and steps related to this pipeline: https://docs.qiime2.org/2020.8/tutorials/overview/ , some QIIME 2 tutorials: https://docs.qiime2.org/2020.8/tutorials/ , and to visit the QIIME 2 forum for questions: https://forum.qiime2.org/

Needed files (input files):

- **A set or sets of multiplexed paired-end read files** (forward, reverse and barcode files).

  NOTE: This pipeline is fixed to work with either a single folder (a single set of raw data corresponding to a single sequencing run) or multiple folders (sets of raw data of several sequencing runs, each set must be contained in a different folder but in the same directory). Also, you don't need to modify the name of your folders with this pipeline.

- **A single or multiple metadata files** (depending on the number of folders you will work with) that you must create because it will be required by QIIME 2. These files contain important information about the samples.
- The **“*classifier.qza*” file** included in the page, needed for the taxonomy classification step. This file is a classifier based on the Silva database version 138 (515f-806r)
- The **seven scripts** included in the page: ***“folders.sh”***, ***“q2-demux.sh”***, ***“q2-denoise.sh”***, ***“q2-merge.sh”***, ***“q2-taxonomy.sh”***, ***“q2-exclude.sh”*** and ***“q2-diversity.sh”***. Each script will carry out each step along the pipeline.

  NOTE: I recommend you to glance at the content of the scripts to be aware of what these include. Every QIIME2 command in the scripts is headed by a description that may help you to understand the steps that will be executed.

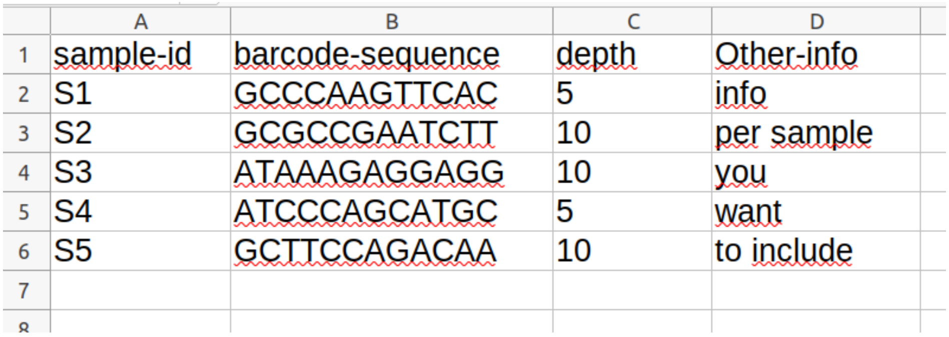
How building the metadata files required by QIIME 2:

NOTE: Create a metadata file for each folder you will work with. Each metadata file consists of a matrix (rows and columns) of information about your samples in a given folder.

1. MANDATORY FILE(S): Within an empty text file or Excel, create a table where the first and second columns must be headed by the labels “sample-id” and “barcode-sequence”, respectively. Thus, the rows of the first column must indicate the identification of your samples, and the rows of the second column must indicate the corresponding barcode sequence of each sample. These columns are indispensable to QIIME 2 for identifying each sample and barcode sequence, respectively, therefore it is mandatory to set up these columns. Moreover, you can include more information about the samples in the next columns for making later comparisons among groups of samples if that is the case.

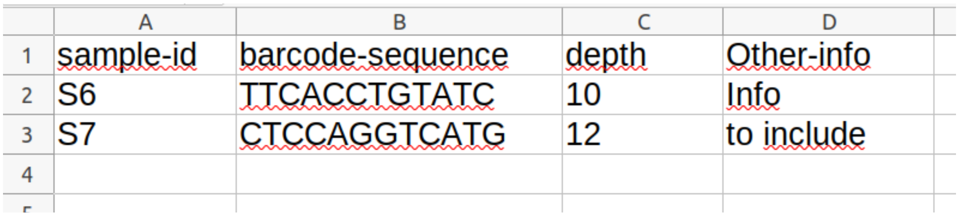
   e.i. if you have 3 folders, the content of your metadata files should look like:

(for the first folder)



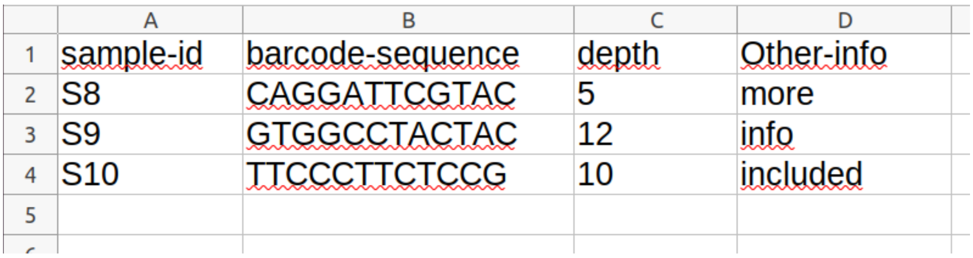
sample-id	barcode-sequence	depth	Other-info
S1	GCCCAAGTTCAC	5	info
S2	GCGCCGAATCTT	10	per sample
S3	ATAAAGAGGAGG	10	you
S4	ATCCCAGCATGC	5	want
S5	GCTTCCAGACAA	10	to include

(for the second folder)



sample-id	barcode-sequence	depth	Other-info
S6	TTCACCTGTATC	10	Info
S7	CTCCAGGTCATG	12	to include

(for the third folder)



sample-id	barcode-sequence	depth	Other-info
S8	CAGGATTCGTAC	5	more
S9	GTGGCCTACTAC	12	info
S10	TTCCCTTCTCCG	10	included

The name of each file must be saved according to the name of the folder as:

metadata-*foldername*.tsv

e.i. if you have 3 folders, you must create 3 metadata files:

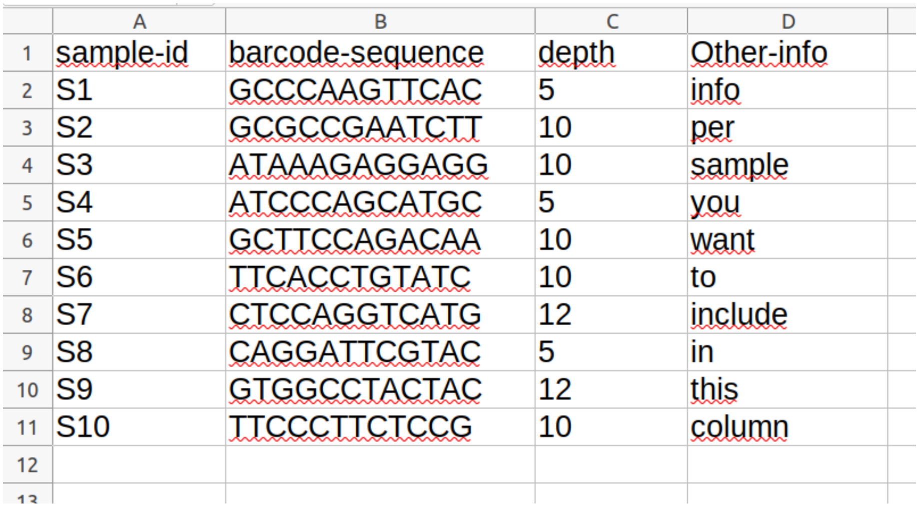
metadata-*foldername1*.tsv

metadata-*foldername2*.tsv

metadata-*foldername3*.tsv

2. OPTIONAL FILE: In the case you are working with multiple folders and you will want to merge the data after the denoising step, create an additional and single metadata file compiling the sample information of all folders (remember that sample IDs and barcodes must not be repeated within each column).

The content of this metadata file must look like:



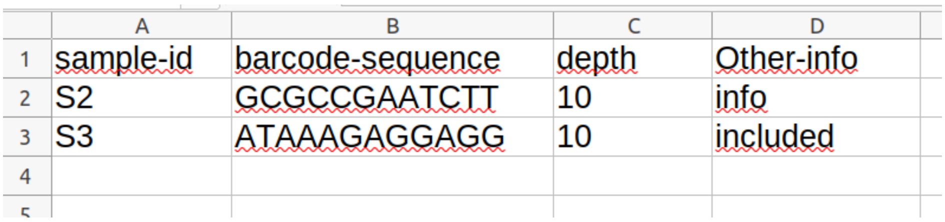
	A	B	C	D
1	sample-id	barcode-sequence	depth	Other-info
2	S1	GCCCAAGTTCAC	5	info
3	S2	GCGCCGAATCTT	10	per
4	S3	ATAAAGAGGAGG	10	sample
5	S4	ATCCCAGCATGC	5	you
6	S5	GCTTCCAGACAA	10	want
7	S6	TTCACCTGTATC	10	to
8	S7	CTCCAGGTCATG	12	include
9	S8	CAGGATTCGTAC	5	in
10	S9	GTGGCCTACTAC	12	this
11	S10	TTCCCTTCTCCG	10	column
12				
13				

This file must be saved as:

metadata-all.tsv

3. OPTIONAL FILE: In the case you will want to filter specific samples after the taxonomy classification step, create an additional metadata file with only the sample(s) you want to exclude (it is no problem if the samples are from the same or different folders).

   The content of this metadata file must look like:



	A	B	C	D
1	sample-id	barcode-sequence	depth	Other-info
2	S2	GCGCCGAATCTT	10	info
3	S3	ATAAAGAGGAGG	10	included
4				
5				

   This file must be saved as:

   metadata-exc.tsv

Resulting files (output files):

- Typical QIIME 2 output files for each step and each folder during the script execution.

- The “denoise.sh” file, containing the commands needed for a customizable denoising step (this will work as an input file too).
- ”*info-*” files (.txt) containing the names of the QIIME 2 output files that were correctly generated and saved.
- “error-” files (.txt) containing information about possible errors produced in any QIIME 2 output file.

PIPELINE

IMPORTANT: Remember to locate all the folders you will work with in the same directory. Within such directory must also be located the metadata file(s), the “*classifier.qza”* file, and the seven scripts. Also, remember to activate QIIME 2 through Conda as:

```
conda activate qiime2-2020.11
```

A. Preparing the data:

Before starting, multiplexed paired-end read files as name_R1_001.fastq, name_R2_001.fastq and name_I1_001.fastq files must be renamed and compressed to "forward.fastq.gz", "reverse.fastq.gz" and “barcode.fastq.gz”, respectively (do this into each folder you will work with). It is recommendable to keep a backup of the files with their original name in another directory you want.

For declaring and loading the folders you will work with in all next steps, execute the script “folders.sh” as:

`bash folders.sh` or `./folders.sh`

The program will ask you for the folder name(s) you will work with, you must only write it/them as the program will indicate to you (separated by spaces if there are multiple folders). In this way, your folders will be recognized and loaded for all next steps. If you made a mistake, you must just execute the script again.

Expected outputs: none

B. Demultiplexing paired-end reads:

From now on, we will work with QIIME 2.

For the demultiplexing step, execute the script “q2-demux.sh” as:

`bash q2-demux.sh` or `./q2-demux.sh`

Expected outputs:

➔ emp-paired-end-sequences-*foldername*.qza
➔ demux-full-*foldername*.qza
➔ demux-details-*foldername*.qza
➔ demux-full-*foldername*.qzv
➔ info-demux.txt
➔ error-demux.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)

NOTE: All “.qzv” output files could be visualized in a browser page by executing the following command (this applies for the rest steps too):

qiime tools view *filename*.qzv

The output file(s) “demux-full-.qza” is the one that will display the quality control graphics of the demultiplexed reads.

C. Denoising poor quality reads

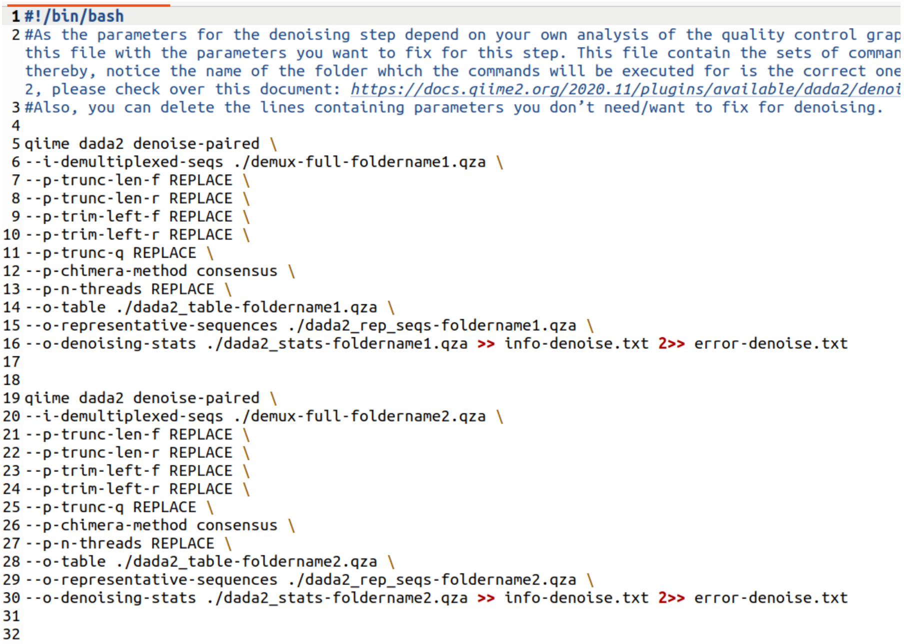
NOTE: DADA2 is used as the denoising method in this pipeline.

IMPORTANT: After you decide which parameters you want to use for denoising poor quality reads, open the output file “denoise.sh”. As the parameters for the denoising step depend on your own analysis of the quality control graphics, you must replace the words “REPLACE” in the commands within this file with the parameters you want to fix for this step, and save the changes. This file will contain the sets of commands needed for denoising the reads of each folder independently, thereby, notice the name of the folder which the commands will be executed for is the correct one. Also, you can delete the lines containing parameters you don't need/want to fix for denoising. If you don't know how to fix the DADA2 parameters within QIIME 2, please check over this document:

https://docs.qiime2.org/2020.11/plugins/available/dada2/denoise-paired/

Example:

(Before replacing)



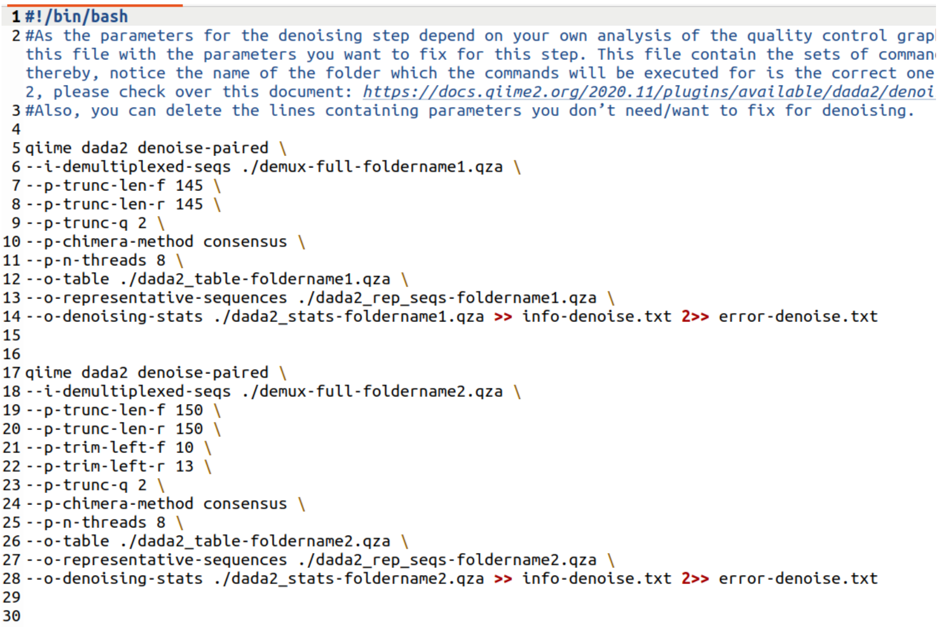
```
#!/bin/bash
#As the parameters for the denoising step depend on your own analysis of the quality control grap
this file with the parameters you want to fix for this step. This file contain the sets of comman
thereby, notice the name of the folder which the commands will be executed for is the correct one
2, please check over this document: https://docs.qiime2.org/2020.11/plugins/available/dada2/denoi
#Also, you can delete the lines containing parameters you don’t need/want to fix for denoising.

qiime dada2 denoise-paired \
--i-demultiplexed-seqs ./demux-full-foldername1.qza \
--p-trunc-len-f REPLACE \
--p-trunc-len-r REPLACE \
--p-trim-left-f REPLACE \
--p-trim-left-r REPLACE \
--p-trunc-q REPLACE \
--p-chimera-method consensus \
--p-n-threads REPLACE \
--o-table ./dada2_table-foldername1.qza \
--o-representative-sequences ./dada2_rep_seqs-foldername1.qza \
--o-denoising-stats ./dada2_stats-foldername1.qza >> info-denoise.txt 2>> error-denoise.txt


qiime dada2 denoise-paired \
--i-demultiplexed-seqs ./demux-full-foldername2.qza \
--p-trunc-len-f REPLACE \
--p-trunc-len-r REPLACE \
--p-trim-left-f REPLACE \
--p-trim-left-r REPLACE \
--p-trunc-q REPLACE \
--p-chimera-method consensus \
--p-n-threads REPLACE \
--o-table ./dada2_table-foldername2.qza \
--o-representative-sequences ./dada2_rep_seqs-foldername2.qza \
--o-denoising-stats ./dada2_stats-foldername2.qza >> info-denoise.txt 2>> error-denoise.txt


```

(After replacing)



```
#!/bin/bash
#As the parameters for the denoising step depend on your own analysis of the quality control grap
this file with the parameters you want to fix for this step. This file contain the sets of comman
thereby, notice the name of the folder which the commands will be executed for is the correct one
2, please check over this document: https://docs.qiime2.org/2020.11/plugins/available/dada2/denoi
#Also, you can delete the lines containing parameters you don’t need/want to fix for denoising.

qiime dada2 denoise-paired \
--i-demultiplexed-seqs ./demux-full-foldername1.qza \
--p-trunc-len-f 145 \
--p-trunc-len-r 145 \
--p-trunc-q 2 \
--p-chimera-method consensus \
--p-n-threads 8 \
--o-table ./dada2_table-foldername1.qza \
--o-representative-sequences ./dada2_rep_seqs-foldername1.qza \
--o-denoising-stats ./dada2_stats-foldername1.qza >> info-denoise.txt 2>> error-denoise.txt


qiime dada2 denoise-paired \
--i-demultiplexed-seqs ./demux-full-foldername2.qza \
--p-trunc-len-f 150 \
--p-trunc-len-r 150 \
--p-trim-left-f 10 \
--p-trim-left-r 13 \
--p-trunc-q 2 \
--p-chimera-method consensus \
--p-n-threads 8 \
--o-table ./dada2_table-foldername2.qza \
--o-representative-sequences ./dada2_rep_seqs-foldername2.qza \
--o-denoising-stats ./dada2_stats-foldername2.qza >> info-denoise.txt 2>> error-denoise.txt


```

Then, for the denoising step, execute the script “q2-denoise.sh” as:

`bash q2-denoise.sh` or `./q2-denoise.sh`

Expected outputs:

- ➔ dada2_table-*foldername*.qza
- ➔ dada2_rep_seqs-*foldername*.qza
- ➔ dada2_stats-*foldername*.qza
- ➔ dada2_table-*foldername*.qzv
- ➔ dada2_rep_seqs-*foldername*.qzv
- ➔ dada2_stats-*foldername*.qzv
- ➔ dada2_summary-*foldername*.qzv
- ➔ info-denoise.txt
- ➔ error-denoise.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)

D. Merging data (optional)

In the case you are working with multiple folders, you might want to merge the denoised data of different files, as tables and representative sequences, to a single table and file of non-redundant representative sequences for the next analyses. If it is not the case, omit this step.

For the merging step, execute the script “q2-merge.sh” as:

`bash q2-merge.sh` or `./q2-merge.sh`

Expected outputs:

- ➔ dada2_table-all.qza
- ➔ dada2_rep_seqs-all.qza
- ➔ dada2_table-all.qzv
- ➔ dada2_rep_seqs-all.qzv
- ➔ dada2_summary-all.qzv
- ➔ info-merge.txt

- ➔ error-merge.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)

E. Taxonomy classification:

NOTE: In this step, the Silva database version 138 (515f-806r) is used.

For the taxonomy classification step, execute the script “q2-taxonomy.sh” along with the percentage of confidence you want to work with (from 0 to 1) as:

bash q2-taxonomy.sh *%number* or ./q2-taxonomy.sh *%number*

(e.g.: ./q2-taxonomy.sh 0.8)

The execution of “q2-taxonomy.sh” will also make a filtering-features step. This consists in filtering representative sequences that during the taxonomy assignment were wrongly labeled as “mitochondria” and/or “chloroplast”, and/or sequences unresolved to at least phylum, labeled as “Unassigned”. You will obtain output files before and after this filtering step.

In addition, a merging step of tables containing the taxa and the sequences will also be carried out.

Expected outputs:

- ➔ taxonomy80-*name*.qza
- ➔ taxonomy80-*name*qzv
- ➔ taxa_barplot80-*name*qzv
- ➔ taxa_barplot80-*name*-filt.qzv
- ➔ table_taxa-*name*-filt.qza
- ➔ summary-*name*-filt.qzv
- ➔ rep_seqs-*name*-filt.qza
- ➔ rep_seqs-*name*-filt.qzv
- ➔ merged-taxa-table-*name*.qzv
- ➔ merged-taxa-table-*name*-filt.qzv
- ➔ info-taxonomy.txt

➔ error-taxonomy.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)

F. Excluding samples (optional):

In the case you want to filter specific samples, this step will exclude the samples in the “metadata-exc.tsv” file to the results of taxonomy classification, filtering of features, merging of tables and for the next analyses. If it is not the case, omit this step.

If you previously executed the merging samples step, now, for excluding samples execute the script “q2-exclude” as:

`bash q2-exclude.sh` or `./q2-exclude.sh`

If you don’t merged samples previously, execute the script “q2-exclude.sh” along with the name of the folder(s) where the samples you want to exclude are (separated by spaces) as:

bash q2-exclude.sh *foldername1 foldername2* or

`./q2-exclude.sh foldername1 foldername2`

In the case you did not merge samples before the taxonomy classification, the program will ask you whether you have a folder(s) where samples were not excluded. If it is the case, you must only write the name(s) as the program will indicate to you (separated by spaces).

Expected outputs:

➔ table_taxa-exc-*name*.qza
➔ summary-exc-*name*.qzv
➔ summary-exc-*name*.qzv
➔ table_taxa-exc-filt-*name*.qza
➔ summary-exc-filt-*name*.qzv
➔ taxa_barplot80-exc-filt-*name*.qzv
➔ rep_seqs-exc-*name*.qza

- ➔ rep_seqs-exc-filt-*name*.qza
- ➔ rep_seqs-exc-filt-*name*.qzv
- ➔ merged-taxa-table-exc-*name*.qzv
- ➔ merged-taxa-table-exc-filt-*name*.qzv
- ➔ info-exclude.txt
- ➔ error-exclude.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)

G. Diversity analysis:

NOTE: In this step, MAFFT and FastTree are used for the alignment and phylogenetic tree construction, respectively.

For making a diversity analysis, execute the script “q2-diversity.sh” along with the number of threads you want to work with the diversity analysis, and the number of the maximum depth which you want to use for the rarefaction curves (separated by spaces), as:

bash q2-diversity.sh *n°threads maxdepth* or

./q2-diversity.sh *n°threads maxdepth*

(e.g.: ./q2-diversity.sh 8 10000)

Expected outputs:

- ➔ ASVs-exc-filt-*name*.qza
- ➔ ASVs-exc-filt-*name*.qzv
- ➔ aligned-*name*.qza
- ➔ masked-aligned-*name*.qza
- ➔ unrooted-tree-*name*.qza
- ➔ rooted-tree-*name*.qza
- ➔ rarefaction_curves-exc-filt-*name*.qzv
- ➔ diversity-metrics-*name* (folder)
- ➔ info-diversity.txt
- ➔ error-diversity.txt (IMPORTANT: Check out this file for any error related to QIIME 2. If it is empty, it means there is no errors)