



# Documentation

Version Alpha

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# 1 Introduction

In 16S rRNA sequence-based diversity analysis, a common practice is the clustering of the sequences based on similarity cutoffs to obtain groups reflecting molecular species, genera or families. Due to the size of the available data, greedy algorithms are preferred for their time efficiency. Such algorithms rely only on pairwise similarities and tend to cluster together sequences with diverse phylogenetic background. Taxonomic classifiers use position specific taxonomic information in assigning probable taxonomy to a given sequence. We developed a tool that uses classifier assigned taxonomy to restrict the clustering among sequences that share the same taxonomic path. We tested “Taxonomy Informed Clustering” with the sequences from SILVA release 138\* and show that TIC outperforms greedy algorithms like Usearch and Vsearch in terms of clusters purity and entropy\*\*. We offer a complete and automated pipeline for use in diversity analysis context, based on this concept, and a pipeline for 16S rRNA amplicon dataset processing. This implementation first process raw reads down to denoised amplicons, taxonomically classify them and apply TIC to cluster them further to sOTUs, gOTUs and fOTUs. The resulting tables offer more accurate insights at different evolutionary levels views that will be useful in microbiome research.

# 2 Organization

TIC-Pipeline is composed of 4 steps that can be run independently or as a set.

1. Samples-Processing
2. Taxonomy-Classification
3. Taxonomy-Informed-Clustering
4. Results\_Reporting

Running them in the given order simplifies the process as the outputs of each step are the inputted to the next. There is also a recommended folder

where the original data are placed to keep the analysis of one study in a compact and organized structure. Before running any script, please make sure you have read and fully understood the corresponding section of the documentation for each step.

## 3 Options Reference

### 3.1 Global Settings

#### TESTING\_MODE:

Flag (YES/NO) enables the step.

TIC Pipeline includes a testing script that verifies all the necessary tools and databases are present and callable in the current system.

#### THREADS:

Number of threads to use in the system. Please avoid selecting more than the available resources in the current system.

### 3.2 Samples-Processing

#### SAMPLES\_PROCESS\_STEP:

Flag (YES/NO) enables the step.

#### USER\_FASTQ\_FOLDER:

the full path of the FASTQ files.

#### TRIM\_SCORE:

the minimum quality score of the FASTQ read last position [3-20] [recommended 20]

#### MAXDIFF:

This is the maximum mismatches during merging of reads allowed.

MINPCTID:

Minimum %identity of alignment. Default 90. Consider decreasing if you have long overlaps.

MINMERGELEN:

200 or 380 or 250 the minimum length allowed after pairing of reads.

MAXMERGELEN:

260 or 440 or 310 the maximum length allowed after pairing of reads.

FORWARD\_TRIM:

length of trimming at the forward side of a read [5-25][recommended 10].

REVERSE\_TRIM:

length of trimming at the reverse side of a read [5-25][recommended 10].

EXPECTED\_ERROR\_RATE:

the maximum rate of expected errors allowed in the assembled paired end reads 0.01 is 1% .

This is the first step of the TIC-Pipeline. It produces unique sequences from your FASTQ files. The FASTQ files should be in the directory specified by the option USER\_FASTQ\_FOLDER. In the same directory, a 'mapping\_file.ssv' must also exist. This file is space-separated and contains the information of which FASTQ files are paired, and what sample they originated from. Please refer to the next table for a short example (the header line is only for comprehension, and should not be included):

Sample Name	Paired Flag	Forward File	Reverse File
SRR2127221	2	SRR2127221_1.fastq	SRR2127221_2.fastq
ERR8971239	1	ERR8971239_1.fastq	

The Sample-Processing step contains quality filtering, trimming and derepli-

cation components of the TIC-Pipeline. We propose that you trim the bases on the FASTQ file that are of lower than 20 on the quality score (TRIM\_SCORE option). The options for the merging of reads are self-explanatory. You can specify the maximum mismatches during the merging of reads and also the percentage of identity of alignment (MAXDIFF and MINPCTID respectively) After the merging of reads, you can control which reads are either too short to be included in the downstream analyses or too long with the options: MIN-MERGELEN and MAXMERGELEN. After the reads are produced, the pipeline continues by trimming the forward and reverse side of each read, this is done principally to remove primers. The EXPECTED\_ERROR\_RATE option sets the maximum rate of expected errors allowed in the assembled paired end reads. All these stages are run by vsearch v2. [6].

### 3.3 Taxonomy Classification

#### ALIGNMENT\_CLASSIFICATION\_STEP:

Flag (YES/NO) enables the step.

#### OUTPUT\_FASTA\_ALI\_CLASS:

Provide the full path where the sina-aligned OTUs will be written to.

#### PDF\_REGION\_OUTPUT:

Graphical representation(PDF) of the sum of the aligned bases in each SINA position in order to view it and select which region you want to extract for the clustering process.

#### EXTRACTION\_STEP:

Flag (YES/NO) enables the step.

#### INPUT\_FASTA\_EXTRACTION:

Provide the full path where the sina-aligned OTUs will be read from.

#### EXTRACTION\_REGION\_START:

start of the extracted region from the sina alignment. [0-49999].

EXTRACTION\_REGION\_END:

end of the extracted region from the sina alignment. [0-49999].

EXTRACTION\_REGION\_LIMIT:

the minimum number of bases that should be present in the sina aligned region selected, so the sequence is kept. This number is multiplied by 0.80 to increase the number of sequences kept.

OUTPUT\_FASTA\_EXTRACTION:

Provide the full path where the OTUs with incorporated taxonomy information will be written to.

ZOTU\_CREATION\_STEP:

Flag (YES/NO) enables the step.

MIN\_ZOTU\_SIZE:

The minimum size of a ZOTU to be counted (trades speed for sensitivity)

OUTPUT\_ZOTUS\_EXTRACTION:

Provide the full path of the extracted region ZOTUs with taxonomic information FASTA file.

Description:

This is the second step of the TIC-Pipeline. Every produced OTU is aligned and classified using SINA [4] and the SILVA ARB [5] file as reference. Each OTU is aligned to 50.000 positions and is classified up to the GENUS taxonomic level with the majority voting rule implemented in SINA, first find the 10 closest neighbors of the input sequence in the ARB file and then vote which is the lowest taxonomic rank that the majority of the neighbors' taxonomy agrees. Also included is a sub-step that sums for each of the 50.000 position how many bases are aligned in that position. This vector is then plotted by the R script 'ggplot\_alignment\_vector.R' and creates a PDF output in the

'PDF\_REGION\_OUTPUT' option, that helps you identify which region should be extracted for the next step and how many bases should be at least aligned in that region so that the OTU is included in your clustering. 1.

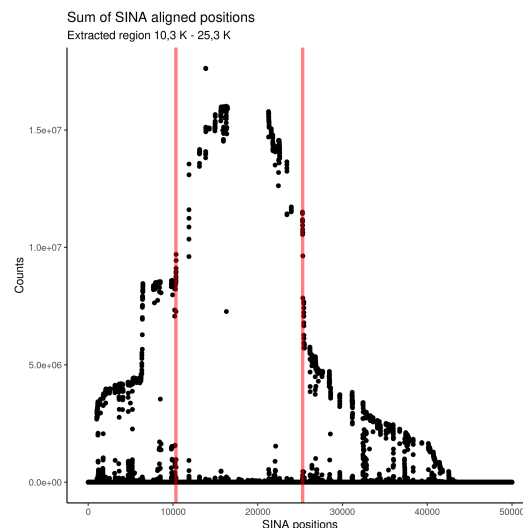


Figure 1: Sum of bases aligned in each position.

The first time you run this command, it will produce an index of the ARB file. This process is slow but it happens only this one time and is expected by the pipeline.

The produced OTUs are checked for chimeras using the SortMeRNA tool [2] with the SILVA bacteria and archaea databases used as references.

For producing the ZOTUs from the non-chimeric reads you only have to specify the MIN\_ZOTU\_SIZE. Any ZOTU that contains less than this number is discarded. Please keep in mind, when specifying this option that smaller values than the default 4 makes the ASVs more sensitive but a lot slower.

### 3.4 Taxonomy Informed Clustering

TAXONOMIC\_CLUSTERING\_STEP:

Flag (YES/NO) enables the step.

CLUSTERING\_DIRECTORY:

Full path directory to save the results of the taxonomic split and taxonomic



clustering.

INPUT\_FASTA\_CLUSTERING:

Full path directory of the FASTA with taxonomic classifications which will be used as input of the step.

FAMILY\_IDENTITY:

Percentage identity on the Family level. [0-GENERA\_IDENTITY].

GENERA\_IDENTITY:

Percentage identity on the GENERA level. [FAMILY\_IDENTITY-SPECIES\_IDENTITY].

SPECIES\_IDENTITY:

Percentage identity on the GENERA level. [0-100].

Description:

This is the third step of the TIC-Pipeline. Naive classifiers look only into sequence similarity as a prerequisite to clustering resulting in impure clusters containing sequences from distant taxonomies. By following the TIC-Pipeline Each ZOTU has a defined even if incomplete taxonomy. To avoid the above caveat of vsearch, we split the ZOTUs into separate files bases on their last known taxonomy level. This counteracts some greediness of vsearch by clustering smaller parts of the ZOTUs alone rather than as a whole. Additionally the taxonomical informed clustering, tries to identify misses from the sina classification. Each ZOTU that misses one or multiple taxonomic levels is searched against the sequences included in the active taxonomic clade of the tree, and only if it does not match with any of those sequences, it is used for the creation of new families, genera etc [3](#). Since there is no specific percentage similarity cutoff for the differentiation of Phyla, Classes and Orders, TIC is limited to the generation of new families, genera and species. In case of missing higher taxonomic levels, the labels of UNKPHULUM, UNKCLASS and UNKORDER are used as a catch-all mechanism.

**Algorithm 1** Taxonomy Informed Clustering

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**Require:** taxonomy folder, limit\_families, limit\_genera, limit\_species  
**Ensure:** limit\_families < limit\_genera < limit\_species  
*curr\_genera*  $\leftarrow$  gather sequences with known genera  
**for all** *curr\_genera* **do**  
  cluster at limit\_species  
**end for**  
creation of new species from centroids  
*curr\_families*  $\leftarrow$  gather sequences with known families but unknown genus  
**for all** *curr\_families* **do**  
  search at limit\_species within all species of the respective family  
  *curr\_unmatched*  $\leftarrow$  gather unmatched sequences  
  cluster at limit\_species  
  creation of new species from centroids  
  *curr\_centroids*  $\leftarrow$  gather representative for each new species  
  search at limit\_genera within all genera of the respective family  
  *curr\_unmatched*  $\leftarrow$  gather unmatched sequences  
  cluster at limit\_genera  
  creation of new genera from centroids  
**end for**  
*curr\_orders*  $\leftarrow$  gather sequences with known orders but unknown families  
**for all** *curr\_orders* **do**  
  search at limit\_species within all species of the respective order  
  *curr\_unmatched*  $\leftarrow$  gather unmatched sequences  
  cluster at limit\_species  
  creation of new species from centroids  
  *curr\_centroids*  $\leftarrow$  gather representative for each new species  
  search at limit\_genera within all genera of the respective order  
  *curr\_unmatched*  $\leftarrow$  gather unmatched sequences  
  cluster at limit\_genera  
  creation of new genera from centroids  
  *curr\_centroids*  $\leftarrow$  gather representatives for each new species but with defined new Genus  
  search at limit\_family within all species of the respective order  
  *curr\_unmatched*  $\leftarrow$  gather unmatched sequences  
  cluster at limit\_families  
  creation of new families from centroids  
**end for**

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Figure 2: Pseudo-code of the TIC algorithm.

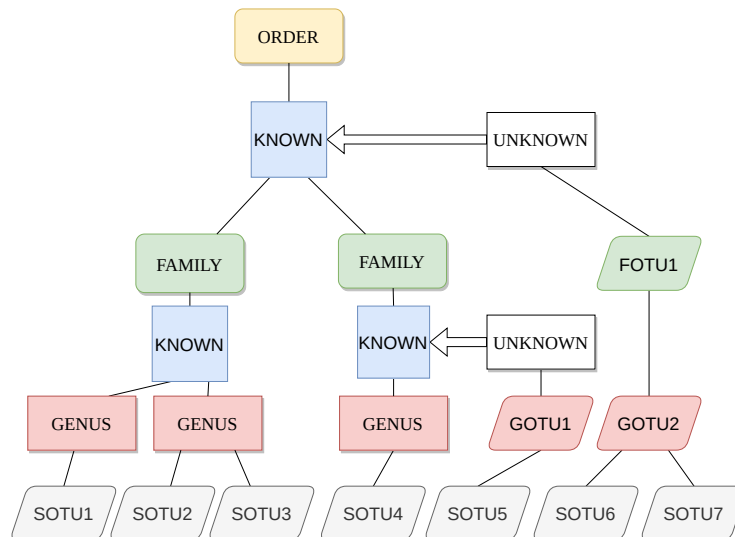


Figure 3: High level abstraction of the TIC algorithm.

### 3.5 Results Reporting

#### RESULTS\_REPORTING\_STEP:

Flag (YES/NO) enables the step.

#### OUTPUT\_FOLDER:

Full path of the directory where all results will be saved.

#### OUTPUT\_ZOTU\_FASTA\_WITH\_TAXONOMY:

Name of the output FASTA file containing the ASVs along with their full taxonomic information.

#### OUTPUT\_ZOTU\_TABLE:

Name of the output TAB file containing the ASVs along with their full taxonomic information and number of reads in each sample of the study.

#### OUTPUT\_SOTU\_FASTA\_WITH\_TAXONOMY:

Name of the output FASTA file containing the SOTU along with their full taxonomic information.

#### Description:

This is the forth and final step of the TIC-Pipeline. In this step, we gather all outputs produced in the previous processes and try to create graphical representations of your data. All outputs are placed in the new folder specified by OUTPUT\_FOLDER in the config\_options.txt. The denoised sequences are assigned to the full taxonomies produced from TIC and written to the file specified by OUTPUT\_ZOTU\_FASTA\_WITH\_TAXONOMY. The OUTPUT\_ZOTU\_TABLE contains the number of reads of each sample in your study as well as their full taxonomy <sup>4</sup>. The centroid sequences for each SOTU are written in the file specified by OUTPUT\_SOTU\_FASTA\_WITH\_TAXONOMY option in the configuration file. Mapping the relationship between species, genera and families is important to the end-users. The files "sotus\_to\_gotus\_map.tab", "gotus\_to\_fotus\_map.tab" detail those ties in TAB-seperated format. A graphlan

taxonomic tree [1] as well as a Krona [3] plot are created and written in the output folder. All red clades in the taxonomic tree represent novel families, orders etc. 5. In addition, two ZOTU-based and SOTU-based phylogenetic trees produced by the Neighbor-Joining method are automatically created by rapindNJ [7].

#ZOTU ID	SAMPLE_NAME_1	SAMPLE_NAME_2	Taxonomy
Zotu13		129	35 Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;Halomonas;SOTU165;
Zotu34		178	86 Bacteria;MBNT15;UNKCLASS;UNKORDER;FOTU209;GOTU537;SOTU1599;

Figure 4: Two lines from the ZOTU tab file.

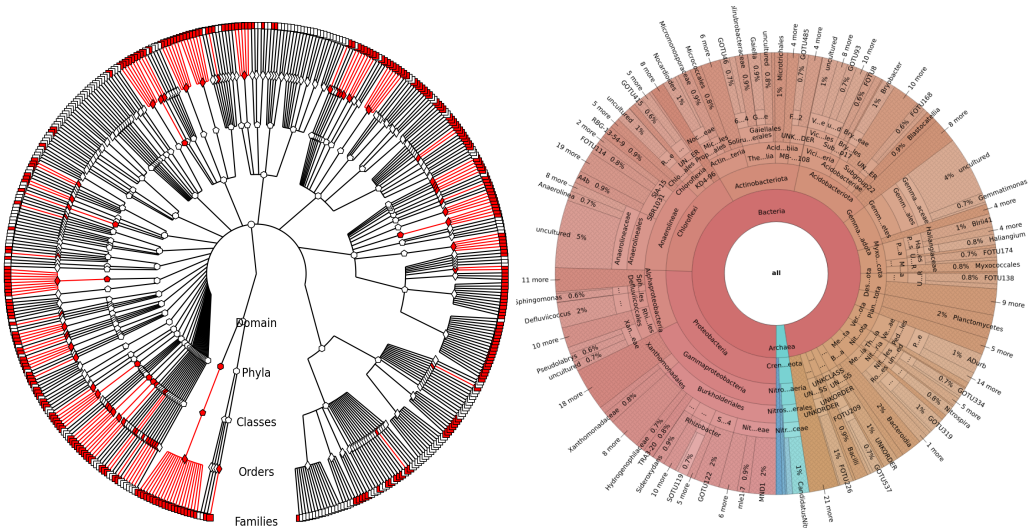


Figure 5: Plots produced from TIC-Pipeline. **(A)** Graphlan plot depicting the taxonomic tree of the denoised sequences after TIC incorporating both novel (red) and known (black) clades up to the family level. **(B)** Krona plot quantifying the size of each taxonomy in the merged study samples. Contains novel and known taxonomies as produced by sina classifier and TIC.

## Bibliography

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