CSC

ICT Solutions for
Brilliant Minds

Bioinformatics for microbiome research (BIO2)

Jyväskylä Summer School 2023

Eija Korpelainen and Heli Juottonen (CSC – IT Center for Science Ltd)

chipster@csc.fi

Schedule

Day 1: Monday 14.8. (10-17) **Amplicon data processing**

Day 2: Tuesday 15.8. (9-16) **Microbial community data analysis**

Day 3: Wednesday 16.8. (9-16) **RNA-seq data analysis**

lunch break 12-13 daily

What will I learn?

- Microbial community analysis of amplicon sequencing data
  - Central concepts
  - Analysis steps
  - File formats
- Ion Torrent 16S data is used in the exercises, but we discuss also how to analyze
  - Illumina MiSeq data
  - ITS data
- How to operate the Chipster software

Understanding data analysis - why?

- Bioinformaticians might not always be available when needed

- Biologists know their own experiments best
  - Potential batch effects etc

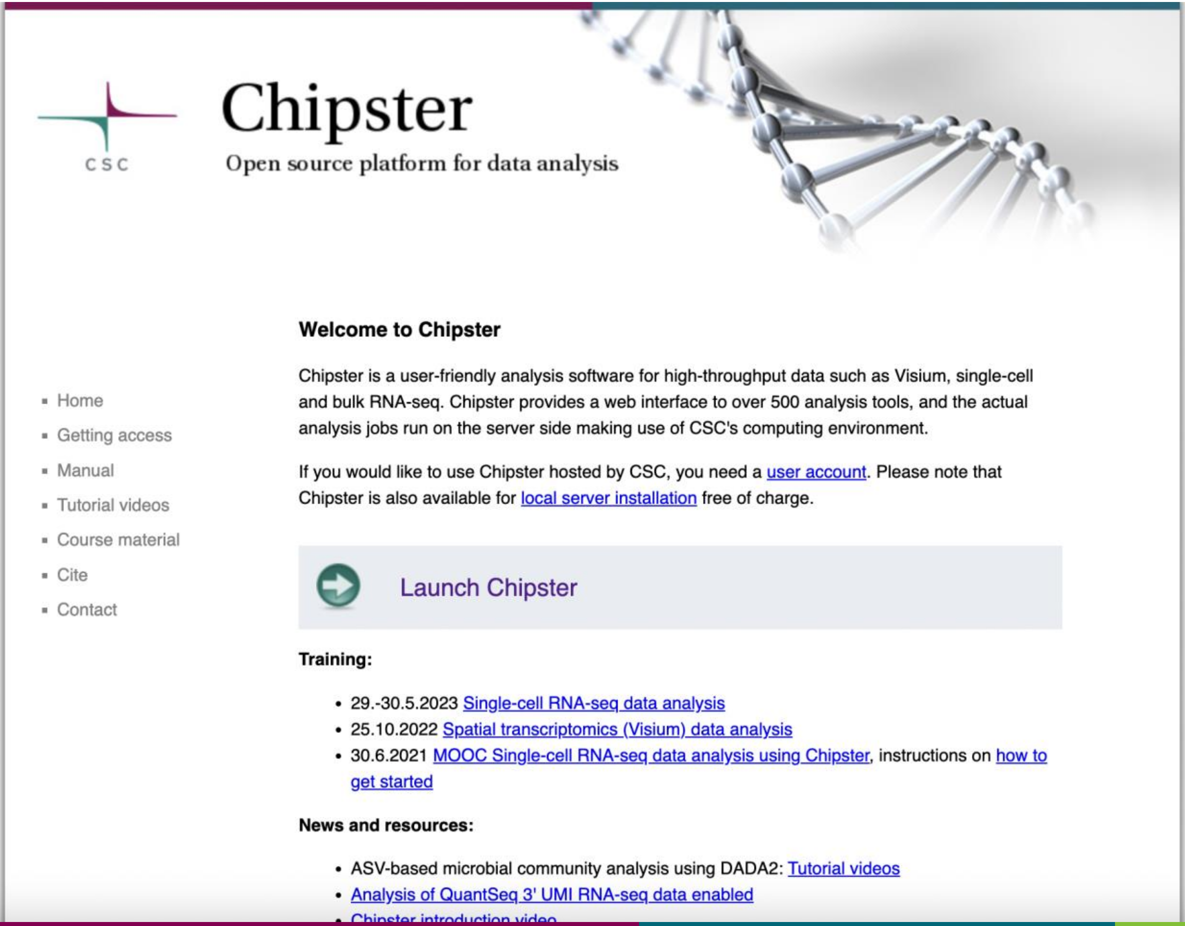
- Allows you to design experiments better → less money wasted

- Allows you to discuss more easily with bioinformaticians

Introduction to Chipster

- User-friendly analysis software for high-throughput data
- Provides an easy access to over 450 analysis tools
    - Command line tools
    - R/Bioconductor packages
- Free, open source software
- What can I do with Chipster?
    - analyze high-throughput data
    - visualize data efficiently
    - share analysis sessions

Chipster website (https://chipster.csc.fi/)



Welcome to Chipster

Chipster is a user-friendly analysis software for high-throughput data such as Visium, single-cell and bulk RNA-seq. Chipster provides a web interface to over 500 analysis tools, and the actual analysis jobs run on the server side making use of CSC's computing environment.

If you would like to use Chipster hosted by CSC, you need a user account. Please note that Chipster is also available for local server installation free of charge.

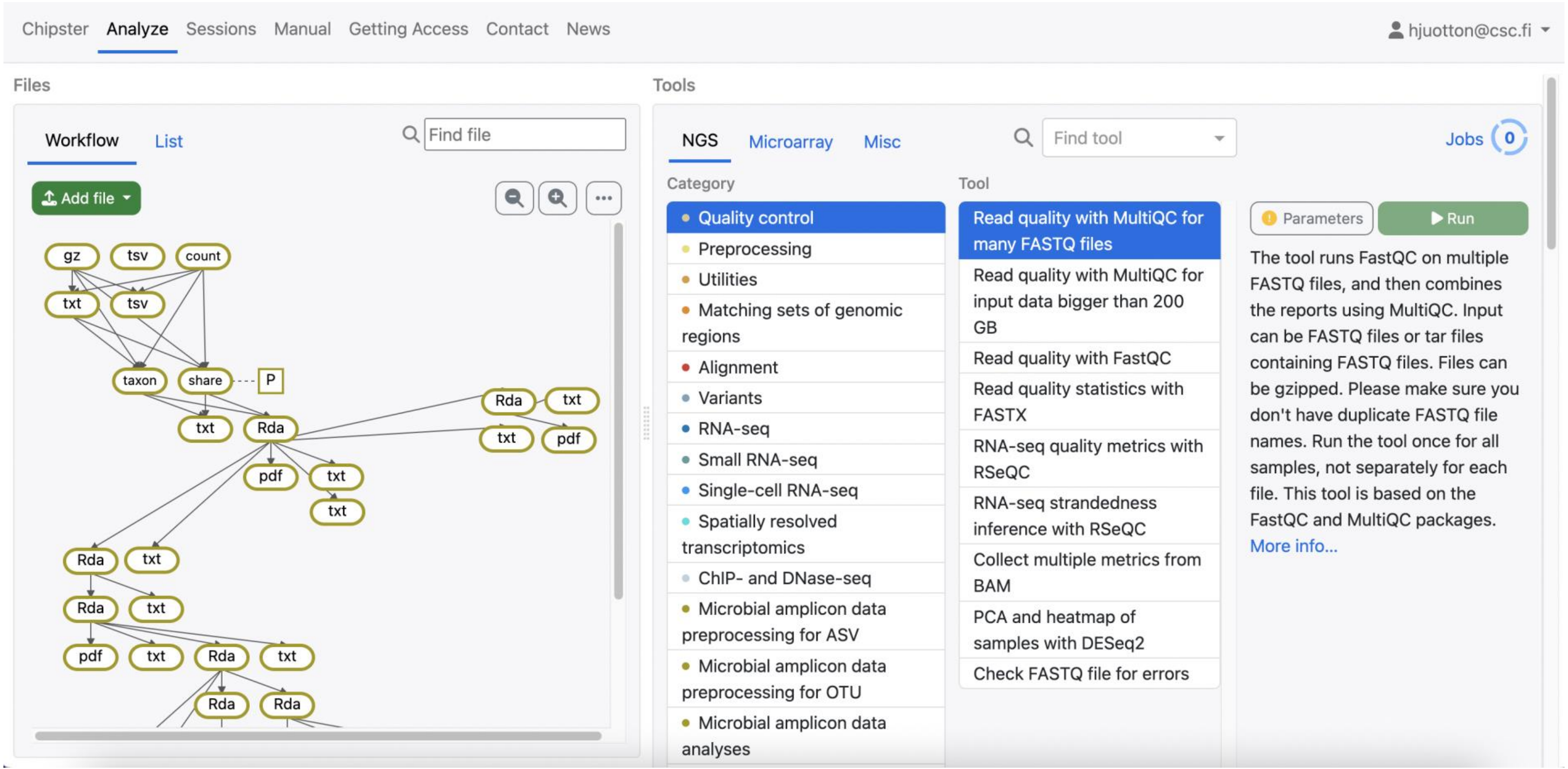
Training:

- 29.-30.5.2023 Single-cell RNA-seq data analysis
- 25.10.2022 Spatial transcriptomics (Visium) data analysis
- 30.6.2021 MOOC Single-cell RNA-seq data analysis using Chipster, instructions on how to get started

News and resources:

- ASV-based microbial community analysis using DADA2: Tutorial videos
- Analysis of QuantSeq 3' UMI RNA-seq data enabled
- Chipster introduction video

Chipster user interface



Category:

- Quality control
- Preprocessing
- Utilities
- Matching sets of genomic regions
- Alignment
- Variants
- RNA-seq
- Small RNA-seq
- Single-cell RNA-seq
- Spatially resolved transcriptomics
- ChIP- and DNase-seq
- Microbial amplicon data preprocessing for ASV
- Microbial amplicon data preprocessing for OTU
- Microbial amplicon data analyses

Tool:

- Read quality with MultiQC for many FASTQ files
- Read quality with MultiQC for input data bigger than 200 GB
- Read quality with FastQC
- Read quality statistics with FASTX
- RNA-seq quality metrics with RSeQC
- RNA-seq strandedness inference with RSeQC
- Collect multiple metrics from BAM
- PCA and heatmap of samples with DESeq2
- Check FASTQ file for errors

The tool runs FastQC on multiple FASTQ files, and then combines the reports using MultiQC. Input can be FASTQ files or tar files containing FASTQ files. Files can be gzipped. Please make sure you don't have duplicate FASTQ file names. Run the tool once for all samples, not separately for each file. This tool is based on the FastQC and MultiQC packages. More info...

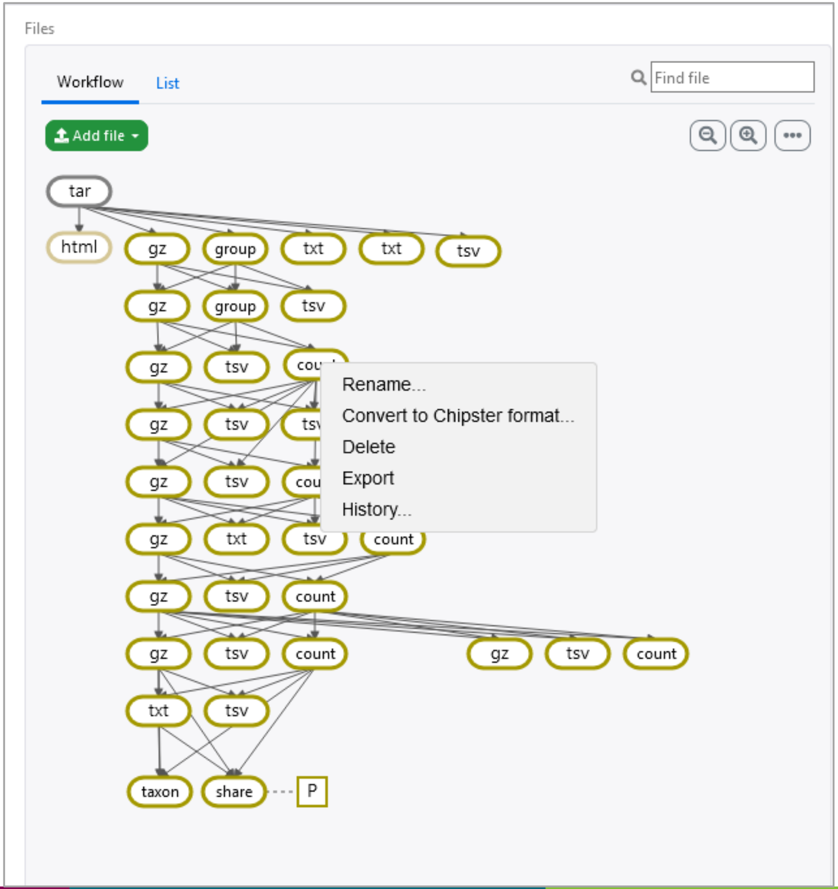
Analysis sessions

- Your analysis is saved automatically in the cloud
  - Session includes all the files, their relationships and metadata (what tool and parameters were used to produce each file).
  - Session is a single .zip file.
  - Note that cloud sessions are not stored forever! Remember to download the session when ready.
- You can share sessions with other Chipster users
  - You can give either read-only or read-write access
- If your analysis job takes a long time, you don’t need to keep Chipster open:
  - Wait that the data transfer to the server has completed (job status = running)
  - Close Chipster
  - Open Chipster later and the results will be there

Workflow view

- Shows the relationships of the files
- You can move the boxes around
- Several files can be selected by
  - keeping the Ctrl key down
  - drawing a box around them

- Right click allows you to
  - download a file ("Export")
  - delete a file
  - view analysis history



Options for importing data to Chipster

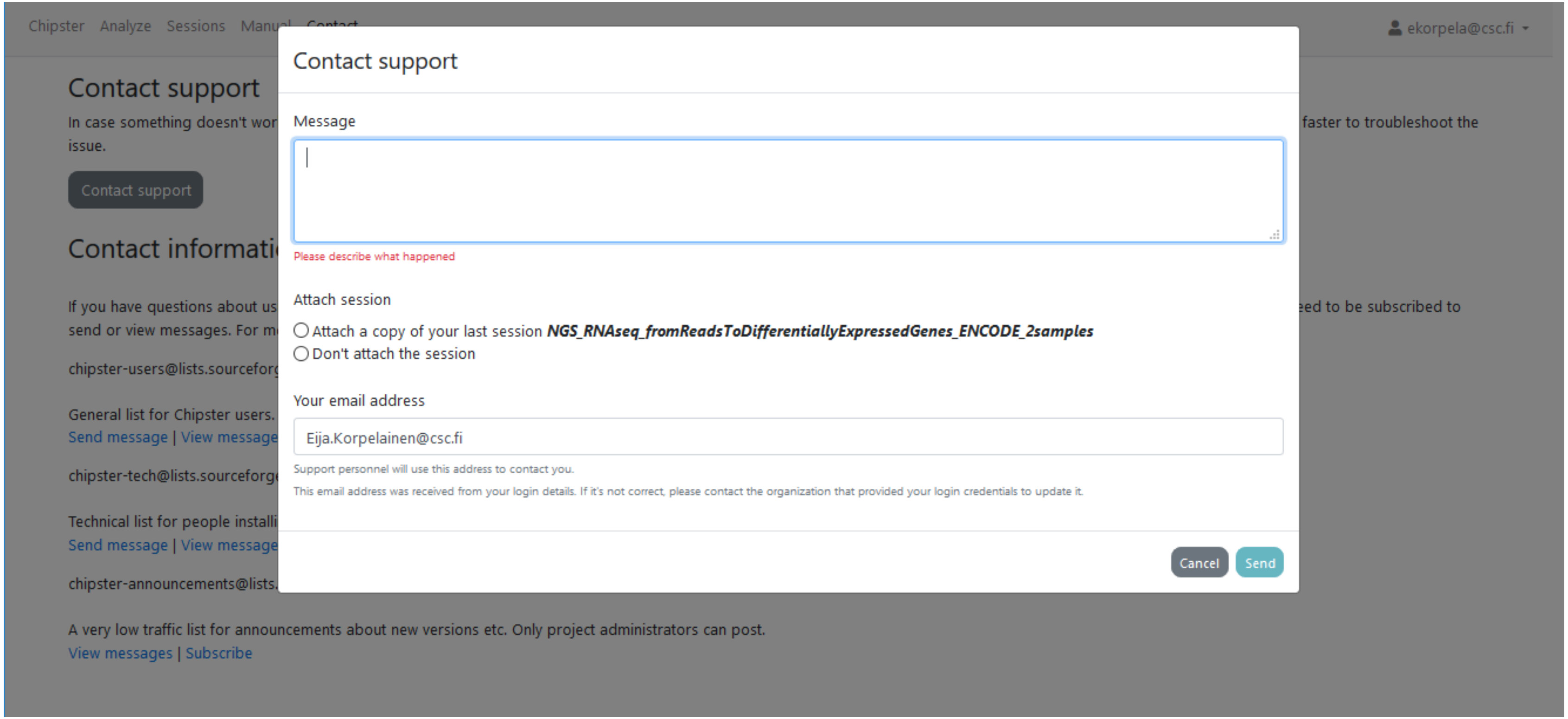
- Add file button
  - Upload files
  - Upload folder
  - Download from URL

- Sessions tab
  - Import session file

- Tools
  - Import from Illumina BaseSpace
    - Utilites / Retrieve data from Illumina BaseSpace
    - Access token needed
  - Import from SRA database
    - Utilities / Retrieve FASTQ or BAM files from SRA
  - Import from Ensembl database
    - Utilities / Retrieve data for a given organism in Ensembl
  - Import from URL
    - Utilities / Download file from URL directly to server

Problems? Send us a support request

-request includes the error message and (optionally) a link to your session



Contact support

Message

Please describe what happened

Attach session

○ Attach a copy of your last session ***NGS_RNAseq_fromReadsToDifferentiallyExpressedGenes_ENCODE_2samples***

○ Don't attach the session

Your email address

Eija.Korpelainen@csc.fi

Support personnel will use this address to contact you.

This email address was received from your login details. If it's not correct, please contact the organization that provided your login credentials to update it.

Contact support

In case something doesn't wor faster to troubleshoot the issue.

Contact support

Contact informati

If you have questions about us eed to be subscribed to send or view messages. For m

chipster-users@lists.sourceforg

General list for Chipster users.
Send message | View message

chipster-tech@lists.sourceforg

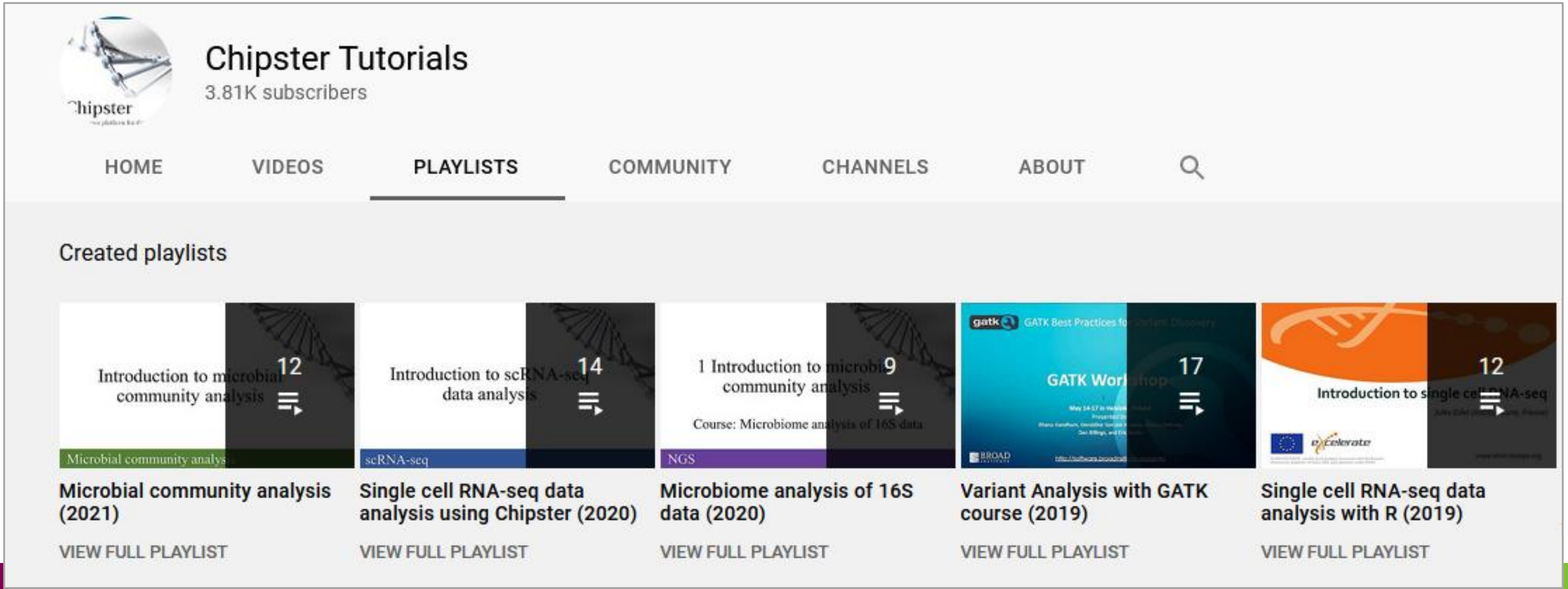
Technical list for people installi
Send message | View message

chipster-announcements@lists.

A very low traffic list for announcements about new versions etc. Only project administrators can post.
View messages | Subscribe

More info

- chipster@csc.fi
- http://chipster.csc.fi
- Chipster tutorials in YouTube
- https://chipster.rahtiapp.fi/manual/courses.html



Chipster Tutorials

3.81K subscribers

Created playlists

Microbial community analysis (2021)

VIEW FULL PLAYLIST

Single cell RNA-seq data analysis using Chipster (2020)

VIEW FULL PLAYLIST

Microbiome analysis of 16S data (2020)

VIEW FULL PLAYLIST

Variant Analysis with GATK course (2019)

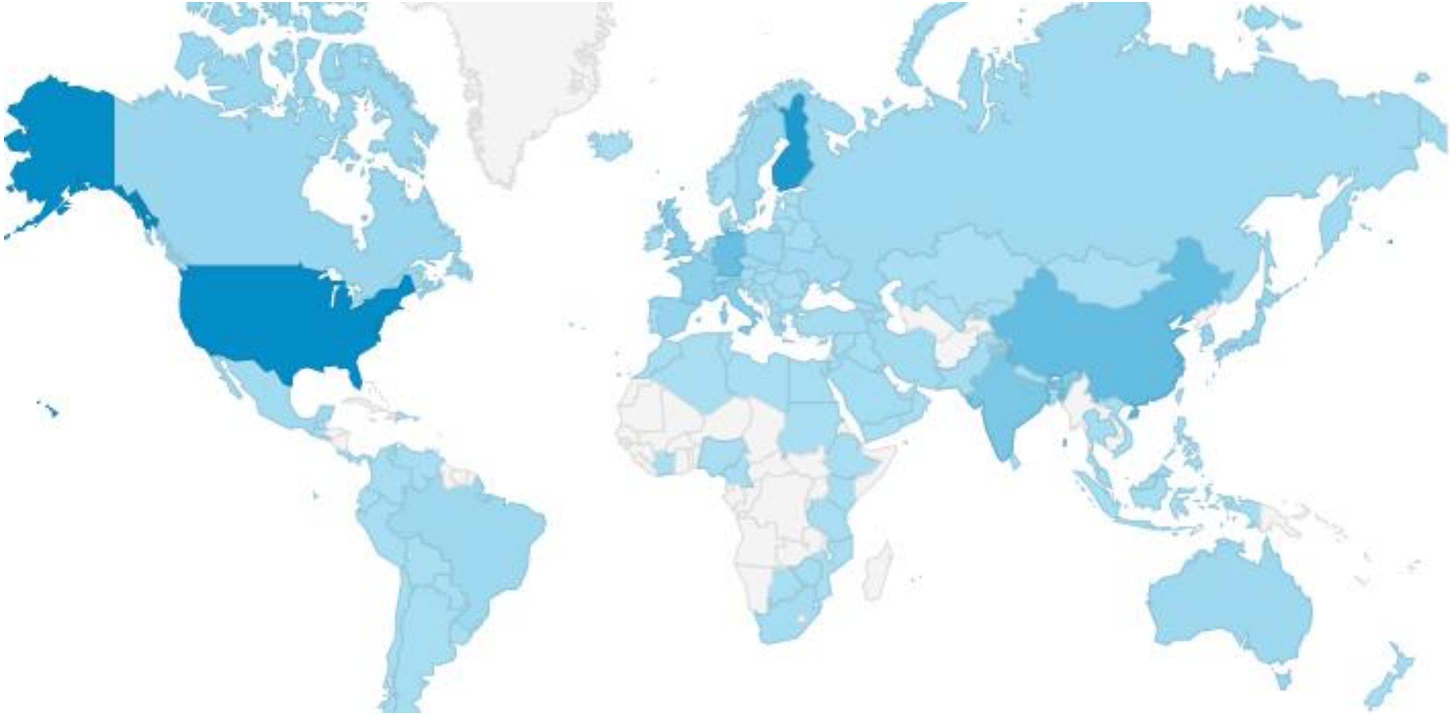
VIEW FULL PLAYLIST

Single cell RNA-seq data analysis with R (2019)

VIEW FULL PLAYLIST

Acknowledgements to Chipster users and contributors

Users' feedback and ideas have helped us to shape the software over the years.
Let us know what needs to be improved!



Introduction to microbial community analysis

Outline

- What questions does it answer
- How is it done
- What are the main steps

Microbial community analysis

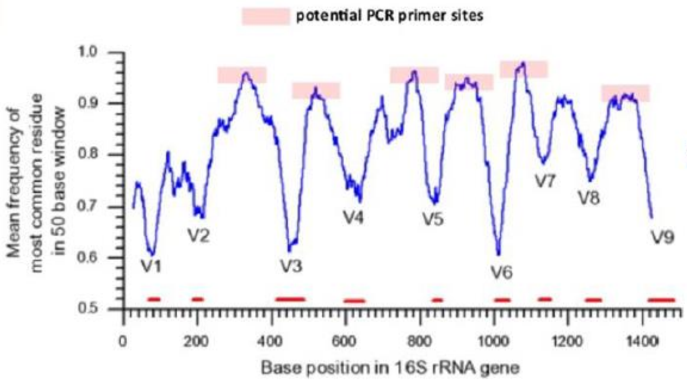
- Answers the questions who are there and in what proportions if compared to your other samples
    - It will not confirm that someone isn’t there (sampling depth, primer/sequencing bias)
- Specific primers are used to amplify a region of one gene
    - Bacterial and archaeal communities: 16S rRNA
    - Fungal communities: ITS (internal transcribed spacer between 18S and 5.8S rRNA genes)
- Sequenced using Illumina MiSeq or Ion Torrent
    - New: PacBio full-length sequencing provides better resolution
- Different from metagenomics, where the aim is to sequence all genes
    - Answers the questions who are there and what are they capable of doing

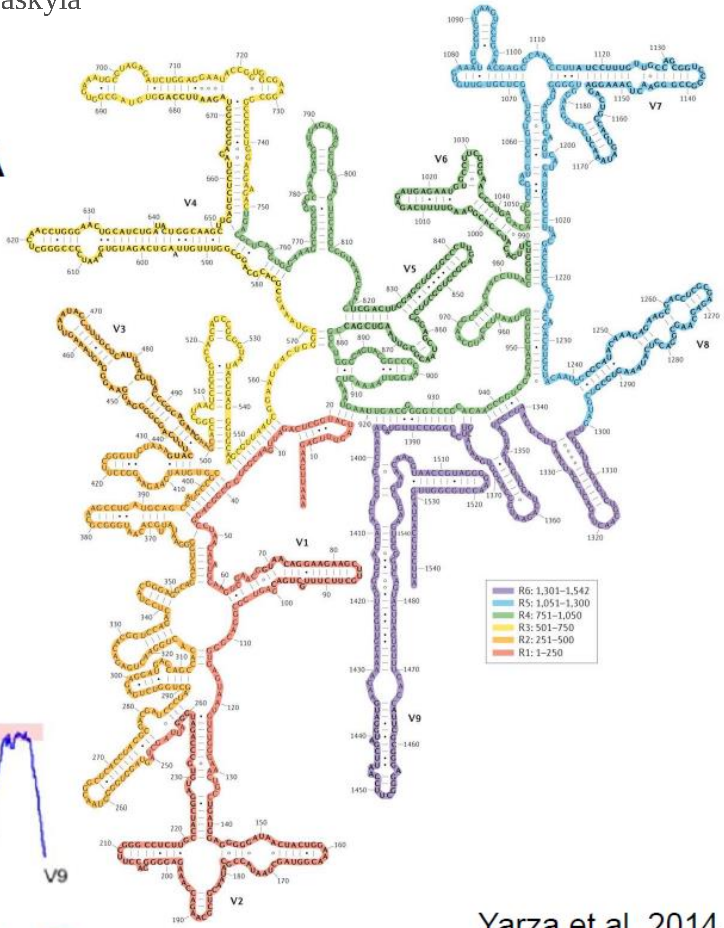
Slide by Dr Anu Mikkonen, University of Jyväskylä

Commonly used 16S rRNA gene amplicons are called by the variable regions they contain

- V1-V2
- (V3-)V4

Variable Regions of the 16S rRNA:





Yarza et al. 2014

Nature Reviews | Microbiology

UNIVERSITY OF JYVÄSKYLÄ

Main parts of microbial community analysis

- **Preprocessing**
  - Quality control, trim primers/adaptors and bad quality ends
  - Depending on data type:
    - MiSeq: Combine paired end reads to contigs
    - Ion Torrent: Single-end reads in one or several FASTQ files
  - Filter out bad quality sequences, remove identical sequences
  - Align sequences to reference template (e.g. SILVA)
  - Filter sequences based on alignment position, trim sequence alignment
  - Remove chimeras and sequencing errors
- **Classification and clustering**
  - Taxonomic assignment of sequences (e.g. SILVA for 16S, UNITE for ITS)
- **Community analysis and visualization**
  - Does community structure differ between sample groups?
  - Which taxa are differentially abundant between sample groups?

How to choose the preprocessing protocol?

- Sequencing technology:
  - Illumina Miseq: paired-end short reads
  - Ion Torrent: single-end short reads
  - PacBio and Nanopore: long reads
- Gene: 16S rRNA, ITS, other?
  - reference database
  - gene characteristics
- Operational taxonomic units (OTUs) vs. amplicon sequence variants (ASVs)
  - OTUs: mothur, QIIME2
  - ASVs: DADA2

How to choose the preprocessing protocol?

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  - **Ion Torrent: single-end short reads**
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- Gene: **16S rRNA**, ITS, other?
  - reference database
  - gene characteristics
- Operational taxonomic units (OTUs) vs. amplicon sequence variants (ASVs)
  - **OTUs: mothur**, QIIME2
  - ASVs: DADA2

Main parts of ITS data analysis

- **Preprocessing**
  - Quality control, trim primers/adaptors and bad quality ends
  - Depending on data type:
    - MiSeq: Combine paired end reads to contigs
    - Ion Torrent: Single-end reads in one or several FASTQ files
  - Filter out bad quality sequences, remove identical sequences
  - ~~Align sequences to reference template (e.g. SILVA)~~
  - ~~Filter sequences based on alignment position, trim sequence alignment~~
  - Remove chimeras and sequencing errors
- **Classification and clustering**
  - Taxonomic assignment of sequences using the UNITE reference

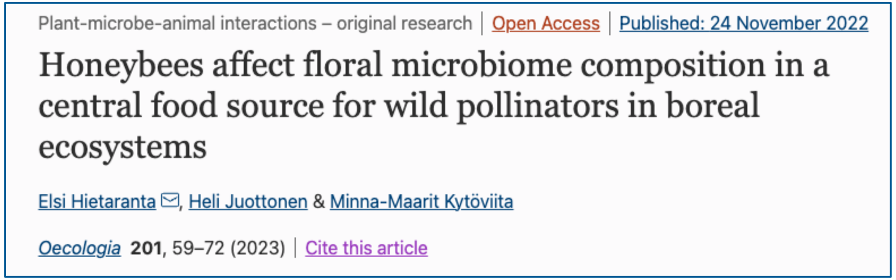
  ! When running Generate input files for phyloseq set Type of data = ITS (AGC instead of OptiClust is used for clustering, because the sequences are not aligned)
- **Community analysis and visualization**

Data set for exercises: willow catkin bacteria

- Subset of 16 samples of willow catkins to study plant-associated bacteria
- Do pollinator visits change bacterial community?
- Two treatments (4 replicates each):

  protected from pollinators

  control visited by pollinators
- Two sites



Plant-microbe-animal interactions – original research | Open Access | Published: 24 November 2022

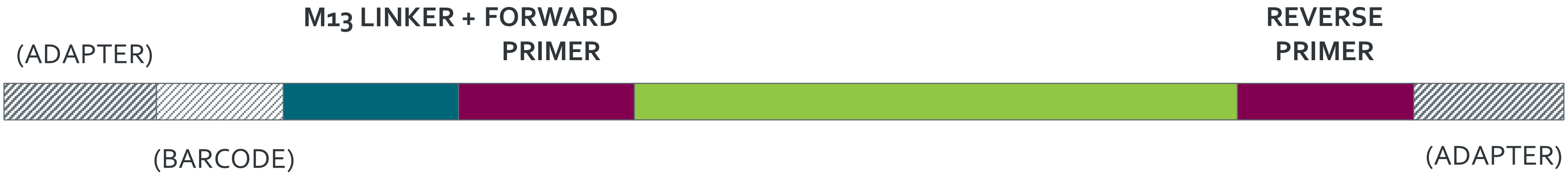
Honeybees affect floral microbiome composition in a central food source for wild pollinators in boreal ecosystems

Elsi Hietaranta, Heli Juottonen & Minna-Maarit Kytöviita

Oecologia **201**, 59–72 (2023) | Cite this article

Data set for exercises: willow catkin bacteria

- PCR amplification of V6-V8 region of the bacterial 16S rRNA gene (ca. 350 bp)
- Sequencing by Ion Torrent
- Demultiplexed and barcodes removed
- Each sample in a separate FASTQ file



Quality control of raw reads

Outline

- Different types of quality problems
- FASTQ file format
- Tools for checking read quality
- Tools for improving read quality

What and why?

- Potential problems
  - low confidence bases, Ns
  - adapters
  - …

- Knowing about potential problems in your data allows you to
  - correct for them before you spend a lot of time on analysis
  - take them into account when interpreting results

FASTQ file format

- Four lines per read:

```
@read name
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+ read name
!"*((((***+))%%%++)(%%%%).1***-+*"))**55CCF>>>>>>CCCCCCC65
```

- http://en.wikipedia.org/wiki/FASTQ_format
- Do **not** unzip FASTQ files, Chipster can cope with .gz files

Base qualities

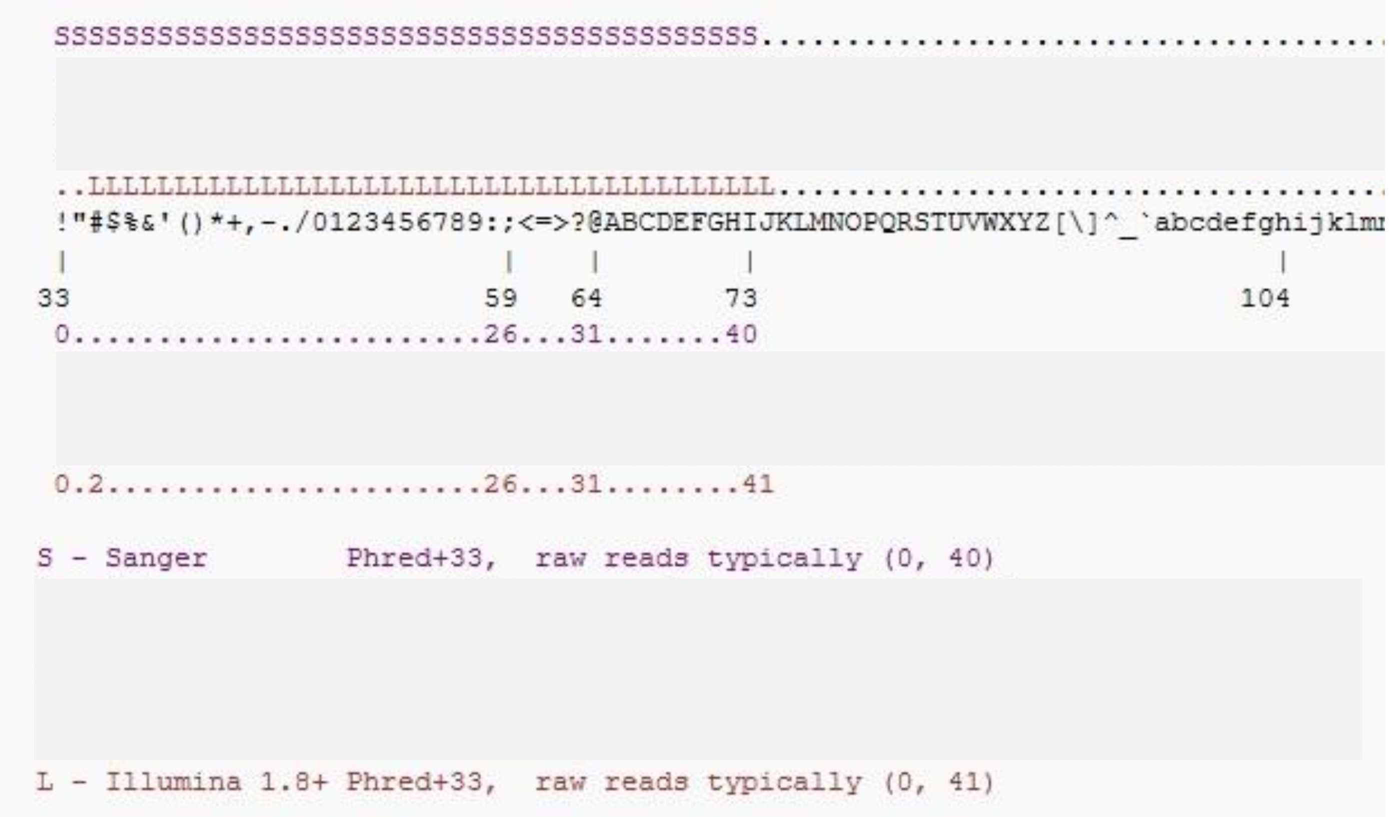
- If the quality of a base is 20, the probability that it is wrong is 0.01.
    - Phred quality score Q = -10 * log10 (probability that the base is wrong)

T C A G T A C T C G

40 40 40 40 40 40 40 40 37 35

- Sanger encoding: numbers are shown as ASCII characters so that 33 is added to the Phred score
    - E.g. 39 is encoded as H, the 72nd ASCII character (39+33 = 72)
    - Note that older Illumina data uses different encoding

Base quality encoding systems

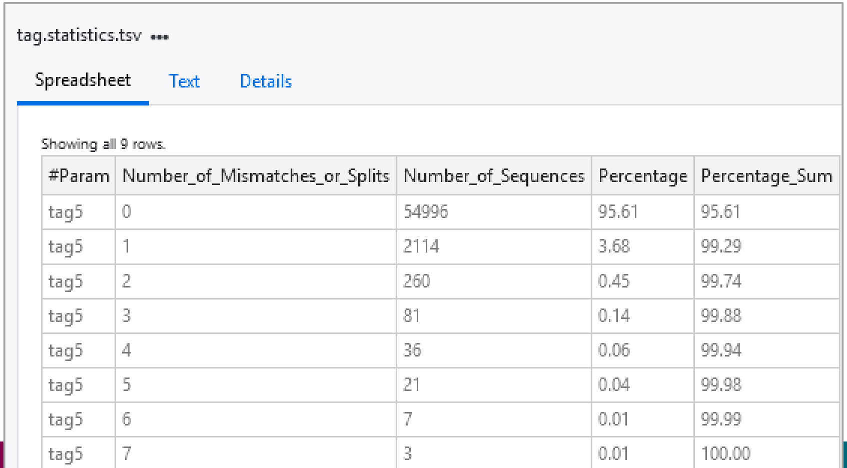


```
  SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS....................................

  ..LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL...................................
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklm
  |                         |    |        |                              |
 33                        59   64       73                            104
  0........................26...31.......40

  0.2......................26...31........41

 S - Sanger        Phred+33,  raw reads typically (0, 40)

 L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

http://en.wikipedia.org/wiki/FASTQ_format

Tools for checking sequence quality

- Read quality with MultiQC for many FASTQ files
  - runs FastQC for all the FASTQ files simultaneously
  - checks base quality and composition, duplication, Ns, k-mers, adapters,…
  - takes a tar package of all the FASTQ files as an input file
- Statistics for primers and adapters with TagCleaner
  - Given an adapter or primer sequence, checks how many reads have it (allowing



Showing all 9 rows.

#Param	Number_of_Mismatches_or_Splits	Number_of_Sequences	Percentage	Percentage_Sum
tag5	0	54996	95.61	95.61
tag5	1	2114	3.68	99.29
tag5	2	260	0.45	99.74
tag5	3	81	0.14	99.88
tag5	4	36	0.06	99.94
tag5	5	21	0.04	99.98
tag5	6	7	0.01	99.99
tag5	7	3	0.01	100.00

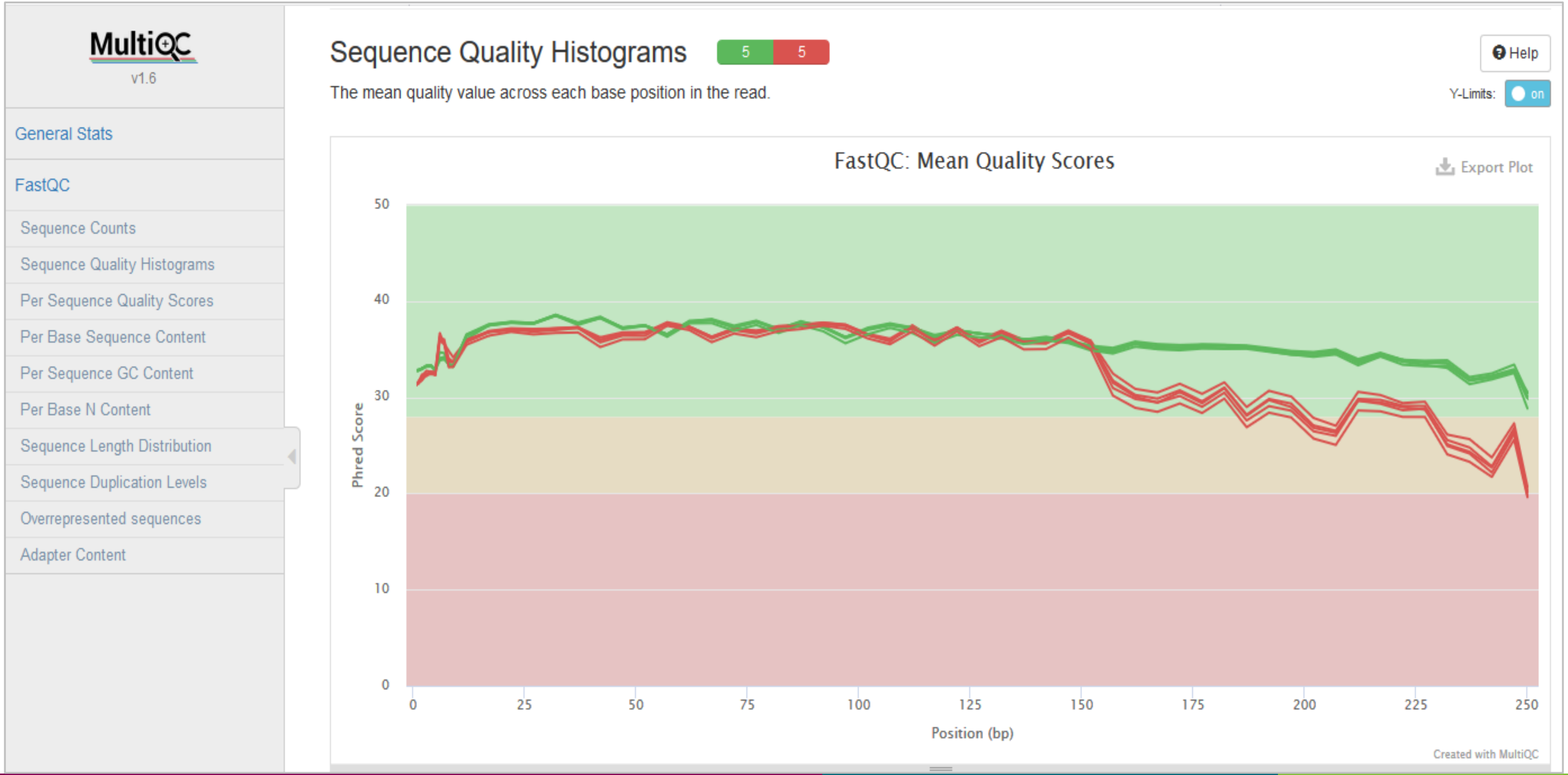
Making a Tar package of FASTQ files

- Use the tool Utilities / Make Tar package
- When your Tar package is ready, you can delete the original FASTQ files
  - If you want to look at the individual FASTQ files later, you can always open the Tar package using the tool Utilities / Extract .tar.gz file

MultiQC features

- Interactive plots
- Plots allow you to view the number or percentage of reads
- Traffic lights (they might not be suitable for your data!)
- Toolbox (click on the right side panel) allows you to
  - Highlight samples
  - Show only selected samples
  - Download plots
  - Rename samples
- Good tutorial video https://www.youtube.com/watch?v=qPbIlO_KWN0

Per position base quality (MultiQC)

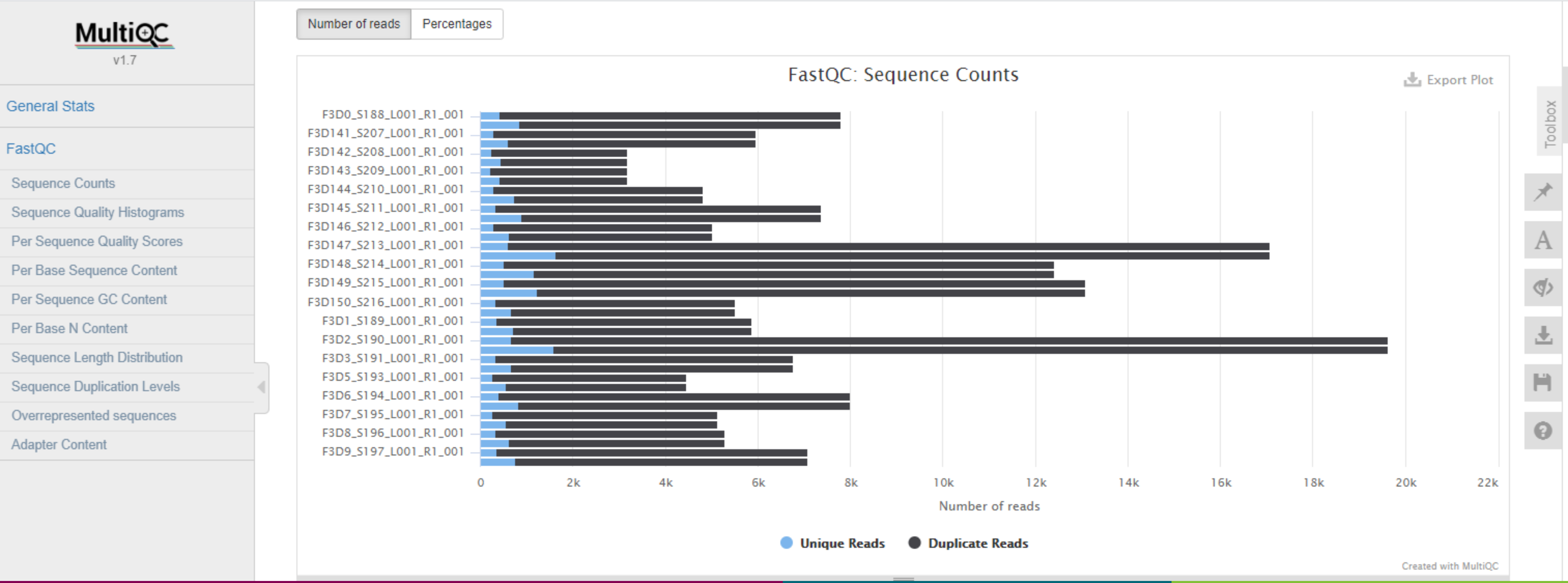


Sequence Quality Histograms

The mean quality value across each base position in the read.

FastQC: Mean Quality Scores

Sequence counts (MultiQC)



What if there is a quality problem?

- You can either trim or filter reads
- Filtering removes the entire read, trimming removes only the bad quality bases
  - Note that trimming can remove the entire read, if all bases are bad
- Trimming makes reads shorter, which is not always optimal
- Paired end data: the matching order of the reads in the two files has to be preserved
  - If a read is removed, its pair has to be removed as well

Preprocessing tools for improving reads

- Trimmomatic and PRINSEQ
    - Filtering based on read quality and length
    - Trimmomatic is faster
- Cutadapt
    - Removes primers and adapters allowing mismatches
- TagCleaner
    - Removes primers and adapters allowing mismatches
- FastX
    - Can be used for trimming a given number of bases from either end of the reads
    - Does not take the pairing of reads into account

Trimmomatic options in Chipster

- Adapters
- Minimum quality
  - Per base, one base at a time or in a sliding window, from 3’ or 5’ end
  - Per base adaptive quality trimming (balance length and errors)
- Minimum mean read quality
- Trim x number of bases from beginning/ end
- Minimum read length after trimming
- Copes with paired end data

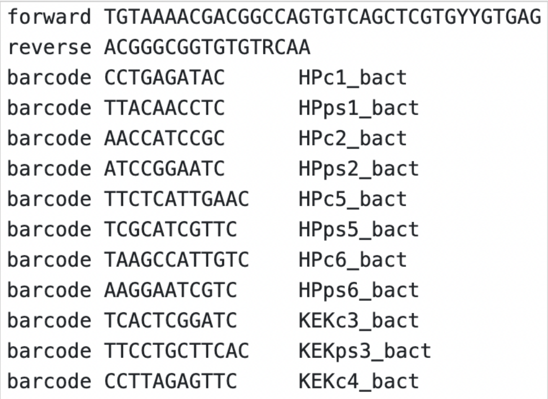
Quality control of single-end reads (Ion Torrent)

1. Remove primers and any adapters
   - if all samples are in a single FASTQ file, also separate samples by barcode and remove barcodes = demultiplexing
2. Remove reads with ambiguous bases (N) and suspiciously long reads
3. Filter reads based on quality
   - starting point for Ion Torrent reads: sliding window of 10 bases, minimum quality in the window 20
4. Remove reads that are too short
5. Remove identical sequences

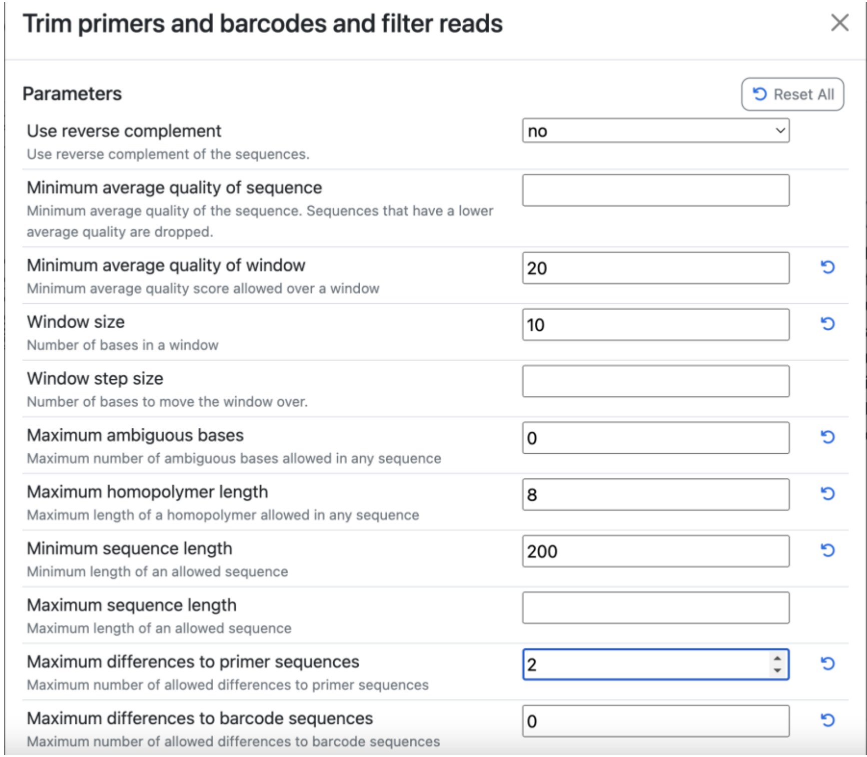
Quality control of single-end reads (Ion Torrent): single FASTQ

If all samples are in a single FASTQ file (not demultiplexed yet): Microbial amplicon data processing for OTU / **Trim primers and barcodes and filter reads** (based on Mothur command trim.seqs)

- ○ Input: single FASTQ file + .**oligos file**



```
forward TGTAAAACGACGGCCAGTGTCAGCTCGTGYYGTGAG
reverse ACGGGCGGTGTGTRCAA
barcode CCTGAGATAC      HPc1_bact
barcode TTACAACCTC      HPps1_bact
barcode AACCATCCGC      HPc2_bact
barcode ATCCGGAATC      HPps2_bact
barcode TTCTCATTGAAC    HPc5_bact
barcode TCGCATCGTTC     HPps5_bact
barcode TAAGCCATTGTC    HPc6_bact
barcode AAGGAATCGTC     HPps6_bact
barcode TCACTCGGATC     KEKc3_bact
barcode TTCCTGCTTCAC    KEKps3_bact
barcode CCTTAGAGTTC     KEKc4_bact
```



Trim primers and barcodes and filter reads

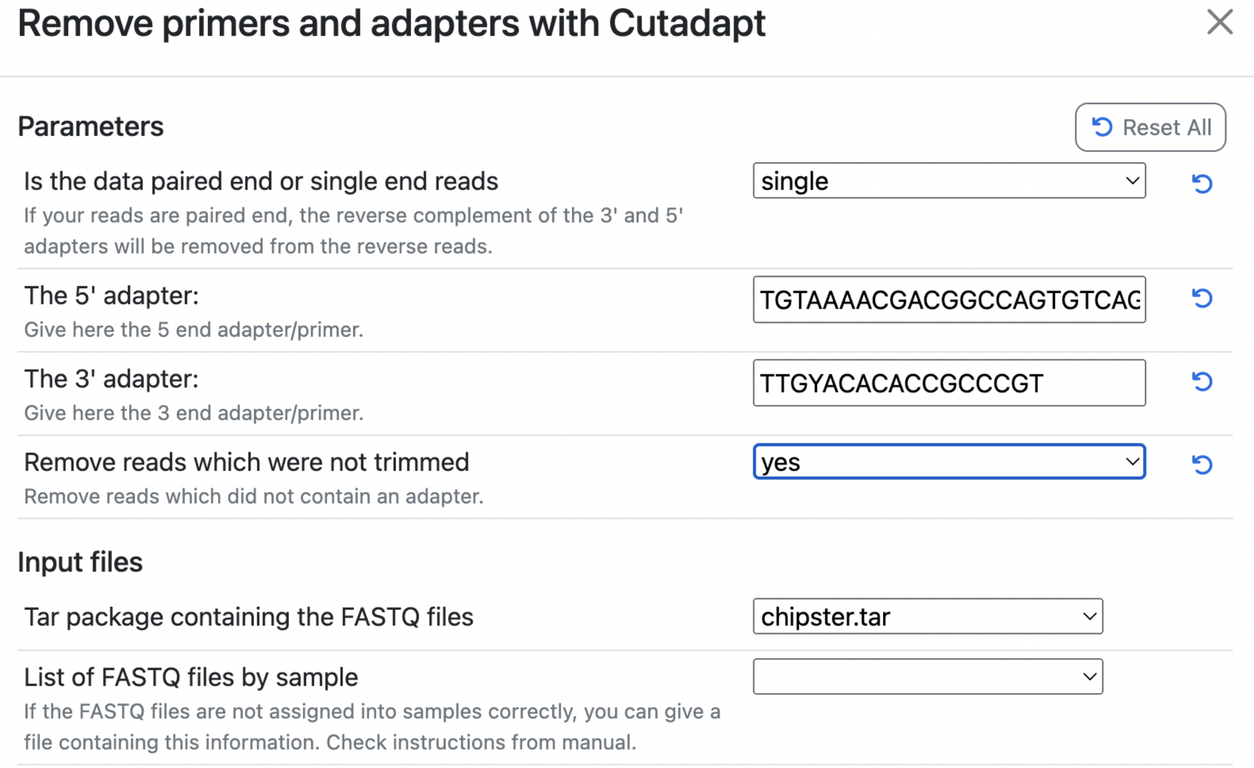
Parameters

Use reverse complement Use reverse complement of the sequences.	no
Minimum average quality of sequence Minimum average quality of the sequence. Sequences that have a lower average quality are dropped.	
Minimum average quality of window Minimum average quality score allowed over a window	20
Window size Number of bases in a window	10
Window step size Number of bases to move the window over.	
Maximum ambiguous bases Maximum number of ambiguous bases allowed in any sequence	0
Maximum homopolymer length Maximum length of a homopolymer allowed in any sequence	8
Minimum sequence length Minimum length of an allowed sequence	200
Maximum sequence length Maximum length of an allowed sequence	
Maximum differences to primer sequences Maximum number of allowed differences to primer sequences	2
Maximum differences to barcode sequences Maximum number of allowed differences to barcode sequences	0

Quality control of single-end reads (Ion Torrent): many FASTQs

If all samples are in separate FASTQ files (already demultiplexed):

- Remove primers and adapters
  - Microbial amplicon preprocessing for ASV / Remove primers and adapters with **Cutadapt**
  - or Preprocessing / Trim primers/adapters with **TagCleaner**
- Filter reads based on quality scores and minimum length
  - Preprocessing / Trim reads with **Trimmomatic**
    - For example: sliding window of 10 bases, minimum quality score 20



Remove primers and adapters with Cutadapt

Parameters

Is the data paired end or single end reads: single

If your reads are paired end, the reverse complement of the 3' and 5' adapters will be removed from the reverse reads.

The 5' adapter: TGTAAAACGACGGCCAGTGTCAG

Give here the 5 end adapter/primer.

The 3' adapter: TTGYACACACCGCCCGT

Give here the 3 end adapter/primer.

Remove reads which were not trimmed: yes

Remove reads which did not contain an adapter.

Input files

Tar package containing the FASTQ files: chipster.tar

List of FASTQ files by sample:

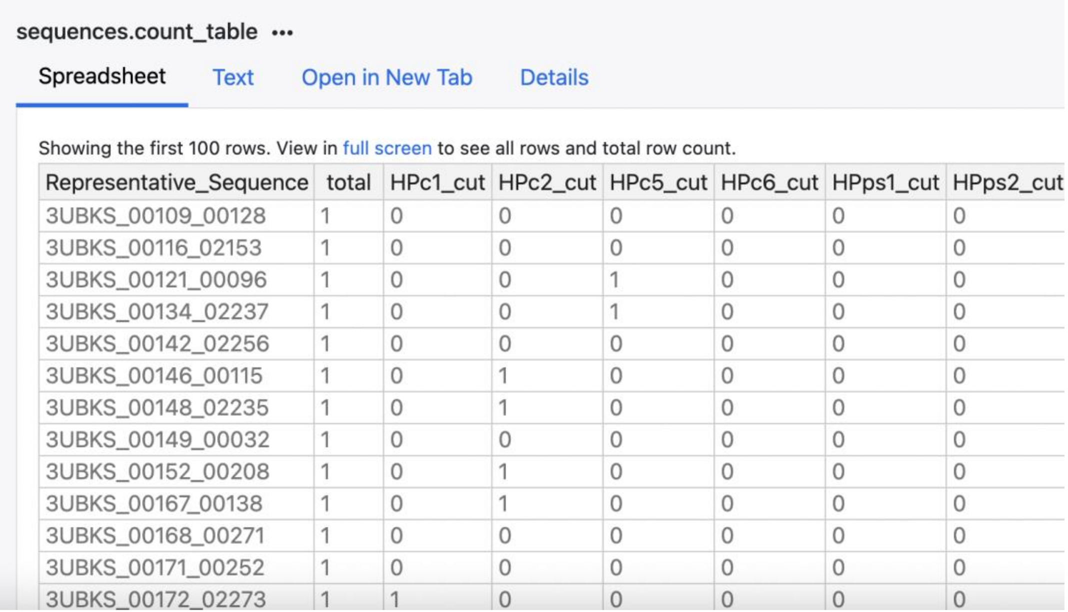
If the FASTQ files are not assigned into samples correctly, you can give a file containing this information. Check instructions from manual.

reverse complement

Combine files and make a count file

- Use the tool Combine FASTQ files into one FASTA file and make a Mothur count file to
  - converts FASTQ to FASTA
  - merges all the samples in one file
  - creates the Mothur count file

count file = keeping track which sequence belongs to which sample



sequences.count_table •••

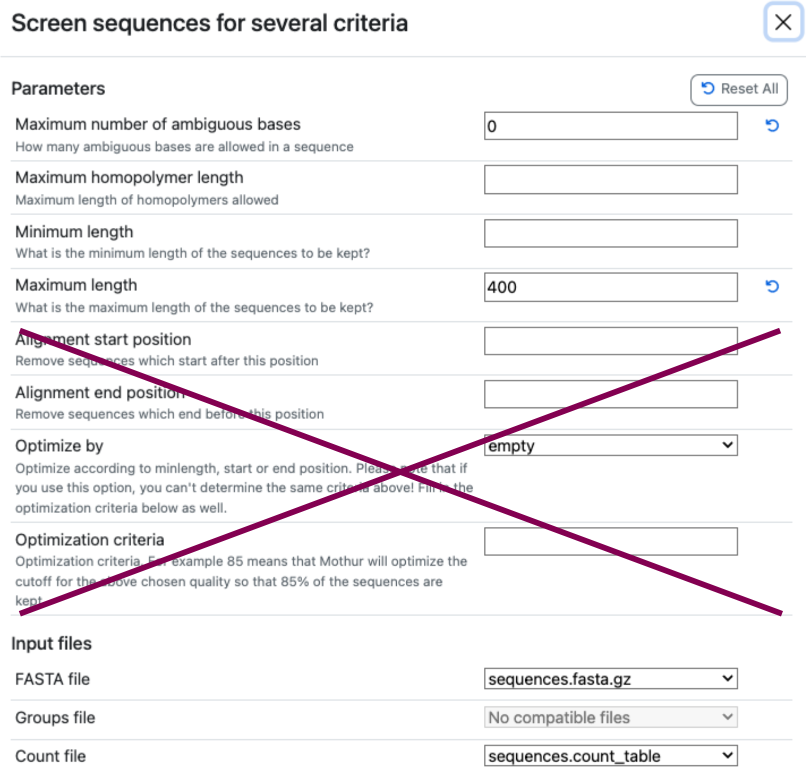
Showing the first 100 rows. View in full screen to see all rows and total row count.

Representative_Sequence	total	HPc1_cut	HPc2_cut	HPc5_cut	HPc6_cut	HPps1_cut	HPps2_cut
3UBKS_00109_00128	1	0	0	0	0	0	0
3UBKS_00116_02153	1	0	0	0	0	0	0
3UBKS_00121_00096	1	0	0	1	0	0	0
3UBKS_00134_02237	1	0	0	1	0	0	0
3UBKS_00142_02256	1	0	0	0	0	0	0
3UBKS_00146_00115	1	0	1	0	0	0	0
3UBKS_00148_02235	1	0	1	0	0	0	0
3UBKS_00149_00032	1	0	0	0	0	0	0
3UBKS_00152_00208	1	0	1	0	0	0	0
3UBKS_00167_00138	1	0	1	0	0	0	0
3UBKS_00168_00271	1	0	0	0	0	0	0
3UBKS_00171_00252	1	0	0	0	0	0	0
3UBKS_00172_02273	1	1	0	0	0	0	0

Screen reads for ambiguous bases and too long reads

- Tool Screen sequences for several criteria
- Input: FASTA file and count file (ends .count_table)

Same tool used later for screening sequence alignment!



Screen sequences for several criteria

Parameters

Maximum number of ambiguous bases
How many ambiguous bases are allowed in a sequence: 0

Maximum homopolymer length
Maximum length of homopolymers allowed

Minimum length
What is the minimum length of the sequences to be kept?

Maximum length
What is the maximum length of the sequences to be kept?: 400

Alignment start position
Remove sequences which start after this position

Alignment end position
Remove sequences which end before this position

Optimize by
Optimize according to minlength, start or end position. Please note that if you use this option, you can't determine the same criteria above! Fill in the optimization criteria below as well.: empty

Optimization criteria
Optimization criteria. For example 85 means that Mothur will optimize the cutoff for the above chosen quality so that 85% of the sequences are kept

Input files

FASTA file: sequences.fasta.gz

Groups file: No compatible files

Count file: sequences.count_table

Remove identical sequences

- The FASTA file contains many identical sequences
- Aligning the same sequence to the reference would be computationally wasteful

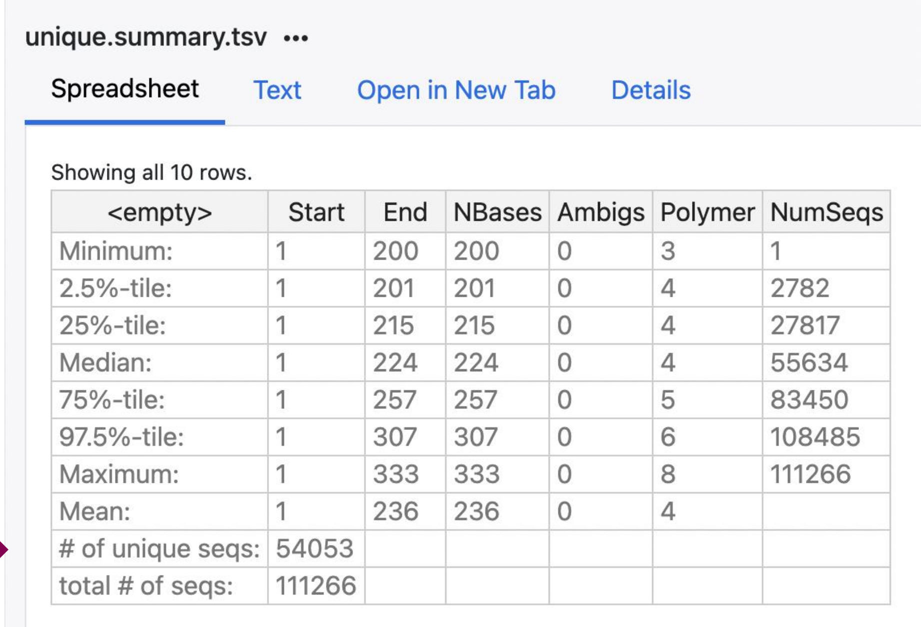
→ We remove identical sequences and keep only one representative in the FASTA file

  - count file keeps track of how many sequences the representative represents in the samples

- Tool Extract unique sequences
- Give fasta file and count file as input
- Output files
  - unique.fasta = unique sequences
  - unique.count_table = how many represented sequences are in each sample
  - unique.summary.tsv = sequence information

unique.summary.tsv

- Number of sequences: total and unique
- Stats (min, max, mean, median and quantiles) of
  - number of bases
  - number of ambiguous bases
  - start and end positions
  - homopolymer length

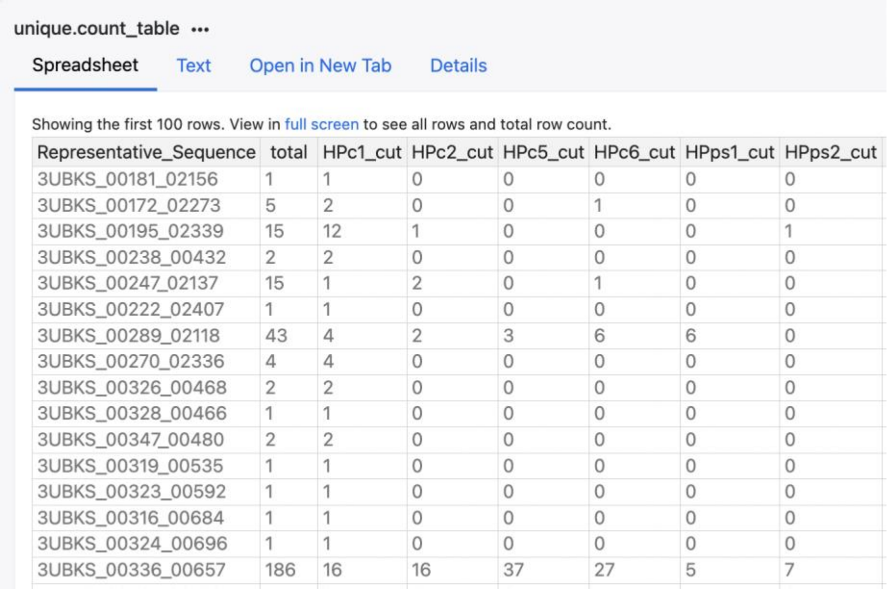


Showing all 10 rows.

<empty>	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	200	200	0	3	1
2.5%-tile:	1	201	201	0	4	2782
25%-tile:	1	215	215	0	4	27817
Median:	1	224	224	0	4	55634
75%-tile:	1	257	257	0	5	83450
97.5%-tile:	1	307	307	0	6	108485
Maximum:	1	333	333	0	8	111266
Mean:	1	236	236	0	4	
# of unique seqs:	54053					
total # of seqs:	111266					

Output of single-end read quality control

- FASTA file: unique.fasta.gz
  - trimmed and filtered unique sequence reads
  - .gz indicates file compression
- Count file: unique.count_table
  - which sequence belongs to which sample
- Next: alignment



unique.count_table •••

Showing the first 100 rows. View in full screen to see all rows and total row count.

Representative_Sequence	total	HPc1_cut	HPc2_cut	HPc5_cut	HPc6_cut	HPps1_cut	HPps2_cut
3UBKS_00181_02156	1	1	0	0	0	0	0
3UBKS_00172_02273	5	2	0	0	1	0	0
3UBKS_00195_02339	15	12	1	0	0	0	1
3UBKS_00238_00432	2	2	0	0	0	0	0
3UBKS_00247_02137	15	1	2	0	1	0	0
3UBKS_00222_02407	1	1	0	0	0	0	0
3UBKS_00289_02118	43	4	2	3	6	6	0
3UBKS_00270_02336	4	4	0	0	0	0	0
3UBKS_00326_00468	2	2	0	0	0	0	0
3UBKS_00328_00466	1	1	0	0	0	0	0
3UBKS_00347_00480	2	2	0	0	0	0	0
3UBKS_00319_00535	1	1	0	0	0	0	0
3UBKS_00323_00592	1	1	0	0	0	0	0
3UBKS_00316_00684	1	1	0	0	0	0	0
3UBKS_00324_00696	1	1	0	0	0	0	0
3UBKS_00336_00657	186	16	16	37	27	5	7

How to start with paired-end MiSeq data?

- Combine paired reads into **contigs** using VSEARCH
- Filter sequences based on expected errors
- Combine FASTQ files into one FASTA file and make a Mothur count file
- Filter contigs based on length, ambigious bases, homopolymers
- Remove identical sequences

➡ Check videos on the Chipster Youtube channel for details

➡ Check tutorial sessions on Chipster

➡ MiSeq exercises: https://github.com/csc-training/chipster-microbial

Align sequences to reference template alignment

Outline

- SILVA reference template alignment
- Alignment steps
- How to improve and speed up the alignment
- Alignment file format

SILVA reference template alignment

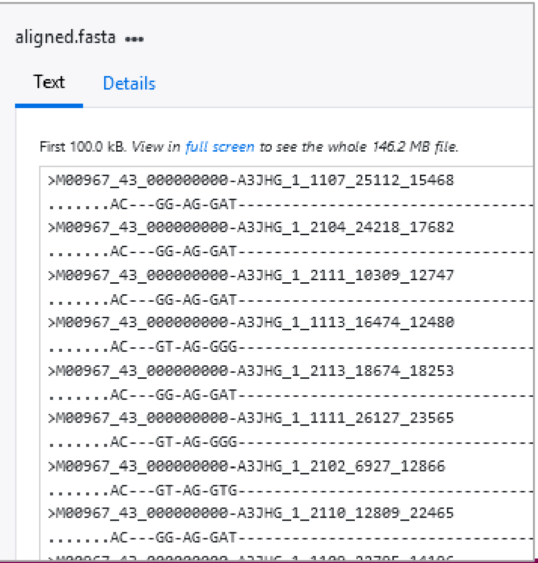
- To identify the sequences we align them to a reference template alignment
- Chipster uses the full SILVA template, but you can also give your own
- The current SILVA version is 138.1
  - Contains 146 601 sequences: 128 884 bacteria, 2846 archaea, and 14 871 eukarya
  - the alignment is 50 000 columns long so that it is compatible with 18S rRNA sequences and archaeal 16S rRNA sequences
  - to make alignment process faster, indicate which region of the SILVA template alignment matches the area you amplified
  - to get the SILVA coordinates of that area, align a small number of samples first
- https://mothur.org/wiki/Silva_reference_files

Aligning sequences to template alignment

- Tool Align sequences to reference, based on Mothur align.seqs and pcr.seqs commands
- Give unique.fasta.gz and unique.count_table as input
- Three steps
  - find the closest template sequence for the query sequence using K-mer search with 8mers
  - align the query and the de-gapped template sequence using Needleman-Wunsch pairwise alignment
  - re-insert gaps to the query and template pairwise alignment using the NAST algorithm so that the query sequence alignment is compatible with the original template alignment
- Speed depends on the number and length of the query and template sequences
- Limit the alignment to the template region which corresponds to the part of the 16S rRNA gene you amplified → better alignment quality, less space needed

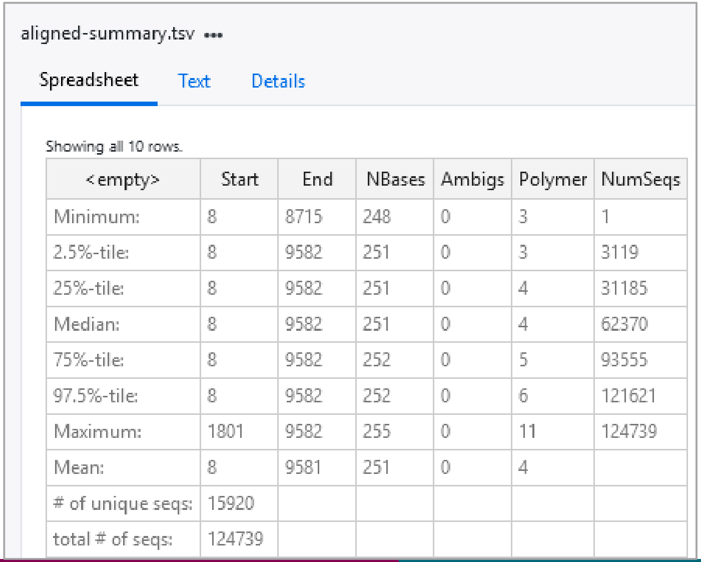
Result files

- aligned.fasta.gz = aligned sequences
    - periods lead to the first base in the sequence and follow the last base of the sequence
- custom.reference.summary.tsv = information on the region of the reference used
- aligned-summary.tsv = aligned sequence information



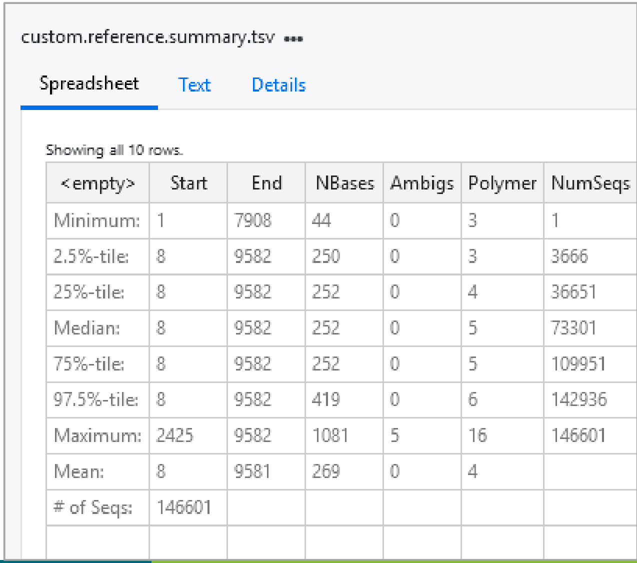
First 100.0 kB. *View in full screen to see the whole 146.2 MB file.*

```
>M00967_43_000000000-A3JHG_1_1107_25112_15468
.......AC---GG-AG-GAT-----------------------------------------
>M00967_43_000000000-A3JHG_1_2104_24218_17682
.......AC---GG-AG-GAT-----------------------------------------
>M00967_43_000000000-A3JHG_1_2111_10309_12747
.......AC---GG-AG-GAT-----------------------------------------
>M00967_43_000000000-A3JHG_1_1113_16474_12480
.......AC---GT-AG-GGG-----------------------------------------
>M00967_43_000000000-A3JHG_1_2113_18674_18253
.......AC---GG-AG-GAT-----------------------------------------
>M00967_43_000000000-A3JHG_1_1111_26127_23565
.......AC---GT-AG-GGG-----------------------------------------
>M00967_43_000000000-A3JHG_1_2102_6927_12866
.......AC---GT-AG-GTG-----------------------------------------
>M00967_43_000000000-A3JHG_1_2110_12809_22465
.......AC---GG-AG-GAT-----------------------------------------
```



Showing all 10 rows.

<empty>	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	8	8715	248	0	3	1
2.5%-tile:	8	9582	251	0	3	3119
25%-tile:	8	9582	251	0	4	31185
Median:	8	9582	251	0	4	62370
75%-tile:	8	9582	252	0	5	93555
97.5%-tile:	8	9582	252	0	6	121621
Maximum:	1801	9582	255	0	11	124739
Mean:	8	9581	251	0	4	
# of unique seqs:	15920					
total # of seqs:	124739					



Showing all 10 rows.

<empty>	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	7908	44	0	3	1
2.5%-tile:	8	9582	250	0	3	3666
25%-tile:	8	9582	252	0	4	36651
Median:	8	9582	252	0	5	73301
75%-tile:	8	9582	252	0	5	109951
97.5%-tile:	8	9582	419	0	6	142936
Maximum:	2425	9582	1081	5	16	146601
Mean:	8	9581	269	0	4	
# of Seqs:	146601					

Filter and trim aligned sequences

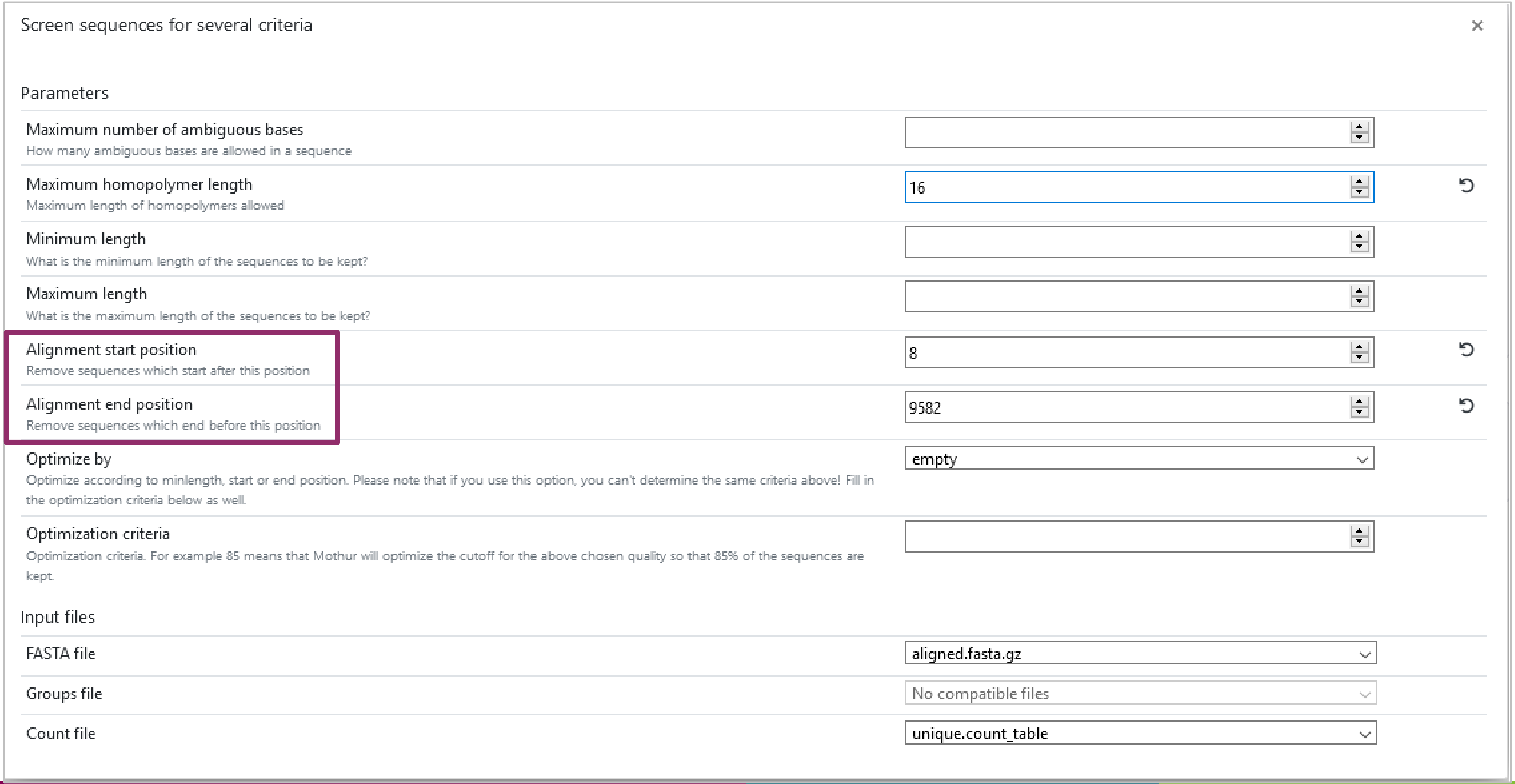
Outline

- Filter sequences based on alignment start and end position
- Trim sequence alignment
- Remove identical sequences

Filter aligned sequences

- All the aligned sequences should overlap the same alignment coordinates
- Remove deviants by filtering based on the alignment start and end position
  - Check aligned-summary.tsv
- Remove also sequences which have homopolymers longer than those in the reference
  - Check custom.reference.summary.tsv
- Tool: Screen sequences for several criteria (based on Mothur command screen.seqs)
- Input files: aligned.fasta.gz and unique.count_table
- Result files
  - screened.fasta.gz = screened sequences
  - screened.count_table = updated count_table
  - summary.screened.tsv = sequence information

Parameters for filtering aligned sequences



Screen sequences for several criteria

Parameters

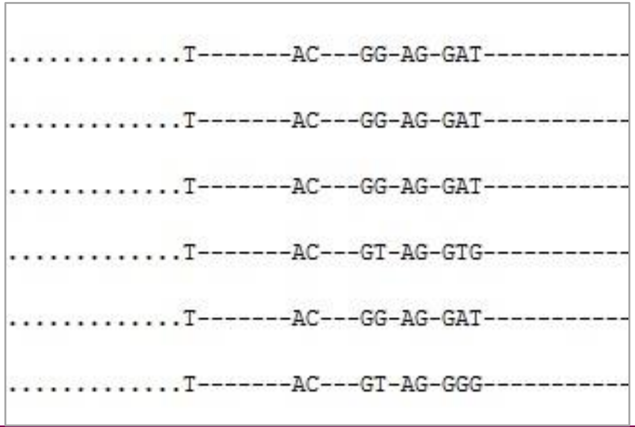
Maximum number of ambiguous bases How many ambiguous bases are allowed in a sequence	
Maximum homopolymer length Maximum length of homopolymers allowed	16
Minimum length What is the minimum length of the sequences to be kept?	
Maximum length What is the maximum length of the sequences to be kept?	
Alignment start position Remove sequences which start after this position	8
Alignment end position Remove sequences which end before this position	9582
Optimize by Optimize according to minlength, start or end position. Please note that if you use this option, you can't determine the same criteria above! Fill in the optimization criteria below as well.	empty
Optimization criteria Optimization criteria. For example 85 means that Mothur will optimize the cutoff for the above chosen quality so that 85% of the sequences are kept.	

Input files

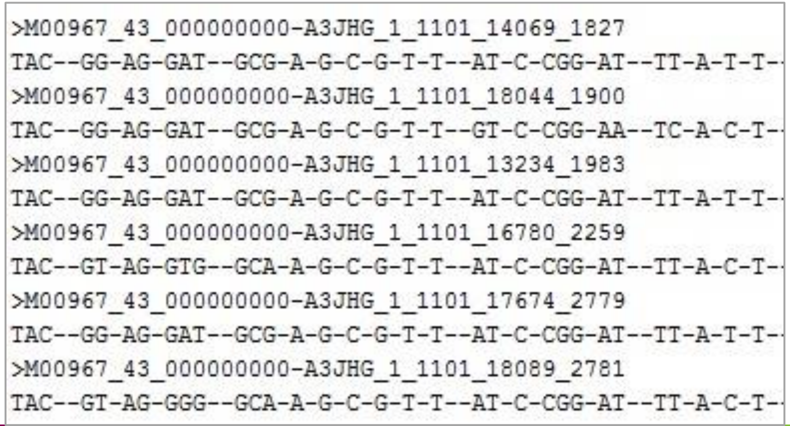
FASTA file	aligned.fasta.gz
Groups file	No compatible files
Count file	unique.count_table

Trim sequence alignment for overhangs and empty columns

- We remove overhangs (columns containing .) and keep the common alignment region
- Gap columns (where all the characters are –) have no information, so we remove them
  - makes distance calculation faster
- Removing alignment columns can create identical sequences → need to remove them
- Tool Filter sequence alignment (based on Mothur commands filter.seqs and unique.seqs)
- Input files: screened.fasta.gz and screened.count_table



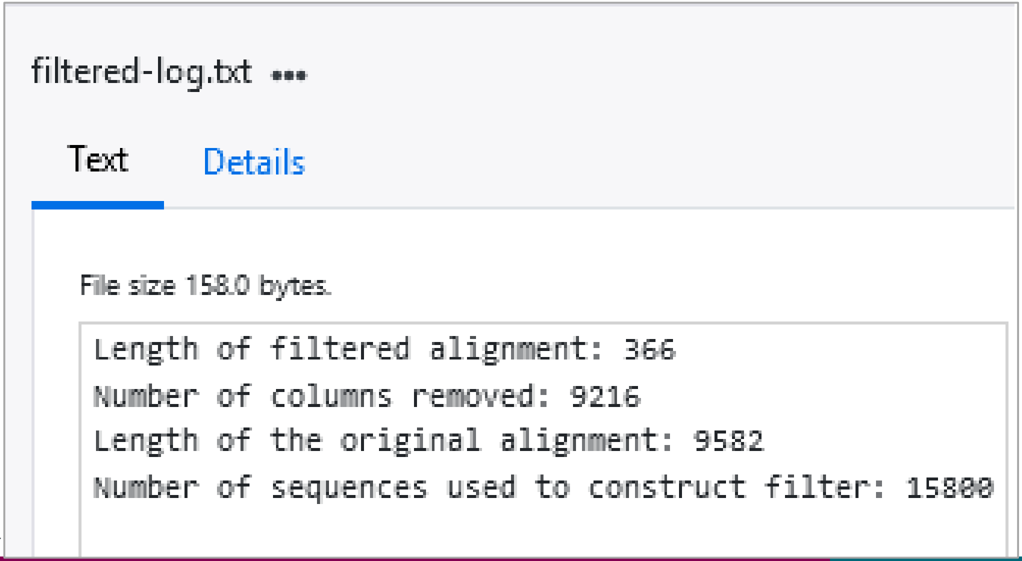
```
.............T-------AC---GG-AG-GAT-----------
.............T-------AC---GG-AG-GAT-----------
.............T-------AC---GG-AG-GAT-----------
.............T-------AC---GT-AG-GTG-----------
.............T-------AC---GG-AG-GAT-----------
.............T-------AC---GT-AG-GGG-----------
```



```
>M00967_43_000000000-A3JHG_1_1101_14069_1827
TAC--GG-AG-GAT--GCG-A-G-C-G-T-T--AT-C-CGG-AT--TT-A-T-T-
>M00967_43_000000000-A3JHG_1_1101_18044_1900
TAC--GG-AG-GAT--GCG-A-G-C-G-T-T--GT-C-CGG-AA--TC-A-C-T-
>M00967_43_000000000-A3JHG_1_1101_13234_1983
TAC--GG-AG-GAT--GCG-A-G-C-G-T-T--AT-C-CGG-AT--TT-A-T-T-
>M00967_43_000000000-A3JHG_1_1101_16780_2259
TAC--GT-AG-GTG--GCA-A-G-C-G-T-T--AT-C-CGG-AT--TT-A-C-T-
>M00967_43_000000000-A3JHG_1_1101_17674_2779
TAC--GG-AG-GAT--GCG-A-G-C-G-T-T--AT-C-CGG-AT--TT-A-T-T-
>M00967_43_000000000-A3JHG_1_1101_18089_2781
TAC--GT-AG-GGG--GCA-A-G-C-G-T-T--AT-C-CGG-AT--TT-A-C-T-
```

Result files

- filtered-unique.fasta.gz = trimmed aligned sequences
- filtered-unique.count_table = updated count_table
- filtered-unique-summary.tsv = sequence information
- filtered-log.txt = how many alignment columns were removed



File size 158.0 bytes.

```
Length of filtered alignment: 366
Number of columns removed: 9216
Length of the original alignment: 9582
Number of sequences used to construct filter: 15800
```

Remove sequencing errors and chimeras

Outline

- How preclustering works
- What are chimeras and how to remove them?

Precluster very similar sequences

- Assumes that abundant sequences are more likely to generate sequencing errors
  - ranks sequences in order of their abundance
  - walks through the list looking for rarer sequences which differ only by x number of bases from the original sequence (allow 1 mismatch for every 100 bp of sequence)
  - merges those that are within the threshold
- Tool: Precluster aligned sequences (based on Mothur command precluster.seqs)
- Input files: filtered-unique.fasta.gz and filtered-unique.count_table
- Result files
  - preclustered.fasta.gz = preclustered aligned sequences
  - preclustered.count_table = updated count_table
  - preclustered-summary.tsv = sequence information

Remove chimeras

- Chimera = artifact sequence formed by two biological sequences
  - incomplete extension during PCR allows subsequent PCR cycles to use a partially extended strand to bind to the template of a similar sequence.
  - the partially extended strand then acts as a primer to extend and form a chimeric sequence.
  - as many as 30% of the sequences from mixed template environmental samples may be chimeric.
- Tool: Remove chimeric sequences (based on Mothur chimera.uchime, chimera.vsearch)
- You can either use a reference or detect chimeras *de novo*
  - Reference is the bacterial subset of the Silva Gold 16S rRNA
  - *De novo* approach uses the more abundant sequences in your data as the reference
- Dereplicate = should we remove a chimera only from the sample where it was spotted?
  - True = only from that sample ("do not replicate")
  - False = from all samples ("replicate to other samples")
- Input files: preclustered.fasta.gz and preclustered.count_table file

Chimera removal results

- Result files
  - chimeras.removed.fasta.gz = aligned sequences
  - chimeras.removed.count_table = updated count_table
  - chimeras.removed.summary.tsv = sequence information
- Results depend heavily on the method and reference used. Example:
  - 6022 unique sequences to start with
  - 5283 after chimera removal with VSEARCH and SILVA gold (29 s)
  - 2467 after chimera removal with VSEARCH and *de novo* (4 s)
  - 5323 after chimera removal with UCHIME and SILVA gold (23 min)
  - 5023 after chimera removal with UCHIME and *de novo* (19 s)

Classify sequences to taxonomic units

Outline

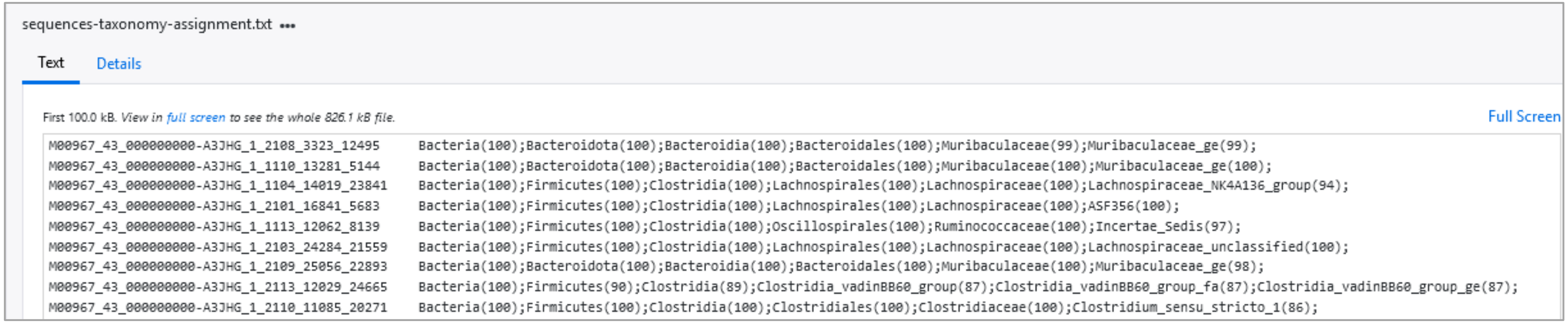
- Tools for assigning sequences to taxonomies
- Wang method
- File formats
    - Taxonomy assignment file
    - Classification summary file

Assign sequences to taxonomy

- Tools Classify 16S or 18S sequences to taxonomic units using Silva and Classify ITS sequences to taxonomic units using UNITE (based on Mothur command classify.seqs)
  - you can also use your own taxonomy by providing reference fasta and taxonomy outline file.
- Wang method
  - looks at the query sequence k-mer by k-mer
  - calculates the probability a sequence from a given taxonomy would contain a specific k-mer
  - calculates the probability a query sequence would be in a given taxonomy based on the k-mers it contains, and assigns the query sequence to the taxonomy with the highest probability
  - calculates bootstrap confidence score for the assignment (chooses randomly 1/8 of the k-mers in the query), by default 100 iterations
  - if the confidence is <80%, assignment will revert to higher level
- Input files: chimeras.removed.fasta.gz and chimeras.removed.count_table

Classification result files

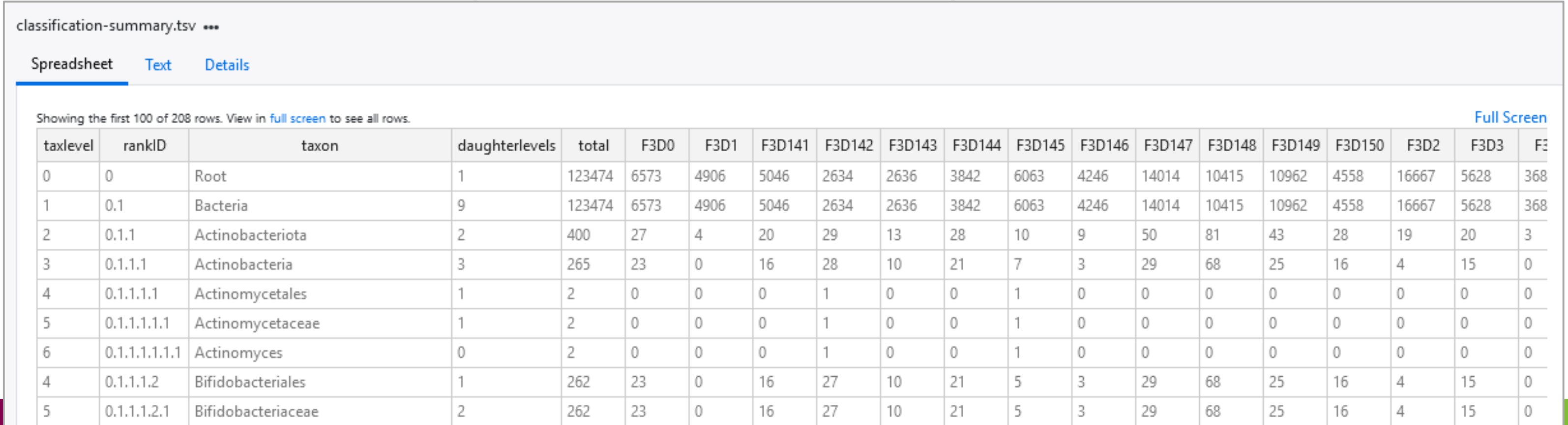
- sequences-taxonomy-assignment.txt = sequence name and taxonomy



First 100.0 kB. *View in* *full screen* *to see the whole 826.1 kB file.*

```
M00967_43_000000000-A3JHG_1_2108_3323_12495     Bacteria(100);Bacteroidota(100);Bacteroidia(100);Bacteroidales(100);Muribaculaceae(99);Muribaculaceae_ge(99);
M00967_43_000000000-A3JHG_1_1110_13281_5144     Bacteria(100);Bacteroidota(100);Bacteroidia(100);Bacteroidales(100);Muribaculaceae(100);Muribaculaceae_ge(100);
M00967_43_000000000-A3JHG_1_1104_14019_23841    Bacteria(100);Firmicutes(100);Clostridia(100);Lachnospirales(100);Lachnospiraceae(100);Lachnospiraceae_NK4A136_group(94);
M00967_43_000000000-A3JHG_1_2101_16841_5683     Bacteria(100);Firmicutes(100);Clostridia(100);Lachnospirales(100);Lachnospiraceae(100);ASF356(100);
M00967_43_000000000-A3JHG_1_1113_12062_8139     Bacteria(100);Firmicutes(100);Clostridia(100);Oscillospirales(100);Ruminococcaceae(100);Incertae_Sedis(97);
M00967_43_000000000-A3JHG_1_2103_24284_21559    Bacteria(100);Firmicutes(100);Clostridia(100);Lachnospirales(100);Lachnospiraceae(100);Lachnospiraceae_unclassified(100);
M00967_43_000000000-A3JHG_1_2109_25056_22893    Bacteria(100);Bacteroidota(100);Bacteroidia(100);Bacteroidales(100);Muribaculaceae(100);Muribaculaceae_ge(98);
M00967_43_000000000-A3JHG_1_2113_12029_24665    Bacteria(100);Firmicutes(90);Clostridia(89);Clostridia_vadinBB60_group(87);Clostridia_vadinBB60_group_fa(87);Clostridia_vadinBB60_group_ge(87);
M00967_43_000000000-A3JHG_1_2110_11085_20271    Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Clostridiaceae(100);Clostridium_sensu_stricto_1(86);
```

- classification-summary.tsv = the number of sequences that were found at each level

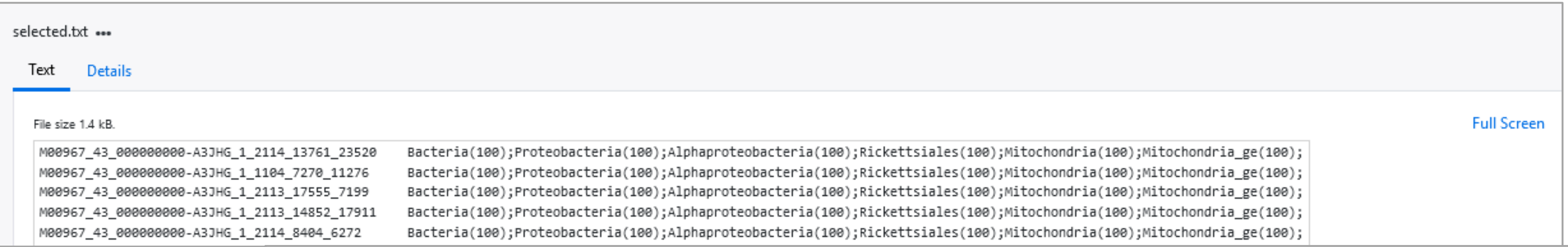


Showing the first 100 of 208 rows. View in full screen to see all rows.

taxlevel	rankID	taxon	daughterlevels	total	F3D0	F3D1	F3D141	F3D142	F3D143	F3D144	F3D145	F3D146	F3D147	F3D148	F3D149	F3D150	F3D2	F3D3	F3
0	0	Root	1	123474	6573	4906	5046	2634	2636	3842	6063	4246	14014	10415	10962	4558	16667	5628	368
1	0.1	Bacteria	9	123474	6573	4906	5046	2634	2636	3842	6063	4246	14014	10415	10962	4558	16667	5628	368
2	0.1.1	Actinobacteriota	2	400	27	4	20	29	13	28	10	9	50	81	43	28	19	20	3
3	0.1.1.1	Actinobacteria	3	265	23	0	16	28	10	21	7	3	29	68	25	16	4	15	0
4	0.1.1.1.1	Actinomycetales	1	2	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
5	0.1.1.1.1.1	Actinomycetaceae	1	2	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
6	0.1.1.1.1.1.1	Actinomyces	0	2	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
4	0.1.1.1.2	Bifidobacteriales	1	262	23	0	16	27	10	21	5	3	29	68	25	16	4	15	0
5	0.1.1.1.2.1	Bifidobacteriaceae	2	262	23	0	16	27	10	21	5	3	29	68	25	16	4	15	0

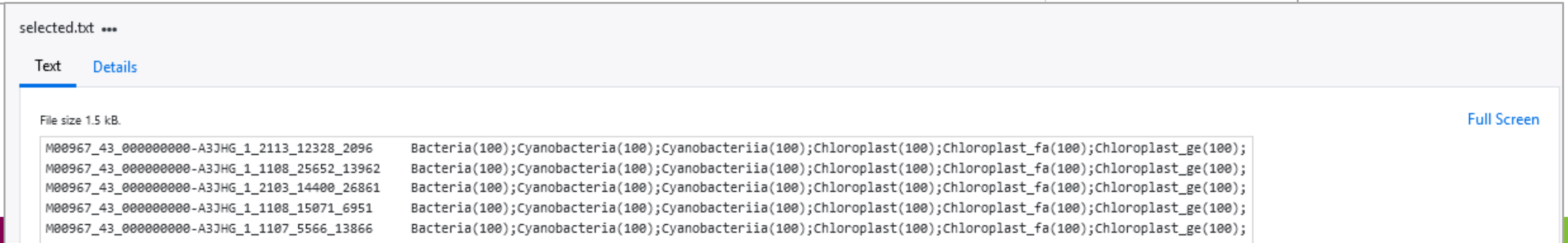
Removing unwanted lineages

- Data may contain assignments to mitochondria, chloroplasts, unknown
- You can remove these after converting Mothur files into phyloseq object
  - Remove selected taxa removes chloroplast and mitochondrial sequences from a phyloseq object, and up to five user-specified taxa at the desired level of biological organization.
  - Filter by taxonomic group tidies a phyloseq object so that OTUs only from the desired taxonomic group (bacteria, archaea, eukaryotes or fungi) are retained. Features with ambiguous phylum-level annotation (e.g. NA, unknown, uncharacterized) are removed.



File size 1.4 kB.

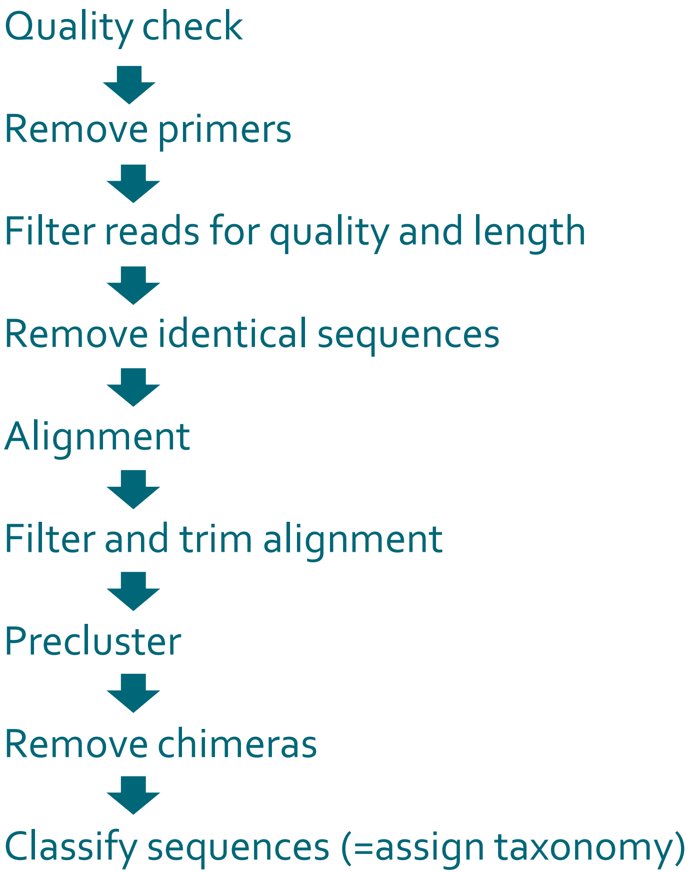
```
M00967_43_000000000-A3JHG_1_2114_13761_23520    Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rickettsiales(100);Mitochondria(100);Mitochondria_ge(100);
M00967_43_000000000-A3JHG_1_1104_7270_11276     Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rickettsiales(100);Mitochondria(100);Mitochondria_ge(100);
M00967_43_000000000-A3JHG_1_2113_17555_7199     Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rickettsiales(100);Mitochondria(100);Mitochondria_ge(100);
M00967_43_000000000-A3JHG_1_2113_14852_17911    Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rickettsiales(100);Mitochondria(100);Mitochondria_ge(100);
M00967_43_000000000-A3JHG_1_2114_8404_6272      Bacteria(100);Proteobacteria(100);Alphaproteobacteria(100);Rickettsiales(100);Mitochondria(100);Mitochondria_ge(100);
```



File size 1.5 kB.

```
M00967_43_000000000-A3JHG_1_2113_12328_2096     Bacteria(100);Cyanobacteria(100);Cyanobacteriia(100);Chloroplast(100);Chloroplast_fa(100);Chloroplast_ge(100);
M00967_43_000000000-A3JHG_1_1108_25652_13962    Bacteria(100);Cyanobacteria(100);Cyanobacteriia(100);Chloroplast(100);Chloroplast_fa(100);Chloroplast_ge(100);
M00967_43_000000000-A3JHG_1_2103_14400_26861    Bacteria(100);Cyanobacteria(100);Cyanobacteriia(100);Chloroplast(100);Chloroplast_fa(100);Chloroplast_ge(100);
M00967_43_000000000-A3JHG_1_1108_15071_6951     Bacteria(100);Cyanobacteria(100);Cyanobacteriia(100);Chloroplast(100);Chloroplast_fa(100);Chloroplast_ge(100);
M00967_43_000000000-A3JHG_1_1107_5566_13866     Bacteria(100);Cyanobacteria(100);Cyanobacteriia(100);Chloroplast(100);Chloroplast_fa(100);Chloroplast_ge(100);
```

Outline of Day 1:



Quality check

↓

Remove primers

↓

Filter reads for quality and length

↓

Remove identical sequences

↓

Alignment

↓

Filter and trim alignment

↓

Precluster

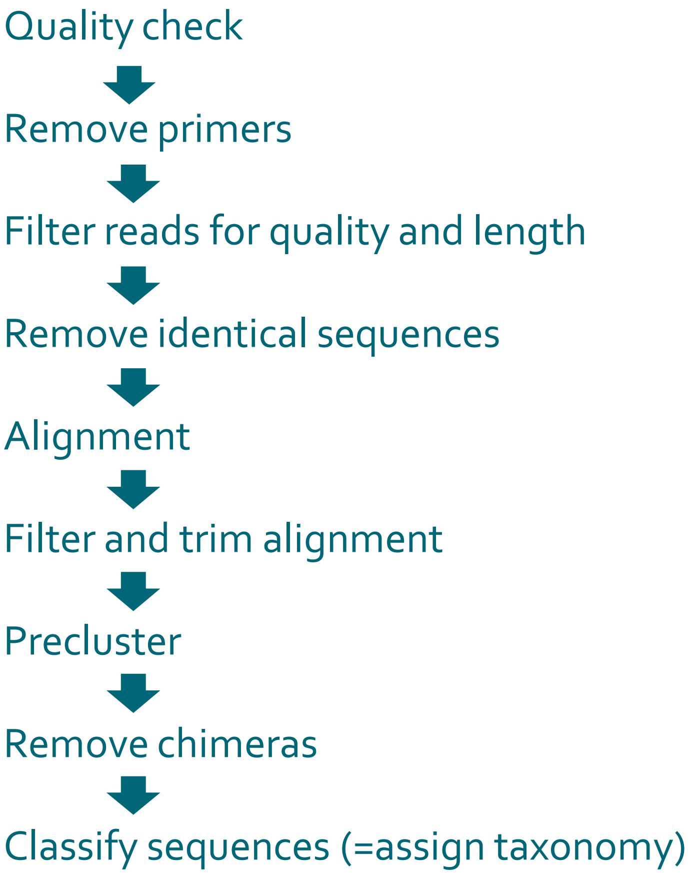
↓

Remove chimeras

↓

Classify sequences (=assign taxonomy)

Outline of Day 1:



Quality check

Remove primers

Filter reads for quality and length

Remove identical sequences

Alignment

Filter and trim alignment

Precluster

Remove chimeras

Classify sequences (=assign taxonomy)

Output so far:

1. FASTA file of processed reads
2. count file (which read in which sample)
3. taxonomy file (taxonomy of each read)

Day 2:

- Clustering into OTUs
- **Phyloseq** object with sample data
- Data tidying & transformations
- Taxonomy plots
- Alpha diversity
- Beta diversity: ordinations & statistics