SCIENCE MEETS LIFE

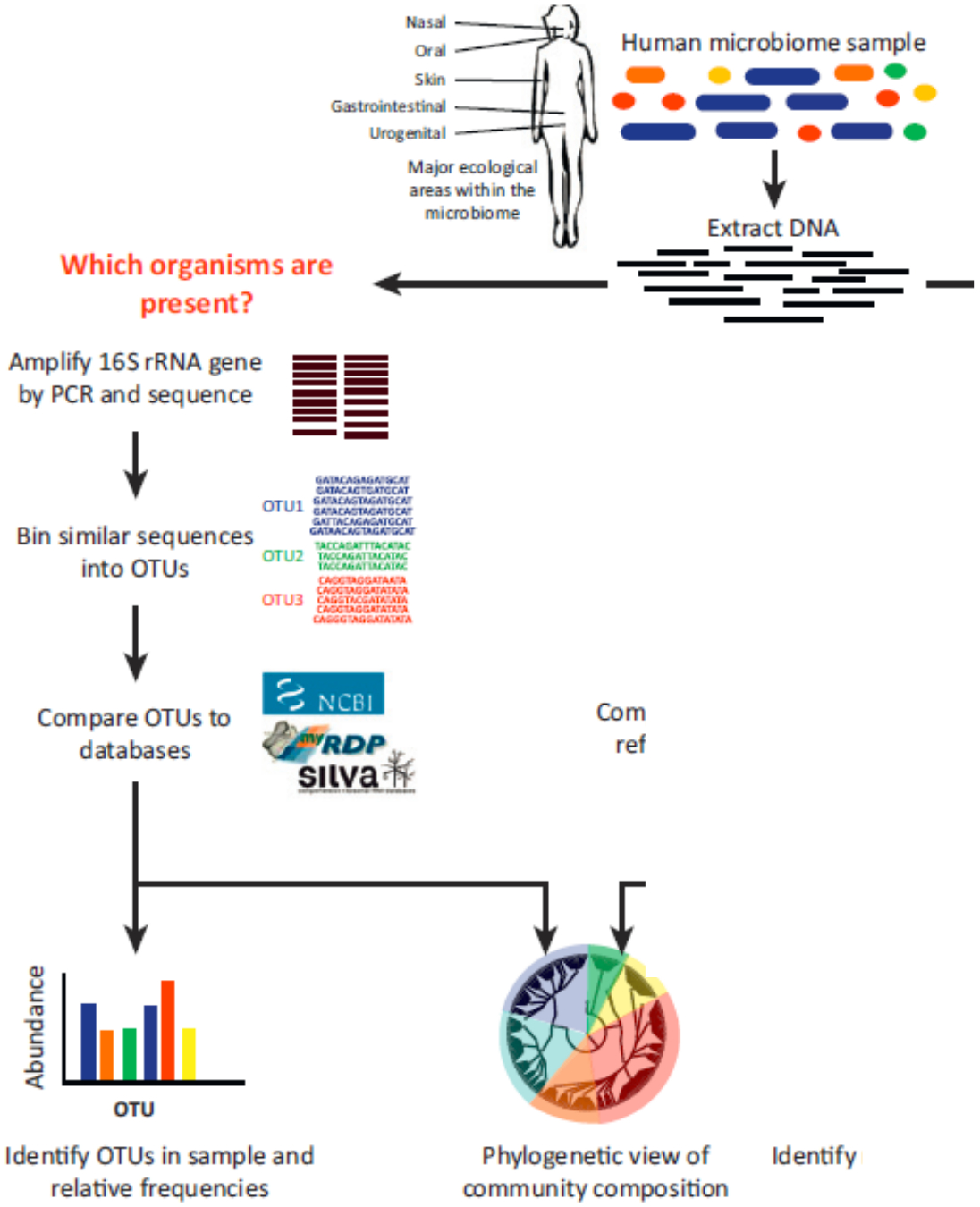
Fundamentals and Overview of bioinformatics pipelines for amplicon sequencing data analysis

Blastocystis COST Action Training School: Blastocystis and the Gut Microbiome

VIB

24-04-2024

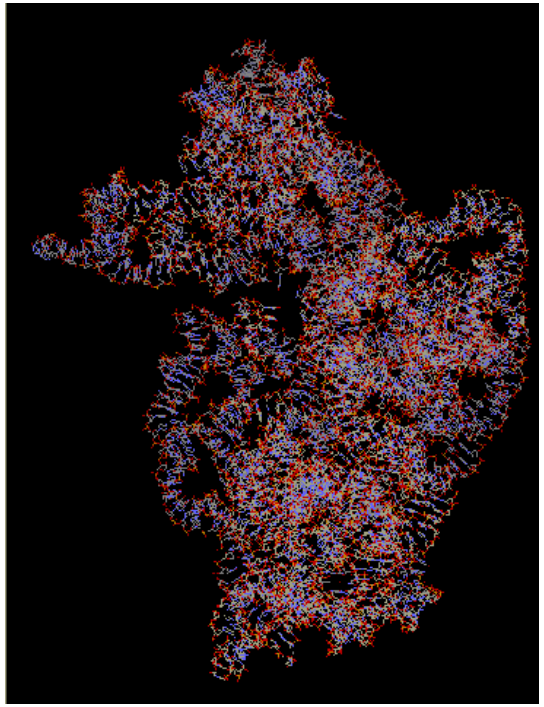
Raul Y. Tito-Tadeo



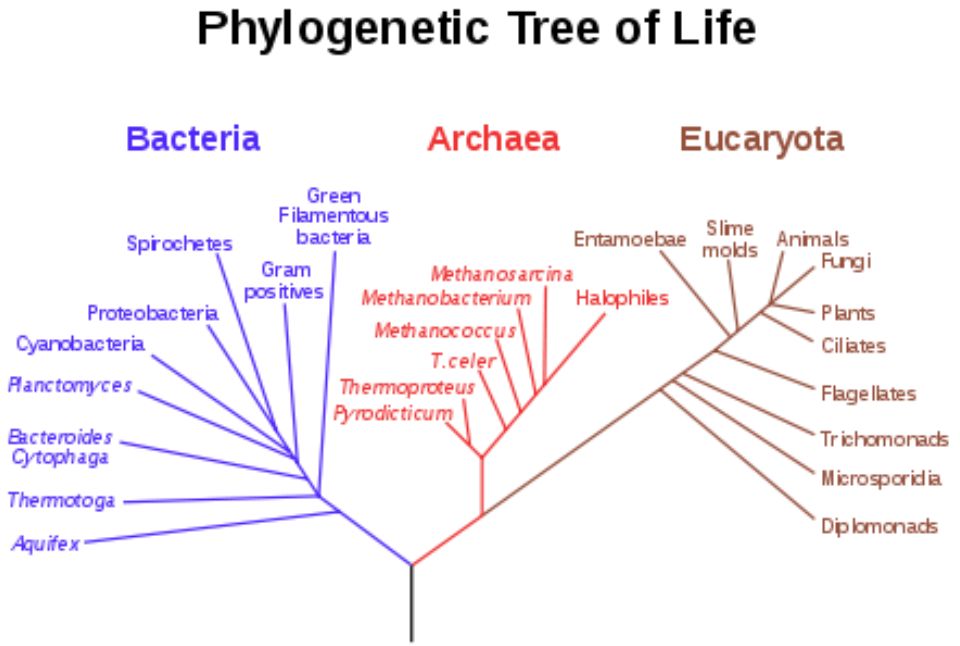
Gut microbiota profiling

Morgan, X. C., Segata, N., & Huttenhower, C. (2013). doi:10.1016/j.tig.2012.09.005

Universal marker gene: SSU rRNA gene



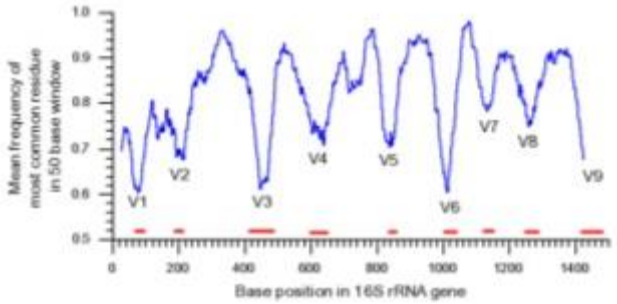
http://www.biochem.umd.edu



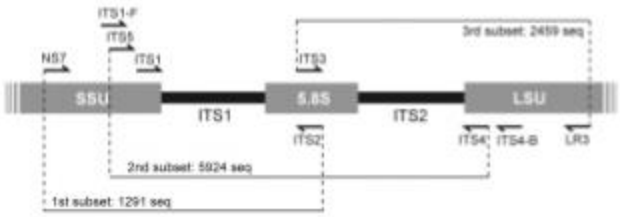
http://en.wikipedia.org/wiki/Carl_Woese

Types of amplicon sequencing

16S rRNA survey of bacterial microbiome



ITS survey of fungal microbiome



- First we need a gene that is universally conserved (also called maker gene)
  - Ribosomal RNA works well
    - SSU (16S, 18S)
    - LSU (23S, 28S)
    - ITS
  - Universally conserved marker
    - E.g. ribosomal proteins
    - Diversity too high to get good primers

Ribosomal marker gene databases

- SILVA – SSU & LSU
  - arb-silva.de
  - Frequent updates
  - Most comprehensive database (SSU & LSU)
  - 598,470 (SSU), 96,642(LSU) sequences
- Greengenes – SSU
  - greengenes.secondgenome.c
  - Last update May 2013
  - 1,262,986 sequences
- Unite – ITS
  - unite.ut.ee/
  - Relatively frequent upd
  - 690,548 sequences
- PR2 – 18S
  - specialized on protists

silva
high quality ribosomal RNA databases

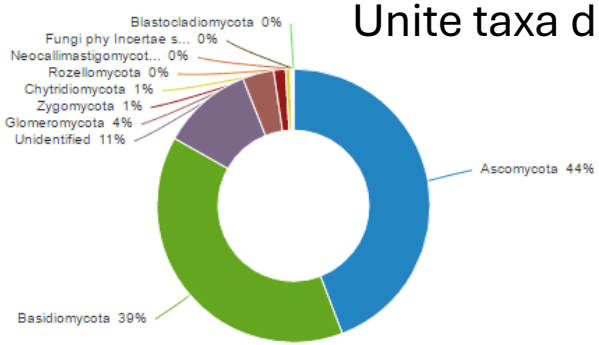
green genes
16S rRNA gene database and workbench compatible with ARB
greengenes.lbl.gov

unite

SILVA SSU / LSU 123 - full release

	SSU Parc	SSU Ref	SSU Ref NR	LSU Parc	LSU Ref
Minimal length	300	1200/900	1200/900	300	1900
Quality filtering	basic	strong	strong	basic	strong
Guide Tree	no	no	yes	no	yes
Release date	23.07.15	23.07.15	23.07.15	23.07.15	23.07.15
Aligned rRNA sequences	4,985,791	1,756,783	597,607	563,332	96,642

Unite taxa distribution



Error rates

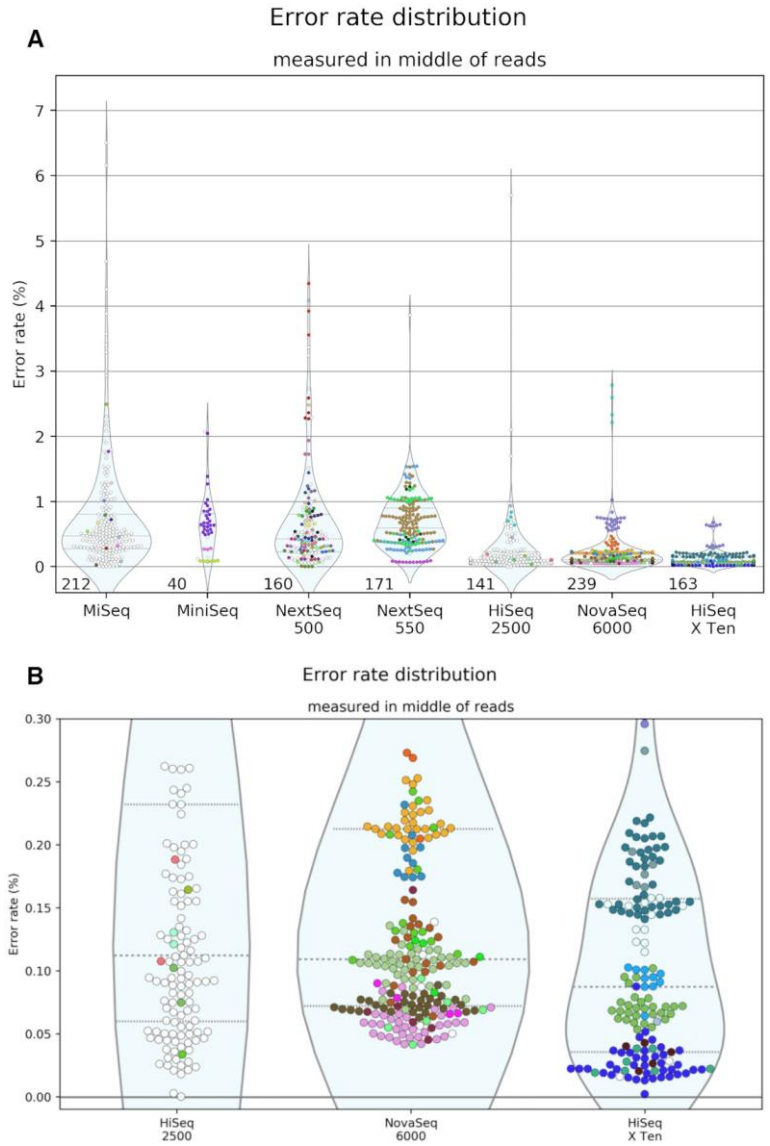


Table 1. Statistics of mappable length and error rates of PacBio and ONT long reads.

Read type	Mappable length (bp)				Error rate (Proportion of overall error) (%)			
	Mean	Median	Standard deviation	Maximum	Overall	Insertion	Deletion	Mismatch
PacBio CCS	1772	1464	1132	8006	1.72	0.087 (5.06)	0.34 (19.48)	1.30 (**75.46**)
PacBio subread	1570	1299	1076	16040	14.20	5.92 (**41.71**)	3.01 (21.17)	5.27 (37.12)
ONT 2D	1861	1754	882	9126	13.40	3.12 (23.30)	4.79 (35.70)	5.50 (**40.99**)
ONT 1D	1695	1602	824	9345	20.19	2.93 (14.51)	7.52 (37.24)	9.74 (**48.25**)

The fractions of each error types are in parenthesis. The fractions of the most predominant error types in each data are in bold.

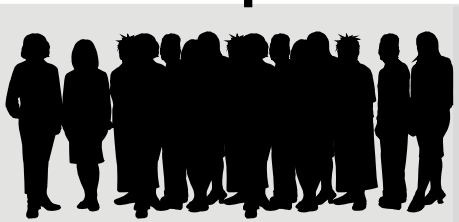
	NS	PacBio	Illumina	Ion Torrent
Read length	Variable (200 bp up to 2 Mbps)	Up to 20 kb	Up to 600 bp (2x300 PE)	Up to 400 bp (SE)
SNV error rate	1%–5%	0.1%*	<0.1%	<0.1%
Indel error rate	5%–10%	4%*	<0.1%	1%

*PE, pair-end; SE, single-end; *Error-rate estimation of PacBio circular consensus sequencing (CCS) method.*

Stoler, N., et al., NAR Genomics and Bioinformatics, 2021

Weirather J., et al.,F1000Research, 2017

Population cohort

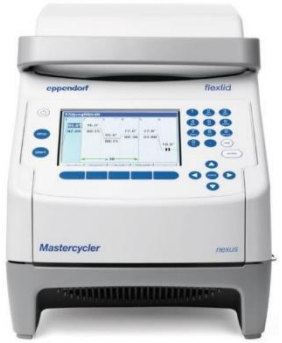


Metadata:
Anthropometrics
Diagnosis
Lifestyle
Medication

Sample collection

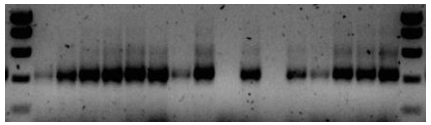
Stool → DNA

Bead-beating!



V4 region, 515f-806r

DI and controls!



Amplicon visualization
Electrophoresis

Amplicon quantification
Fragment Analyzer

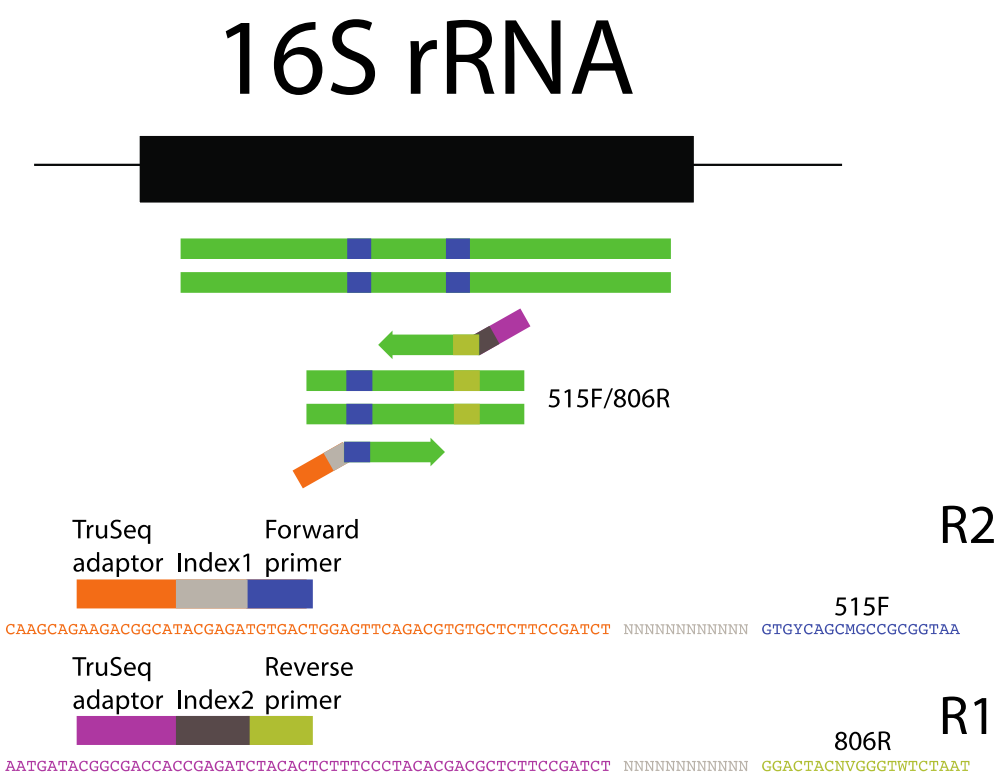
Pooling and library purification

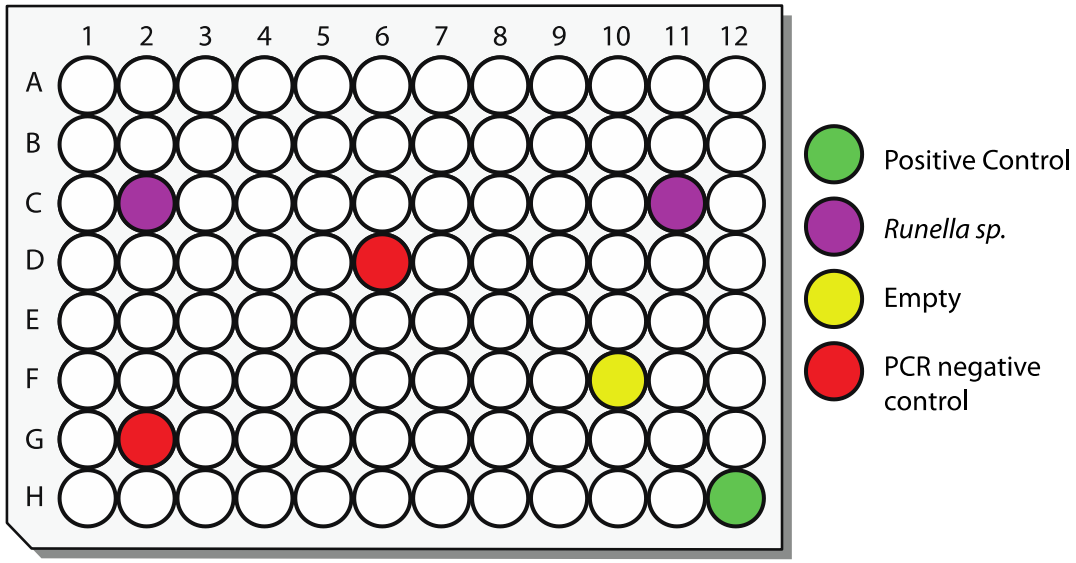
MiSeq / HiSeq 2x250

Data crunching
Amplicon
NGS pipelines

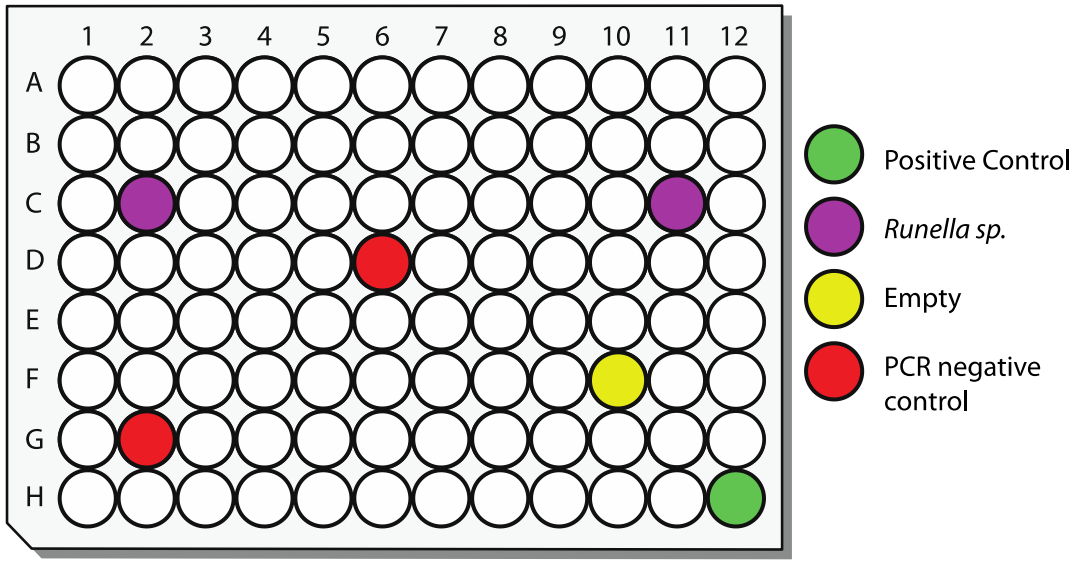
Analysis:
R libraries

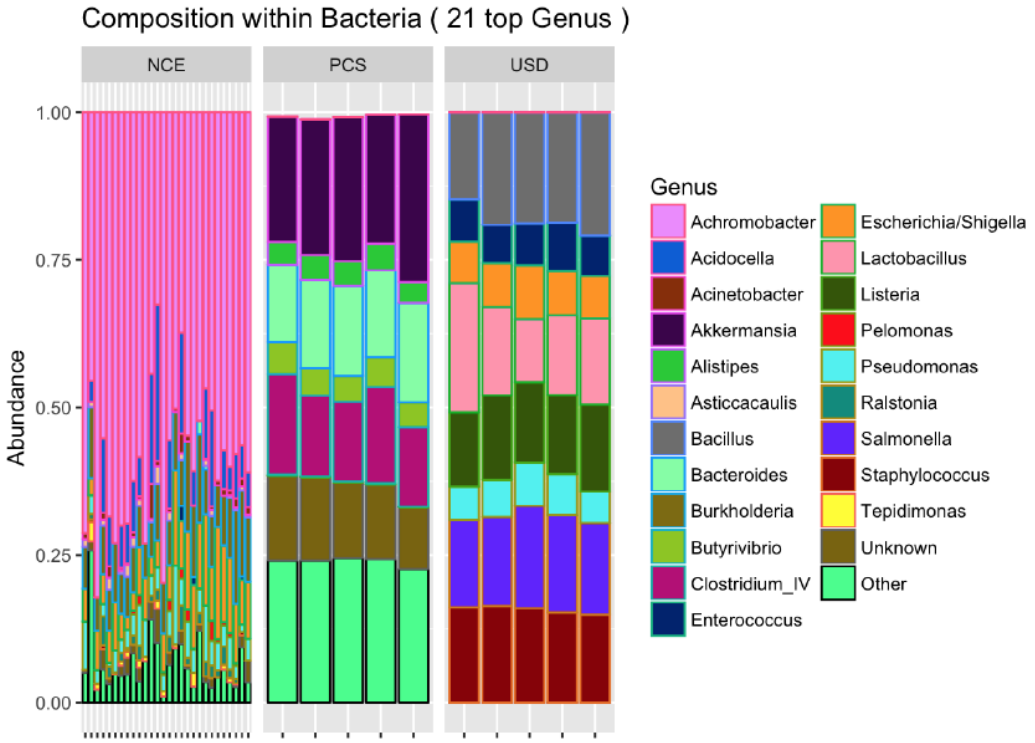
Dual Index and controls!



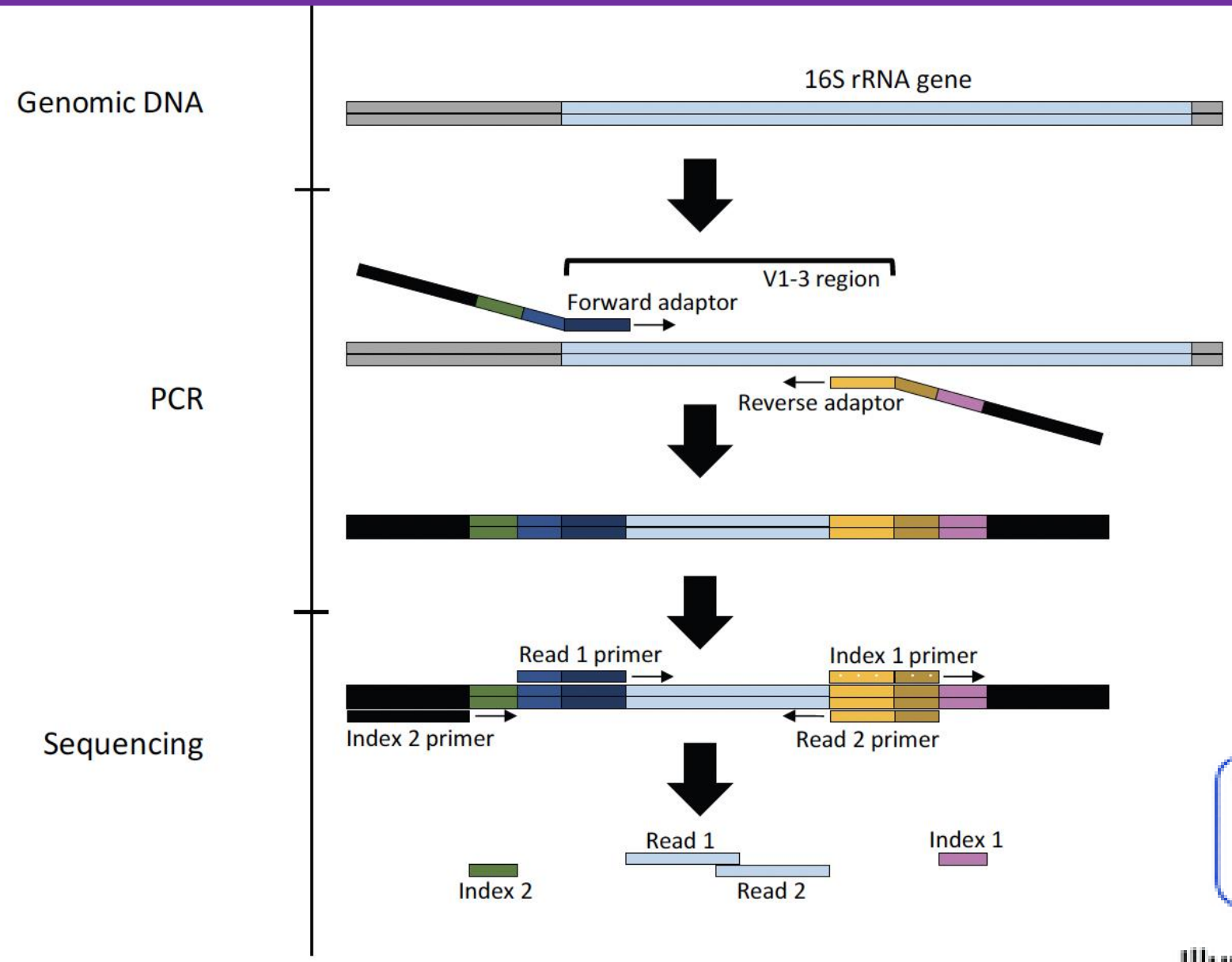


Dual Index and controls!





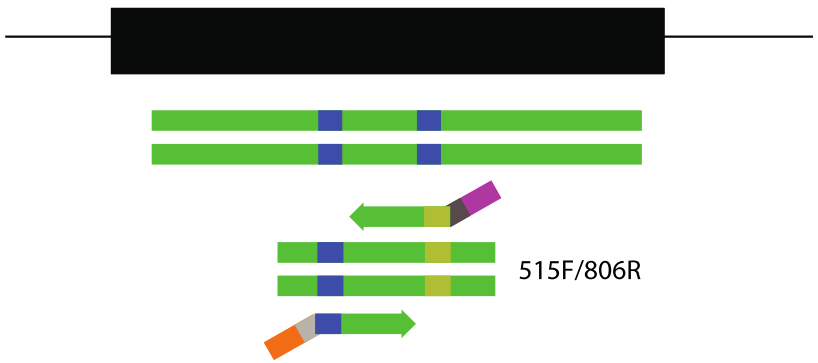
General pre-processing flow



Illumina MiSeq sequencing

Sequencing format (MiSeq, HiSeq, Novaseq)

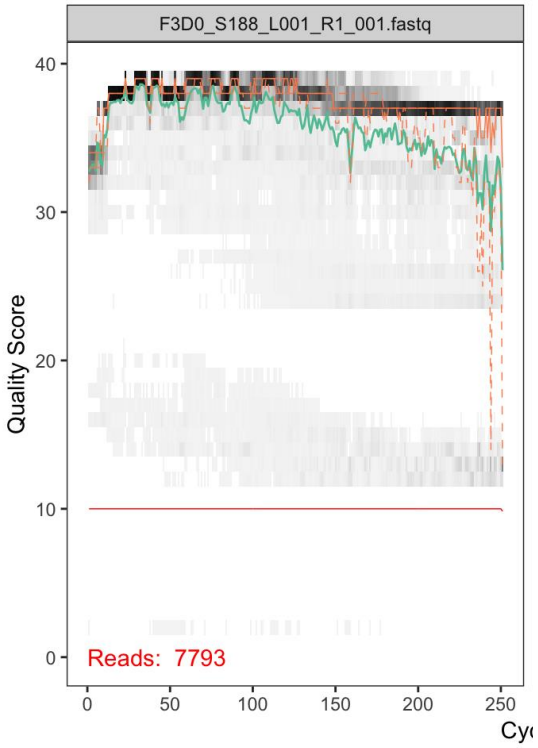
16S rRNA

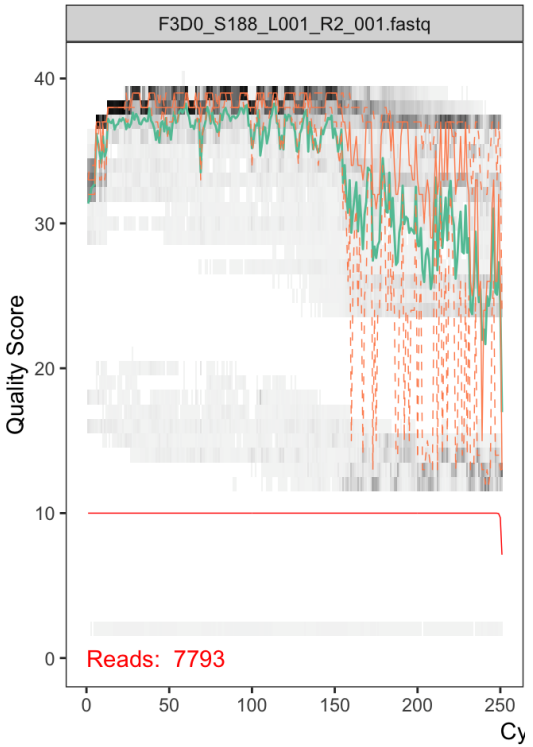


MiSeq PE 2X250
~ 12-18 millions of reads PE
R1 and R2
Fastq files

Table 1: Quality Scores and Base Calling Accuracy

Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%





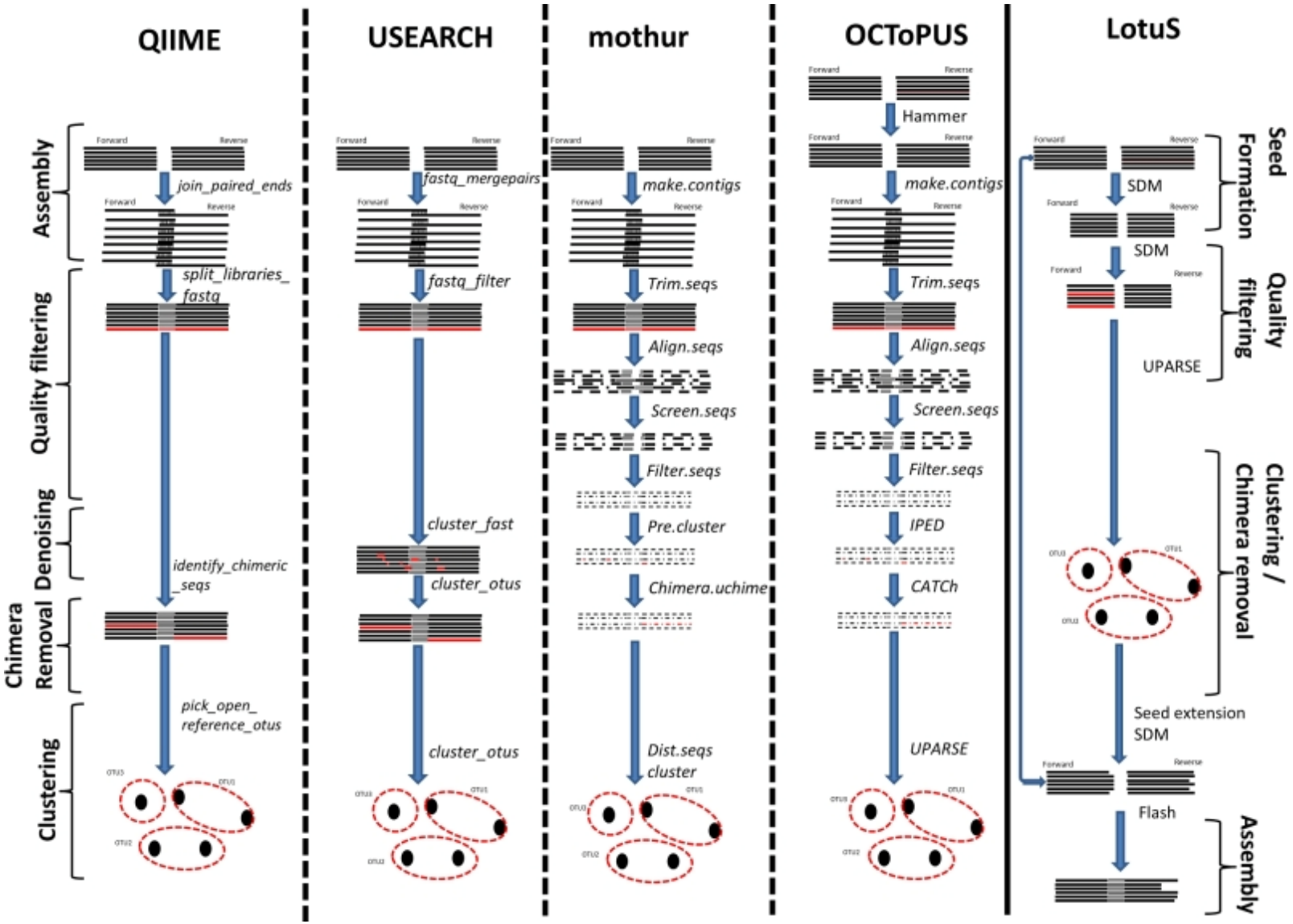
Data crunching: general steps

Basic pipeline for 16S data preprocessing

1. **Demultiplexing**
2. **Quality control and trimming**
3. **Merging R1 and R2**
4. **Chimera removal**
5. **Closed-reference OTU picking**
6. **De novo OTU picking**
7. **Taxonomic annotation**
8. **Tree building**

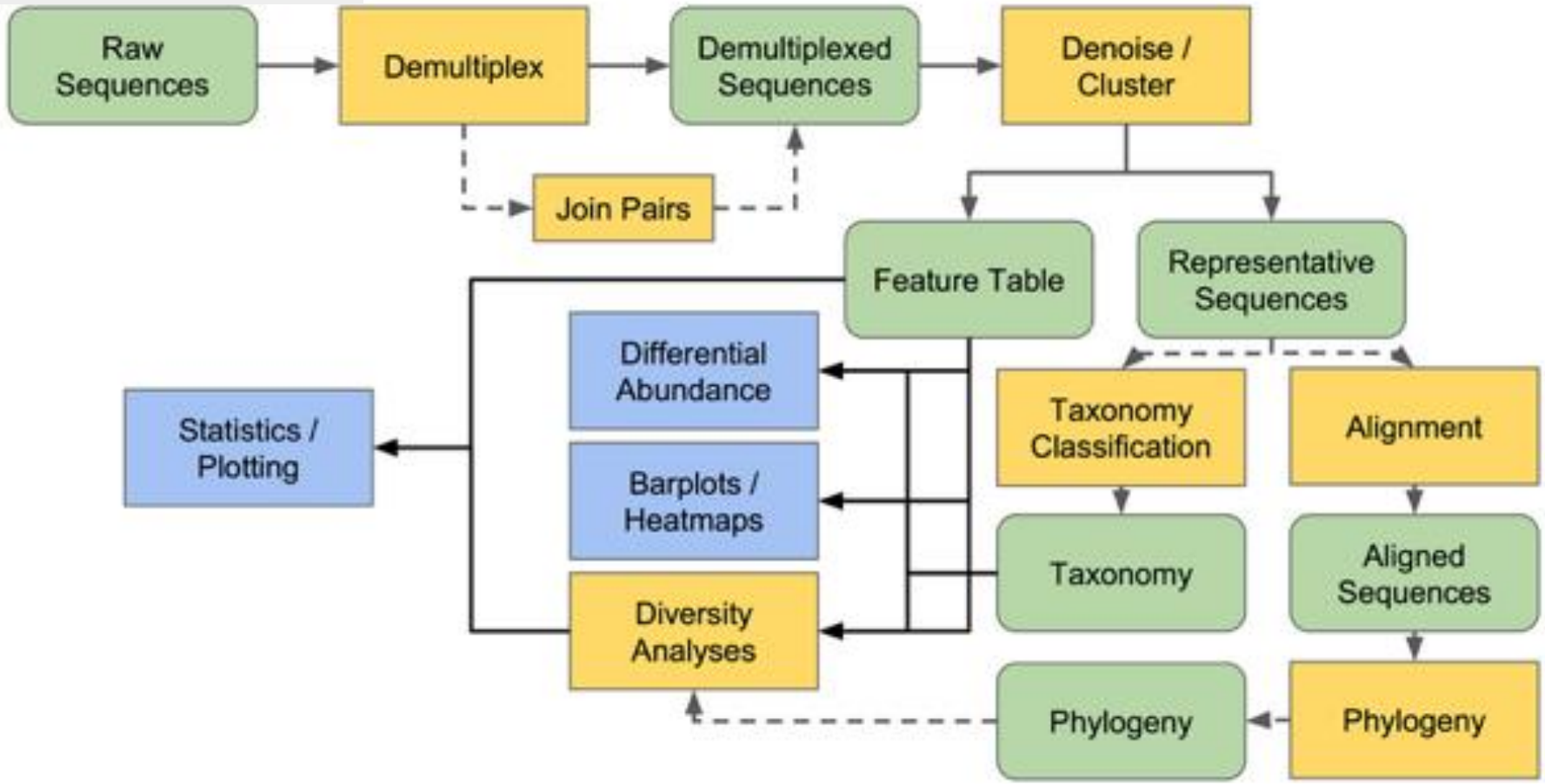
Pipelines to analyzed microbiota amplicon sequencing data (ribosomal markers genes)

Different flavors



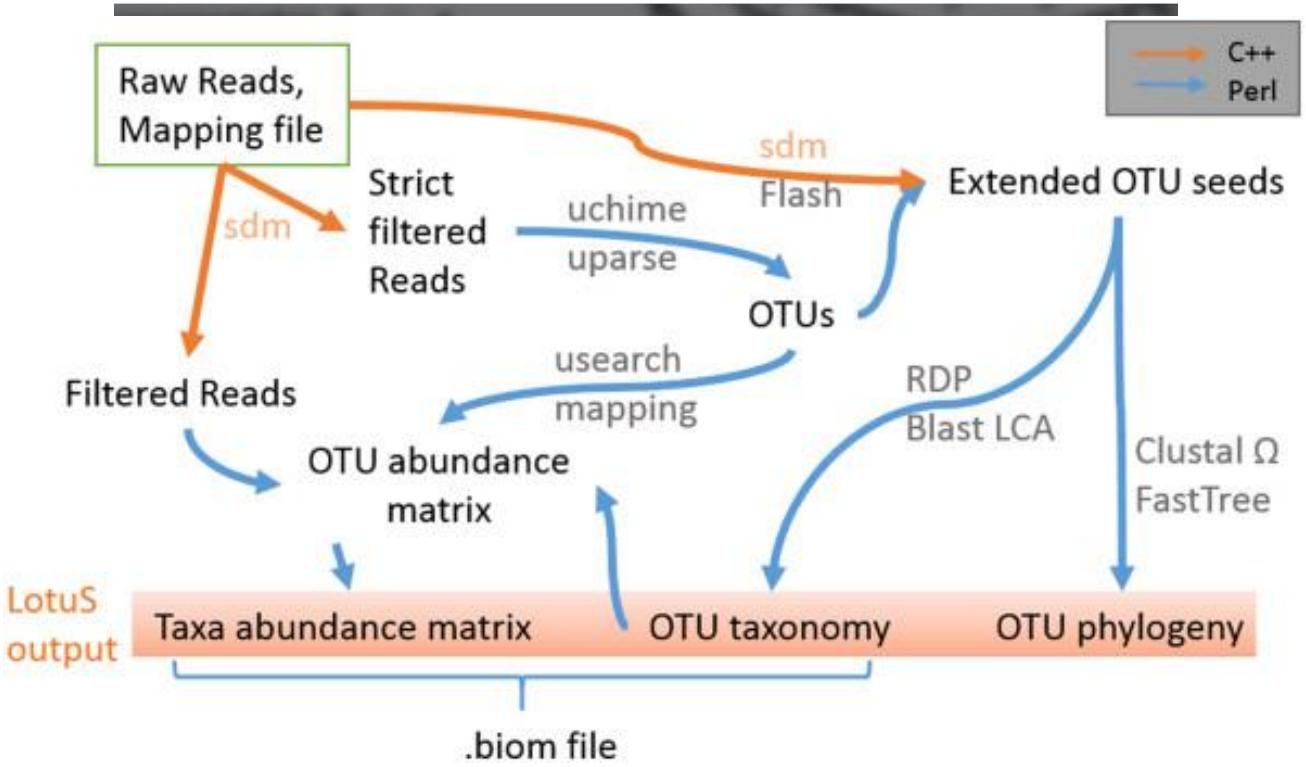
DADA2

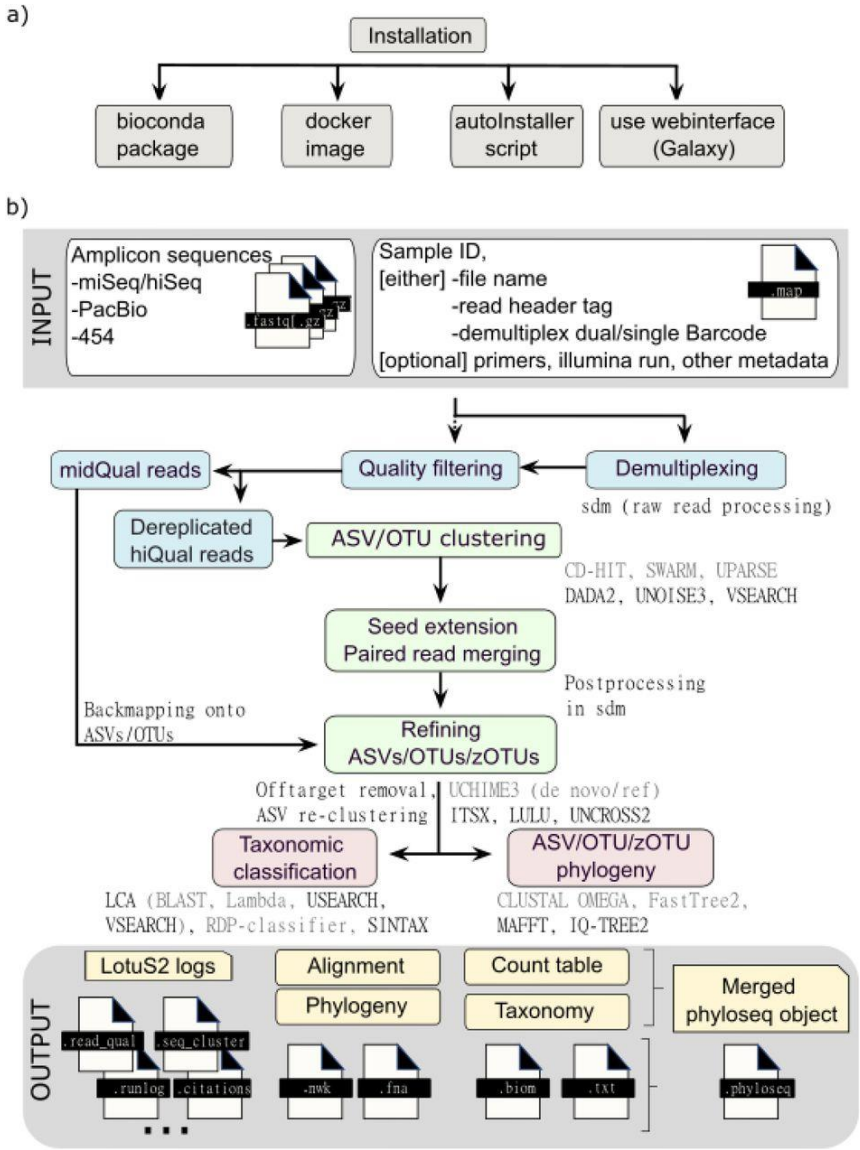
Qiime2 Pipeline



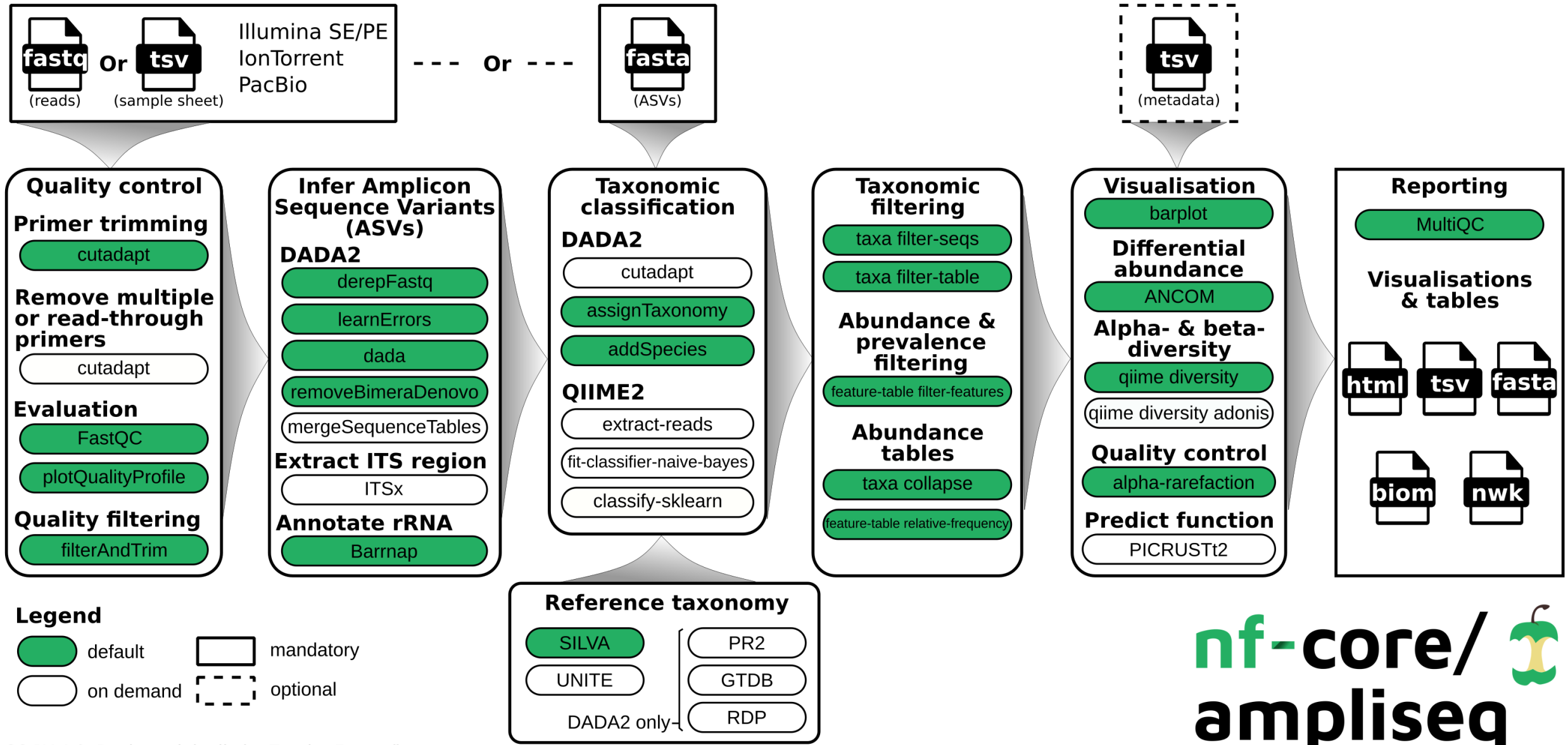
https://qiime2.org/

LotuS





https://github.com/hildebra/lotus2?tab=readme-ov-file

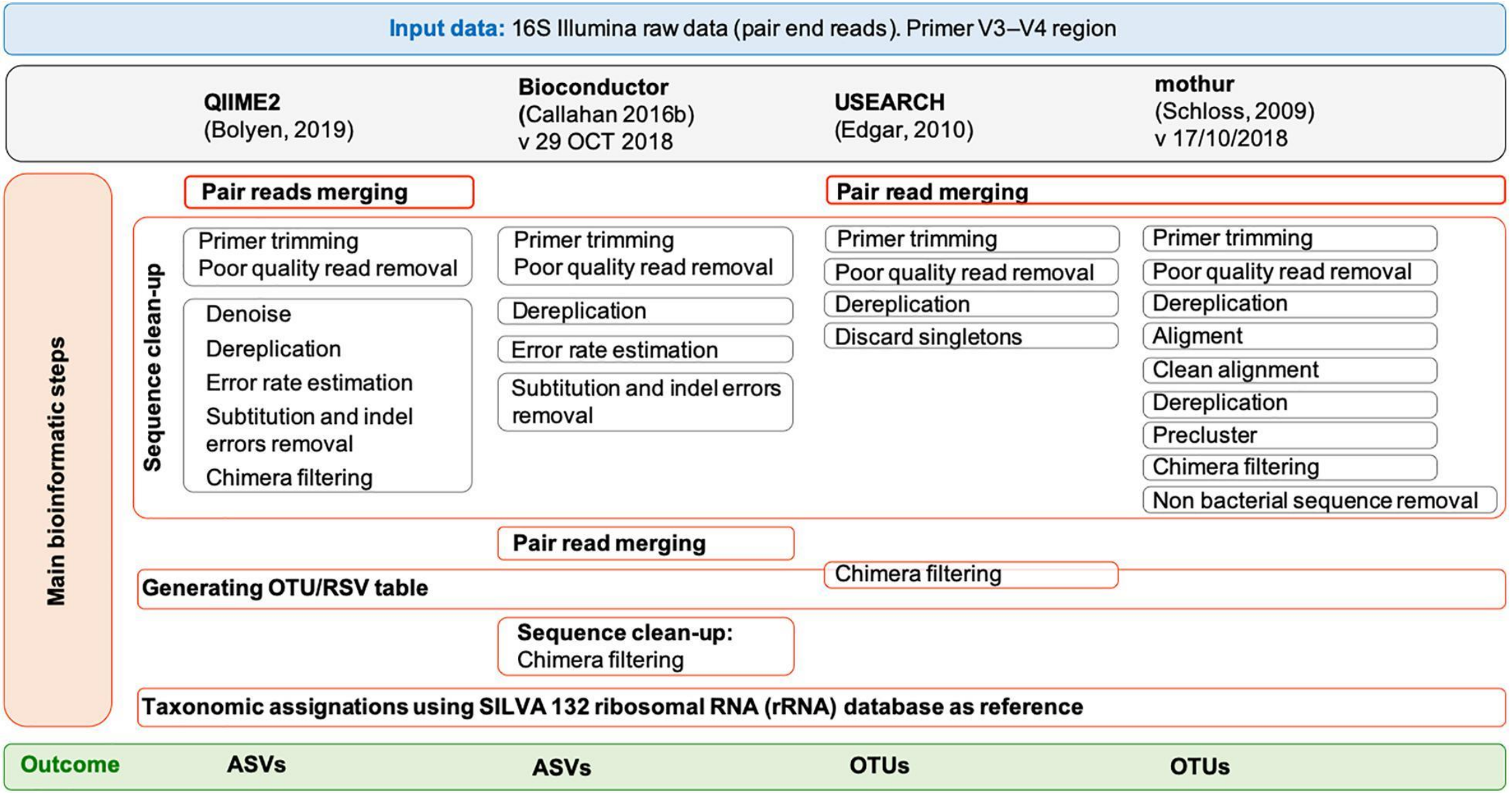


CC-BY 4.0. Design originally by Zandra Fagernäs

nf-core/ampliseq

https://nf-co.re/ampliseq/2.3.1

Pipelines

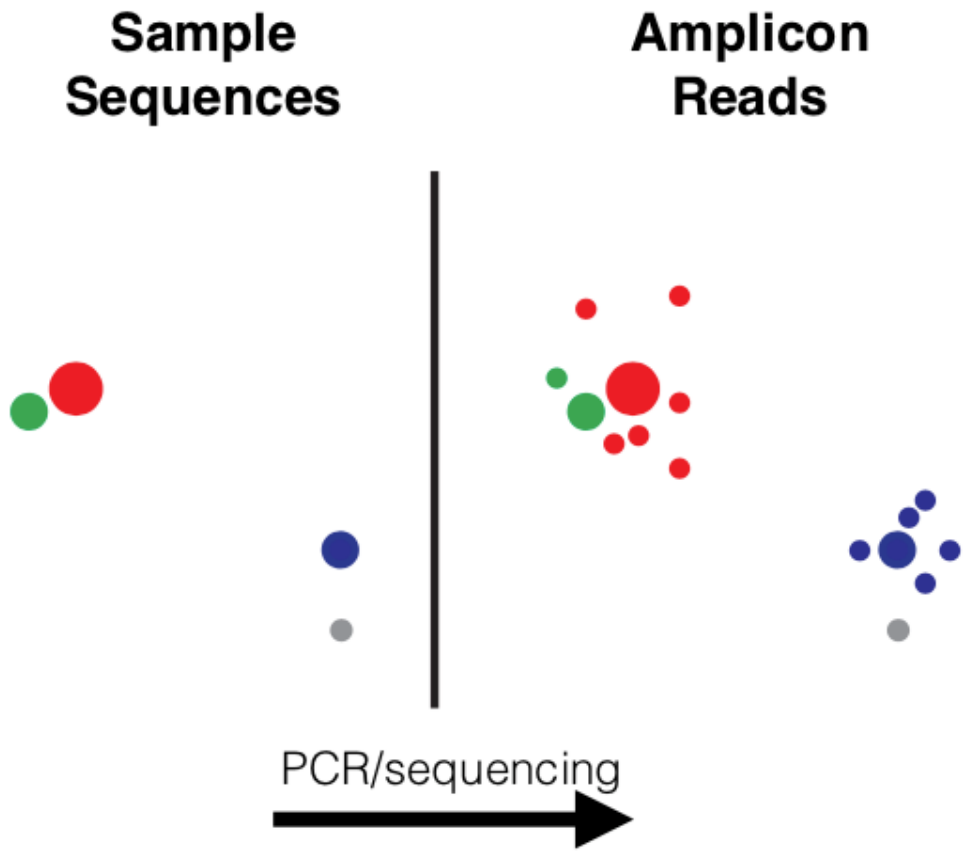


Marizzoni et al., 2020, Front. Microbiol.

Original sequences and amplified sequences

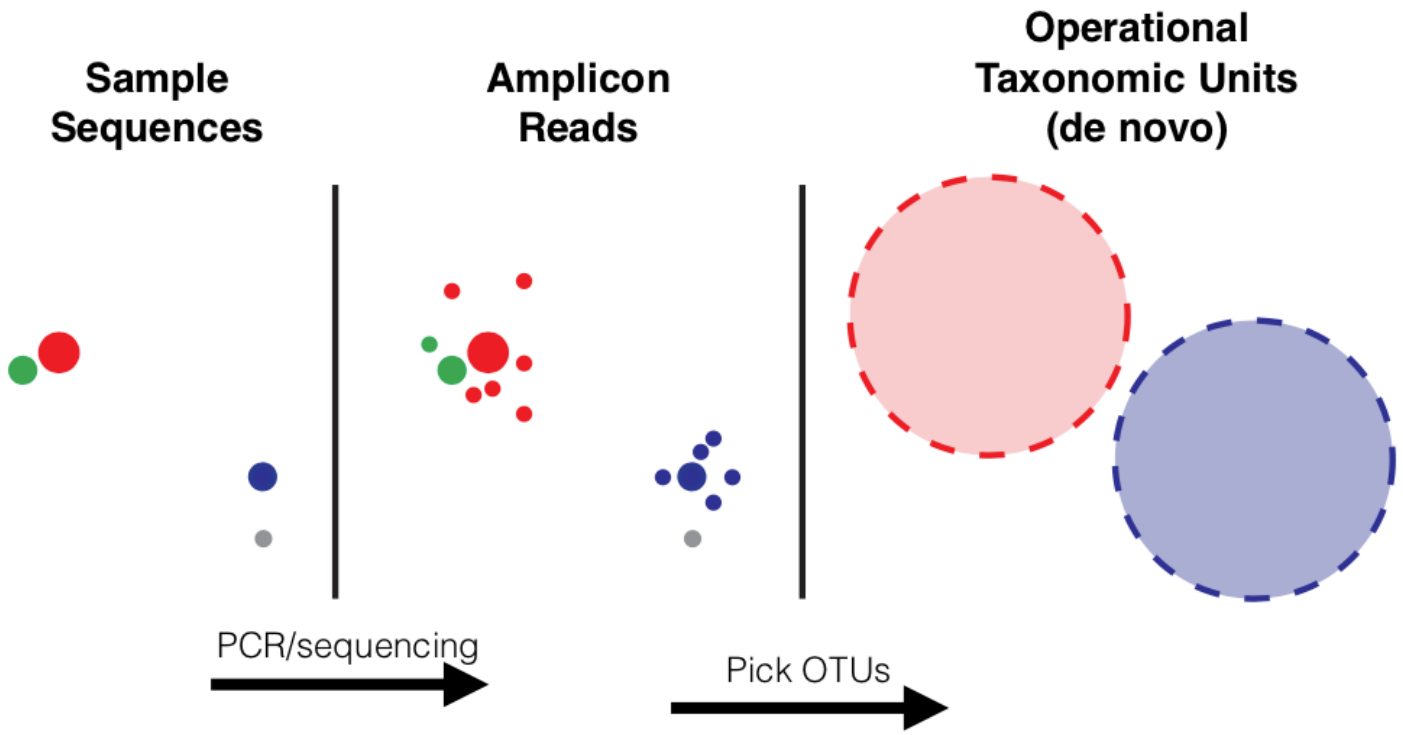
```
GCGGC TCAAC CGTAA AATTG CAGTT
GCGGC TCATC CGTAA AGTTG CAGTT
.....  ...Y.  .....  .R...  .....

GGTGC TTAAC CCTAA AATCG CAGTT
GGTGC TTAAC CGTAA AATCT CAGTT
.....  .....  .S...  ....K  .....
```



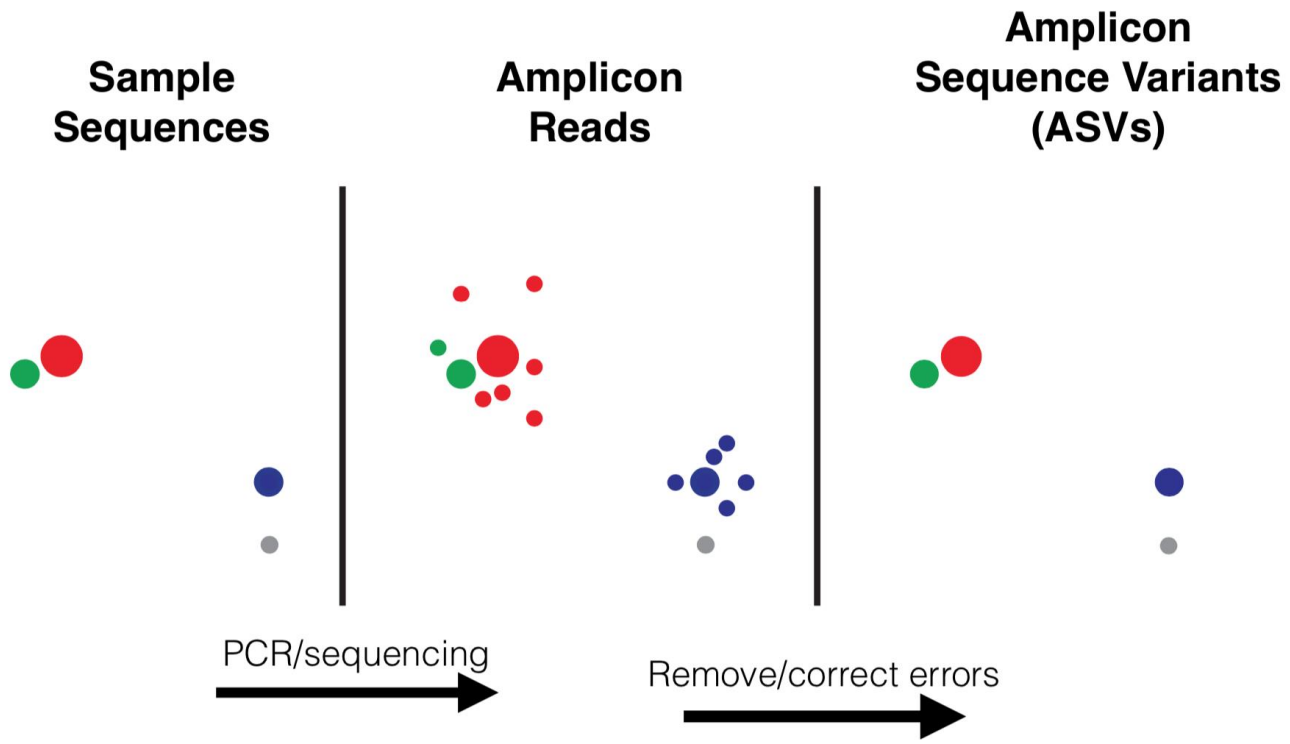
Operational Taxonomic Unit (OTU)

OTUs are defined as a cluster of sequences with an identity above a given threshold, often set to 97% .



Amplicon Sequence Variant (ASV)

‘True’ biological sequences after a denoising procedure to control for errors of the sequencing technology per batch/run.



OTUs vs ASVs

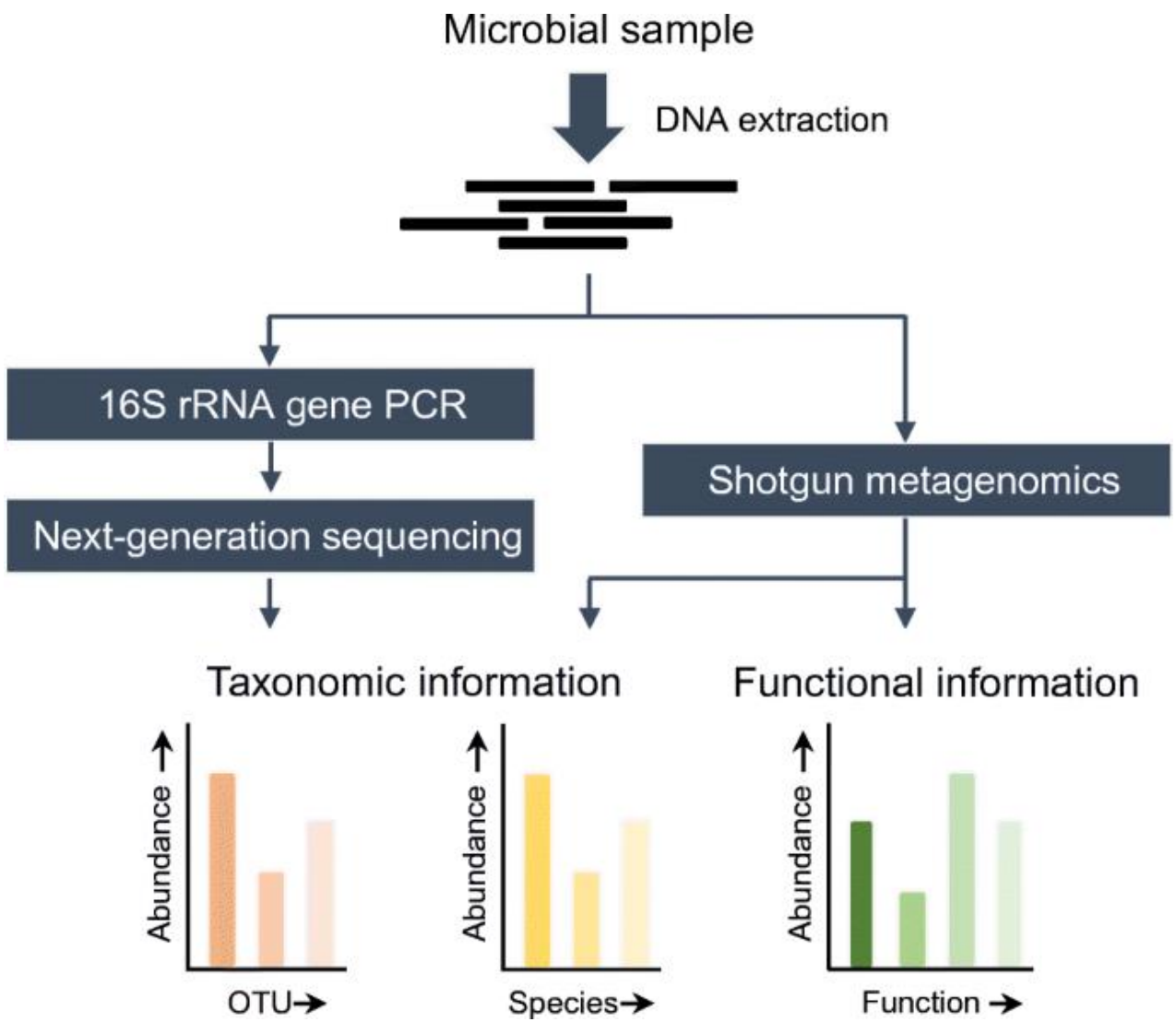
OUT @ 97%	ASV
Can be subject to reference bias	A reference is not used until after taxonomy assignment
OTU-tables cannot be combined between studies	ASV-tables can be combined across studies
Represented by a consensus sequence	Represented by an exact sequence
A consensus sequence can represent multiple species with different sequences	If it represents multiple species, it is because they share an identical sequence

Limitations and biases

Experimental	Mitigation
Step 1: sample collection	
Transport and storage conditions	Immediate freezing at − 20 °C or lower, followed by long-term storage at − 80 °C.
Step 2: DNA extraction	
Different methods	The same method should be used in a whole project
Step 3: PCR amplification	
No 16S rRNA gene PCR primer pair is truly ‘universal’ and different primer pairs may have different proportions of ‘conserved’ sequences.	The same method should be used in a whole project
All protocols are sensitive to contaminating DNA throughout the process	Negative (extraction) controls should be included
Step 4: Next-generation sequencing	
Short sequences (few hundred bases)	Keep method consistent within a project
Step 5: Bioinformatics analysis	
16S rRNA gene NGS results are generally presented as proportional abundances of OTUs/ASVs	The use of protocols that determine the absolute quantity of OTUs/ASVs improves the interpretation.

Adapted from https://link.springer.com/article/10.1007/s10096-019-03520-3

Limitations and biases



Take home message

- There are numerous options for analysis of amplicon sequencing, be consistent within a project.
- Whichever method is selected, be aware of the limitations and acknowledge them as a part of your discussion.
- There are multiple free web resources/pipelines for omics data processing, filtering and analysis, be aware of default settings and options as they may not be comparable from tool to tool.