16s Microbiome Analysis workshop

International Microbiome Centre

October 7-9, 2019

Disclaimer: These slides are taken from difference sources on the web and some are modified. I have added sources but if I have missed some, I apologize in advance to the original content creators.

THE HUMAN MICROBIOME PROJECT

CONTAINS **10** TIMES **MORE** MICROBIAL CELLS THAN HUMAN CELLS.

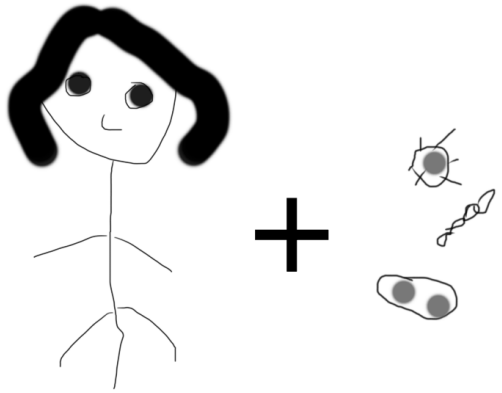
MICROORGANISMS, THEIR GENOMES & ENVIRONMENTAL INTER ACTIONS

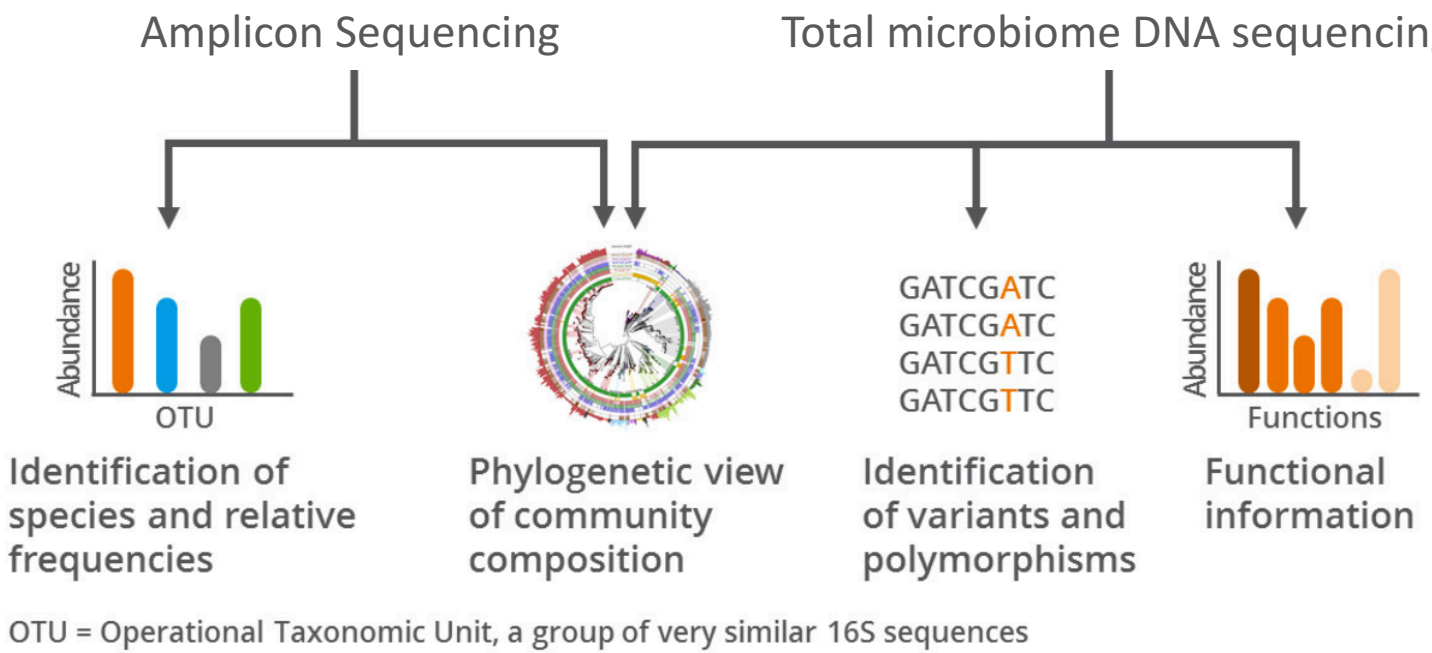
250 HEALTHY PEOPLE

SPOTS SAMPLED:

Who lives here?

Over time + Treatment



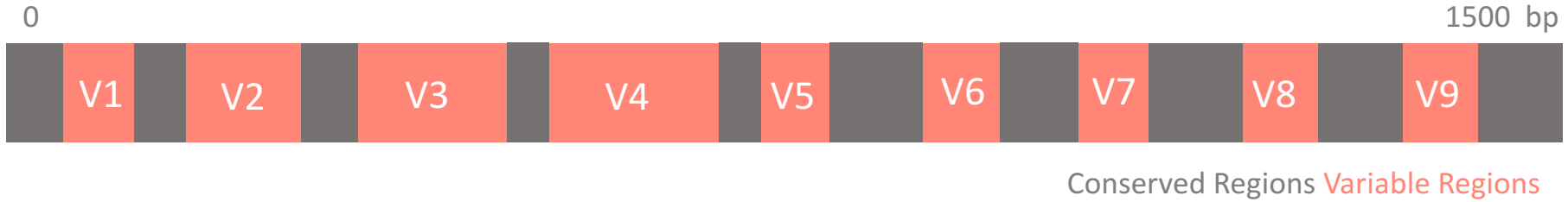


https://www.gatc-biotech.com/en/expertise/targeted-sequencing/16s-rrna-analysis.html

Amplicon Sequencing

What should we amplify?

The Small Subunit ’16s’ ribosomal RNA gene

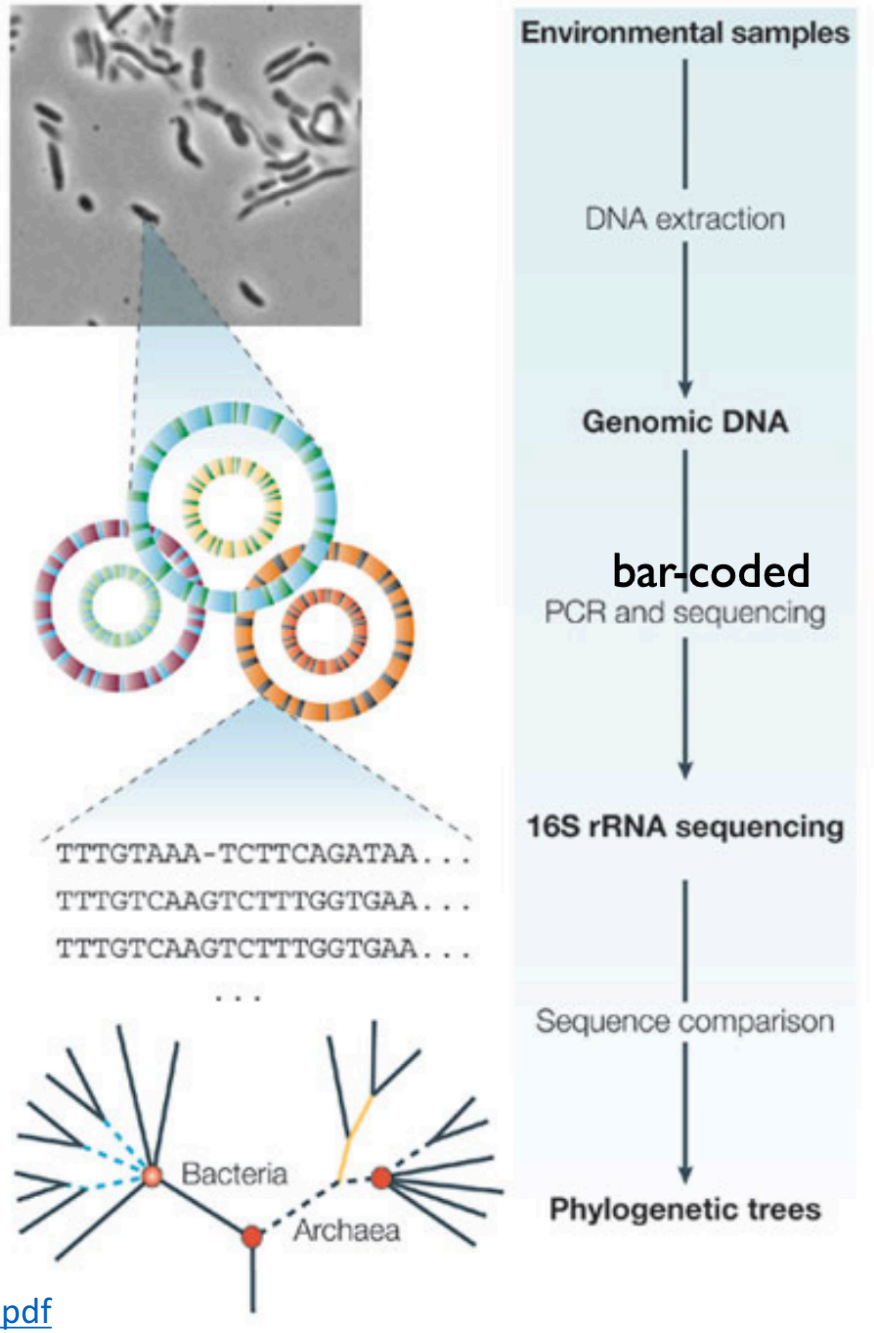


Because this gene is conserved and found in most bacteria, it is used for reconstructing phylogeny.

Depending on the hypervariable region picked, you can classify to different level of taxonomy

Many microbiomes in parallel

1. Break all cells, extract all DNA
2. PCR-amplify 16s rRNA genes using bar-coded primers
3. Sequence samples
4. Cluster sequences after De-multiplexing for each sample
5. Count each species



https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

Any pitfalls ?

Bioinformatics to the rescue !!!

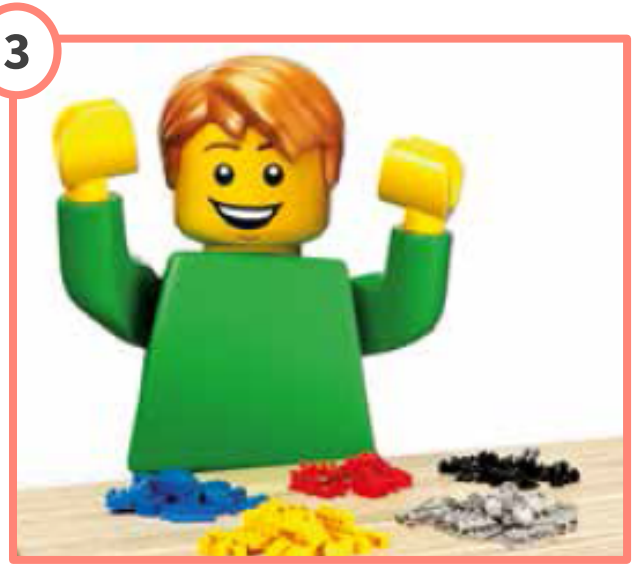
1

Sequencing results

2

Woah! That is a lot of data

3



Bioinformatics Tools

4

Biological Knowledge

Source: LEGO+Johnathan Irish

Thoughtful data analysis is critical for successful taxonomic assignment

What do we know about our data?

16s Region

Which region was sequenced, read length & depth

Negative Controls

Was a negative control included?

Feasibility

Will this data answer the questions asked by the investigator?

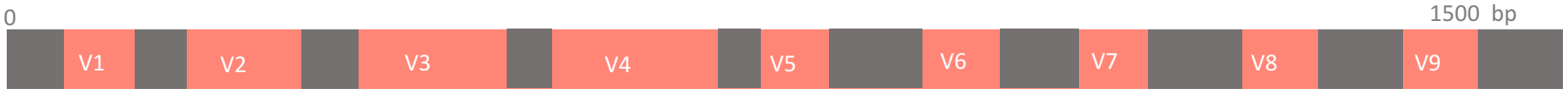
Read Assignment

Are the reads assigned correctly to each sample?

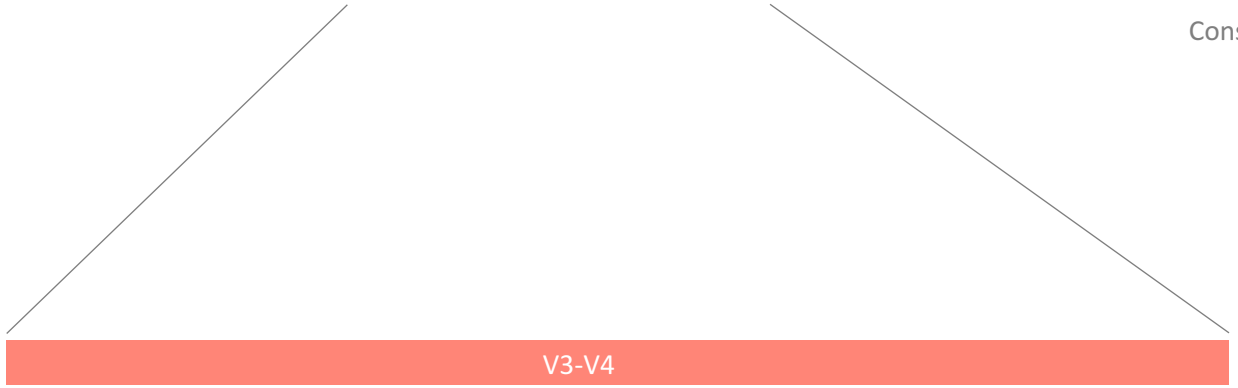
Sample size

Are there enough samples for down stream analysis

16 rRNA



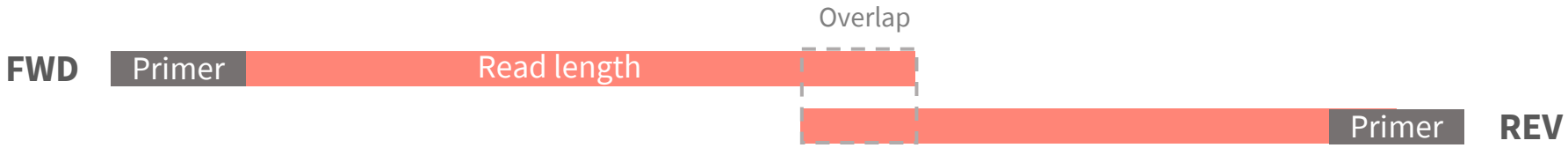
Pick a variable region



Pick a sequencing technique

Illumina (MiSeq)

Select primers & Read lengths

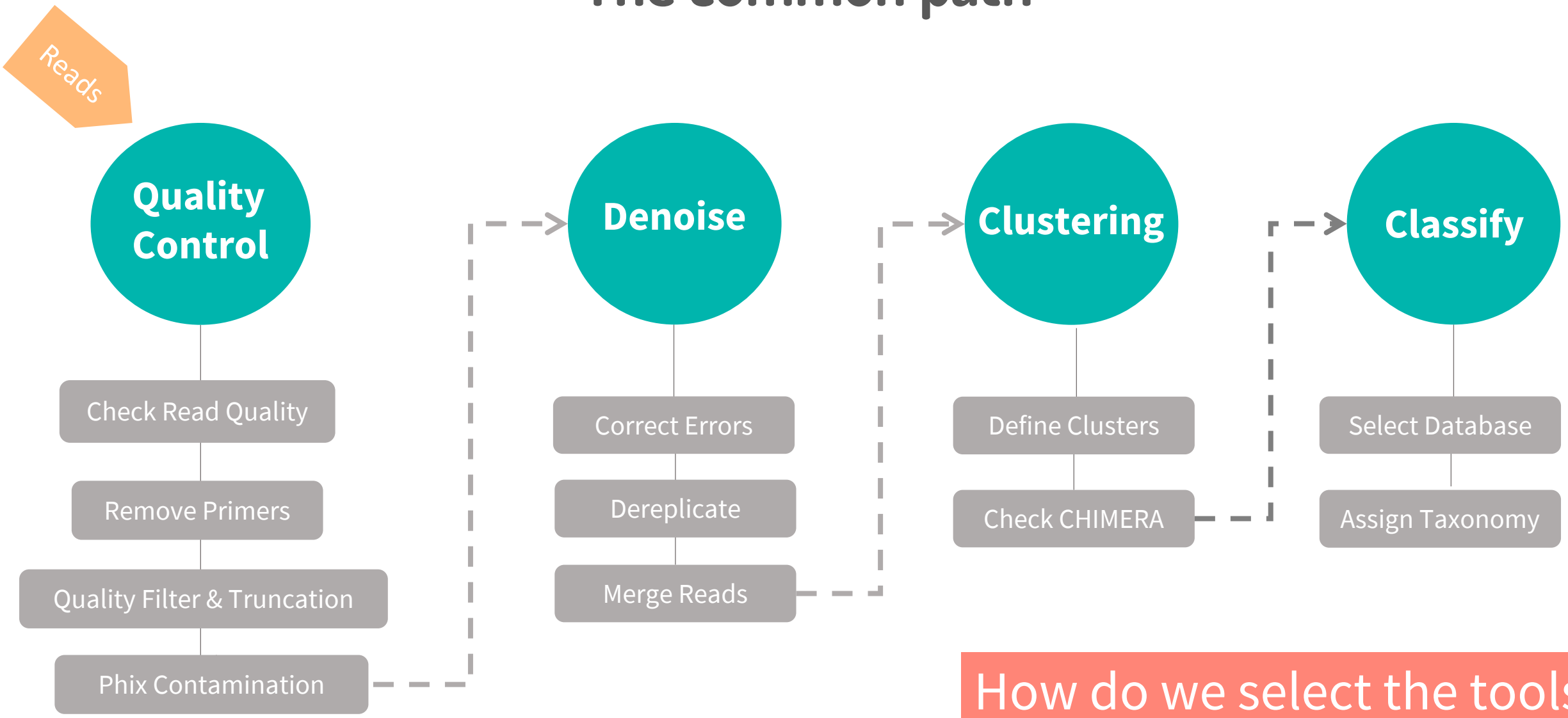


Make sure there is an overlap!!!!

Always have a blank sample sequenced with your samples

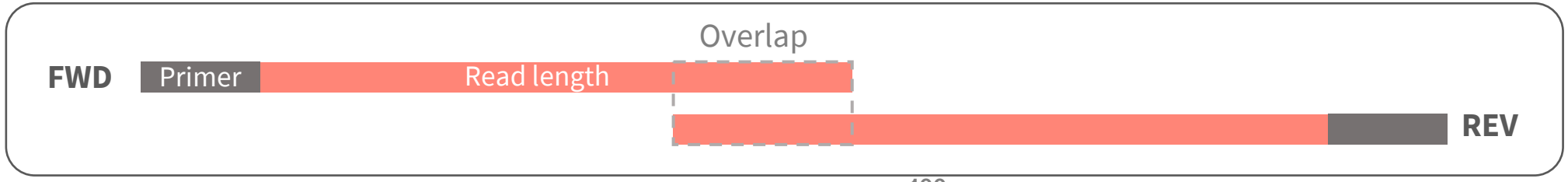
Typical Workflow

The common path

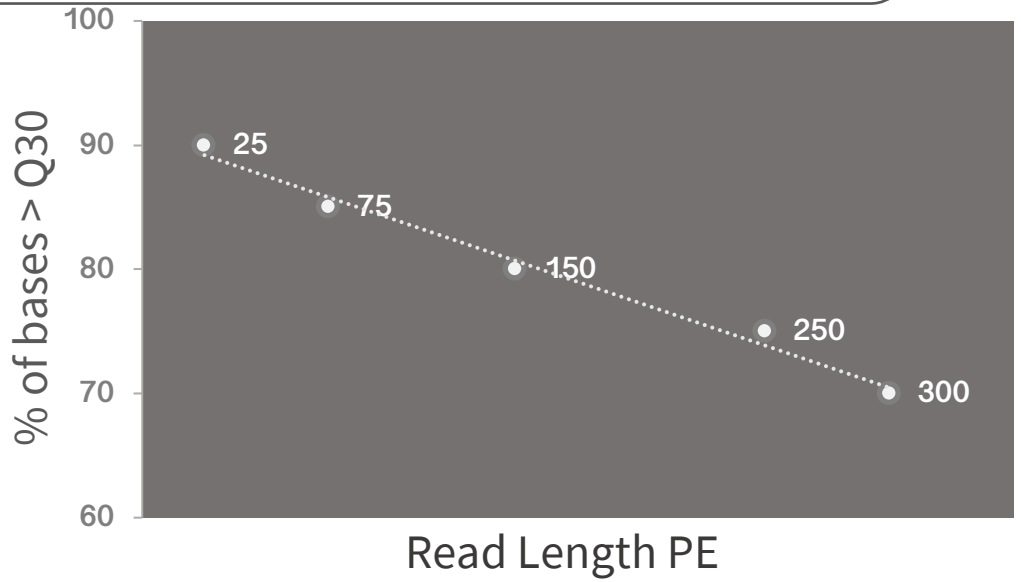


How do we select the tools and use them well ?

Paired End Reads



Region	Read length	Amplicon Length	Overlap
V3	150	~170	130
V3-V4	300	~462	133
V4	150	~254	46
V4	250	~254	246



Source: Illumina

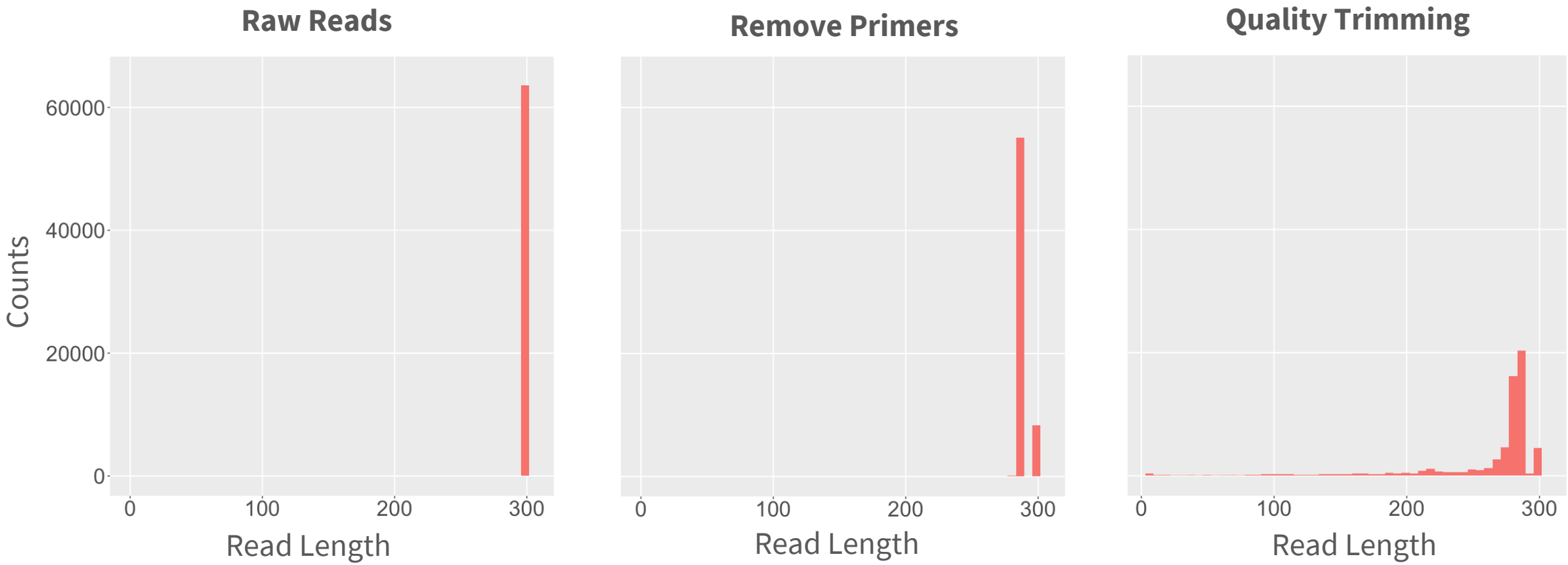
Base Quality differs between Fwd and Rev Reads

Tools

FastQC FastQp MultiQC & Specialized 16s rRNA packages

Source: Dada2 R package

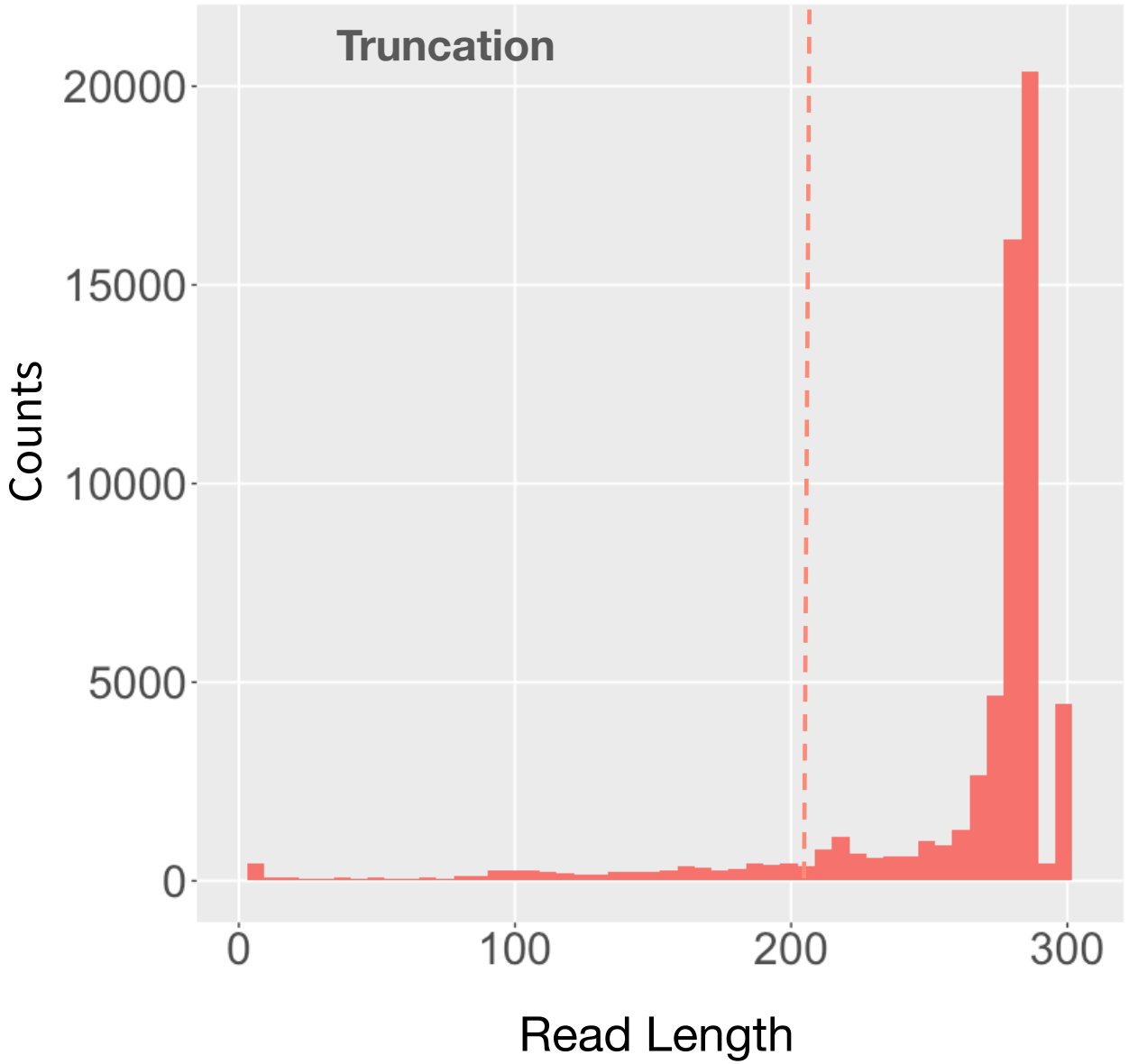
Trimming & Quality Filtering



Tools

CutAdapt

Trimming & Quality Filtering



Available Methods

- Minimum Q (Q >= 20)
- Truncate if 3 consecutive bases are Q < 3
- Expected Errors

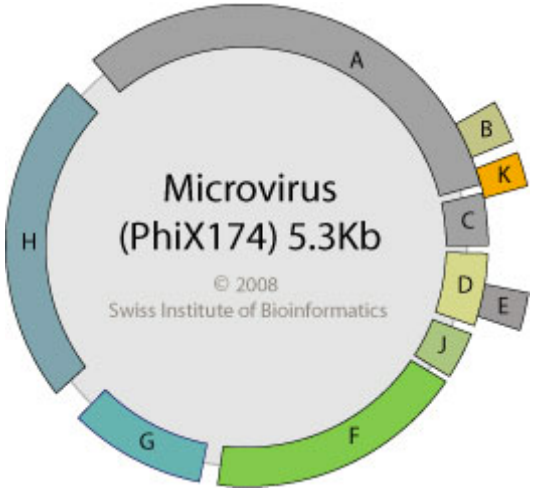
Read length	% of reads
10	98.96
100	96.97
200	89.3
250	80.5

Source: Edgar et al., 2015

Why PhiX?

Low diversity

Significant number of reads have the same sequence



Quality Control

Cluster Generation
Phasing & Prephasing

Source: Illumina

Caution

PhiX Contamination

Illumina removes PhiX Assigned to samples (1%-12%)

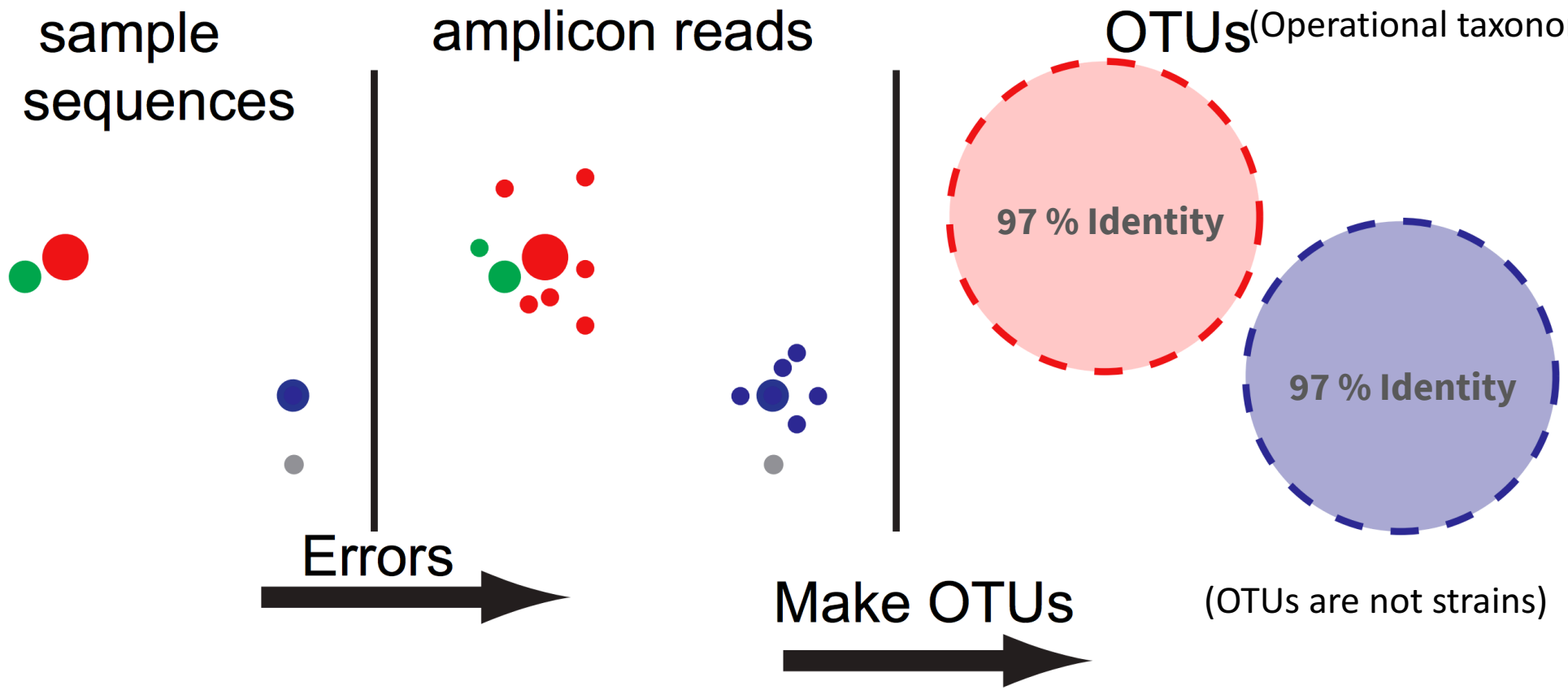
Removal

Blast sample reads against PhiX genome

Source: Mukherjee et al.,2015

Denoising reads

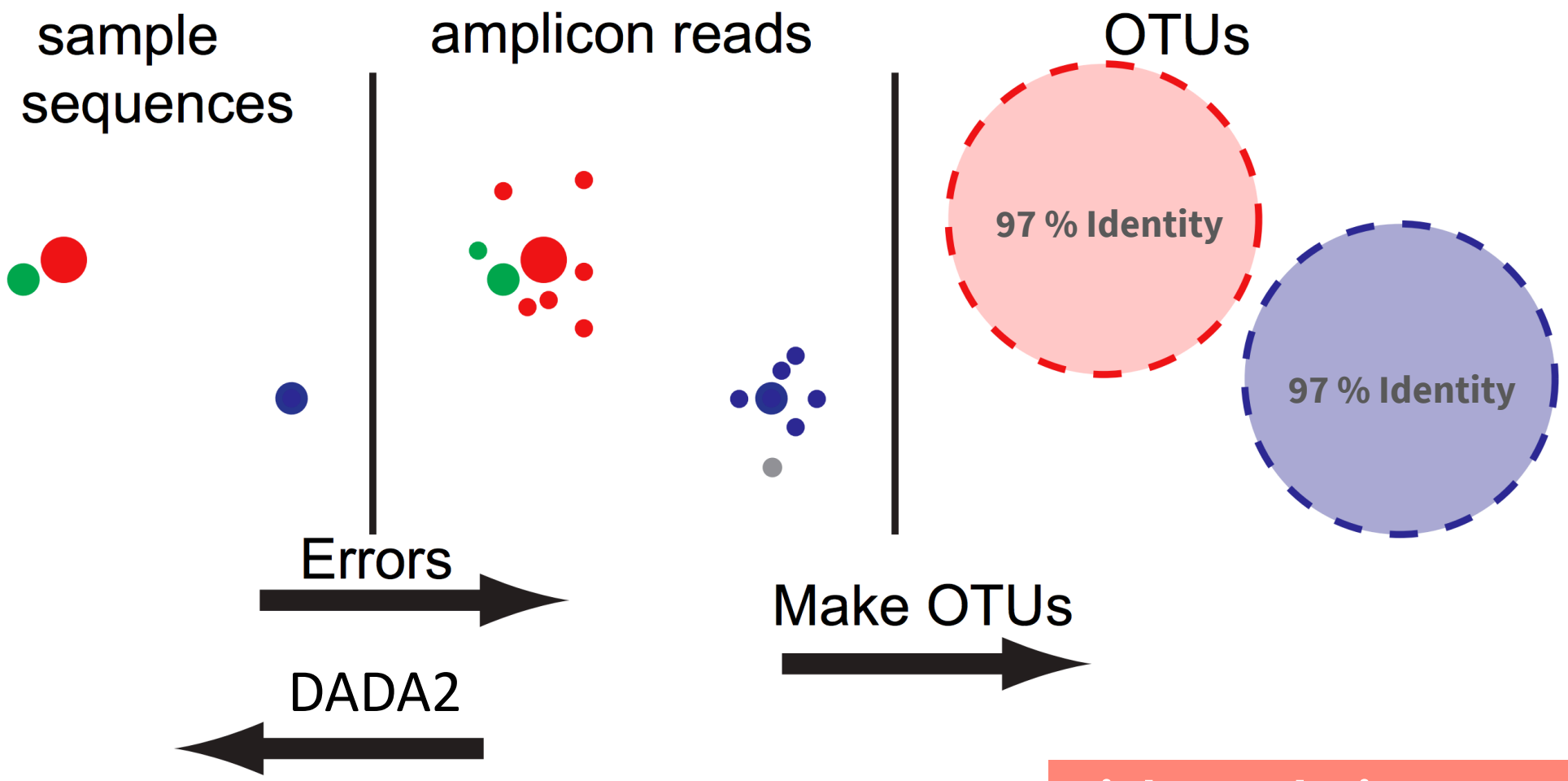
Clustering



OTUs: Lump similar sequences together
DADA2: Statistically infer the sample sequences (Amplicon sequence variants: ASVs)

Source: Callahan et al.,2016

Clustering

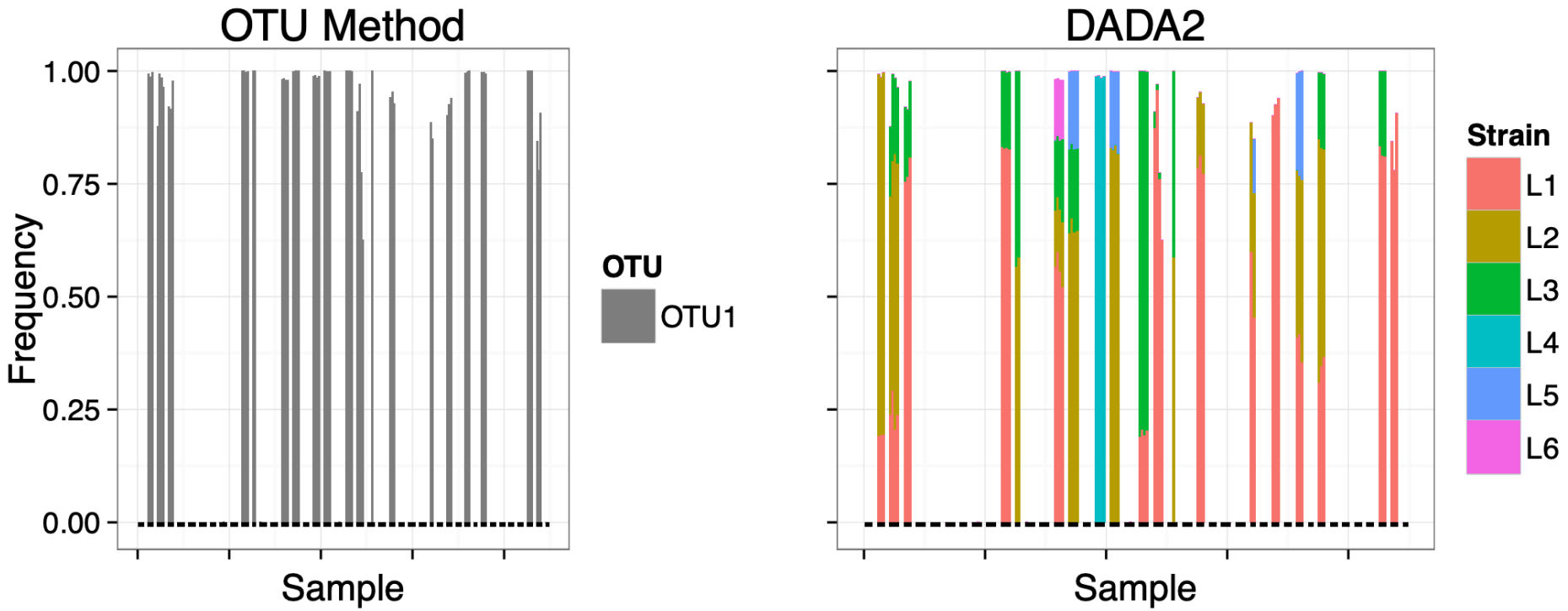


High Resolution & Accuracy

Source: Callahan et al.,2016

Real example, exact sequence resolution

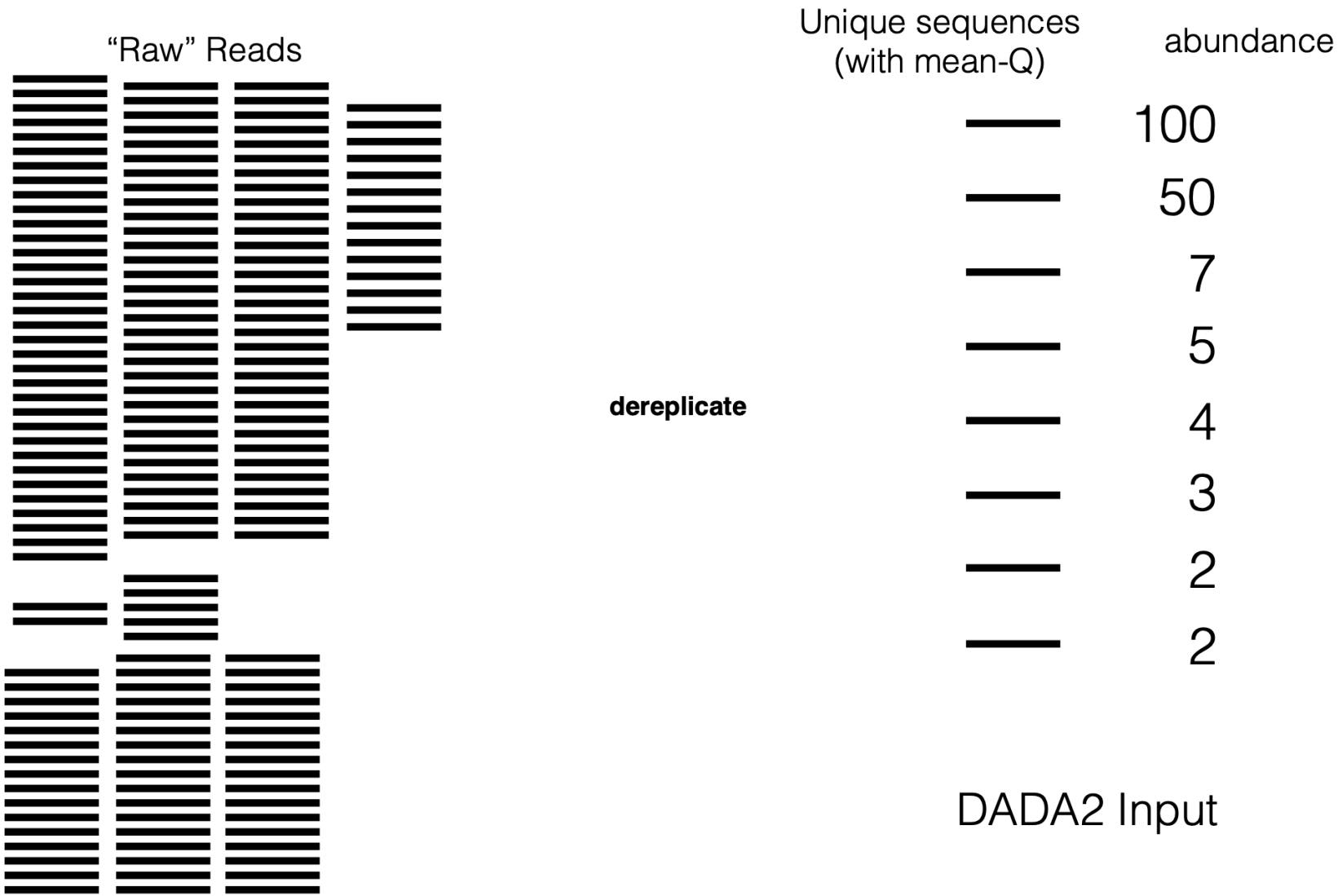
Lactobacillus crispatus sampled from
vaginal microbiome 42 pregnant women



Data: MacIntyre et al. Scientific Reports, 2015.

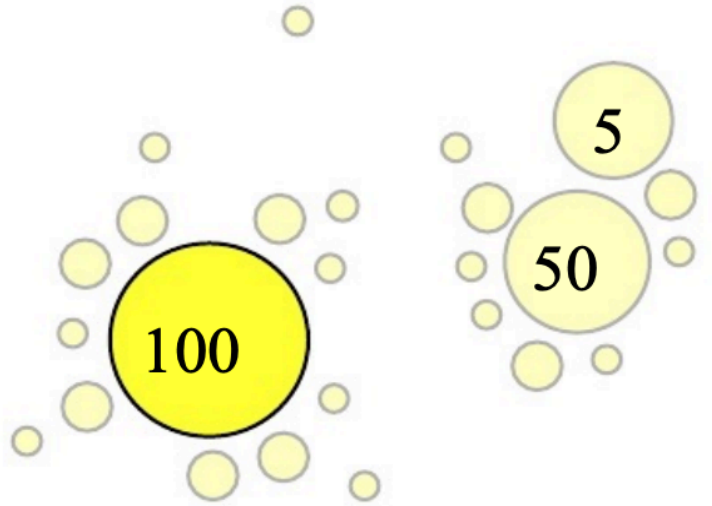
DADA2 algorithm cartoon

Input: unique sequences, their quality values, and abundances



https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

DADA2 algorithm cartoon



Infer initial *error model* under this assumption.

$$\Pr(i \rightarrow j) =$$

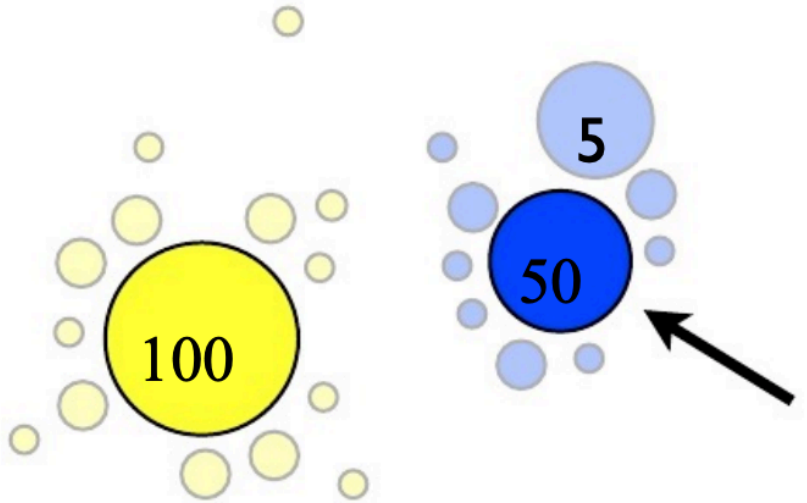
	A	C	G	T
A	0.97	10^{-2}	10^{-2}	10^{-2}
C	10^{-2}	0.97	10^{-2}	10^{-2}
G	10^{-2}	10^{-2}	0.97	10^{-2}
T	10^{-2}	10^{-2}	10^{-2}	0.97

Two models:

- Error Model
- Abundance model (p-value

https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

DADA2 algorithm cartoon



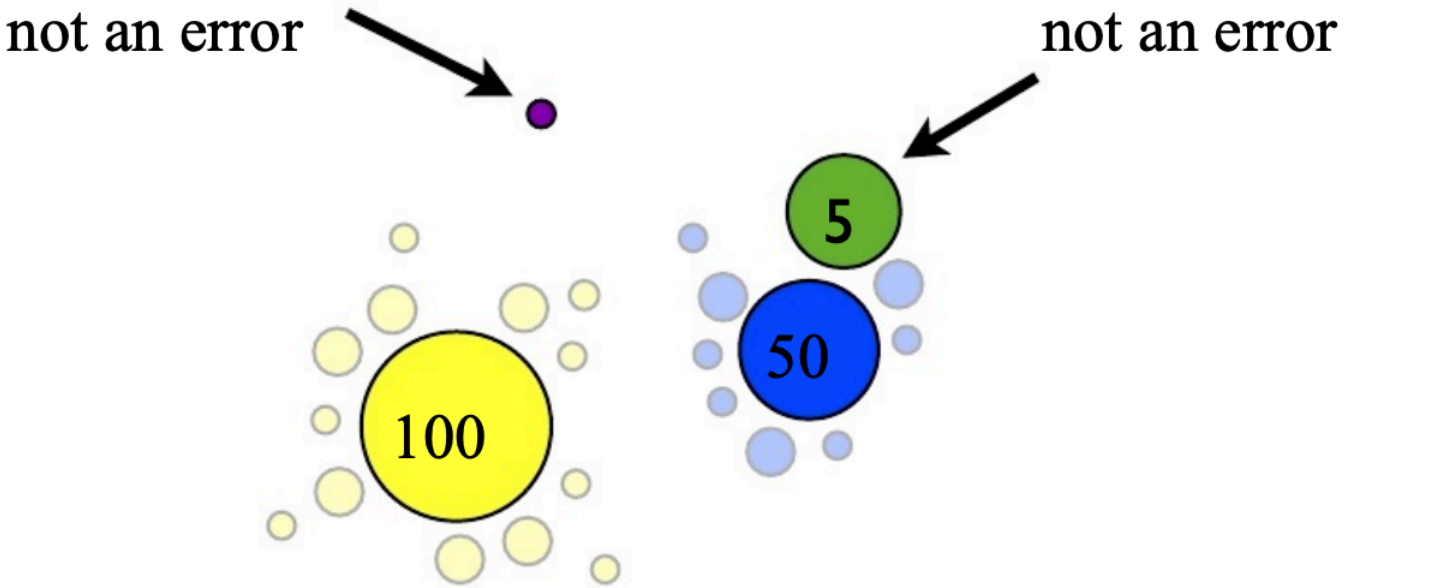
not an error

Reject unlikely error under model. **Recruit** errors.

	A	C	G	T
A	0.97	10^{-2}	10^{-2}	10^{-2}
C	10^{-2}	0.97	10^{-2}	10^{-2}
G	10^{-2}	10^{-2}	0.97	10^{-2}
T	10^{-2}	10^{-2}	10^{-2}	0.97

https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

DADA2 algorithm cartoon

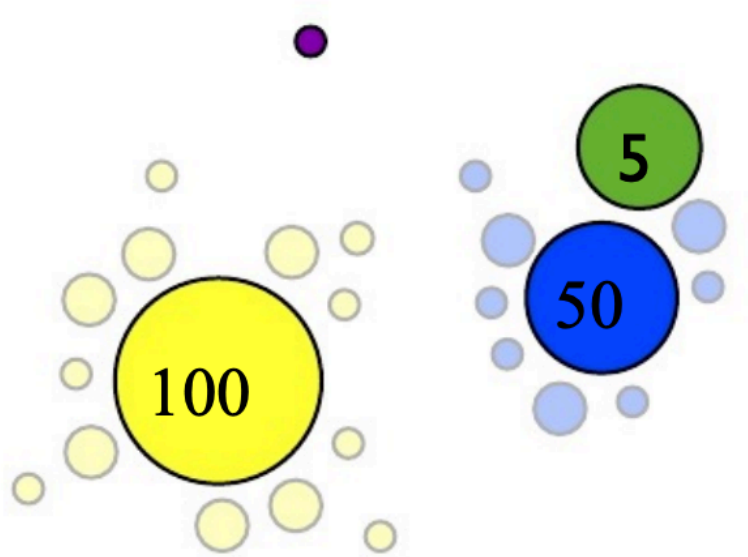


Reject more sequences under ***new*** model

	A	C	G	T
A	0.997	10^{-3}	10^{-3}	10^{-3}
C	10^{-3}	0.997	10^{-3}	10^{-3}
G	10^{-3}	10^{-3}	0.997	10^{-3}
T	10^{-3}	10^{-3}	10^{-3}	0.997

https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

DADA2 algorithm cartoon

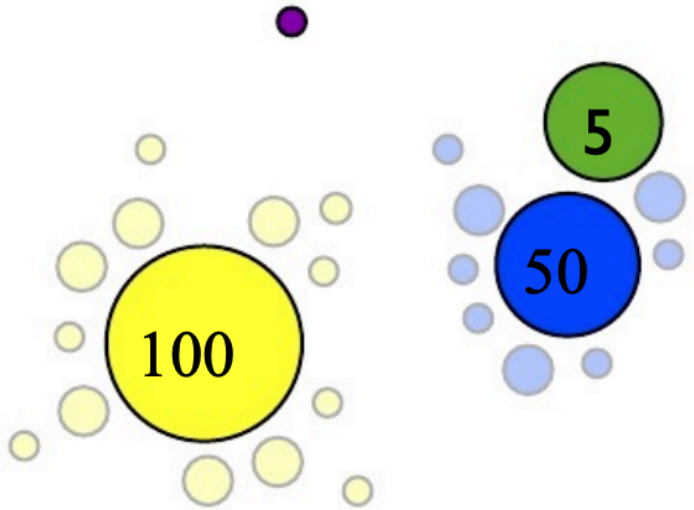


Update model again

	A	C	G	T
A	0.998	$1x10^{-4}$	$2x10^{-3}$	$2x10^{-4}$	
C	$6x10^{-5}$	0.999	$3x10^{-6}$	$1x10^{-3}$	
G	$1x10^{-3}$	$3x10^{-6}$	0.999	$6x10^{-5}$	
T	$2x10^{-4}$	$2x10^{-3}$	$1x10^{-4}$	0.998	

https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

DADA2 algorithm cartoon

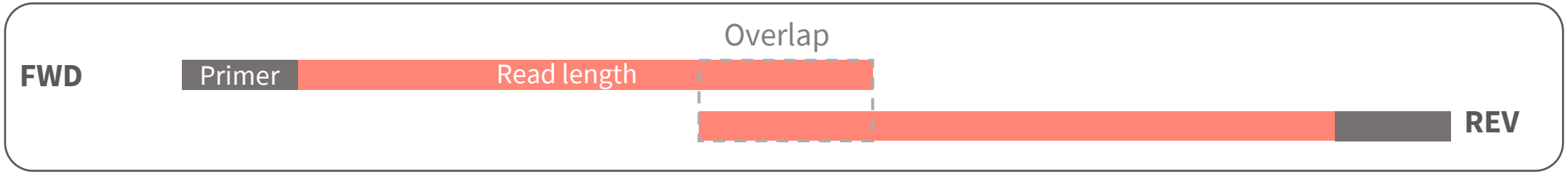


Convergence: all errors are plausible

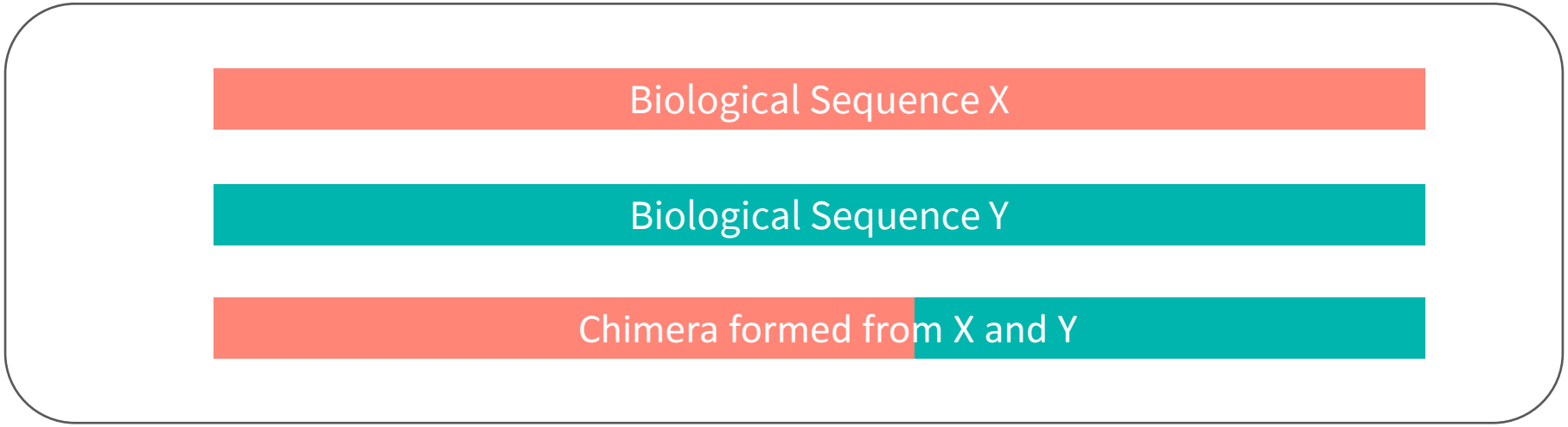
	A	C	G	T
A	0.998	$1x10^{-4}$	$2x10^{-3}$	$2x10^{-4}$	
C	$6x10^{-5}$	0.999	$3x10^{-6}$	$1x10^{-3}$	
G	$1x10^{-3}$	$3x10^{-6}$	0.999	$6x10^{-5}$	
T	$2x10^{-4}$	$2x10^{-3}$	$1x10^{-4}$	0.998	

https://www.biostat.washington.edu/sites/default/files/modules/2018-SISMID-01b.pdf

Merge Paired End Reads



Chimeric Sequences



Created during PCR

Fragment primes different extension

About 1-5 % of reads are chimeric

Source: Lahr & Katz, 2009

Important:

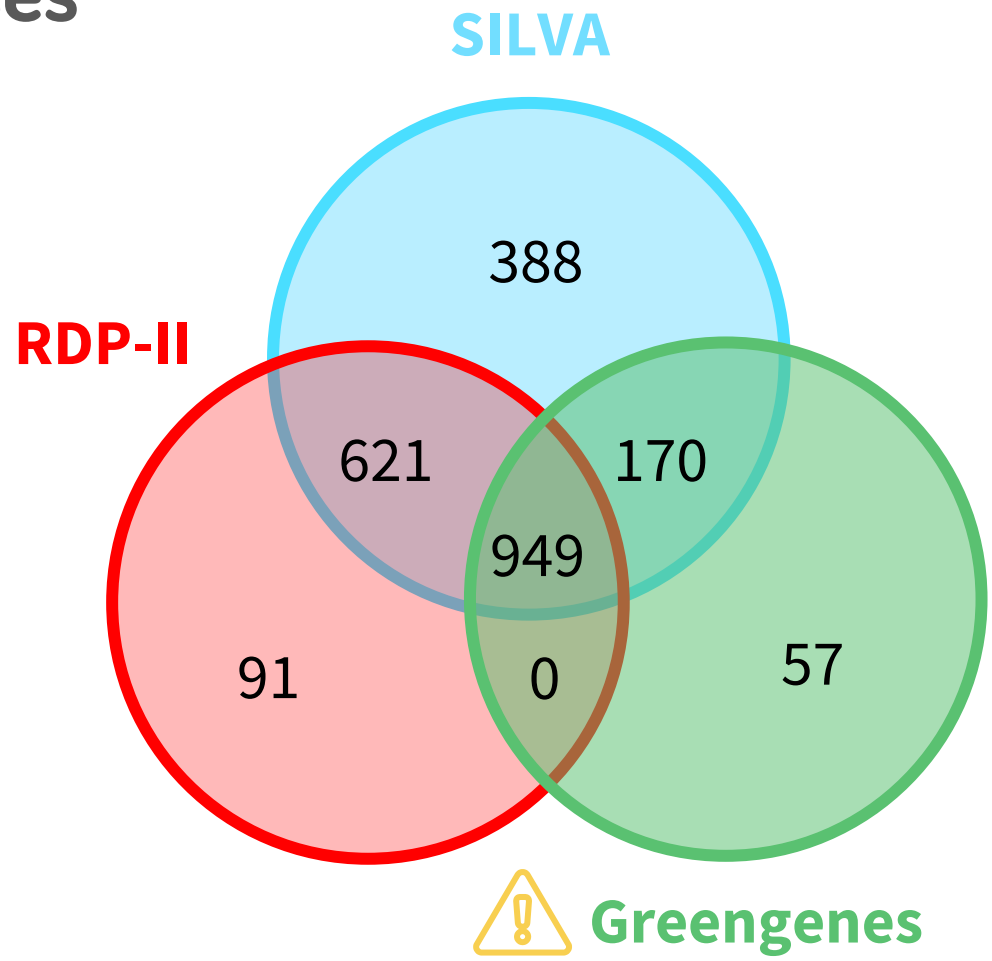
DADA2 gives us Amplicon sequence Variants (ASVs) not operational taxonomic units OTUs

Reference Databases
&
Taxonomy Assignment

Reference Databases

Taxonomy	Type	No. of nodes	Lowest rank	Latest release
SILVA	Manual	12,117	Genus	Dec 2017
RDP-II	Semi	6,128	Genus	Sep 2016
Greengenes	Automatic	3,093	Species	May 2013
GTDB	Automatic	143,512	Species	Today[a]

Source: Balvočiūtė et al 2017



Source: Yilmaz et al 2013

Use a small set of authoritatively classified sequences such as RDP training set or SILVA LTP subset

Taxonomy Assignment

	Alignment-Based	Composition-Based
Method of reference	Sequence Alignment	Shared Feature Vectors (K-mers)
Data used for classification	Reference Sequences	Shared Feature Vectors & probability of taxonomic inclusion
Taxonomic Inference	Identity thresholds	Quantity or proportion of feature vectors shared between reference and query sequence
Available Tools	MEGAN5, RTAX	RDP Classifier, UTAX

Source : Richardson et al., 2016

SUGGESTIONS

Length & Depth
Know which region, read length & depth suits your experiment

Negative Controls
Helps identify contaminants

Sample size
Are there enough samples for down stream analysis

Denoise (DADA2)
Remove errors

Avoid C.R. Clustering
Unless you have a very good reason, avoid.

Be consistent
It is much better to use the same methods than to change methods frequently

Know Why
Understand why you are using a tool or method

"If you **torture** the data long enough, it will confess."

– Ronald Coase, *Economist*