UNIVERSITY OF CALGARY | International Microbiome Centre

Microbiome Workshop

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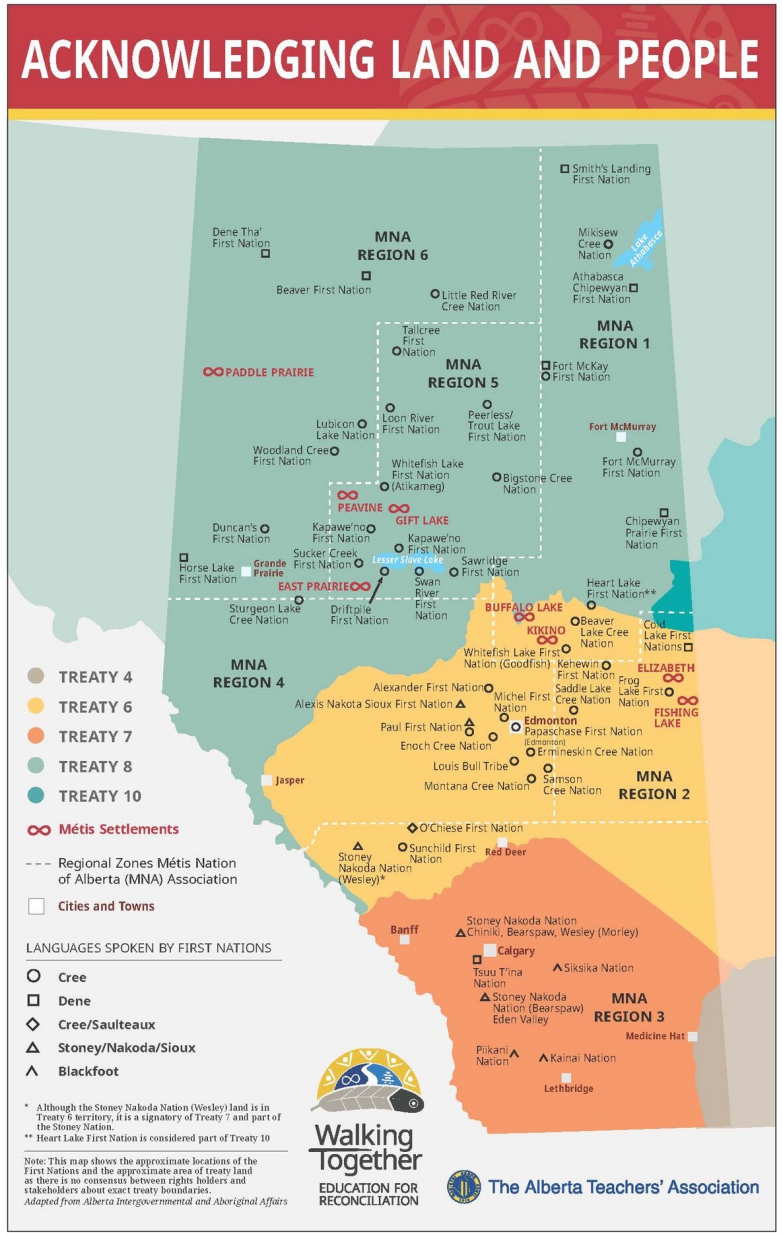
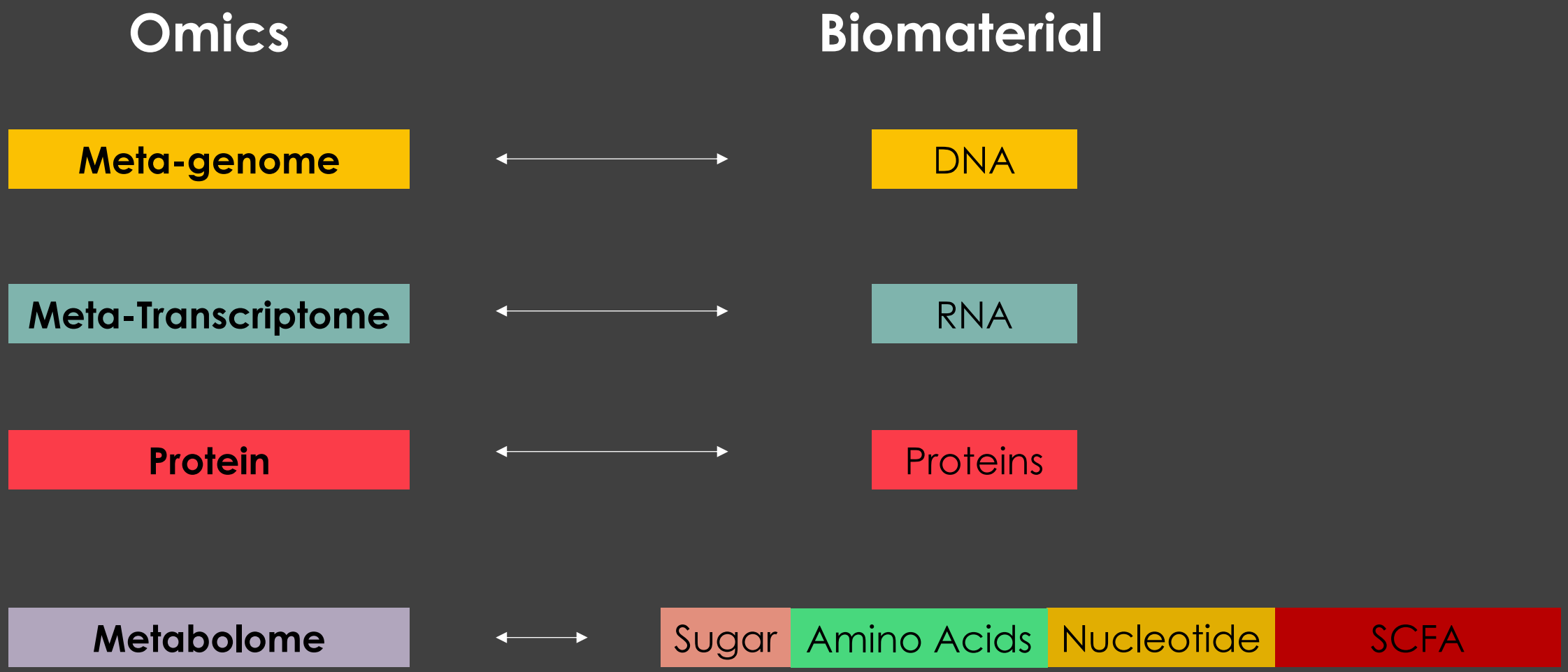
@henaramay

I'd like to acknowledge that we are on Treaty 7 territory, the traditional territories of the Blackfoot Nations, including Siksika (Sick-sick-ah), Piikani (Pee-can-ee), and Kainai (Kigh-a-nigh), the Tsuut'ina (Soot- ina), Nation and Stoney Nakoda First Nations. We acknowledge all the many First Nations, Métis, and Inuit whose footsteps have marked these lands for centuries.



Why do we care about microbes?

How are they communicating with us?

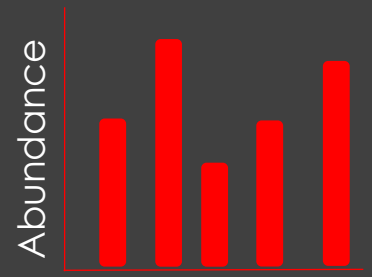


Source: https://slideplayer.com/slide/16950853/

Meta-genome

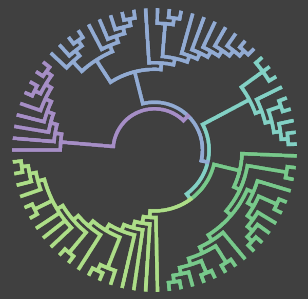
Shotgun Sequencing Output

Amplicon Sequencing Output



ASV

Taxonomy

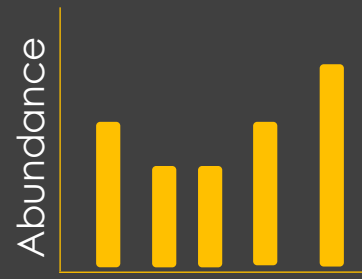


Phylogeny

\+

GATCGATC
GATCGATC
GATCCATC
GATCCATC

Polymorphism



Functions

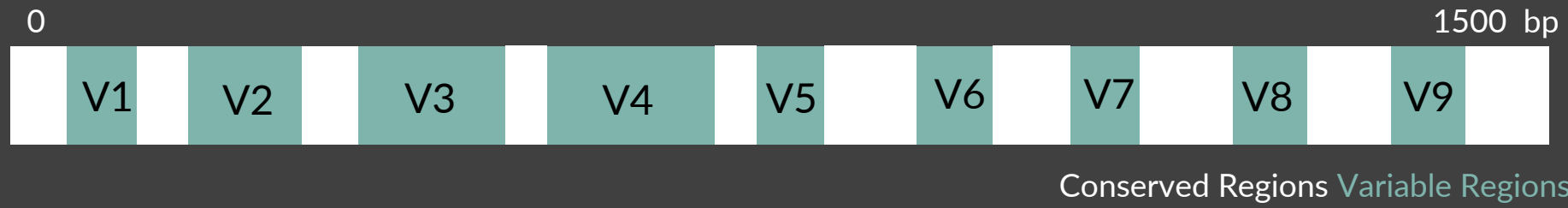
Functional Annotation

ASV: Amplicon Sequence Variant

Amplicon Sequencing

What should we amplify?

The Small Subunit 16s ribosomal RNA gene



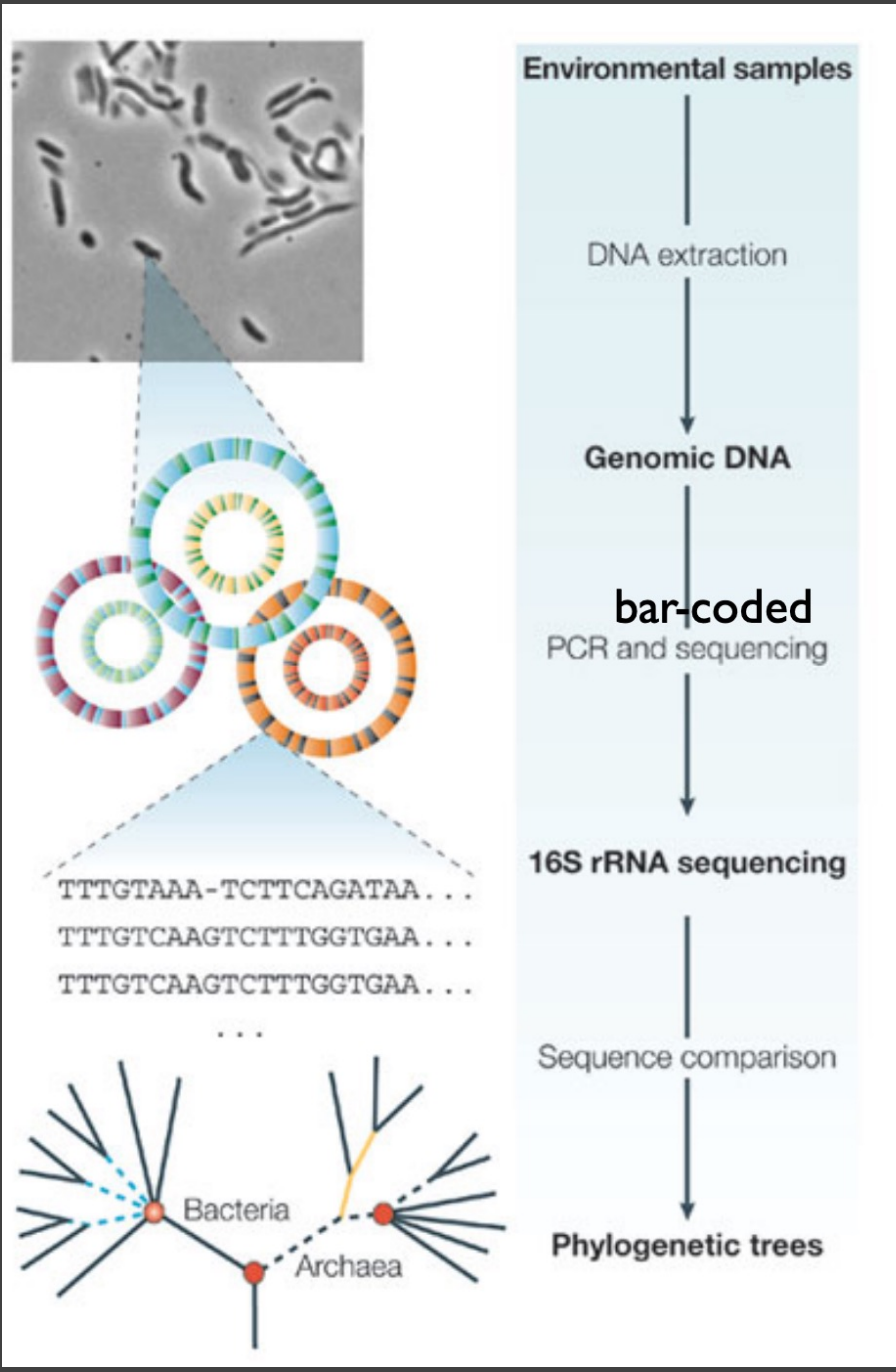
Highly conserved gene found in most bacteria

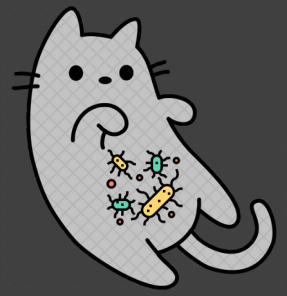
A variety of hypervariable region

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





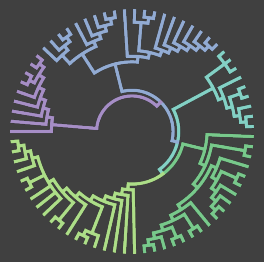
Amplify 16s region

ASV Table

	Sample 1	Sample 2	Sample 3
ASV 1	0.5	0.4	0.6
ASV 2	0.2	0.3	0.1
ASV 3	0.2	0.1	0.2
ASV 4	0.1	0.2	0.1

Amplicon Sequencing Cycle

Bacteria + Archaea



Phylogeny

Samples

ASV 1 = Bug X

ASV: Amplicon Sequence Variant is a sequence detected with a certain abundance in one or more samples

Typical Workflow

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

Controls
Did you use negative (extraction) and positive (mocks) ?

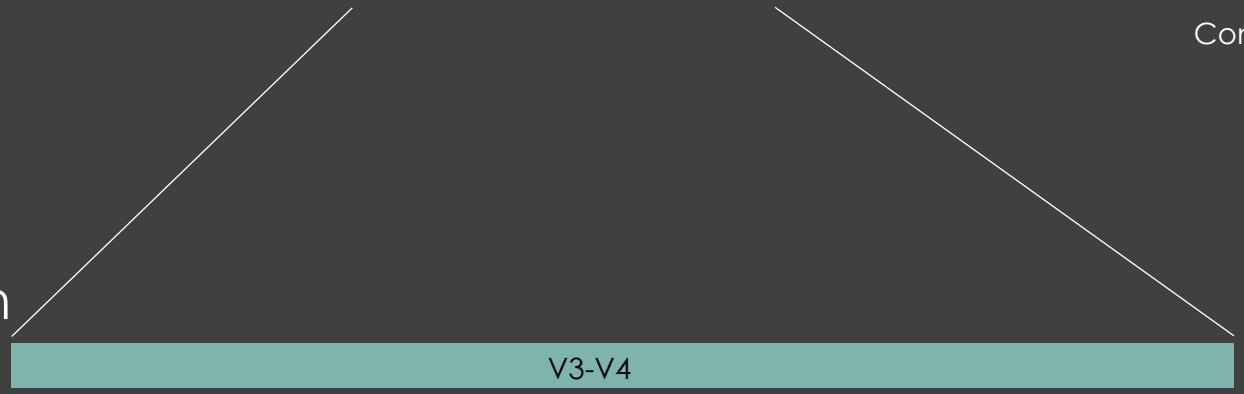
Feasibility
Will this data answer the questions asked by the investigator?

Read Assignment
Are the reads assigned correctly to each sample?

Sample size
Are there enough samples for down stream analysis

16 rRNA

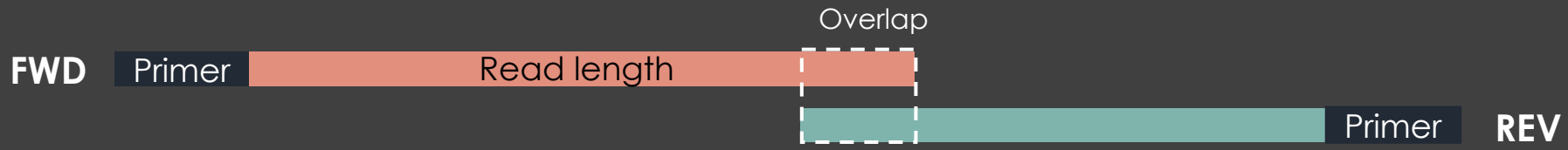
Pick a variable region



Pick a sequencing technique

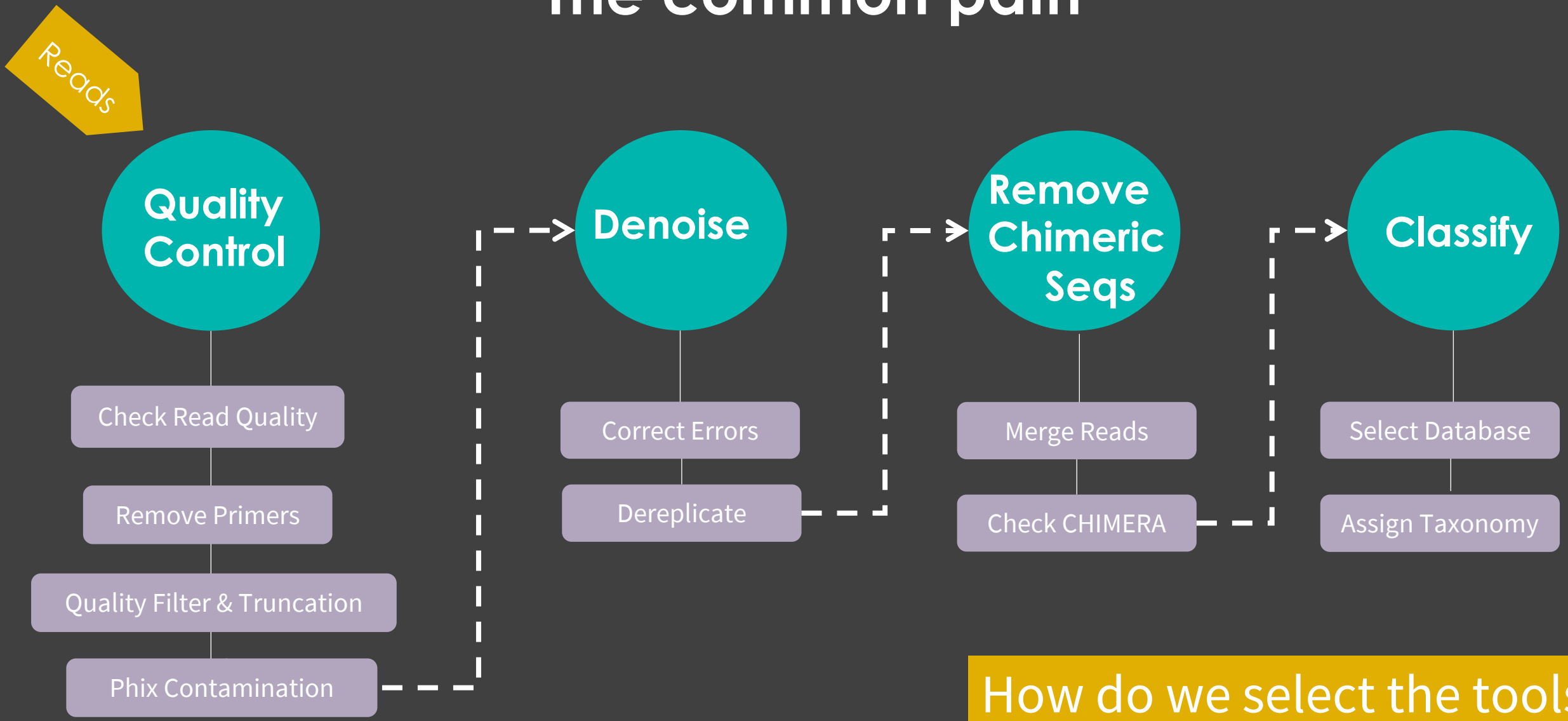
Illumina (MiSeq)

Select primers & Read lengths



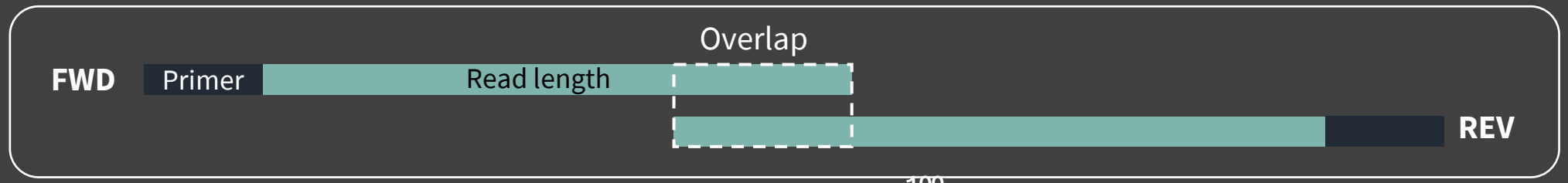
Make sure there is an overlap!!!!

The common path

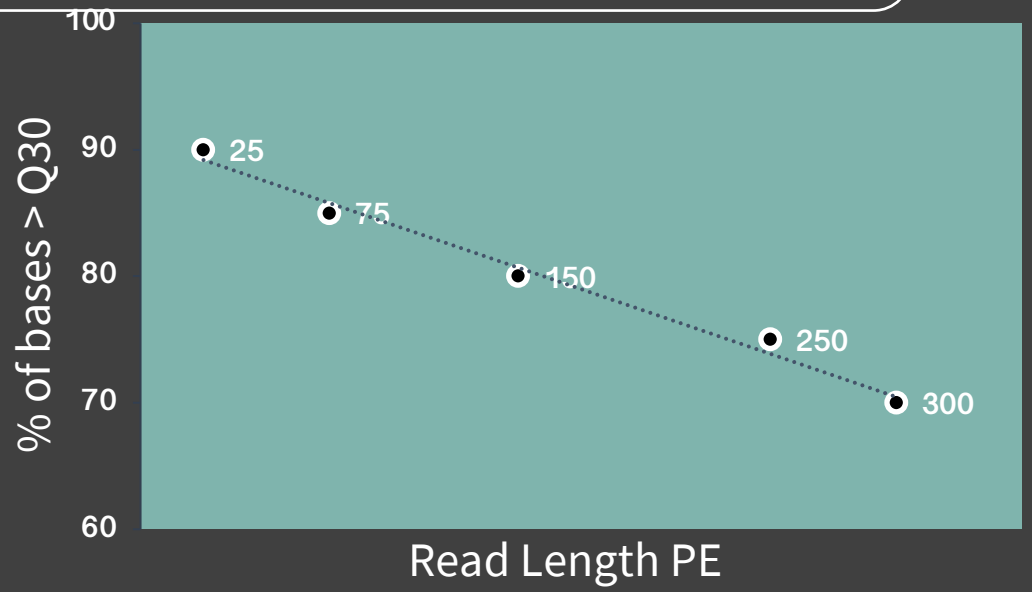


How do we select the tools and use them well ?

Paired End Reads

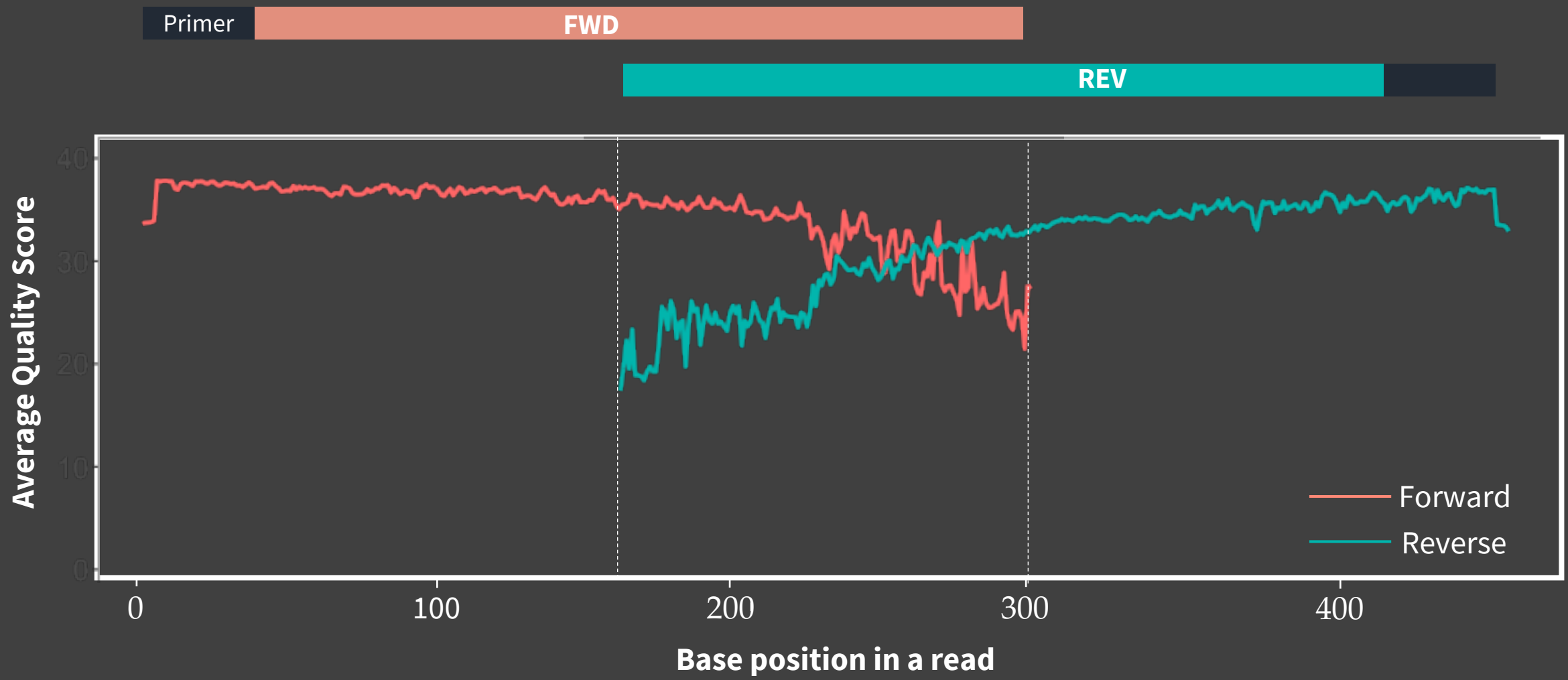


Region	Read length	Amplicon Length	Overlap
V3	150	~170	130
V3-V4	300	~462	133
V4	150	~254	46
V4	250	~254	246



Source: Illumina

Base Quality differs between Fwd and Rev Reads

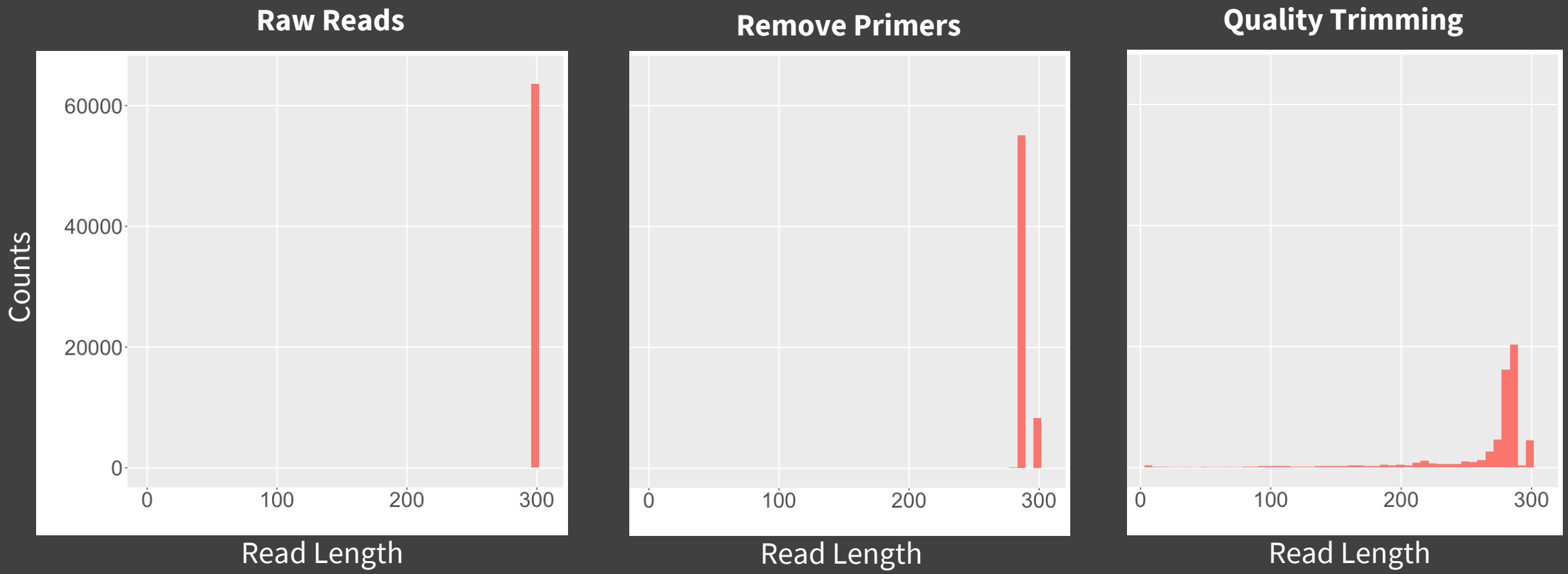


Tools

FastQC FastQp MultiQC & Specialized 16s rRNA packages

Source: Dada2 R package

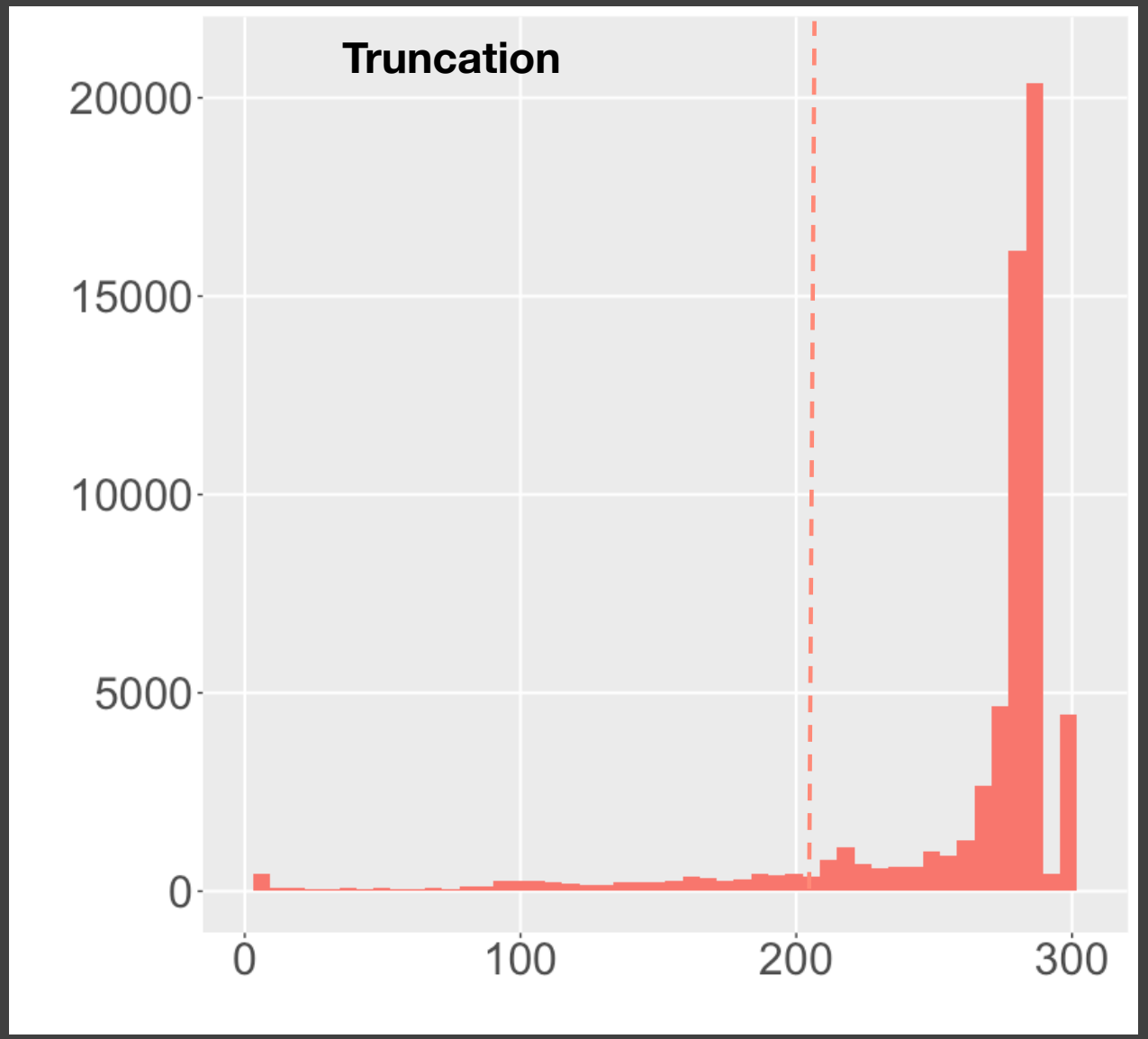
Trimming & Quality Filtering



Tools

CutAdapt

Trimming & Quality Filtering



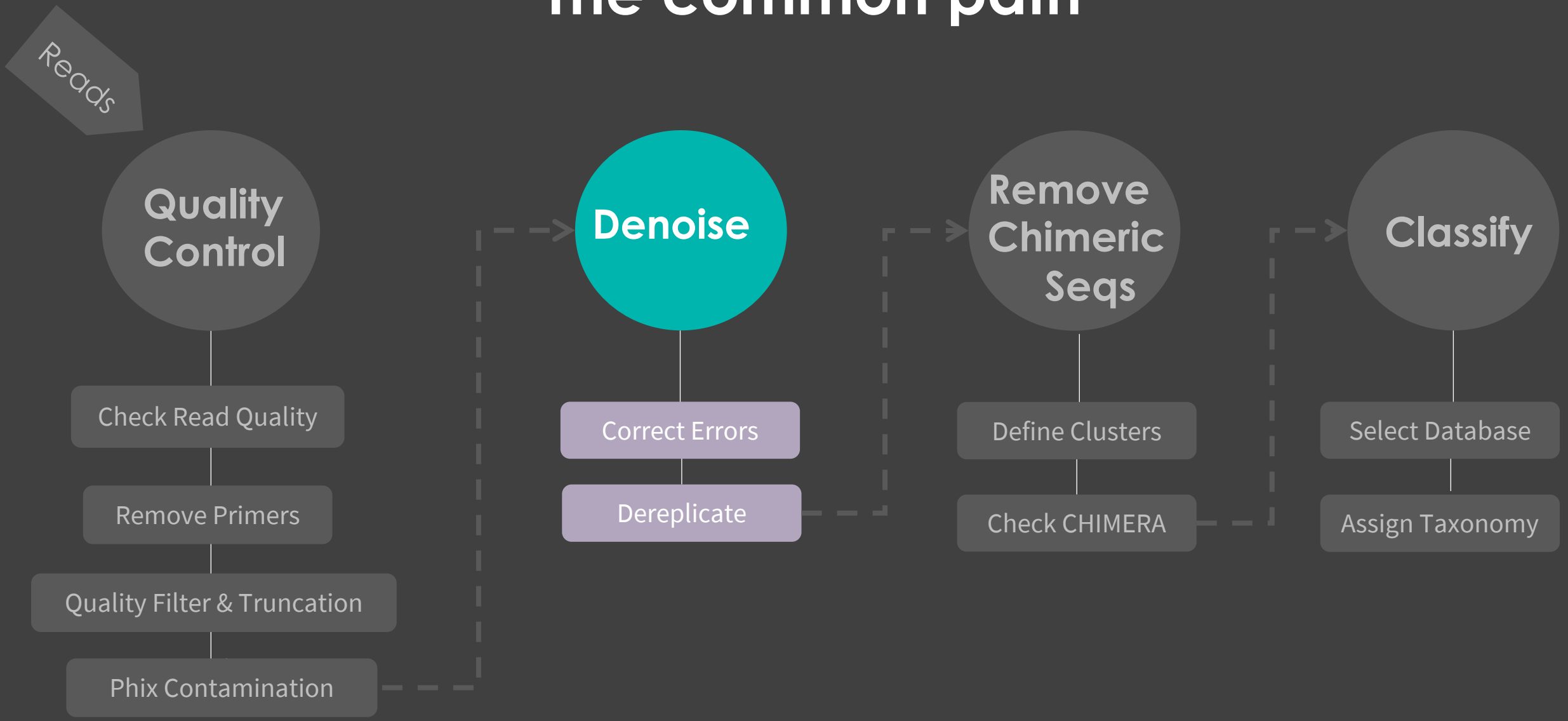
Available Methods

- 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

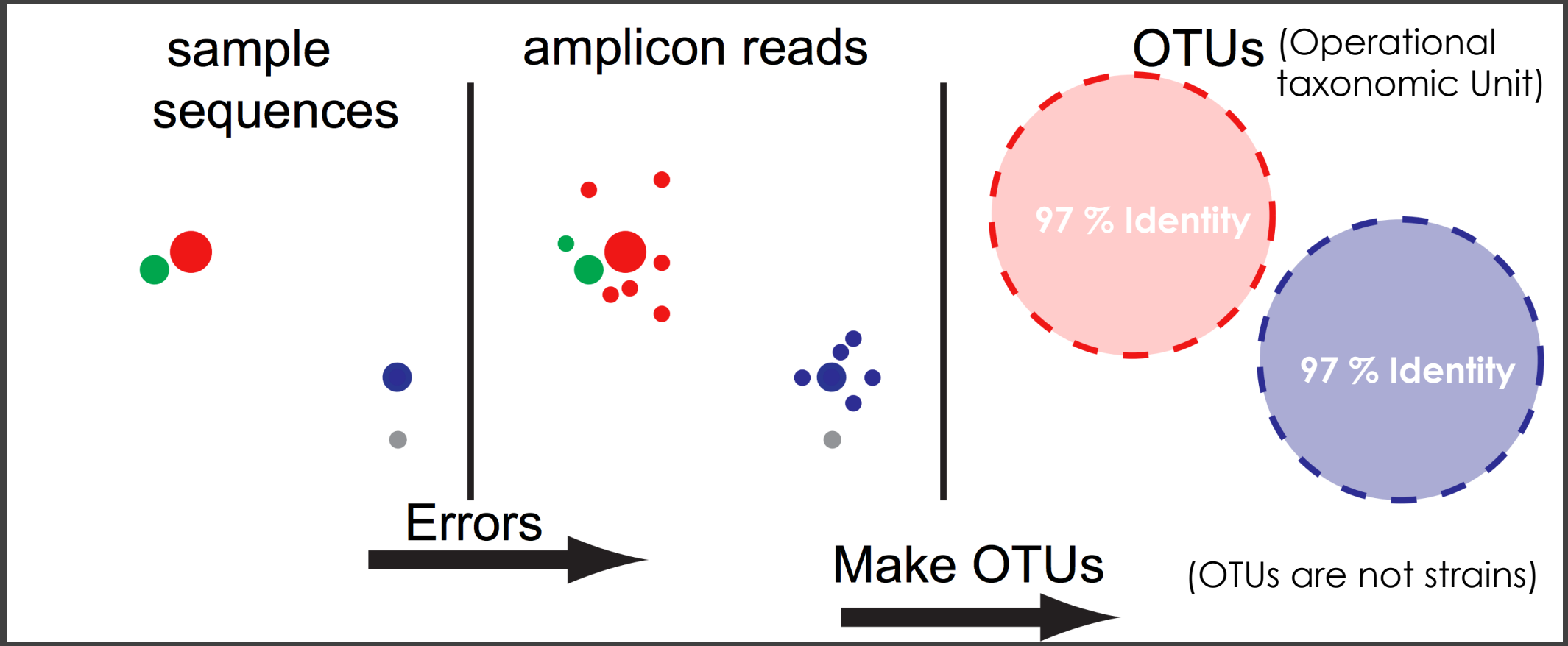
Source: Edgar et al., 2015

The common path



Denoising reads

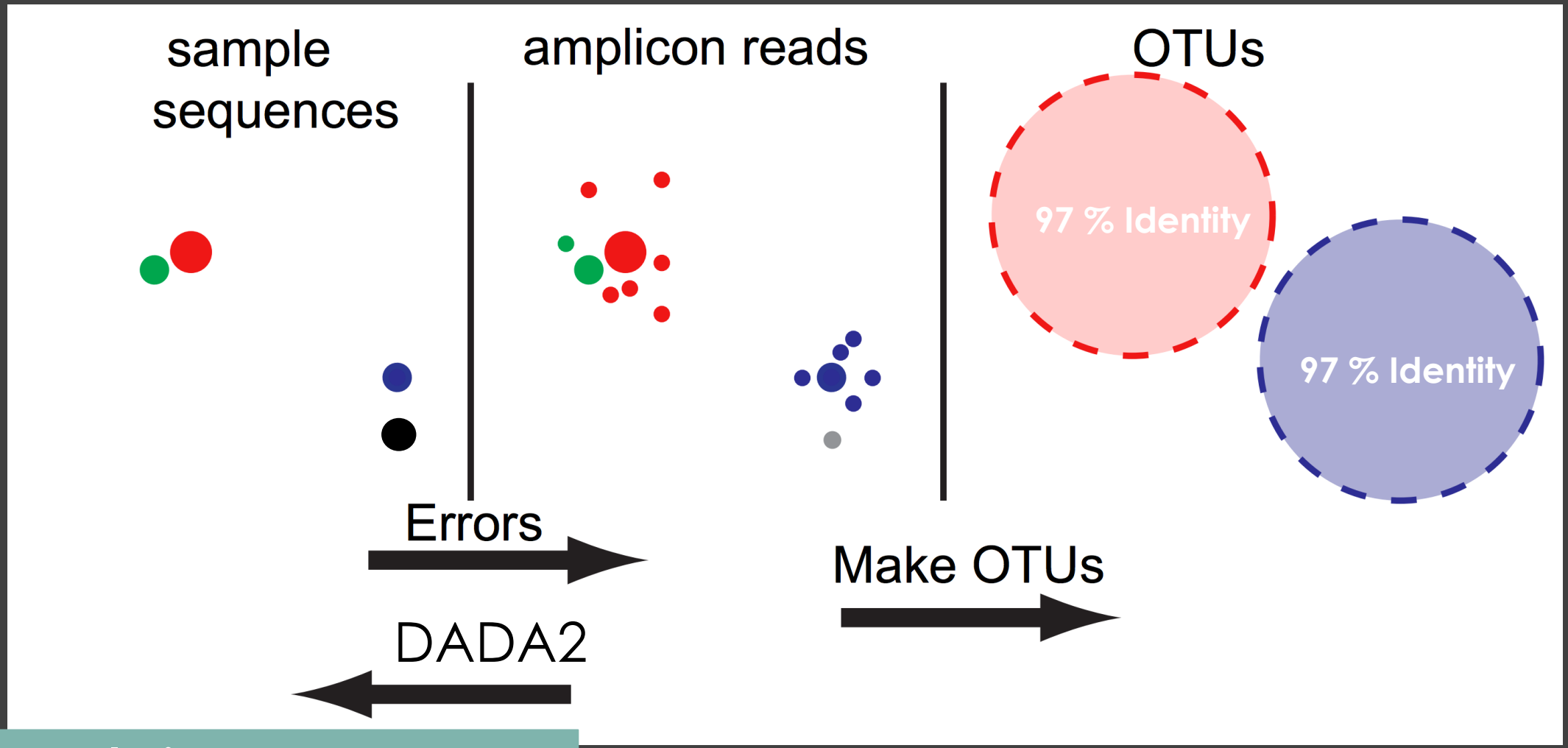
Clustering



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

Source: Callahan et al.,2016

Clustering

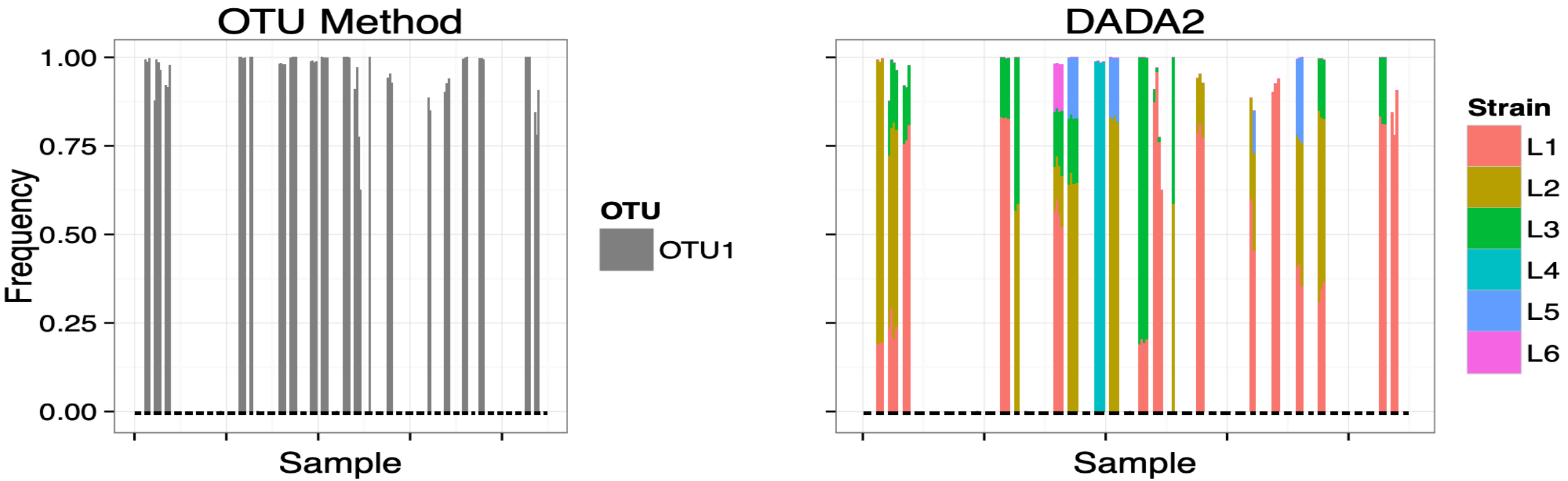


High Resolution & Accuracy

Source: Callahan et al., 2016

Real example, exact sequence resolution

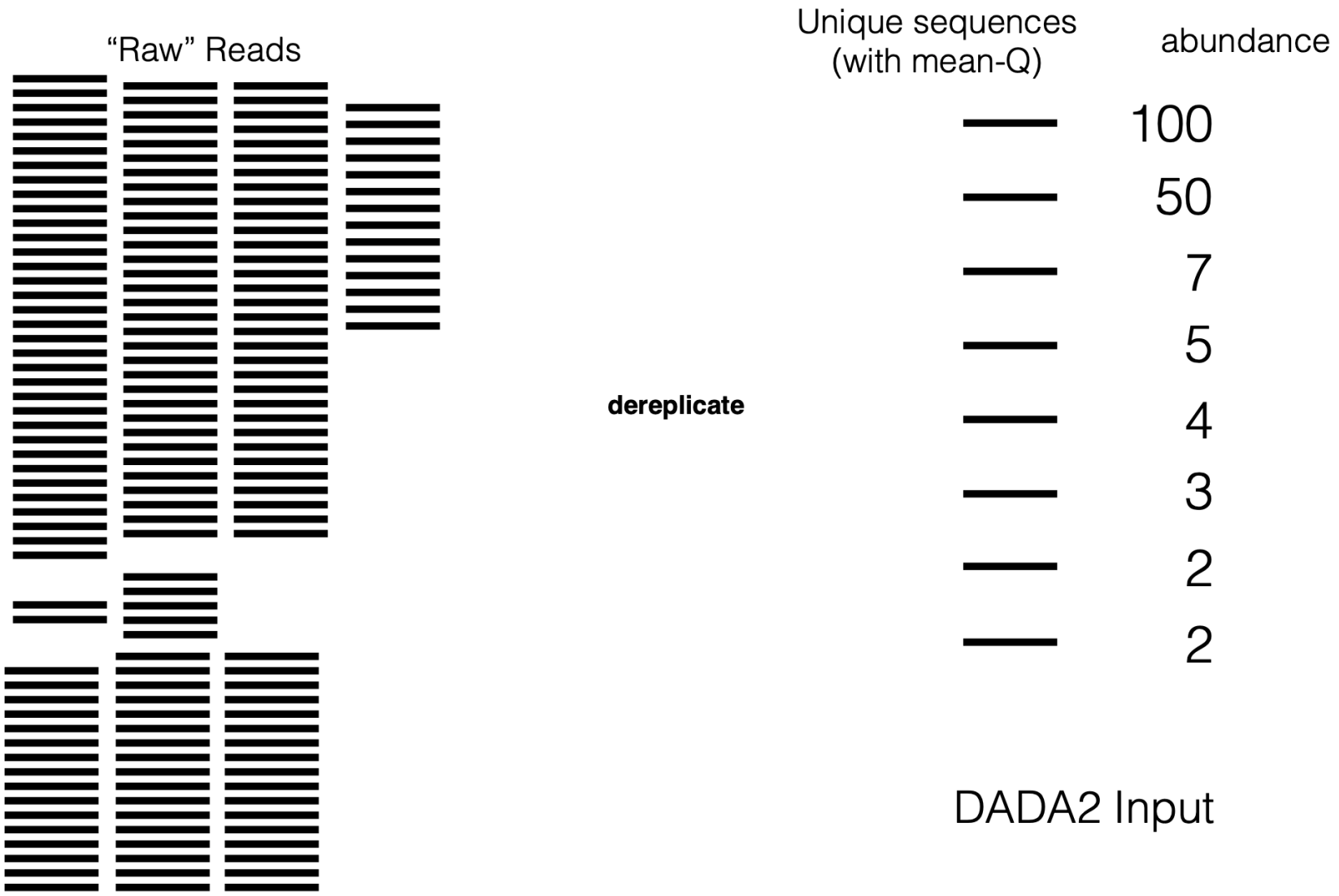
Lactobacillus crispatus sampled from vaginal microbiome 42 pregnant women



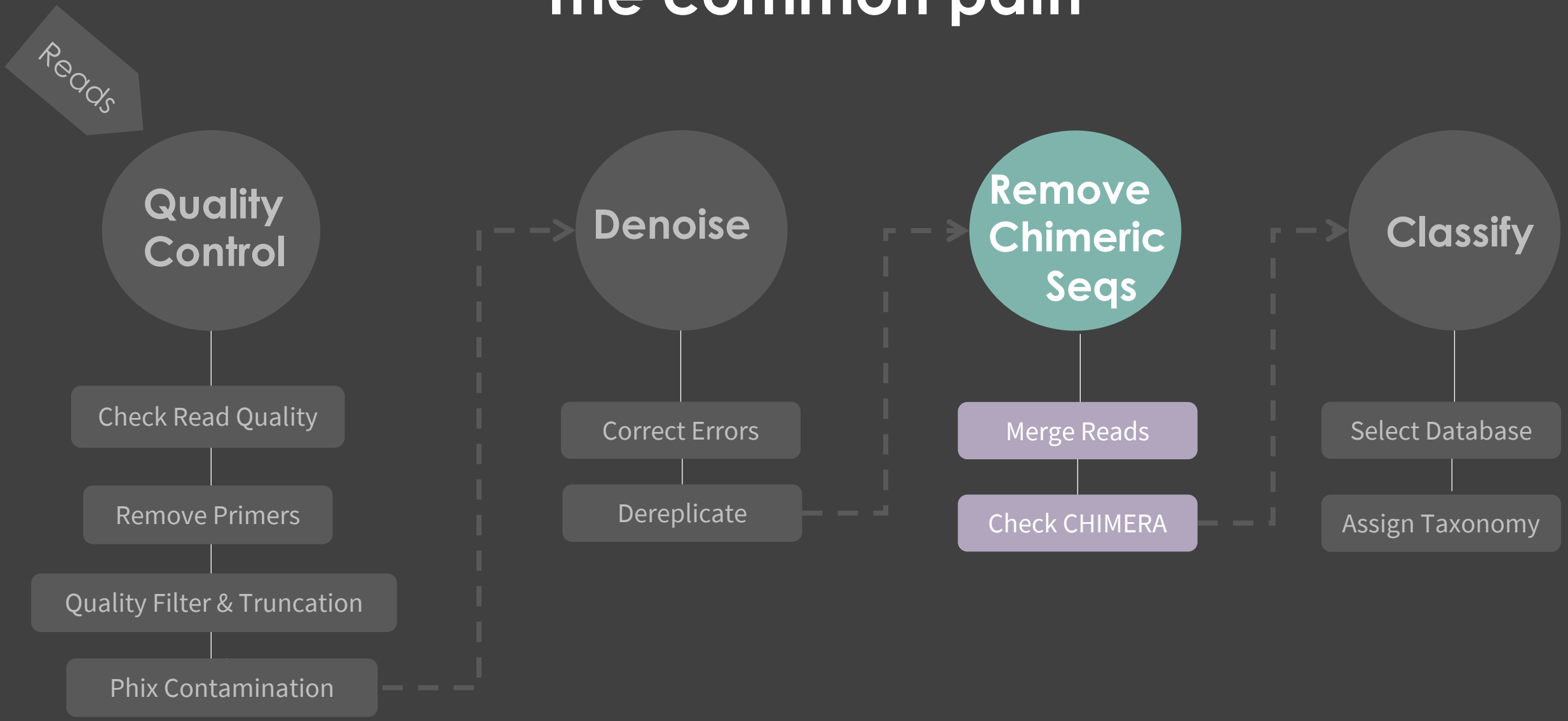
Data: MacIntyre et al. Scientific Reports, 2015.

DADA2 algorithm cartoon

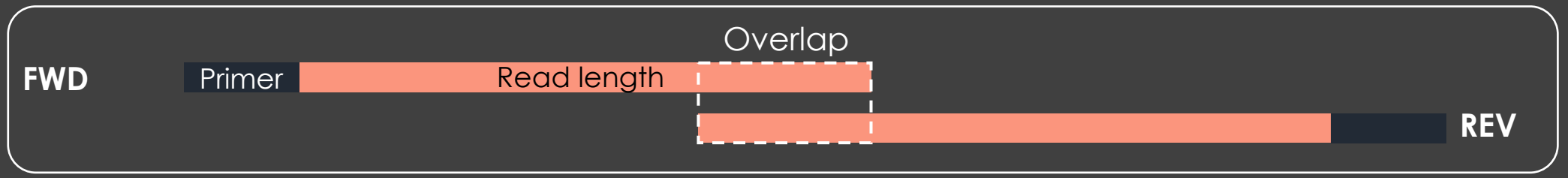
Input: unique sequences, their quality values, and abundances



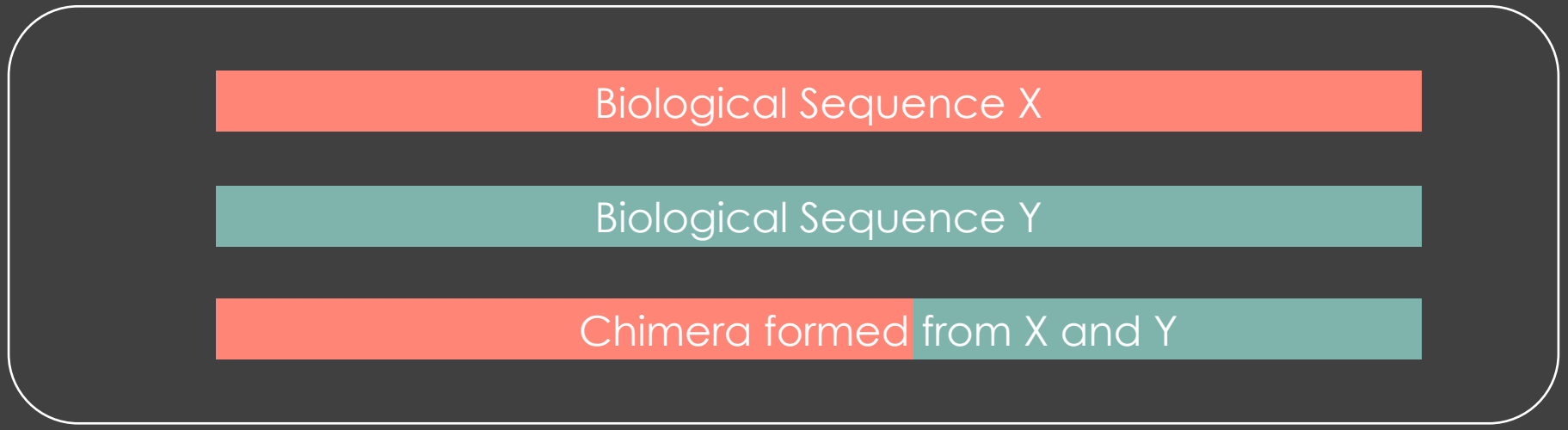
The common path



Merge Paired End Reads



Chimeric Sequences



Created during PCR
Fragment primes different extension

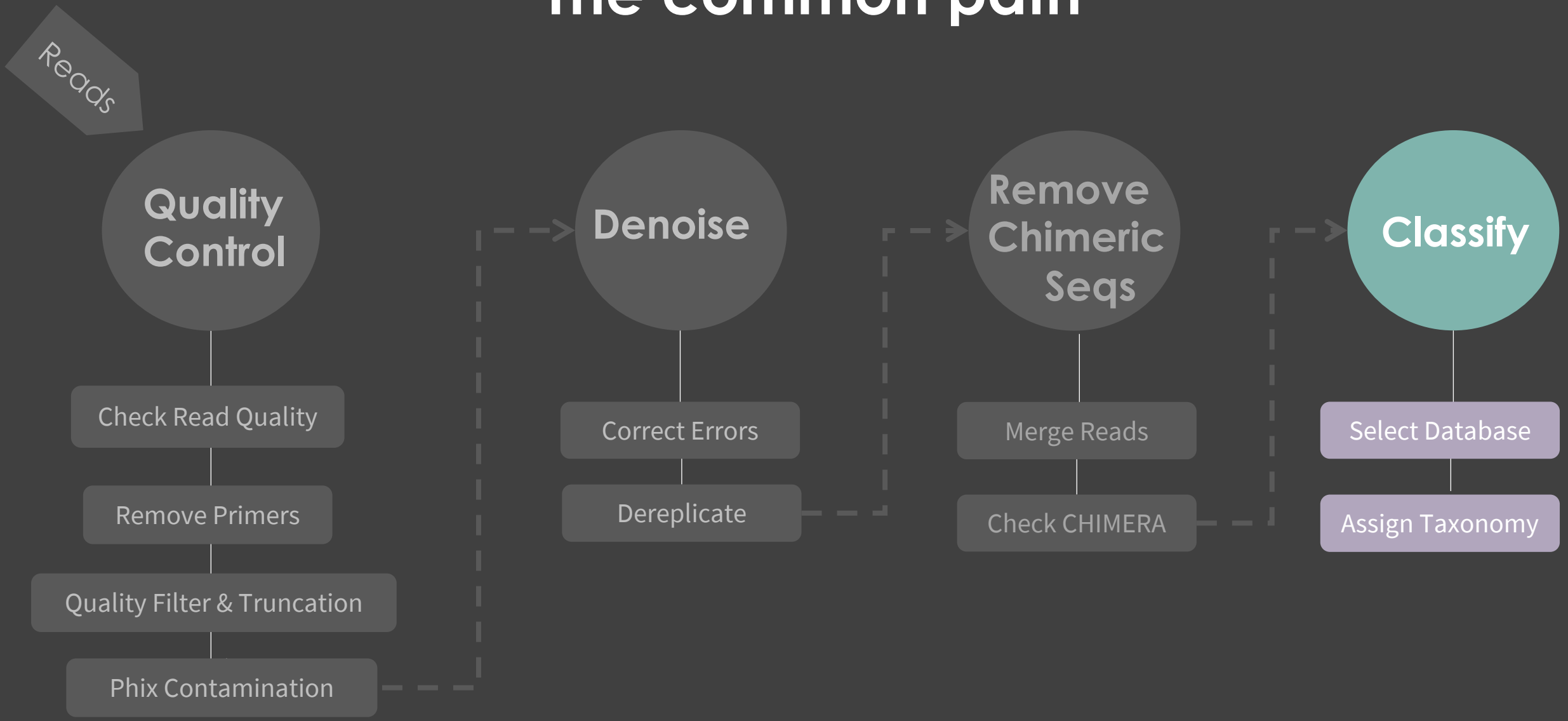
About 1-5 % of reads are chimeric

Source: Lahr & Katz, 2009

Important

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

The common path



Taxa levels

Kingdom
Phylum
Class
Order
Family
Genus
Species

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

Use frequently updated and well curated databases!

Diversity

Alpha diversity

Within sample diversity
Who is in the sample

Beta diversity

Between sample diversity
How similar at the samples

Uses Abundance and/or observed number of each ASV

Distance metric & clustering

Shannon, Simpson, Chao1

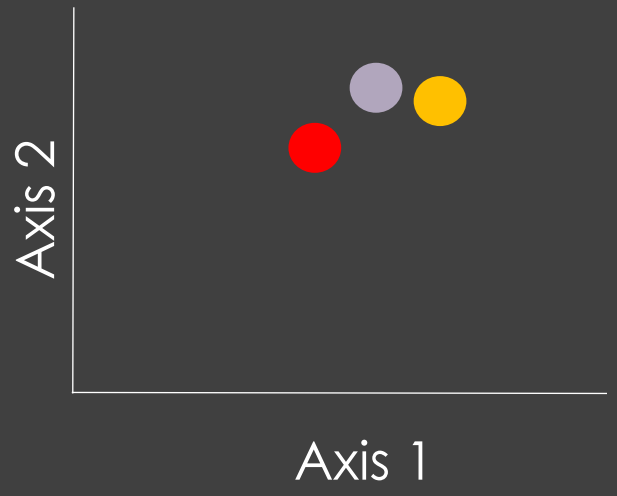
Richness , Evenness

Jaccard	**Unifrac**	Absent/Present
Bray-Curtis	Weighted-Unifrac	Abundance

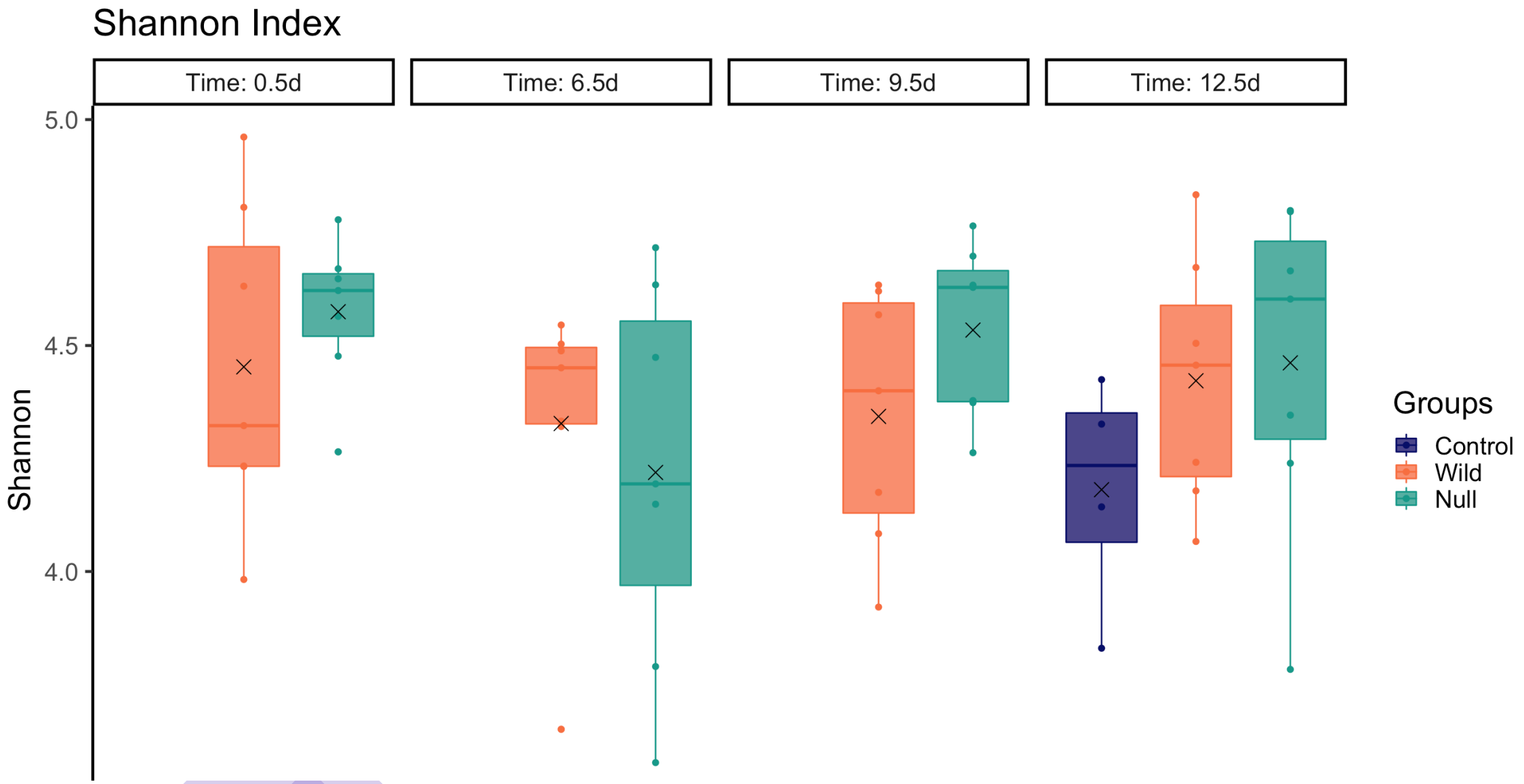
Beta Diversity

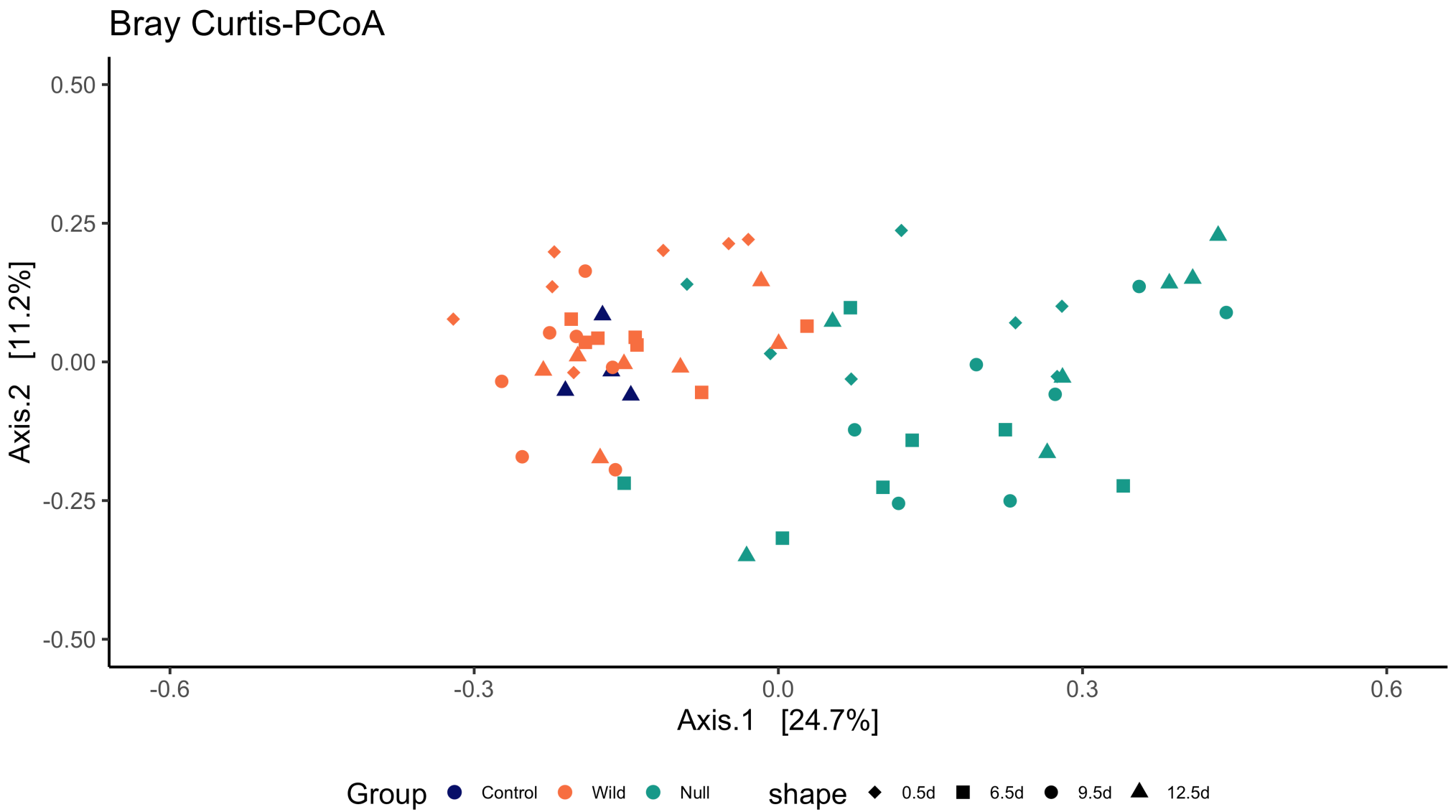
	Sample 1	Sample 2	Sample 3
Sample 1		0.2	0.6
Sample 2	0.2		0.5
Sample 3	0.6	0.5	

Each Axis represents a percentage of variability it explains of the data.



Methods
PCoA
NMDS
CCA


Shannon Index
Time: 0.5d
Time: 6.5d
Time: 9.5d
Time: 12.5d
Shannon
5.0
4.5
4.0
Groups
Control
Wild
Null


Bray Curtis-PCoA
0.50
0.25
0.00
-0.25
-0.50
Axis.2 [11.2%]
-0.6
-0.3
0.0
0.3
0.6
Axis.1 [24.7%]
Group
Control
Wild
Null
shape
0.5d
6.5d
9.5d
12.5d

What next?

Differential abundance analysis
Network analysis
Predictive functional analysis

Differential Abundance Analysis

Are there any taxa that are present in different abundance in the two types of cat?

Methods (to name a few)

1. DESeq2
2. Maaslin2
3. edgeR
4. metagenomeSeq
5. ANCOM2
6. ANCOM-BC
7. corncob
8. ALDEx2

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

– Ronald Coase, *Economist*

Tips

Sample size
Are there enough samples for down stream analysis

Controls + & -
Helps identify contaminants

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

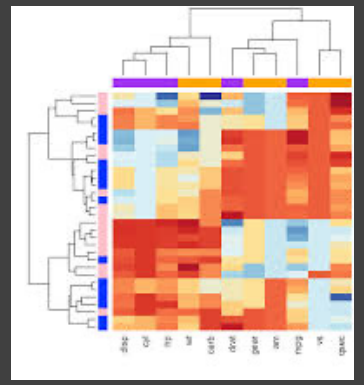
Be consistent
It is much better to use the same methods than to change methods frequently

Think before you start and ask why!

Shotgun Metagenomics

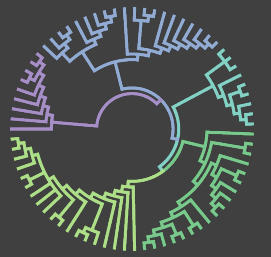
Total DNA

Functional Annotation
+ Metabolite prediction



Assembly

Phylogeny



Taxonomic profile

Marker Gene
Identification or
Kmer exact match
classification

Samples

Taxa levels

Kingdom
Phylum
Class
Order
Family
Genus
Species
Strain

Limitations

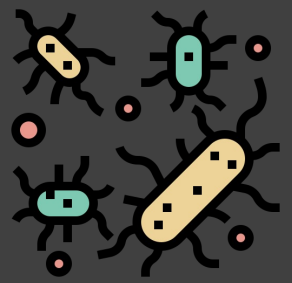
- Avoids PCR bias but has it's own
- Computationally intense
- Did we get enough data to identify the whole community ?

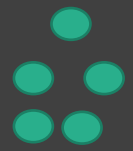
Metabolomics

Identification and quantification of metabolites

Who

How





Microbes

Metabolites

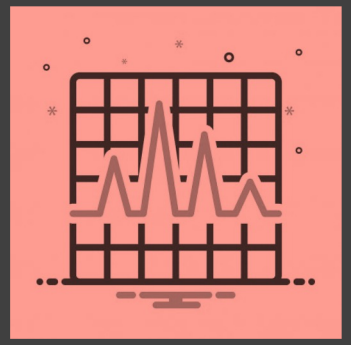
Host

Sugar	Amino Acids	Nucleotide	SCFA

Metabolomics

Untargeted	Targeted
Global profiling Qualitative	Measure a specific known set of metabolites Quantitative

Separate

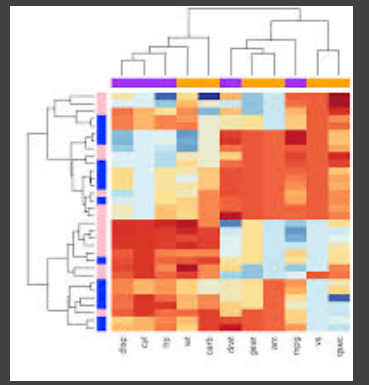


Chromatography
By size and charge

Detect

NMR or Mass spectrometry

Analyze



Detection options

LC/MS	NMR
Separation is required	No separation required
Can resolve metabolites in complex mixtures	Smaller number of biomarkers is detected
High Sensitivity and range	Lower sensitivity
Expensive and serious batch effects	Reproducible and free of batch effects

Typical Analysis

Data Acquisition: Spectra, NMR

Data Processing: QC, normalization and statistical analysis

Data Investigation: Enrichment analysis or pathway analysis

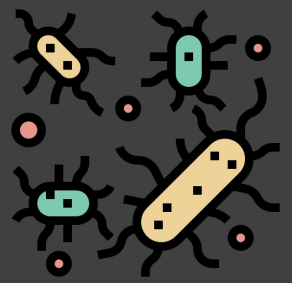
Data Integration: Putting together with other omics data

Source: https://slideplayer.com/slide/16950853/

Multi-Omics Approach

Putting it all together

Who



Microbes

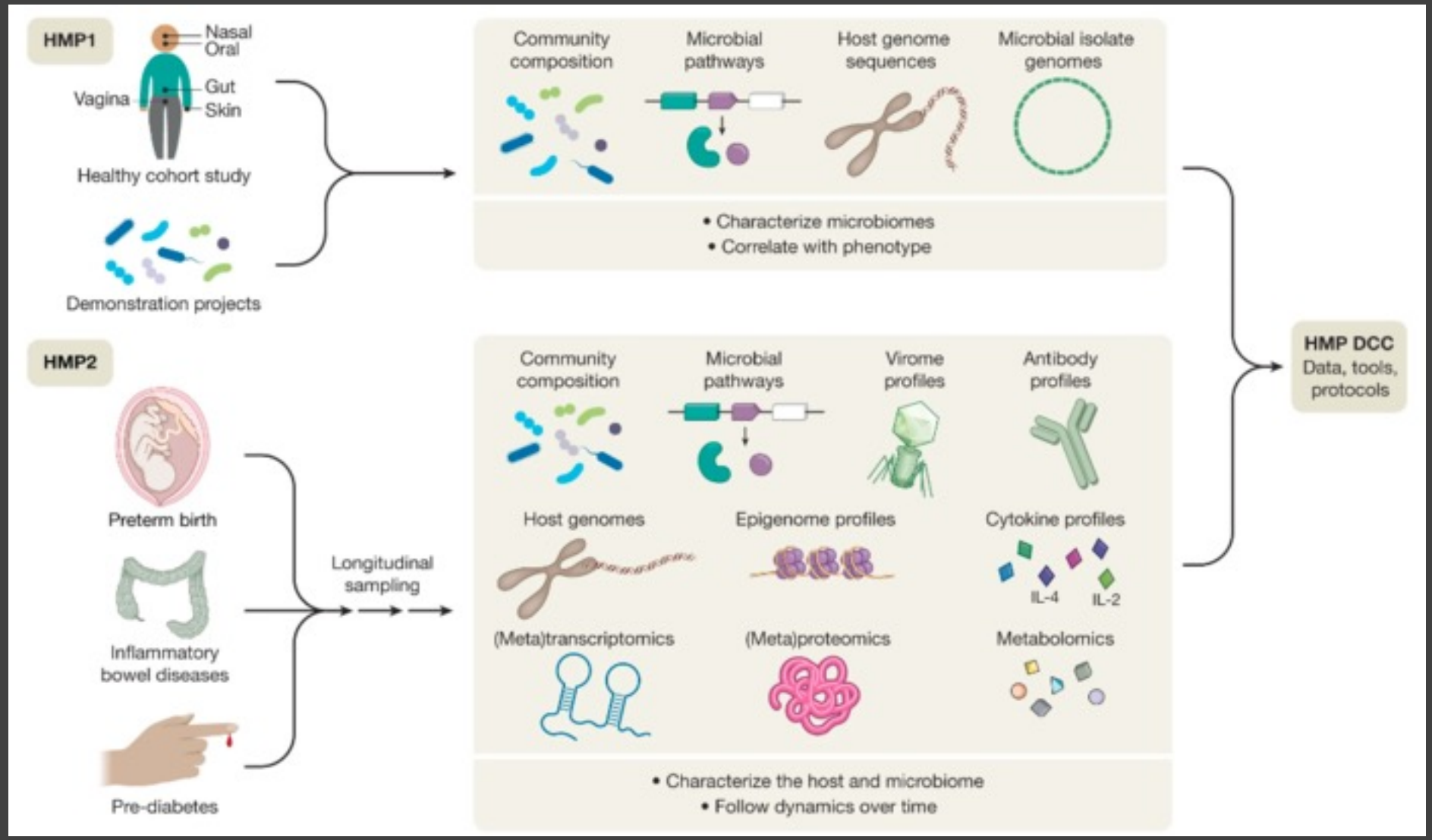
Meta-Transcriptome

Metabolites

Host

Transcriptome

Immune Markers


HMP1
Nasal
Oral
Vagina
Gut
Skin
Healthy cohort study
Demonstration projects
Community composition
Microbial pathways
Host genome sequences
Microbial isolate genomes
• Characterize microbiomes
• Correlate with phenotype
HMP2
Preterm birth
Inflammatory bowel diseases
Pre-diabetes
Longitudinal sampling
Community composition
Microbial pathways
Virome profiles
Antibody profiles
Host genomes
Epigenome profiles
Cytokine profiles
IL-4
IL-2
(Meta)transcriptomics
(Meta)proteomics
Metabolomics
• Characterize the host and microbiome
• Follow dynamics over time
HMP DCC
Data, tools, protocols