Characterization of Microbial Diversity using Metabarcoding

MIO, oct 2020

Different names … same meaning!

16S amplicon sequencing

16s rDNA Gene Tag Sequencing

Metabarcoding

barcoding

16S metagenomics

eDNA metabarcoding

Definitions

A new name for an old concept

"**DNA Barcoding**" appears recently in literature (*Floyd et al. 2002*)

but was firstly reported in 1993 (*Arnot et al. 1993*)

- *sensus stricto*: Use of a standardized DNA region as a **tag** for rapid and accurate **species** identification

- *sensus lato*: Identification of **any taxonomical level** using any DNA fragment

Mitochondrial Cytochrome Oxydase 1 → Animal

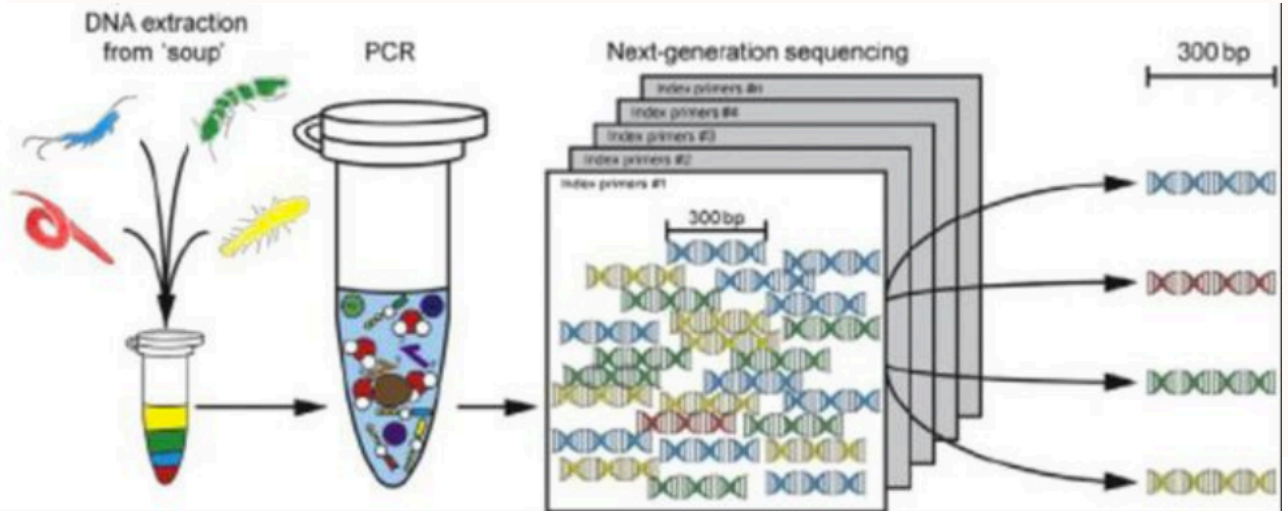
Chloroplast part → Plant

16S rDNA → Bacteria/Archaea

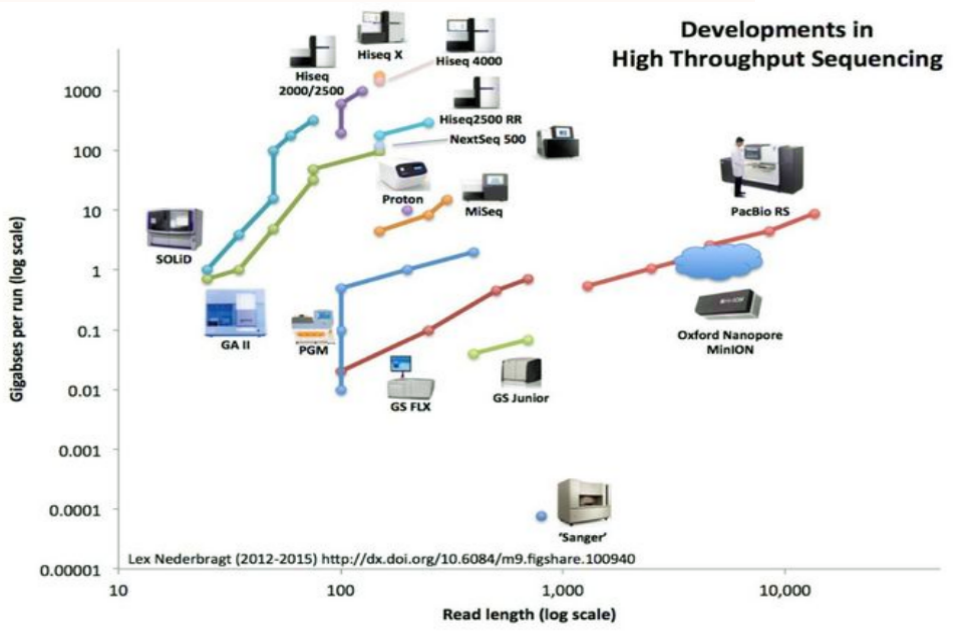
Metabarcoding…

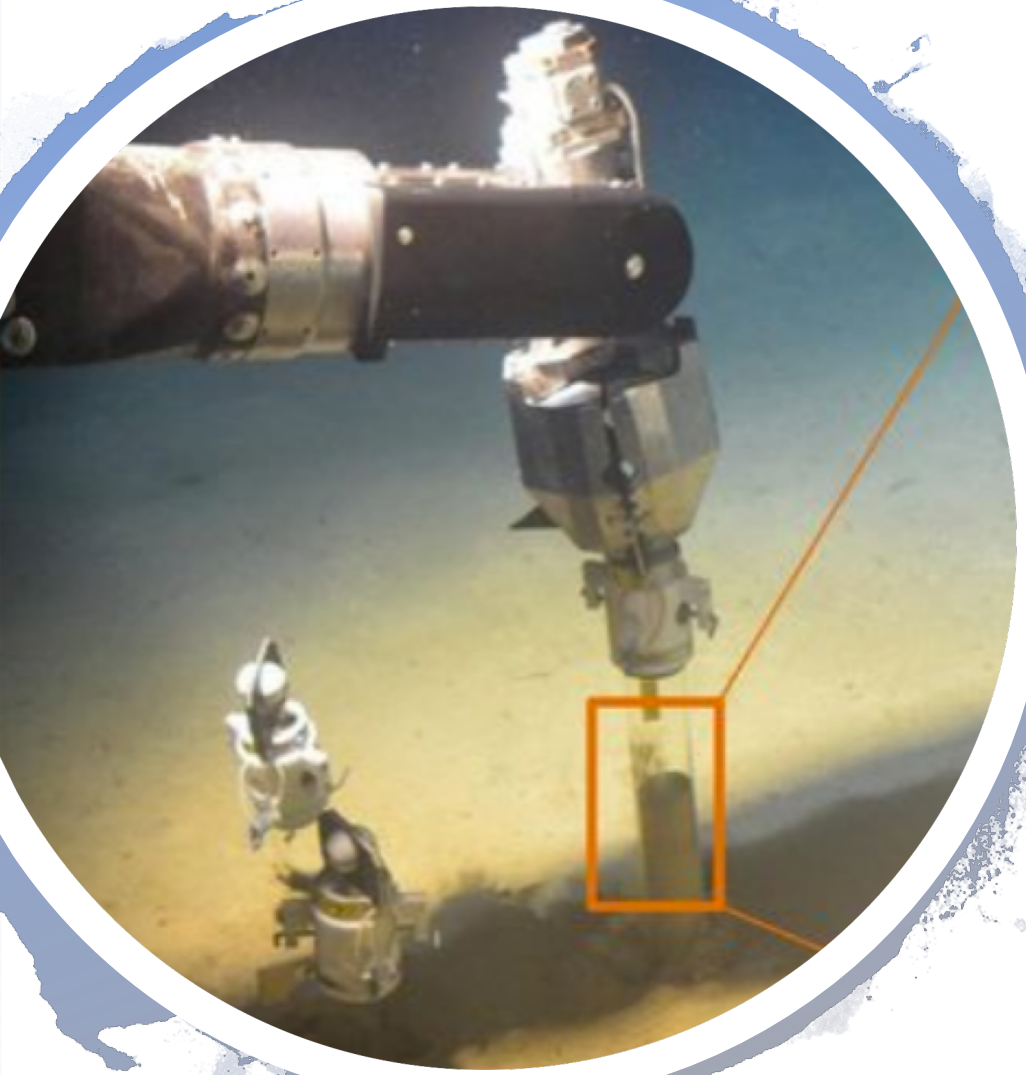
Use to designate **high-throughput multispecies identification** using DNA extracted from an **environmental sample** or from bulk samples of entire organisms (*Taberlet et al. 2009*)

→ Take advantage of Next Generation Sequencing (NGS)



Gill et al. 2016





Environmental DNA (eDNA)

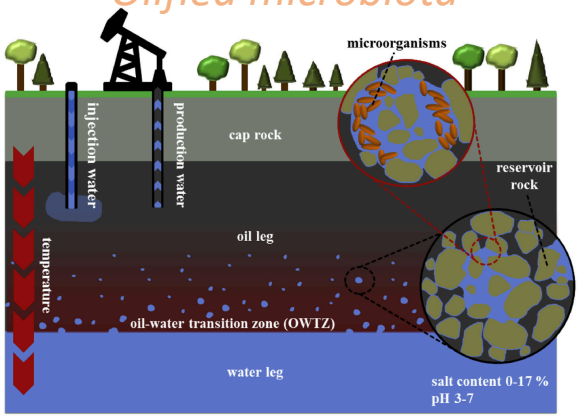
DNA extracted from air, water, soil without isolating any microorganism

Goal of Metabarcoding: Who is there?

Characterization of the **taxonomic diversity** inhabiting various ecosystems using direct eDNA

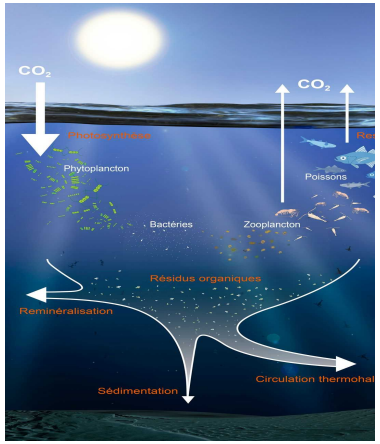
→ Multispecies sample
→ Target 16S rDNA gene marker for Bacteria/Archaea identification
→ Use high-throughput sequencing (= massive data→ complex ecosystem)

Oilfied microbiota



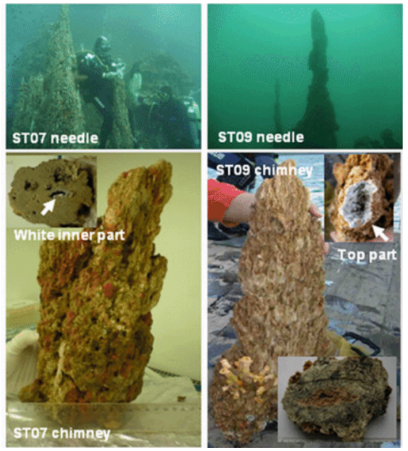
Pannenkens et al. 2019

Biological pump of CO2



Tamburini et al.

Submarine hydrothermal sources



Erauso et al.

More than "Who is there?"....

- **Relationships Taxonomic Composition – Environmental Factors (EF)**

EF: ph, temperature, salinity, trace element, hydrostatic pressure, nutrient availability etc

- **How environmental factors impact community composition/structure**?

→Abundance shifts? Absence/Presence? Gradient? Diversity level?

Pollution Response

- **How similar/different are your samples**

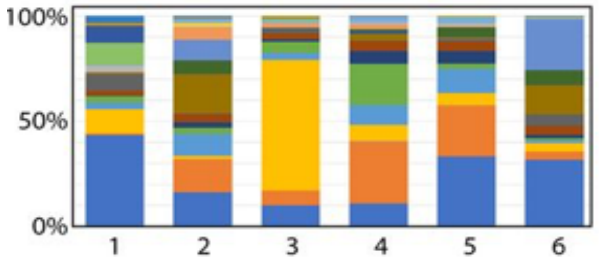
→ What are the major actors driving these similarities?

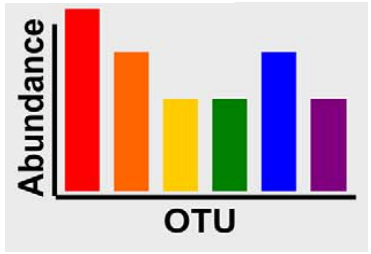
- **Dynamics of communities distribution at time and/or spatial scale**

i.e. Seasons, geographic location, ocean depth, perturbations etc

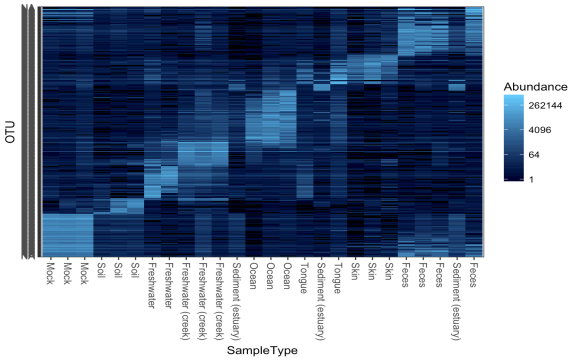
What kind of results you can expect?

Taxonomic profile

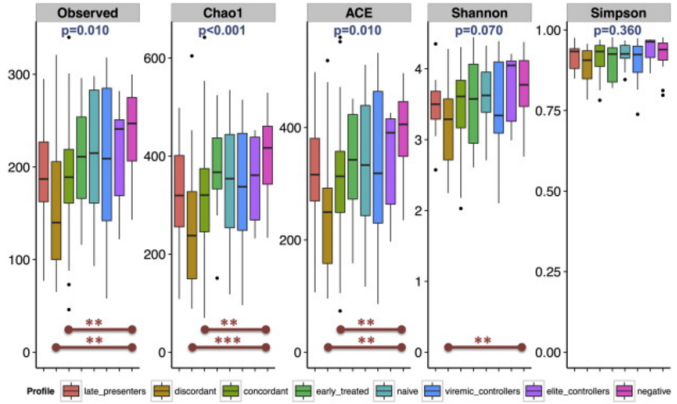




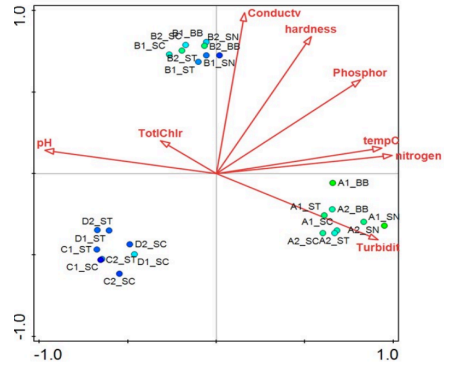
Time or space Gradients



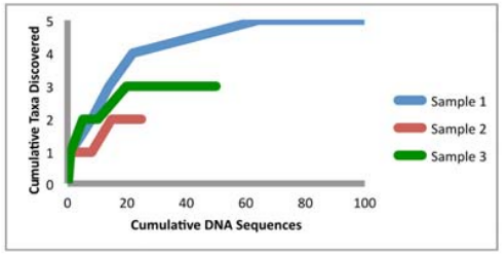
Within-sample alpha diversity



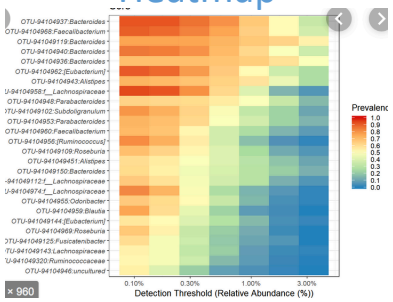
Multivariate analysis



species richness



Heatmap

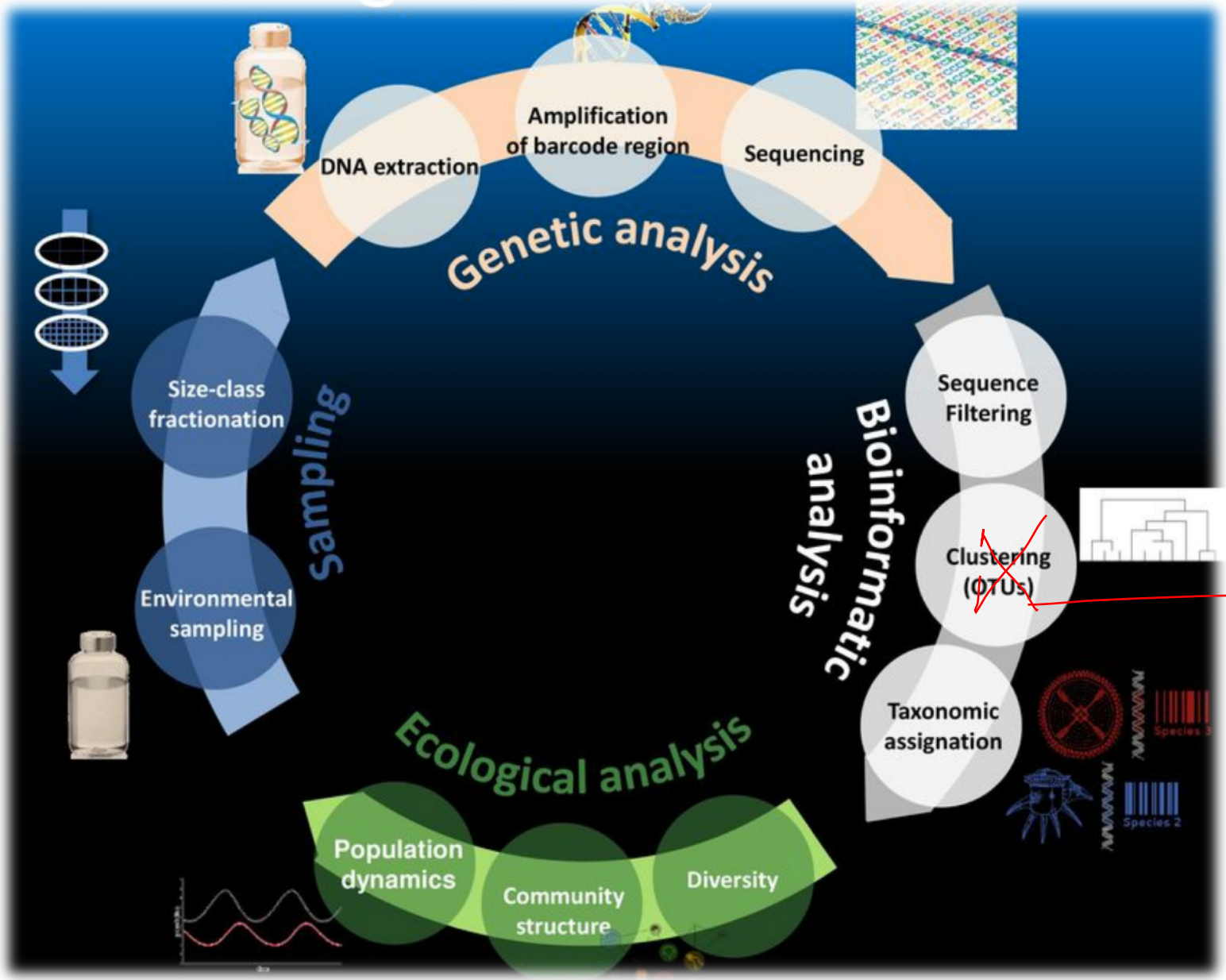


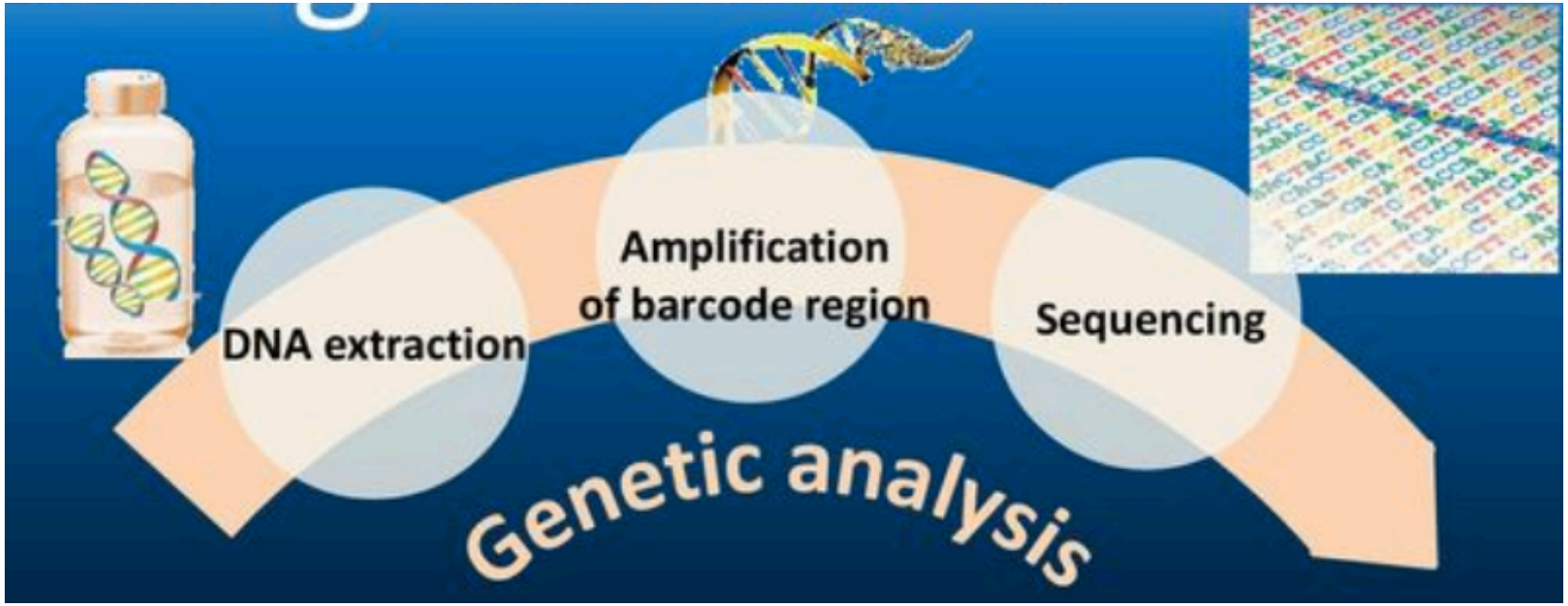
Who is there? Easy?

- **You can not use the full length of 16S gene** ~ 1500 bp
  - → Have to work with a short 16S region ~ 200 bp – 400 bp
  - → Loss of resolutive power for taxonomic identification

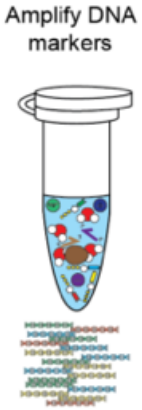
- **PCR & Sequencing introduce errors in sequences/reads**
  - How to deal with that??
  - → Increase taxonomic misidentification

Metabarcoding Global Overview

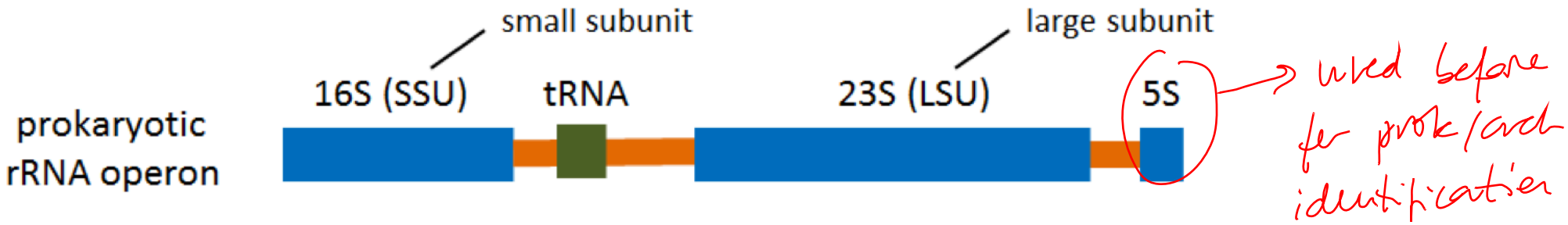




- **Choice of gene Marker**
- **Choice of the Region to amplify**
- **Choice of Sequencing Technology (NGS)**



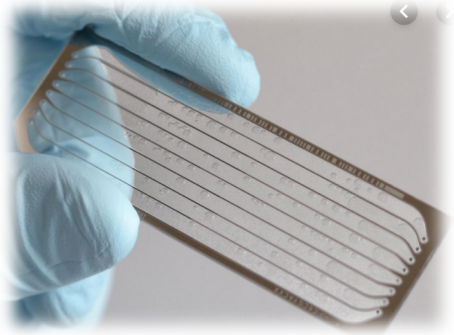
16S marker for bacterial/Archaeal identification

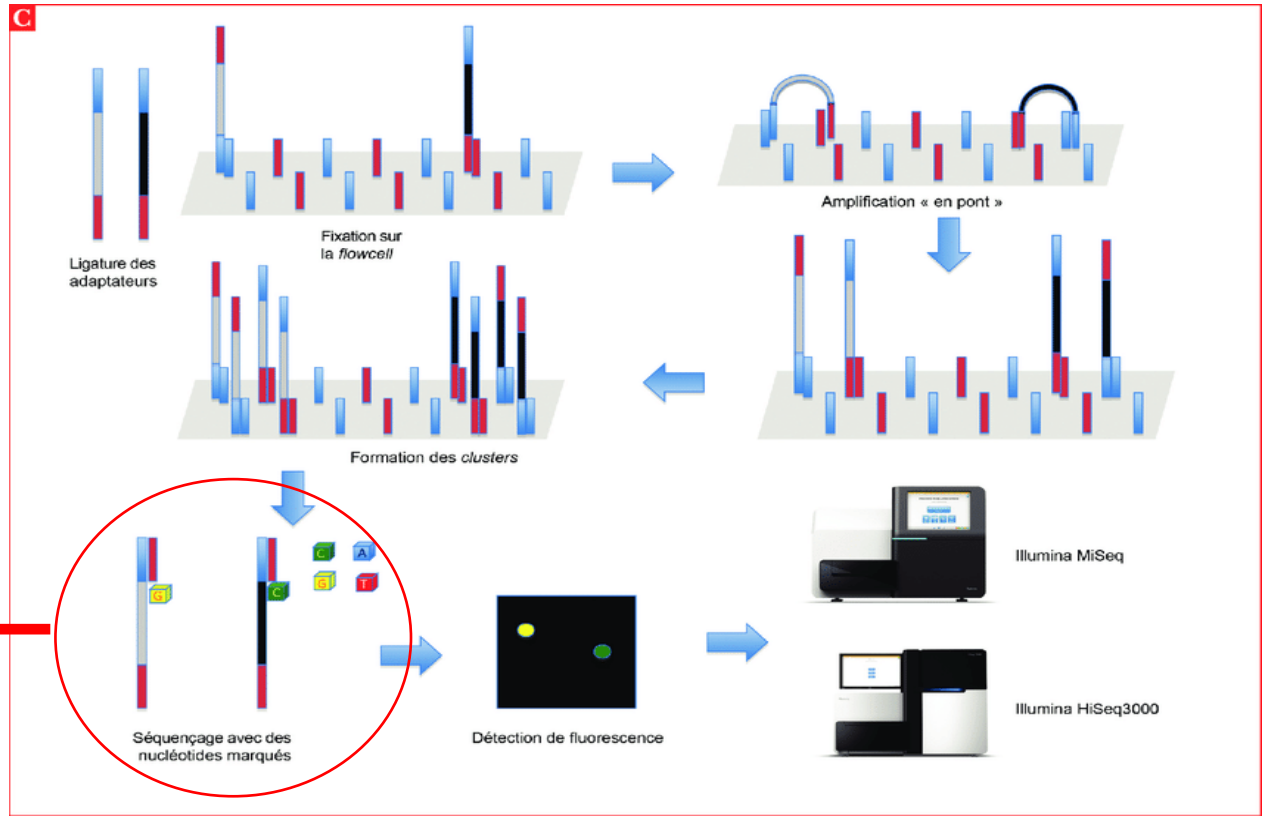


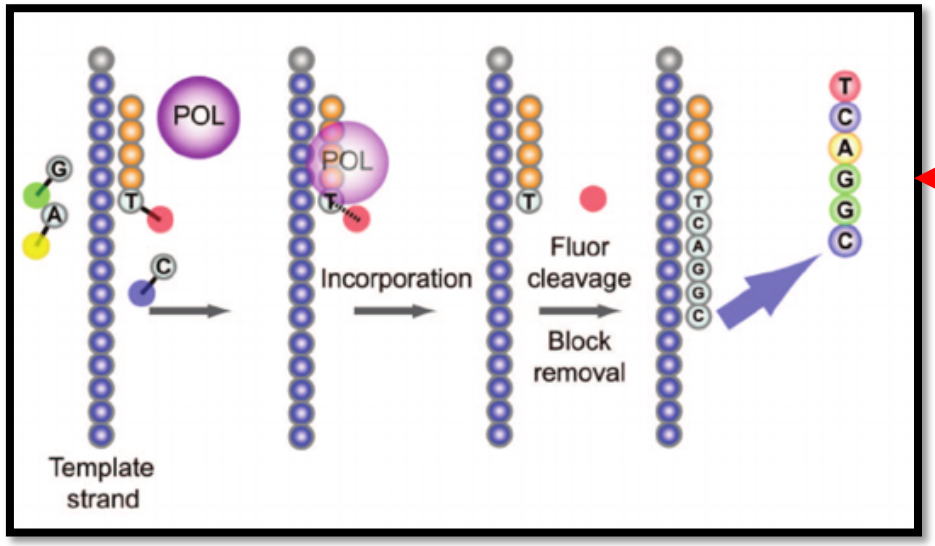
Type	LSU	SSU
prokaryotic	5S - 120 bp 23S - 2906 bp	16S - 1542 bp

1. **Use of 5S : low phylogenetic power**
2. **Use of 23S: too long**
3. **Use of 16S : ideal length 1500 pb (adapted for Sanger sequencing)**

Next Generation Sequencing (NGS): Illumina Miseq

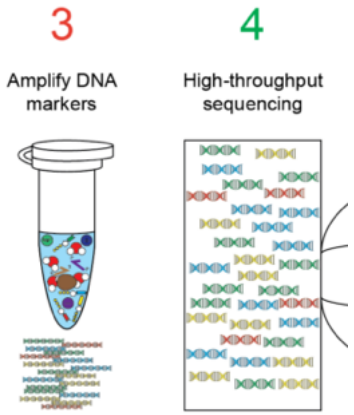






Only remember → Sequencing by reversible dye terminators

Illumina Miseq Sequencing Performance



Run Time	4–55 hours
Maximum Output	15 Gb
Maximum Reads Per Run	25 million †
Maximum Read Length	2 × 300 bp

Massive data for complex ecosystem! good!

V3 Kit

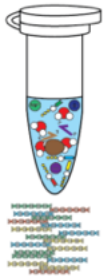
300 allele / 300 nt

Technology limit! oops!! Loss of resolutive power

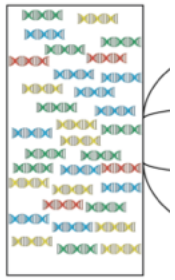
	MiSeq Reagent Kit v2				MiSeq Reagent Kit v3	
Read Length	1 × 36 bp	2 × 25 bp	2 × 150 bp	2 × 250 bp	2 × 75 bp	2 × 300 bp
Total Time*	~4 hrs	~5.5 hrs	~24 hrs	~39 hrs	~21 hrs	~56 hrs
Output	540–610 Mb	750–850 Mb	4.5–5.1 Gb	7.5–8.5 Gb	3.3–3.8 Gb	13.2–15 Gb

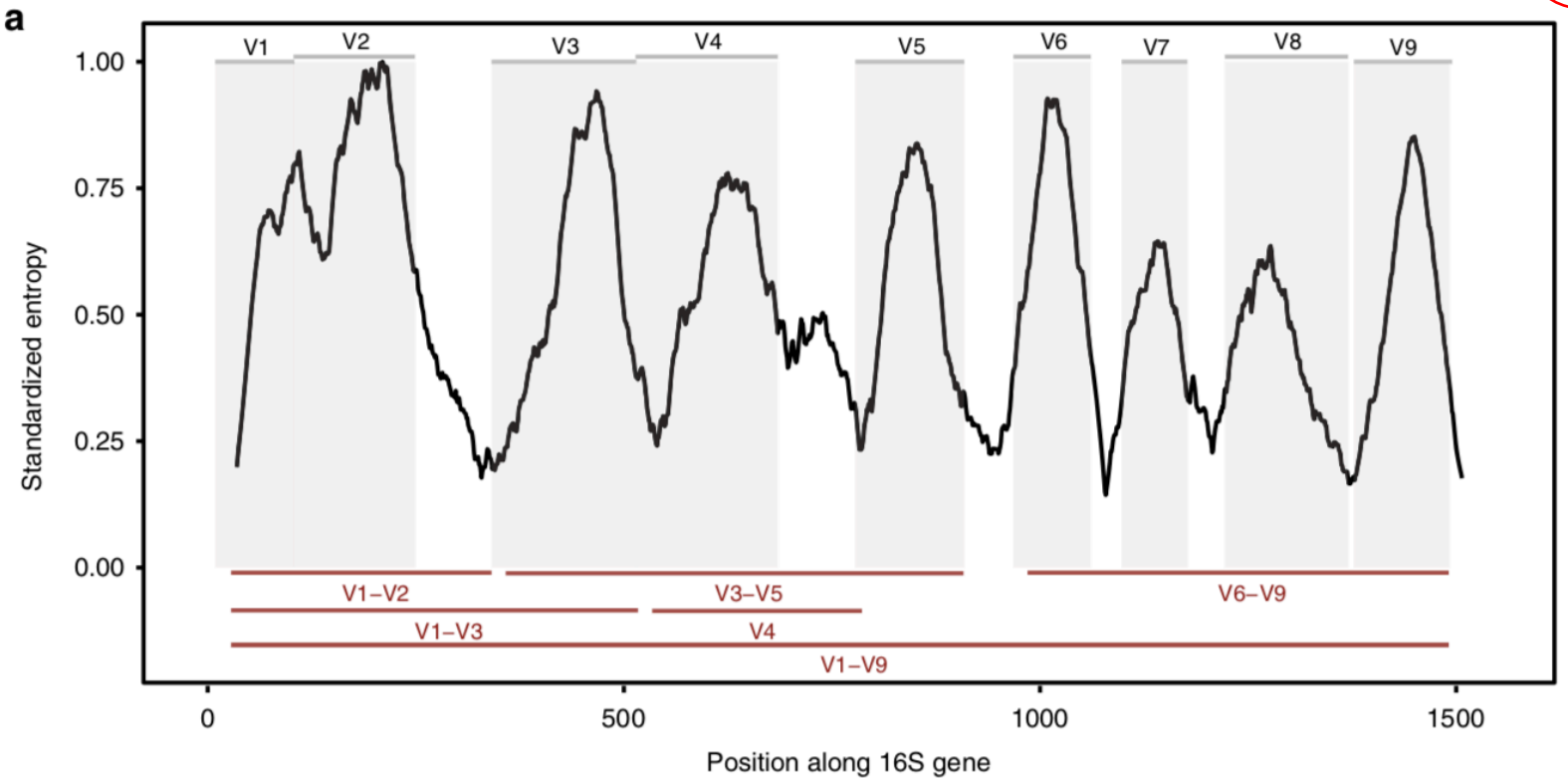
Phylogenetic resoltive power & variable regions of 16S

3 Amplify DNA markers



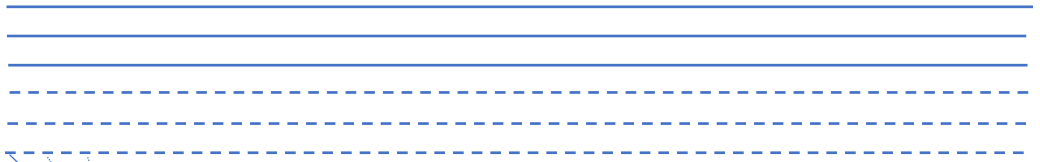
4 High-throughput sequencing





a gene of variable/invariable regions

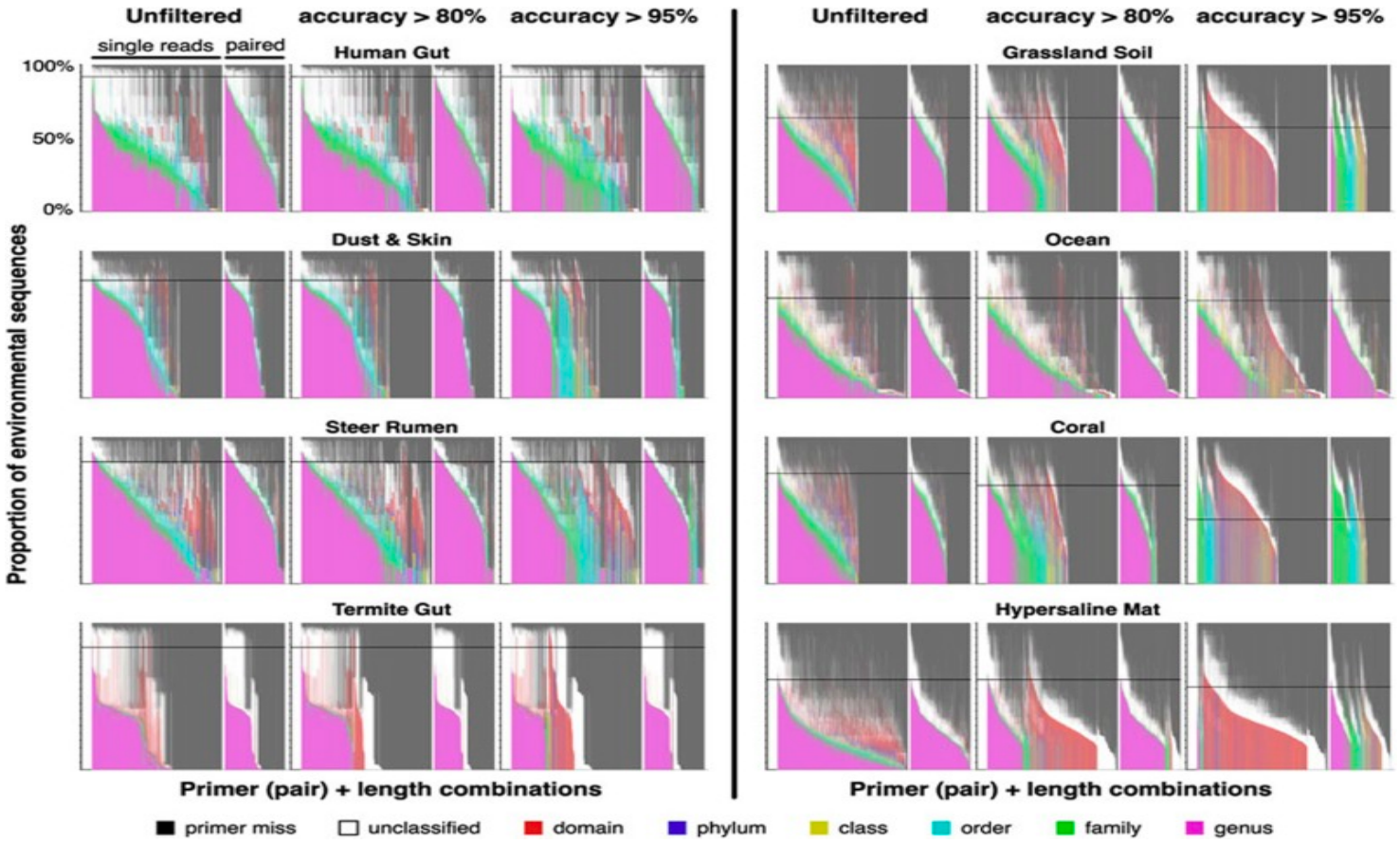
- More entropy (diversity) + influence to da



Multiple Sequence Alignment (MSA)
Of ALL 16S

Inspect each Nucleotide Position in MSA :
Mainly Conserved? -> low Entropy
Variable nucleotide? -> High entropy

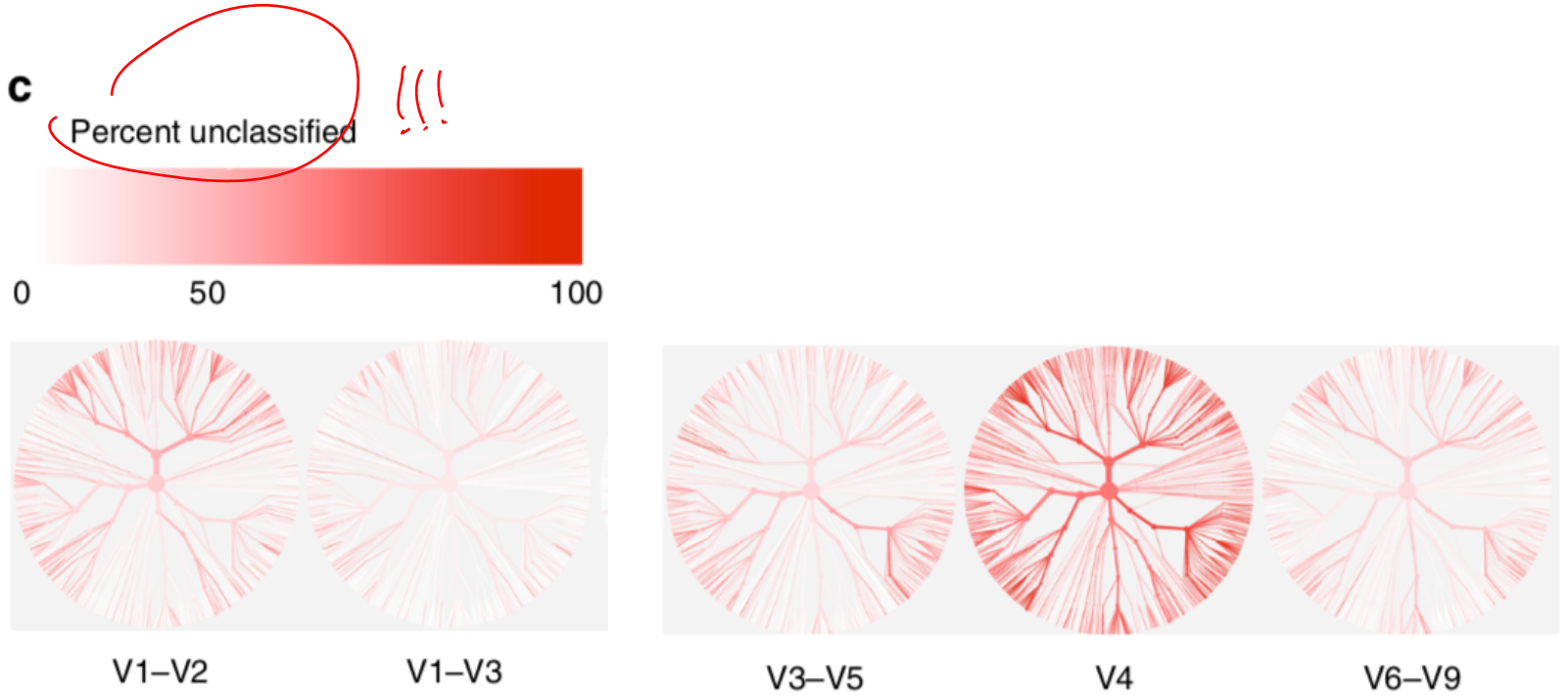
BUT, The most informative region may differ by **environment**



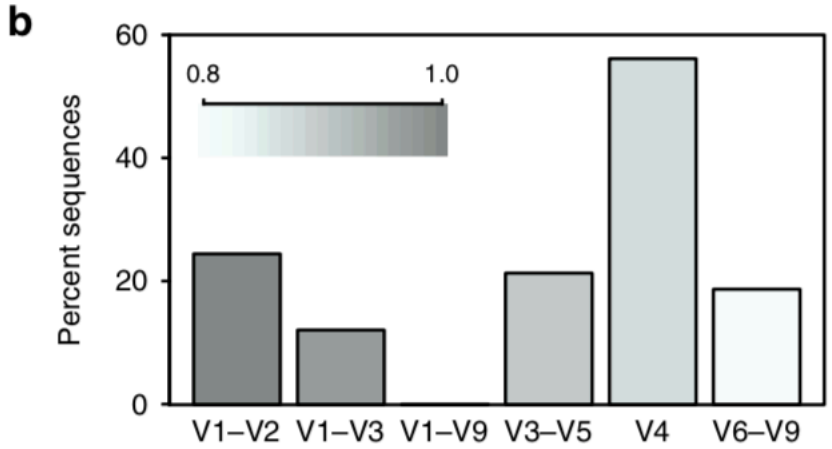
David A W Soergel, ISME J. 2012

→ paired-end sequencing provides substantial benefit in some environments

Variable 16S region: Fail to classify at the species taxonomic level



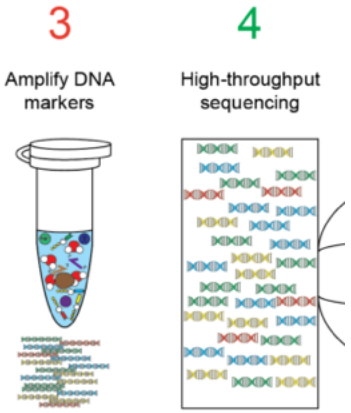
Johnson et al. Nature 2019



56% not assigned to species level with targeted V4 region

Greengene database
RDP classifier (Threshold 80)

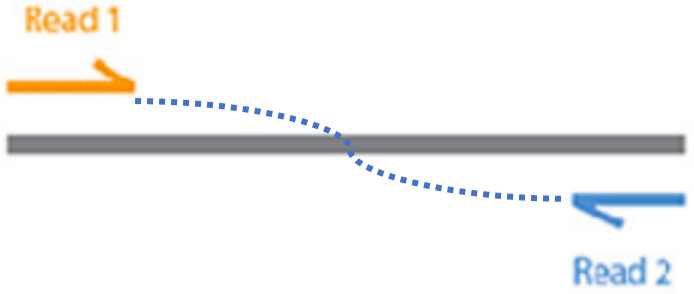
16S paired-end sequencing



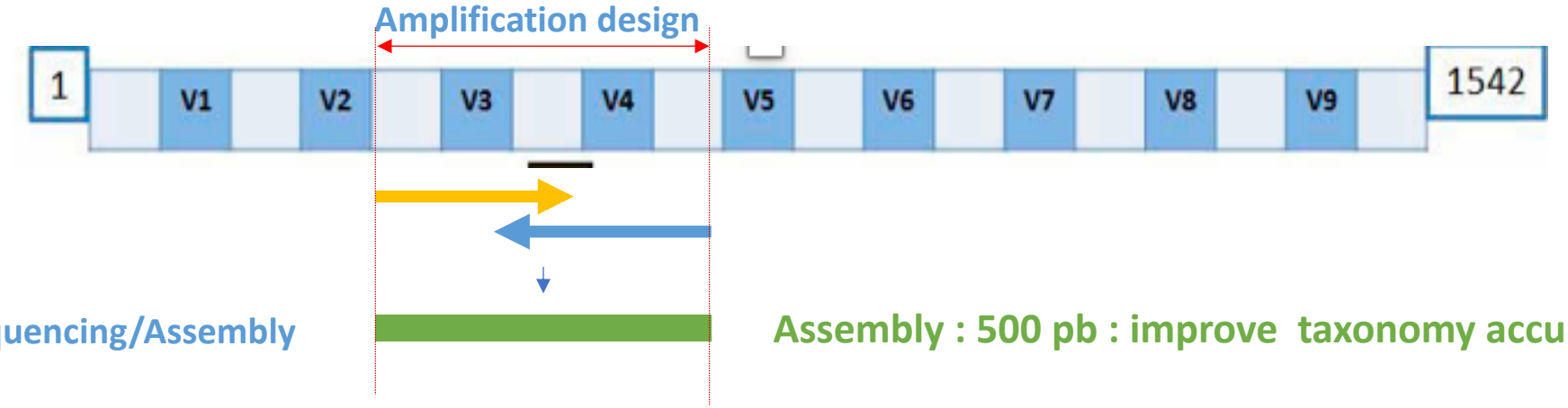
Run Time	4–55 hours
Maximum Output	15 Gb
Maximum Reads Per Run	25 million †
Maximum Read Length	2 × 300 bp

- **Miseq Illumina: Max read length is 300 bp**
- **Paired-end strategy increase the size of your target 16S region (Amplicon)**

Paired end : 2 X 300bp (Forward, Reverse)

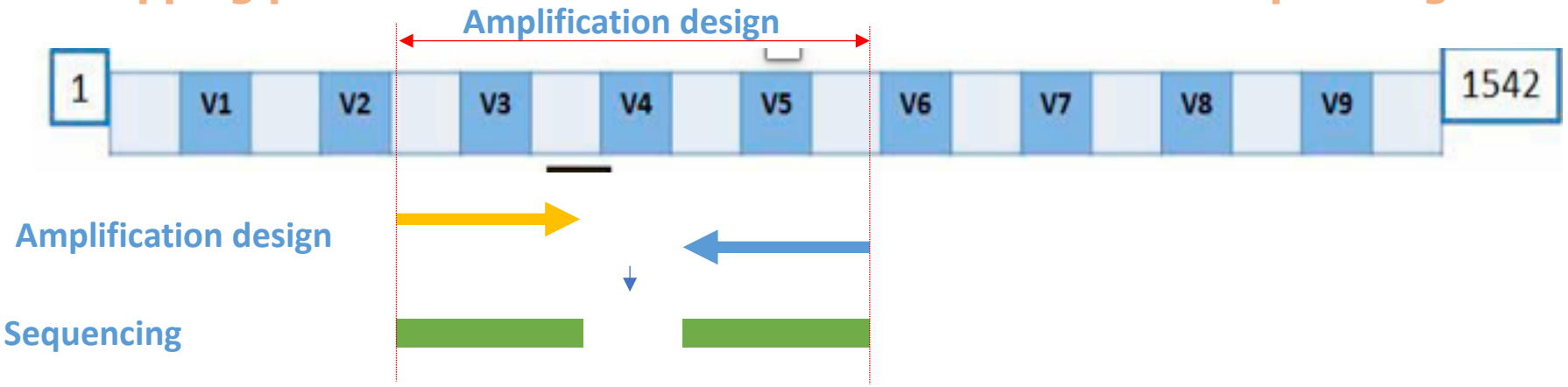


Overlapping paired-end reads : Assembly is possible = increase amplicon size



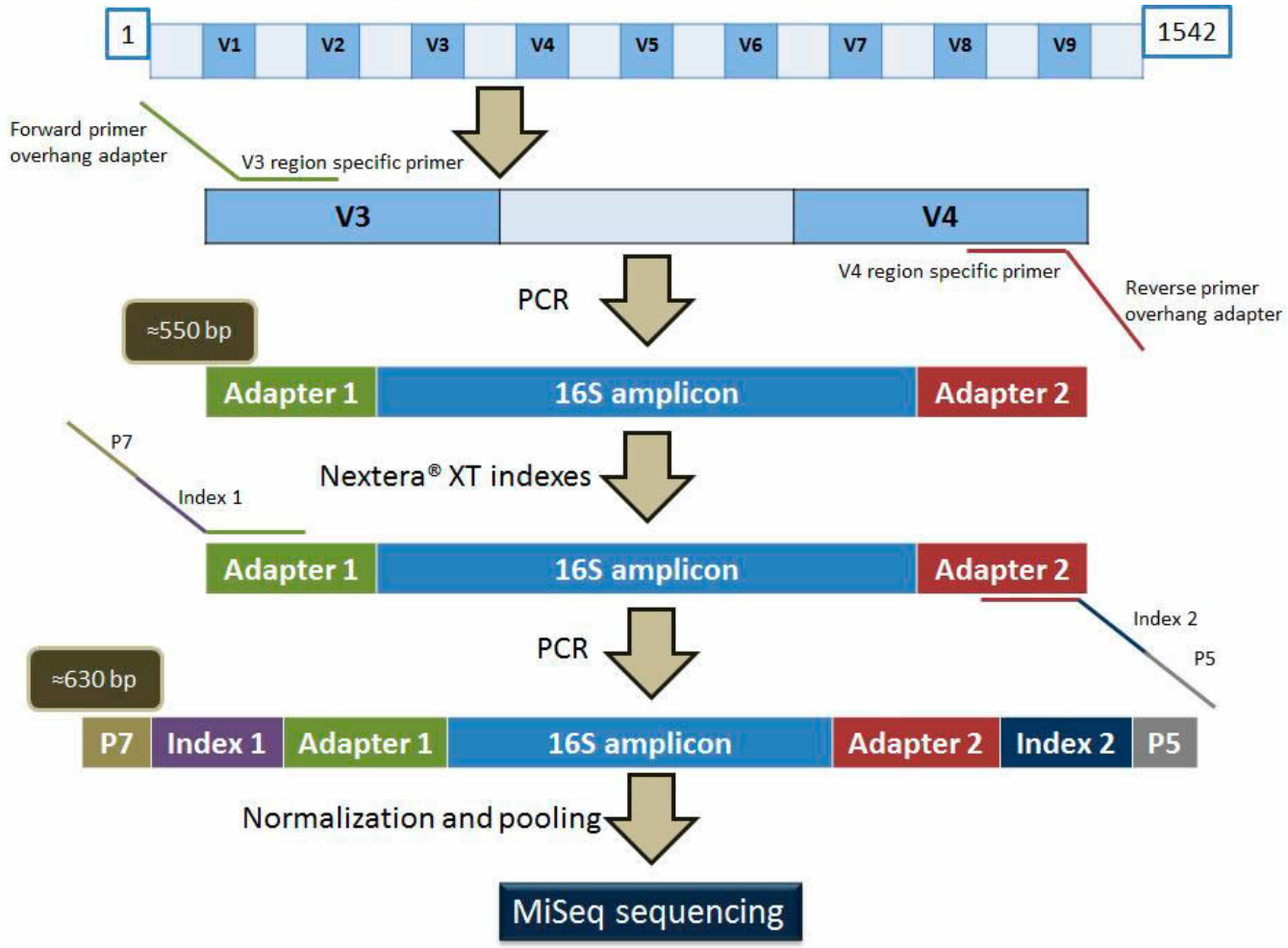
Assembly : 500 pb : improve taxonomy accuracy

Non Overlapping paired-end reads: You can not assemble the sequencing reads!!!

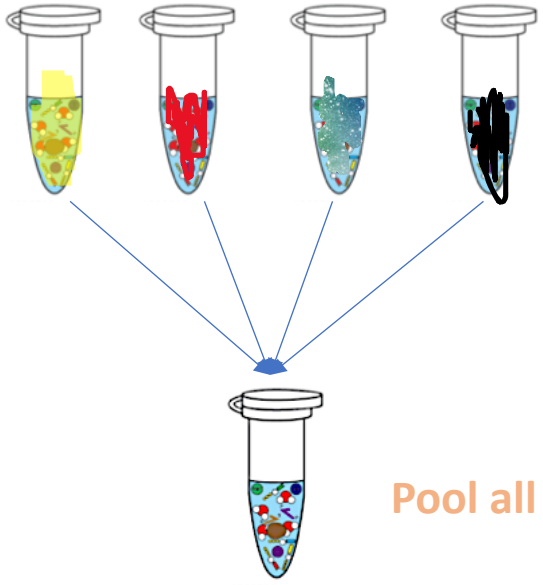


Wrong strategy

16S Miseq paired-end

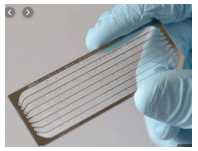


Index concept = Sample multiplexing on the same lane



16S Amplification for each Sample

Pool all samples!!!!!

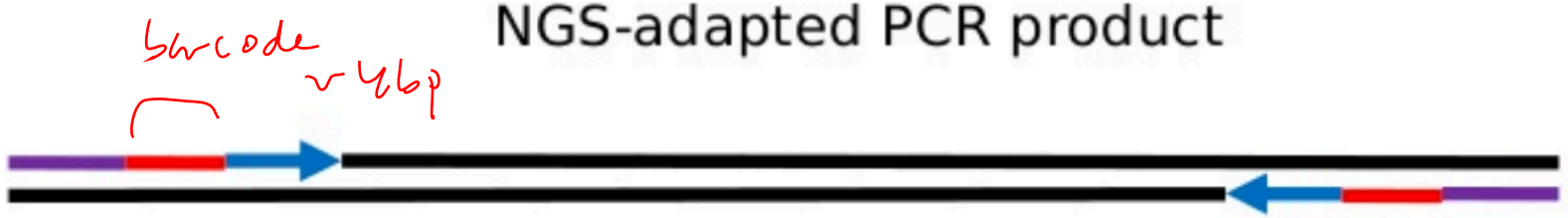


Sequencing

Analyses : How to discriminate sequences according to sample?
Which sequence belongs to which sample??????
→ Index/barcode strategy

Normal PCR product

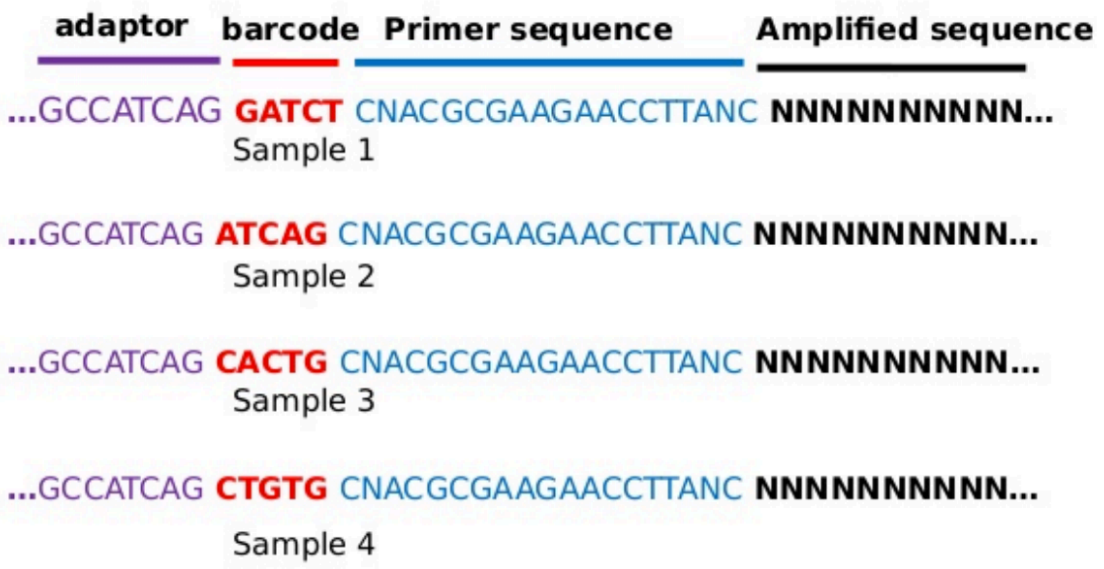
NGS-adapted PCR product



Barcoded PCR product for NGS

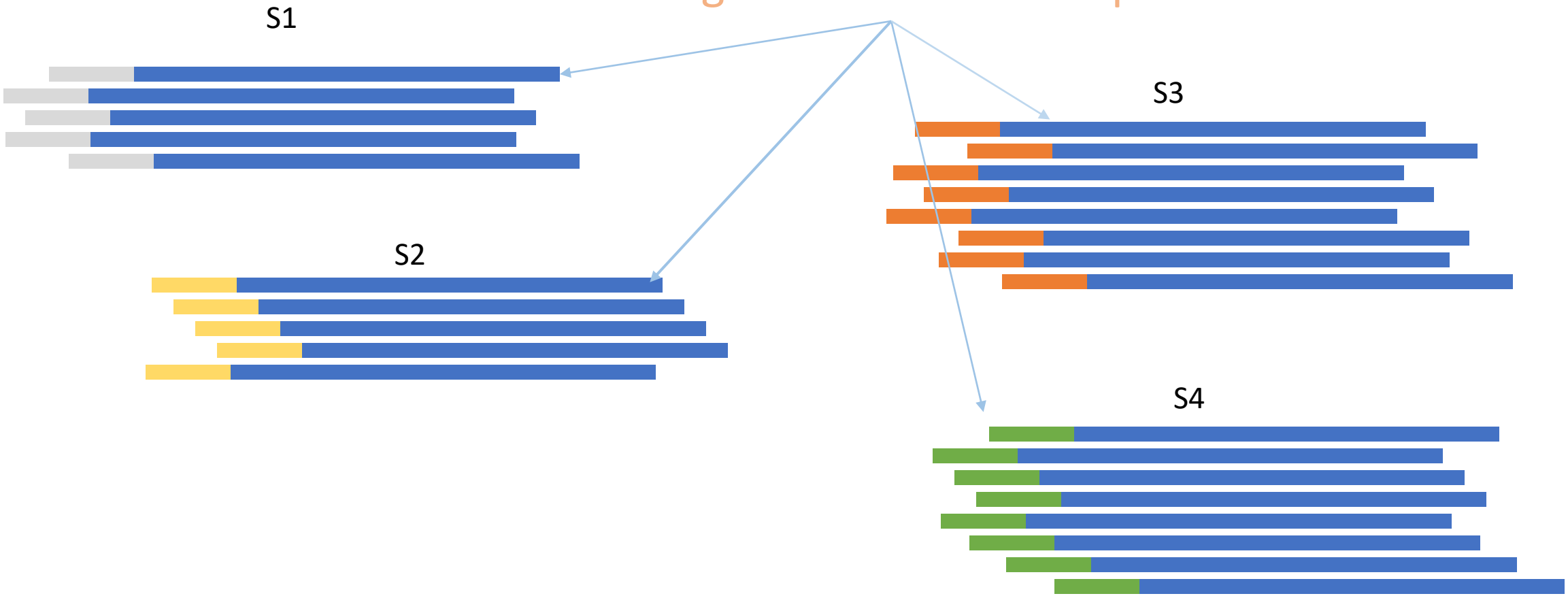
Add a different Index/barcode for each sample

Barcode

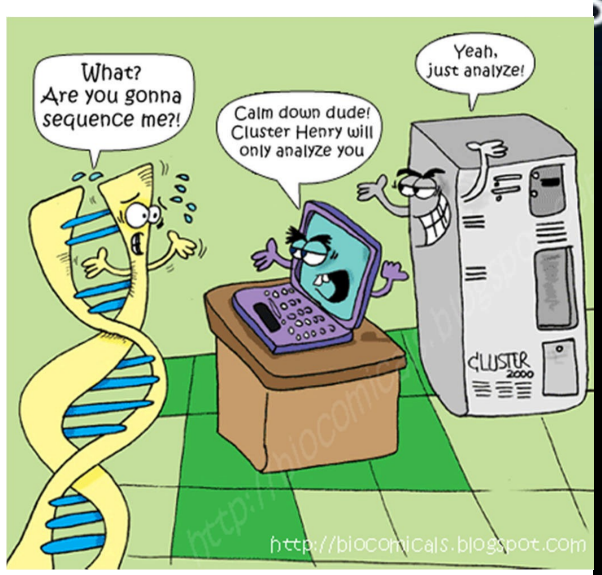


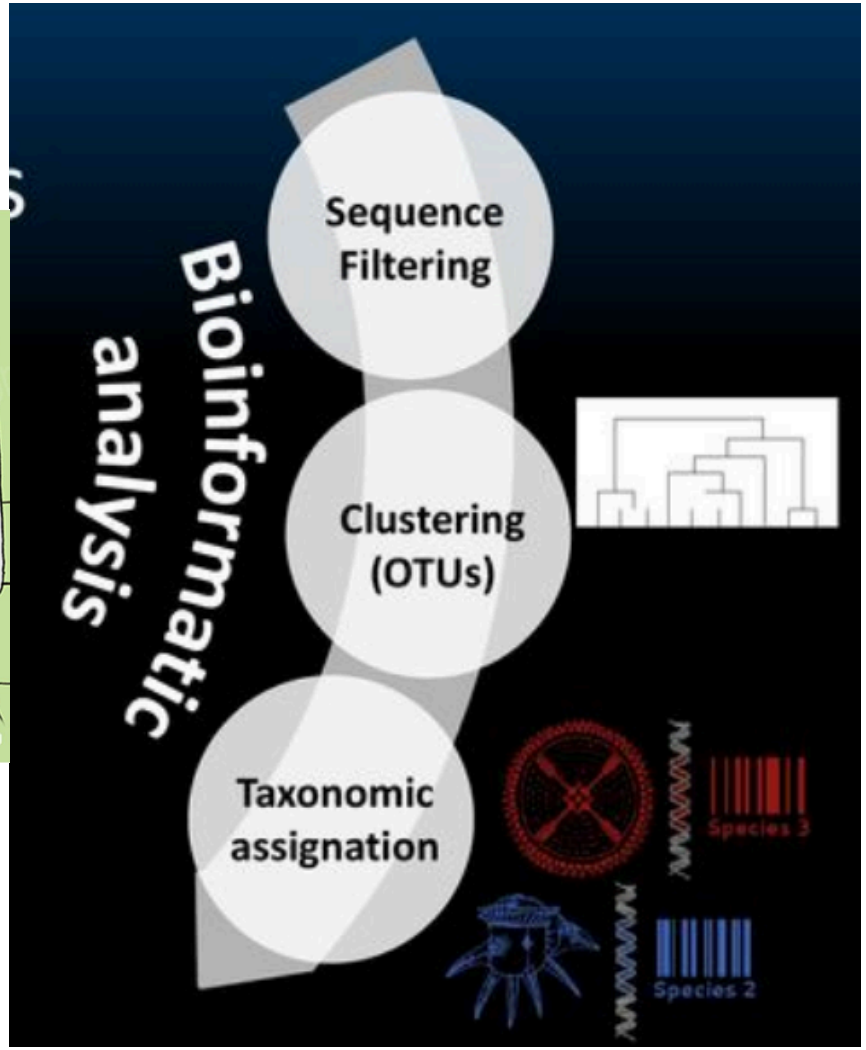
Bioinformatics

Sort sequencing reads of each Sample according to the barcode sequences



Bioinformatics processing…



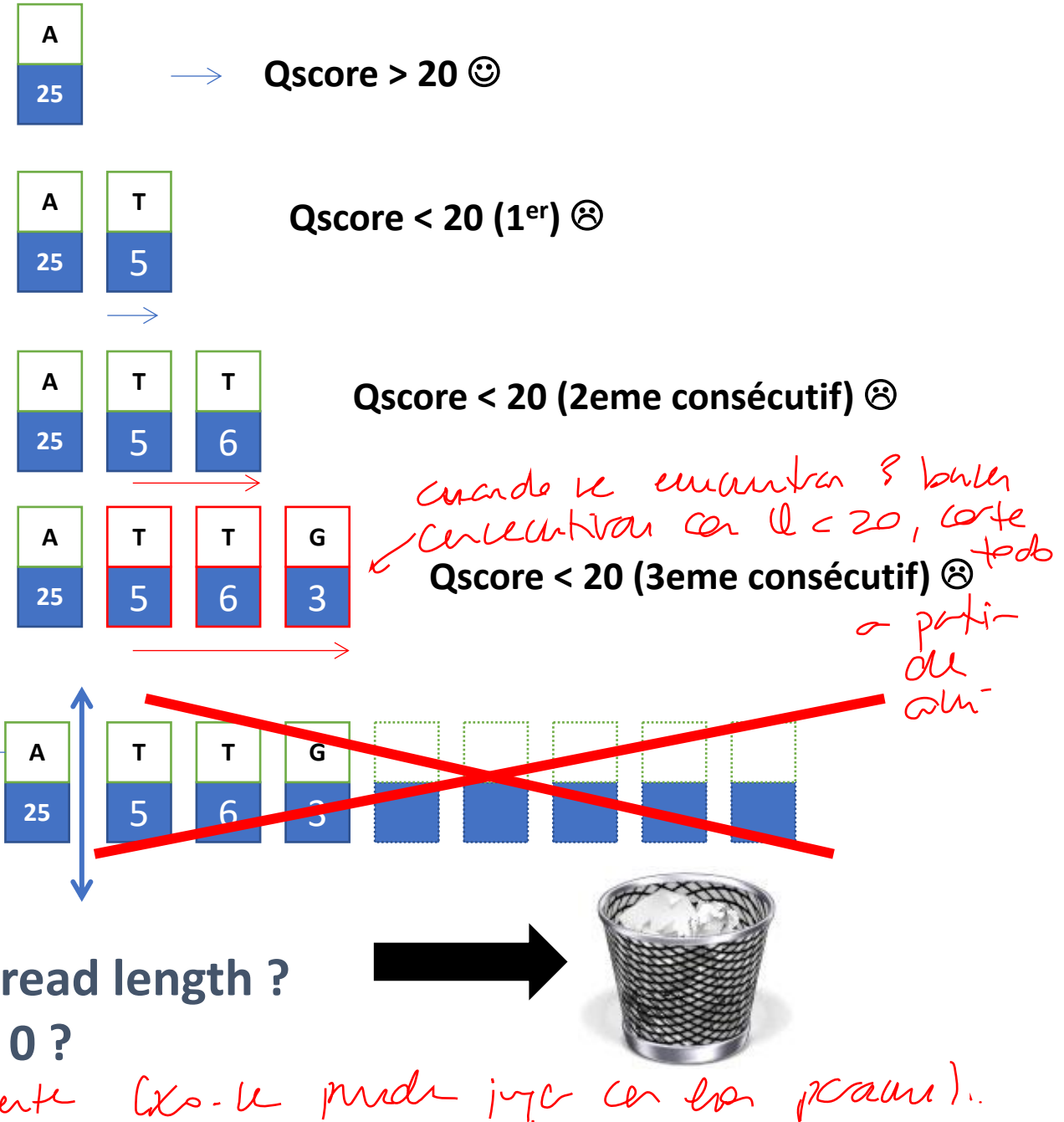


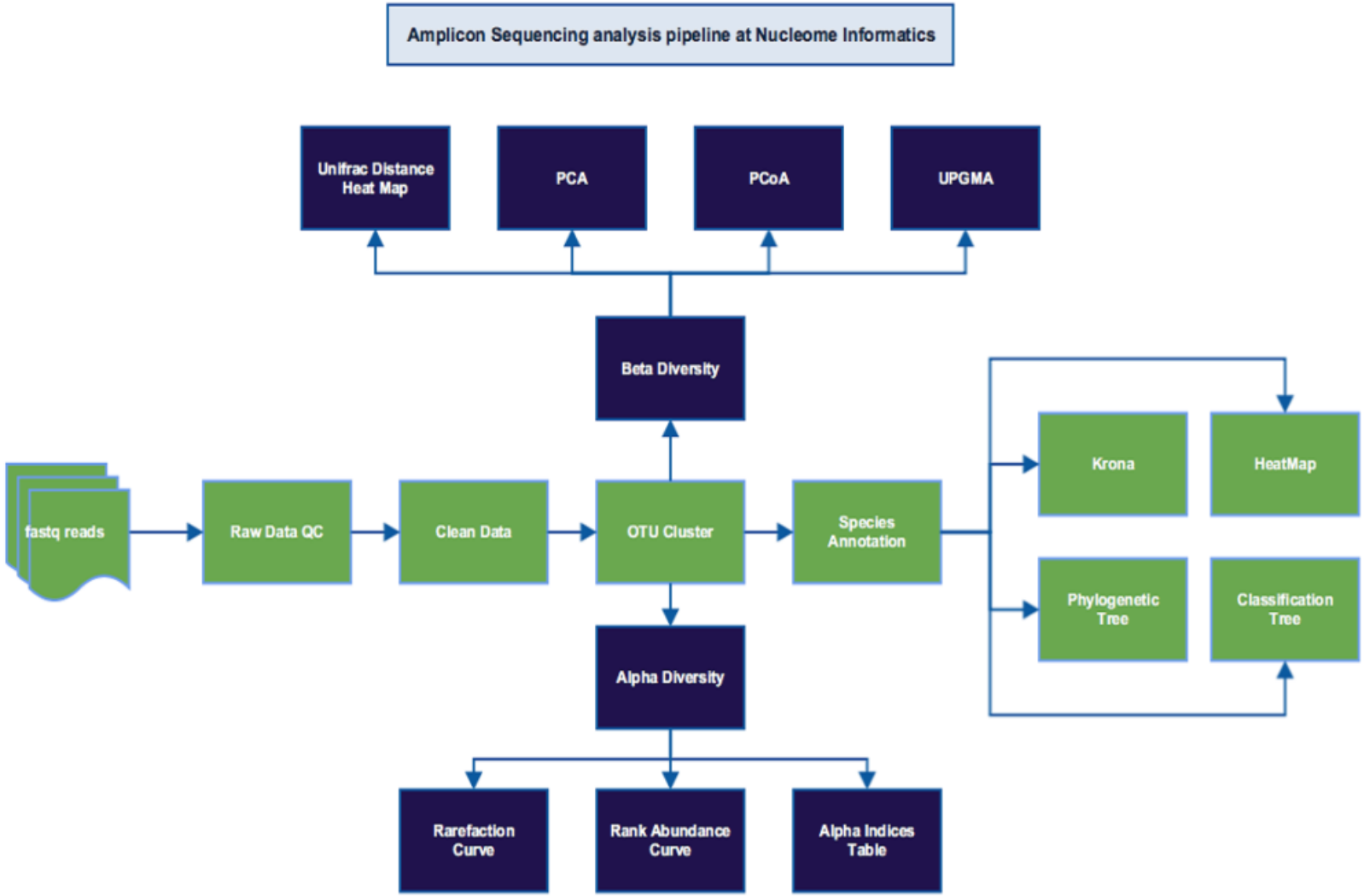
Quality Filtering

Remove bad quality sequencing reads:

- Avoid ambiguous bases « N »
- Define Min/max length of reads
- define cut-off for base Quality (Qscore max=40; min=0)

quality score




Amplicon Sequencing analysis pipeline at Nucleome Informatics
Unifrac Distance Heat Map
PCA
PCoA
UPGMA
Beta Diversity
fastq reads
Raw Data QC
Clean Data
OTU Cluster
Species Annotation
Krona
HeatMap
Phylogenetic Tree
Classification Tree
Alpha Diversity
Rarefaction Curve
Rank Abundance Curve
Alpha Indices Table