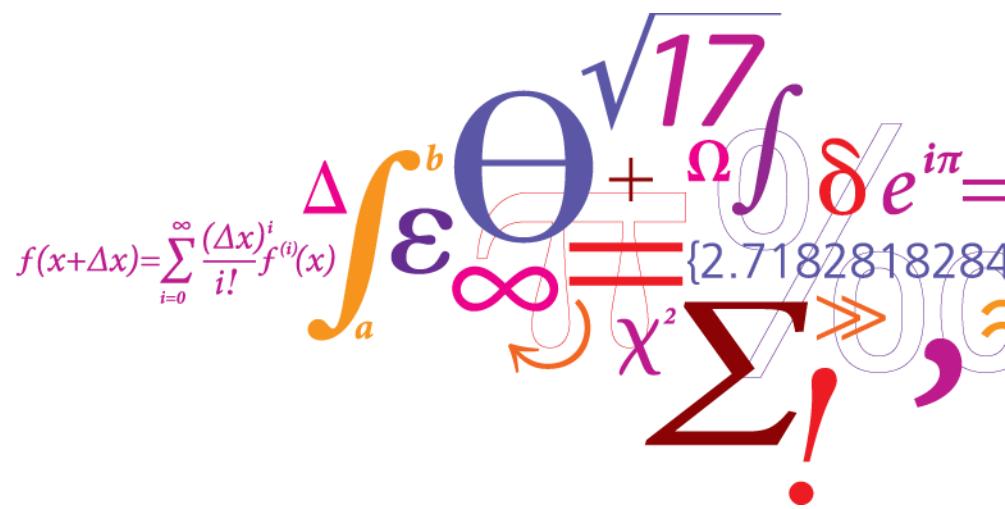


From Organisms to Genomics: From Alive to Live

Saria Otani, Assist. Prof.
MRes., MSc., PhD

Applied Methods in Metagenomics - 23260
Denmark, 30/08/2022

$$f(x+\Delta x) = \sum_{i=0}^{\infty} \frac{(\Delta x)^i}{i!} f^{(i)}(x)$$

$$\Theta^{\sqrt{17}} + \Omega \int \delta e^{i\pi} =$$
$$\{2.718281828459045\}$$

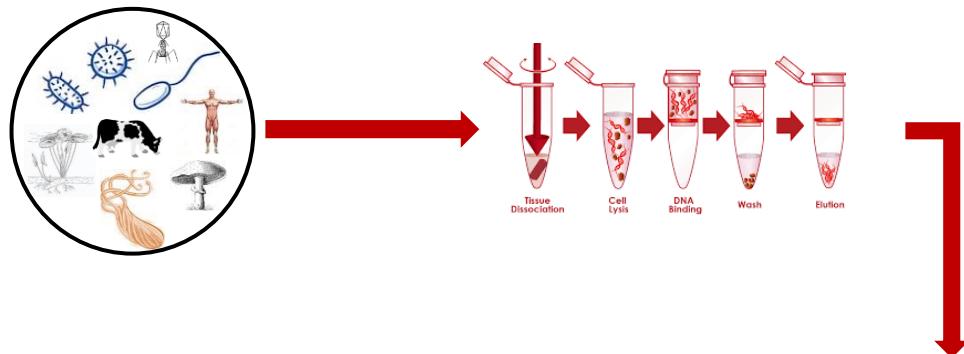
By next week you will be familiar with

Sequencing is only a tool!



1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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PACBIO®

Oxford
NANOPORE
Technologies



Examples

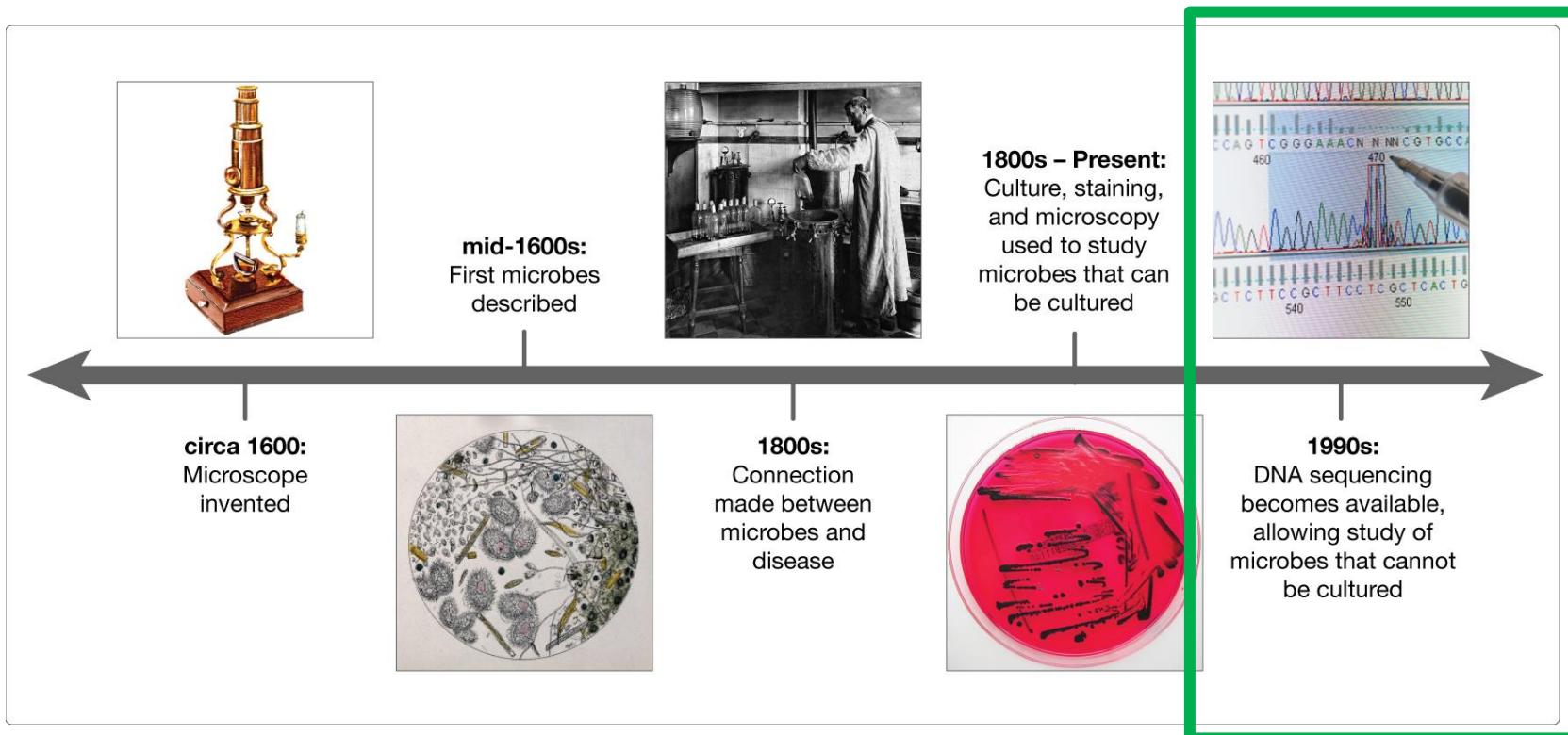


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How do we detect/study microbes

Microbial research in history

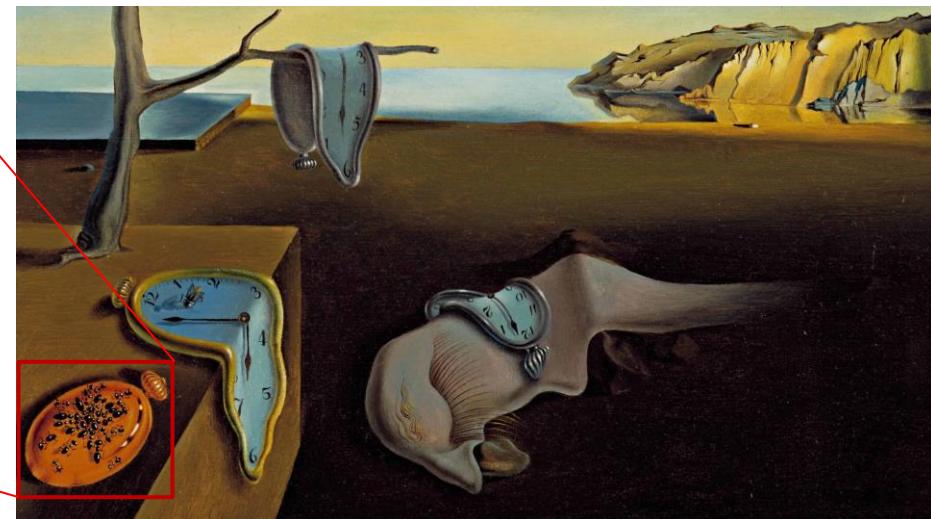
Taxonomy and function



Do I need to change! A transition from conventional to NGS methods.

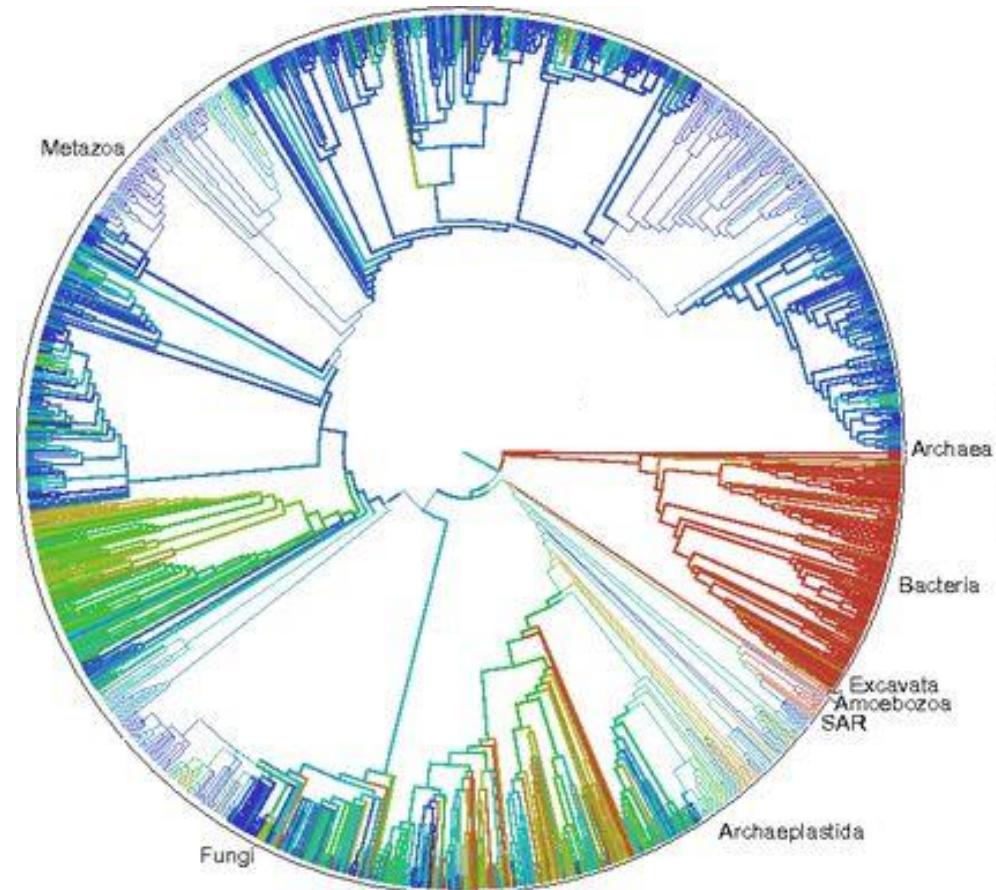
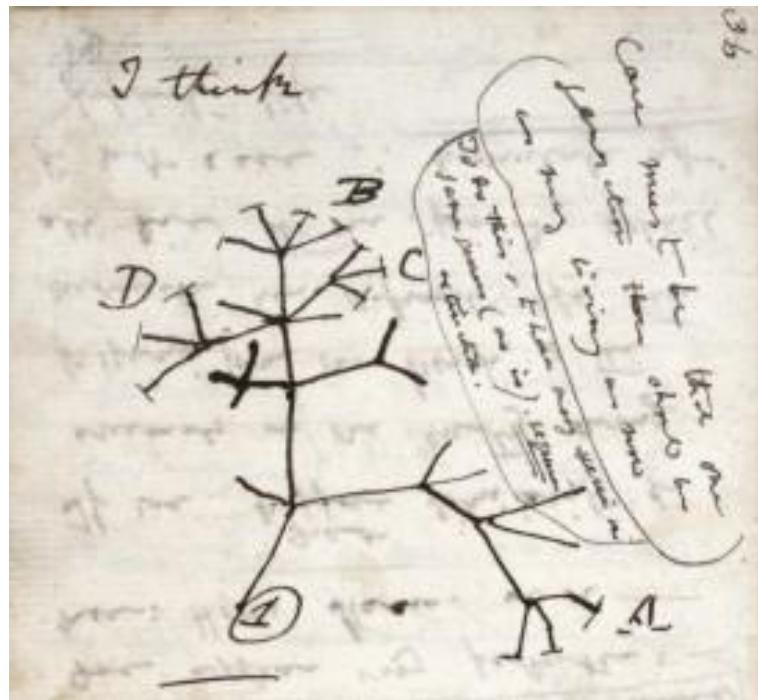
Next-Generation sequencing in Microbiology

What does this different tool provide?



Next-Generation sequencing as a diagnostic tool, why bother!

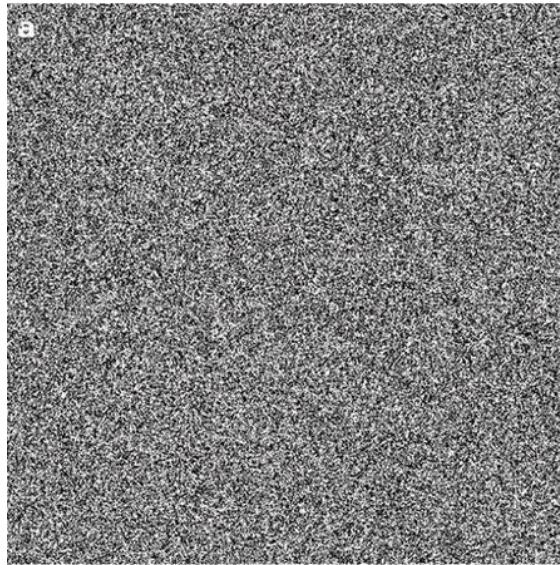
A transition from conventional to NGS methods. High resolution



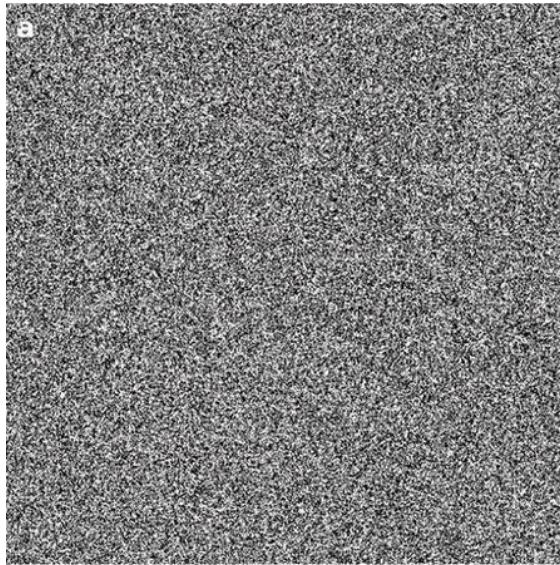
Next-Generation sequencing as a diagnostic tool – TOOL DEPENDENT



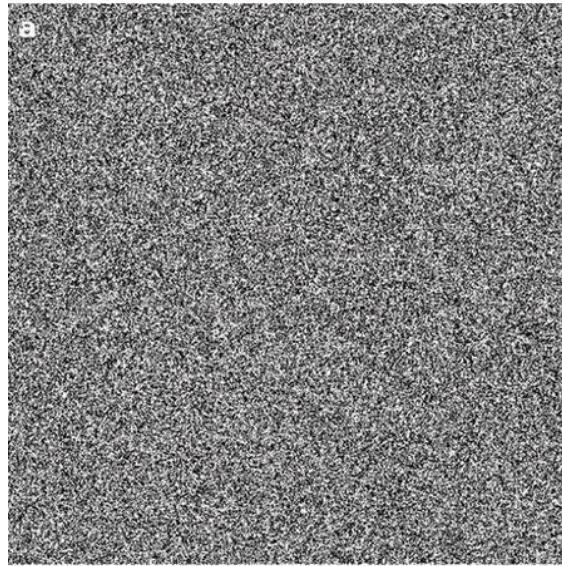
Enhancement
process,
depends on:
the object
and the tools



Enhancement
process,
depends on:
the bacteria and
the sequencing
technology
(the object
and the tools)

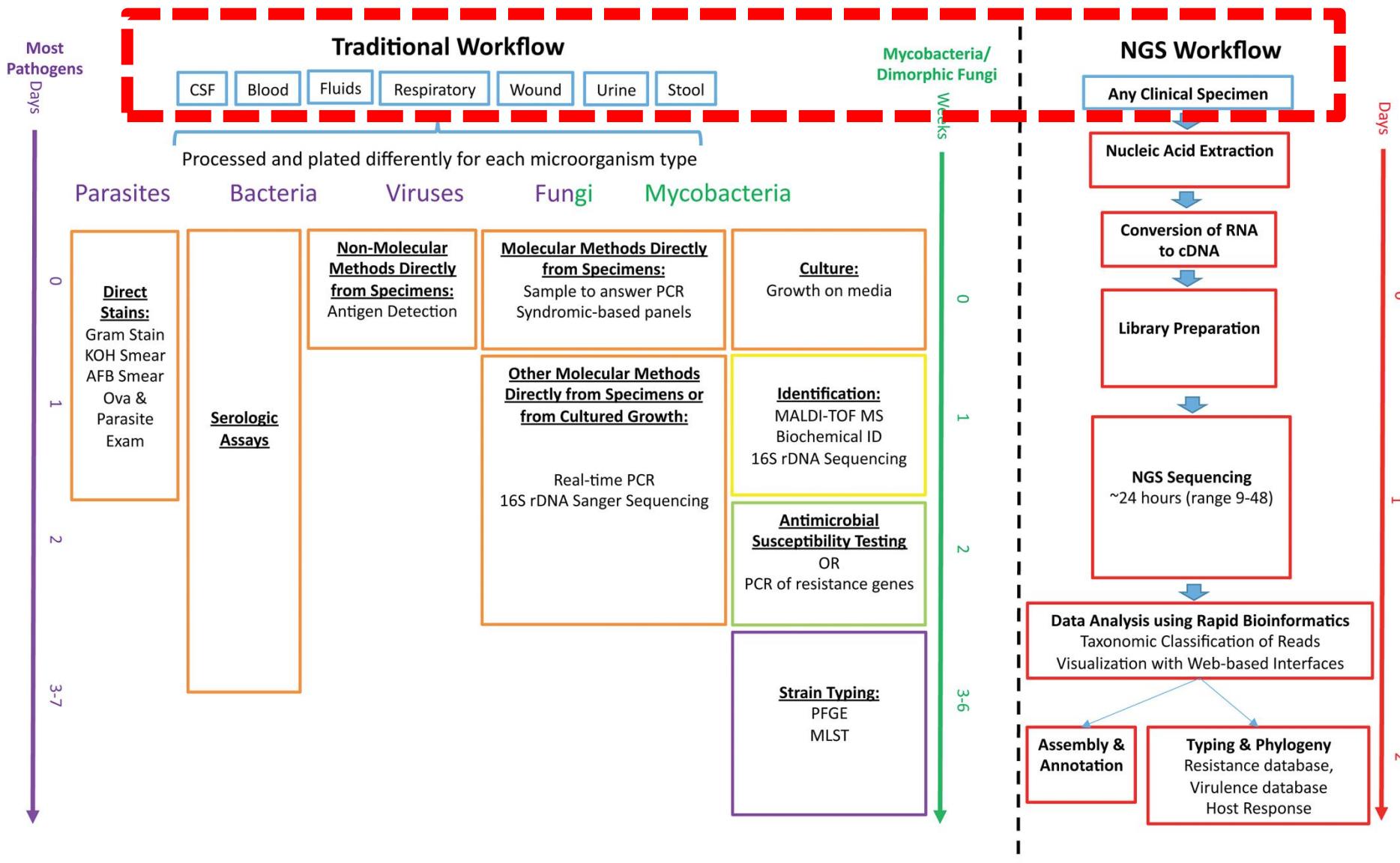


Enhancement
process,
depends on:
the bacteria and
the sequencing
technology
(the object
and the tools)



time: how quickly.

Comparison between NGS and traditional methods as a diagnostic tool



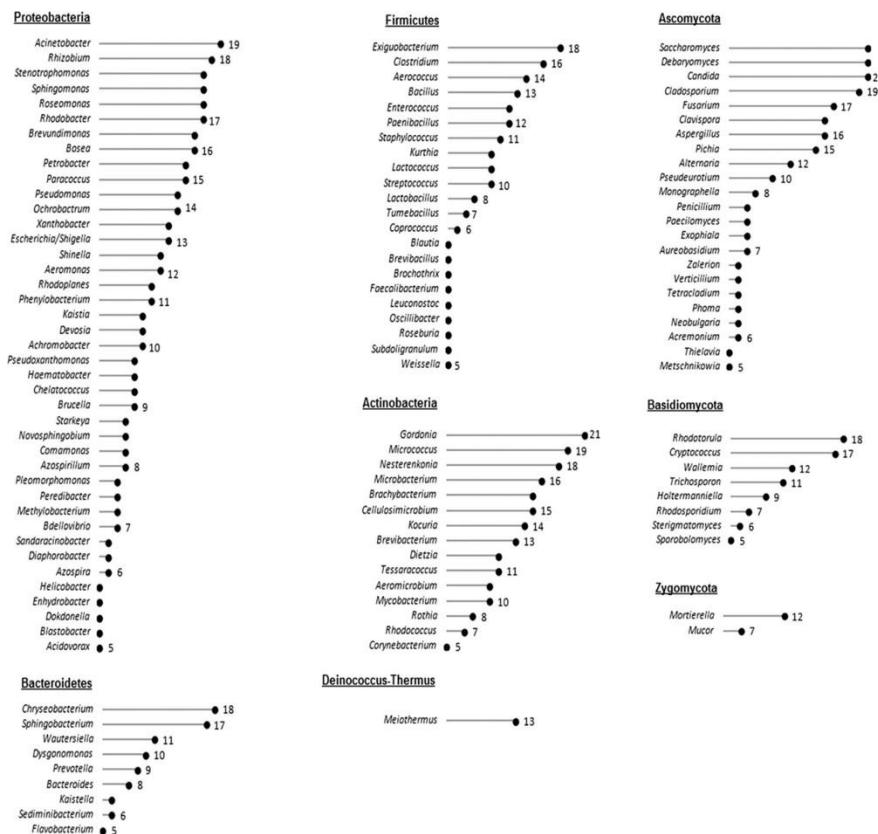
Next-Generation sequencing as a diagnostic tool, why bother!

Until 2018, no bacterial isolate has been detected.

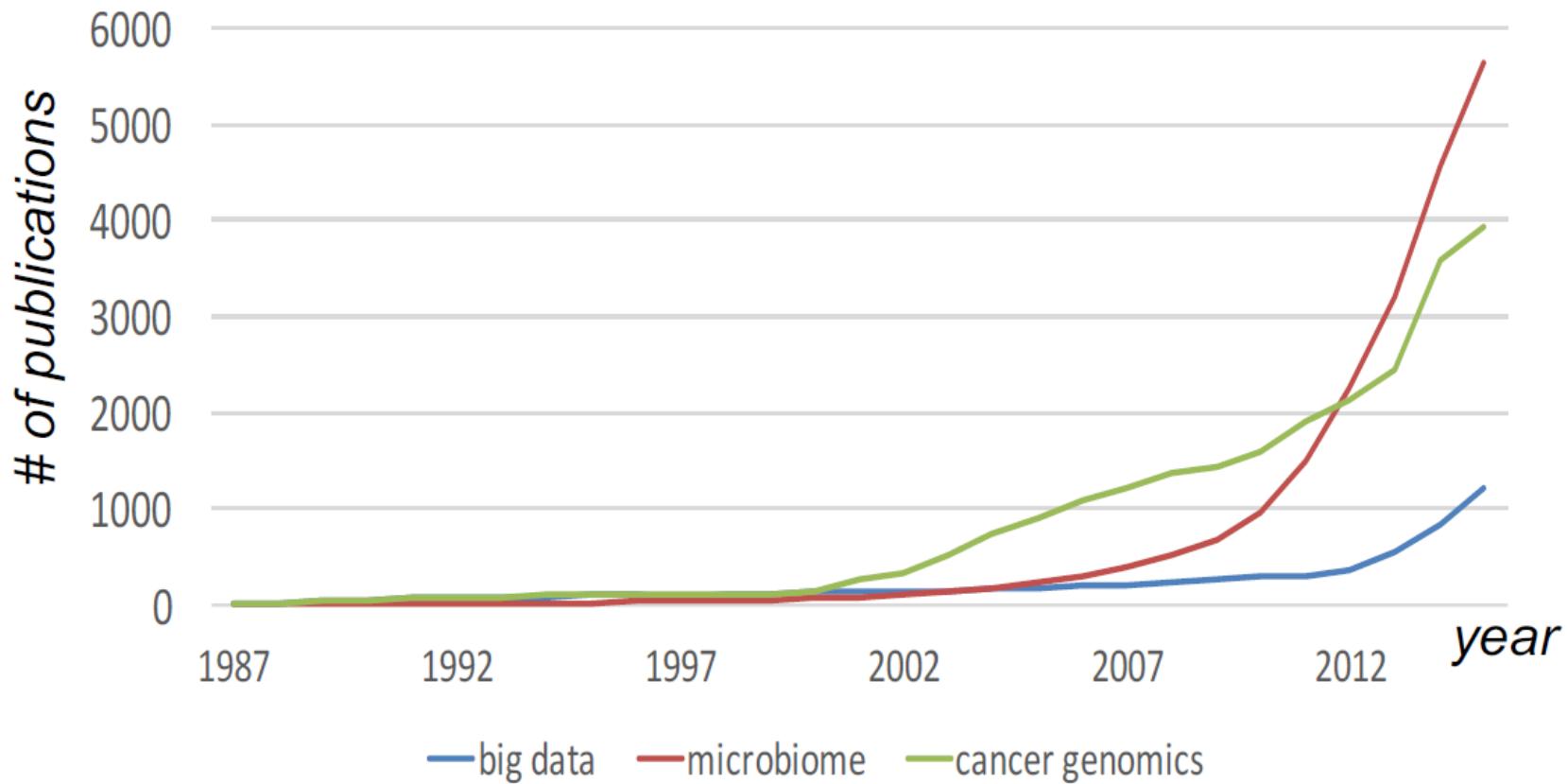


NGS was used in 2017:

> 300 bacterial genera have been detected. A good deal is pathogenic



Microbiome research



What is metagenomics



The study of all genetic material in an environmental sample.

What is microbiome

The collection of microbes that exist in/on a given habitat, tissue or another organism. It refers to all microbes including bacteria, fungi, eukaryotic parasites and viruses.

The microbiome, e.g., human microbiome

How human are we, really?

Human

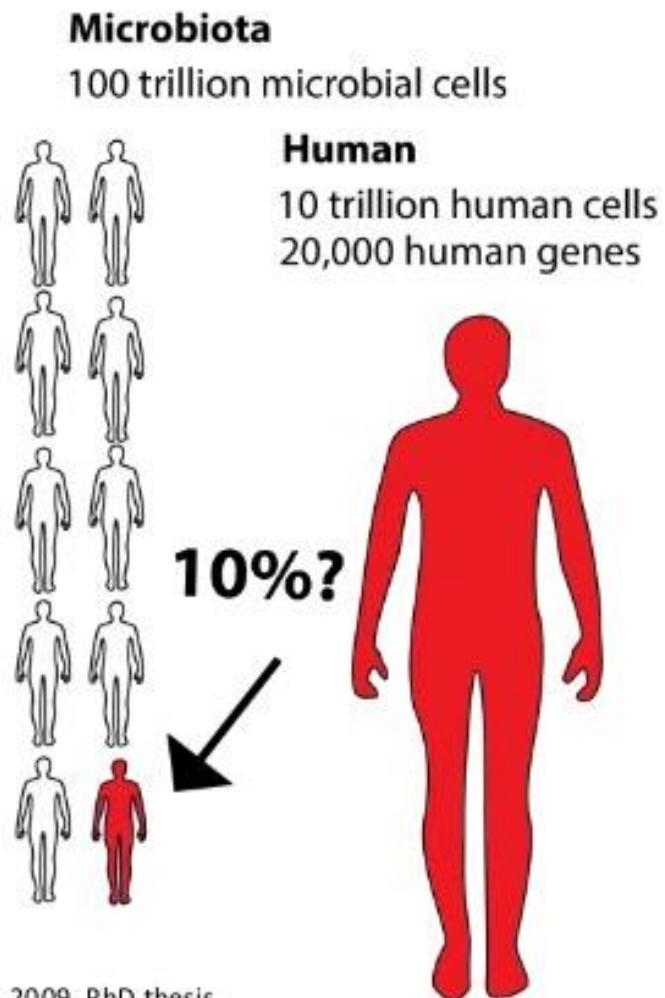
10 trillion human cells
20,000 human genes



Hamady, 2009, PhD thesis

The microbiome, e.g., human microbiome

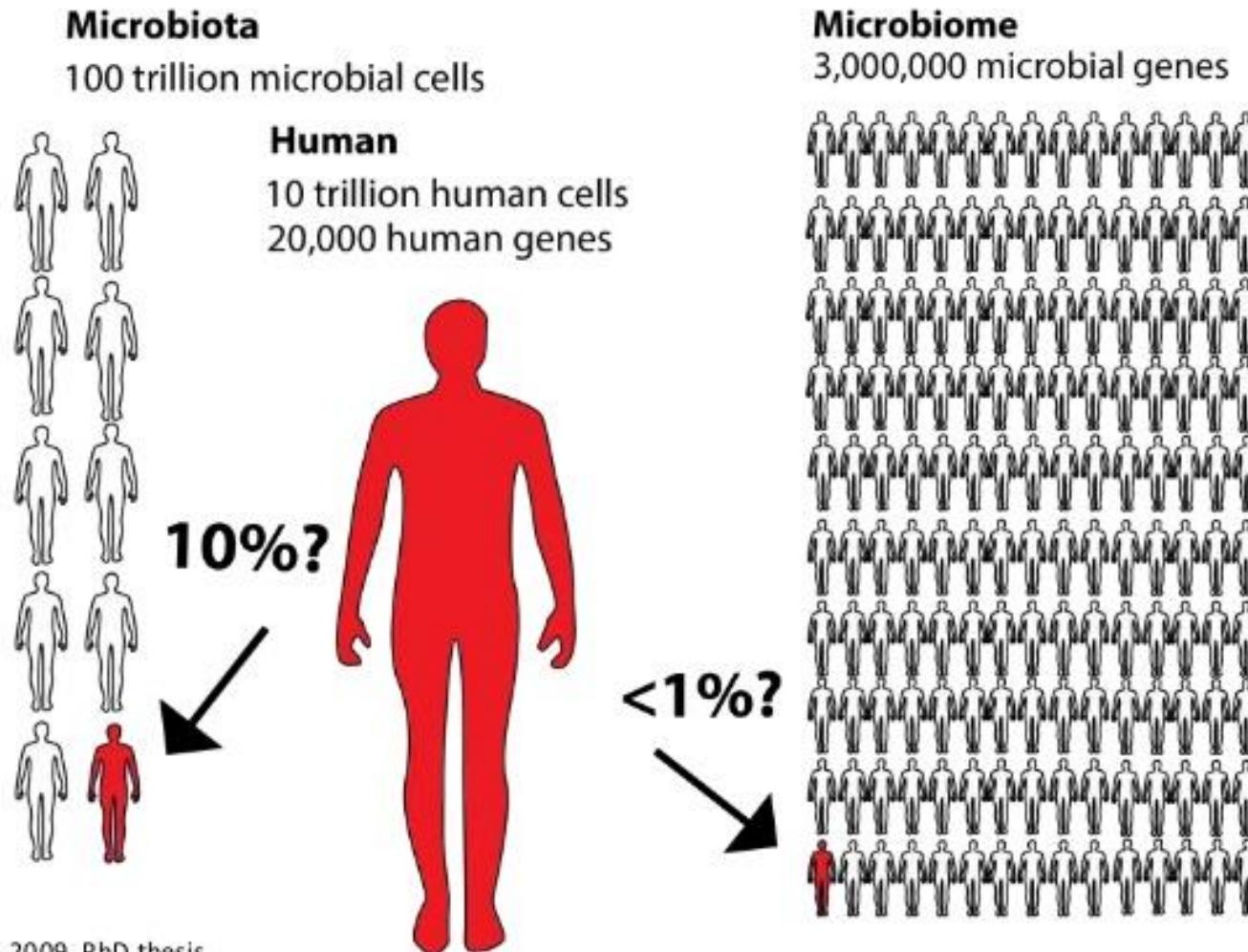
How human are we, really?



Hamady, 2009, PhD thesis

The microbiome, e.g., human microbiome

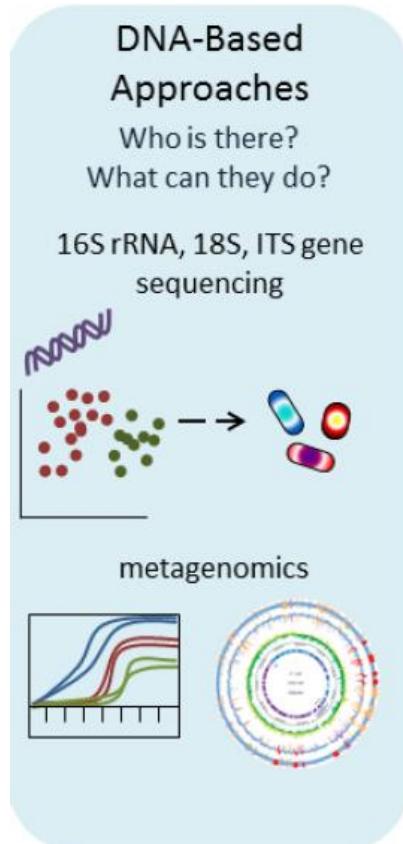
How human are we, really?



Hamady, 2009, PhD thesis

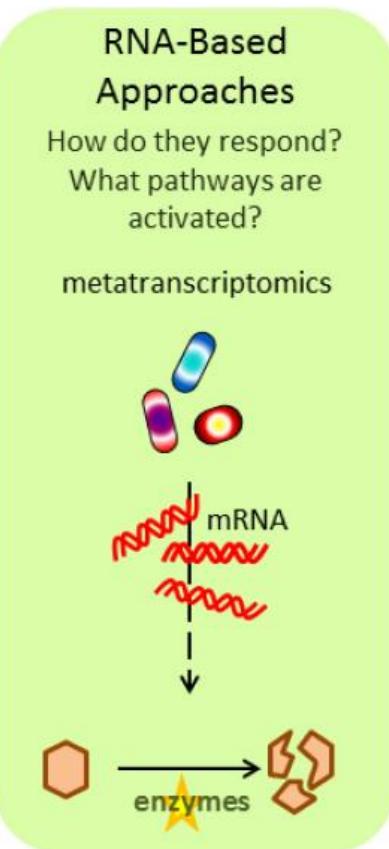
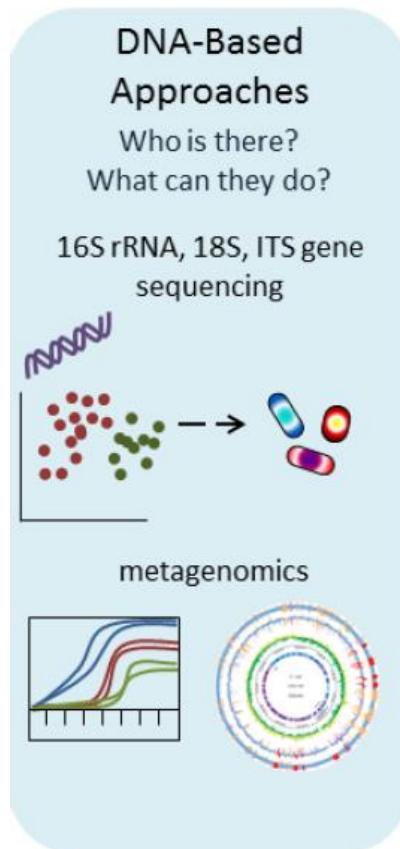
How do we detect/study a microbiome

Current approach



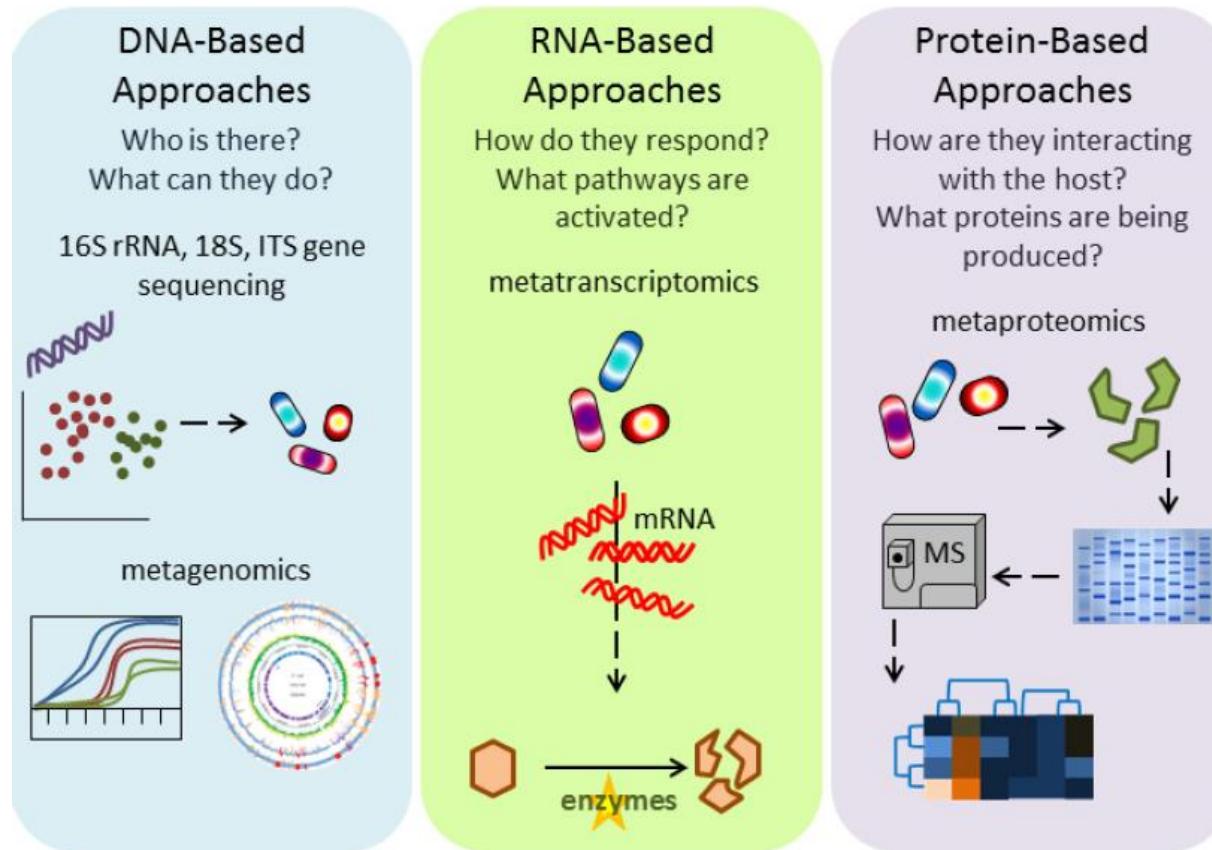
How do we detect/study a microbiome

Current approach



How do we detect/study a microbiome

Current approach



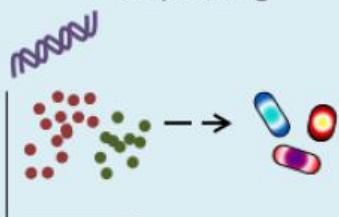
How do we detect/study a microbiome

Current approach

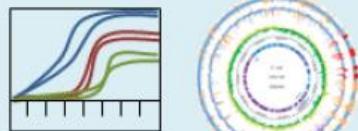
DNA-Based Approaches

Who is there?
What can they do?

16S rRNA, 18S, ITS gene sequencing



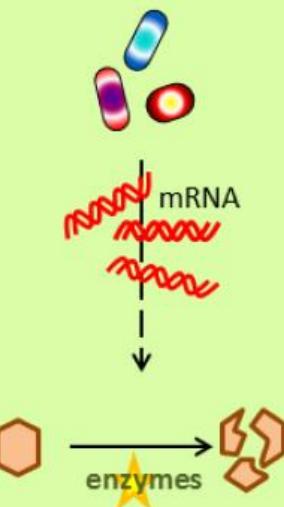
metagenomics



RNA-Based Approaches

How do they respond?
What pathways are activated?

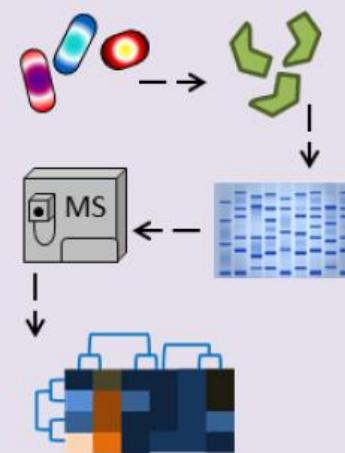
metatranscriptomics



Protein-Based Approaches

How are they interacting with the host?
What proteins are being produced?

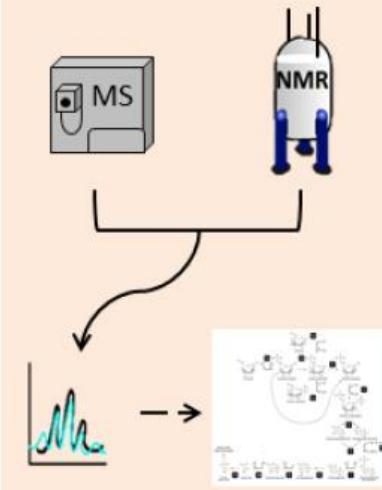
metaproteomics



Metabolite-Based Approaches

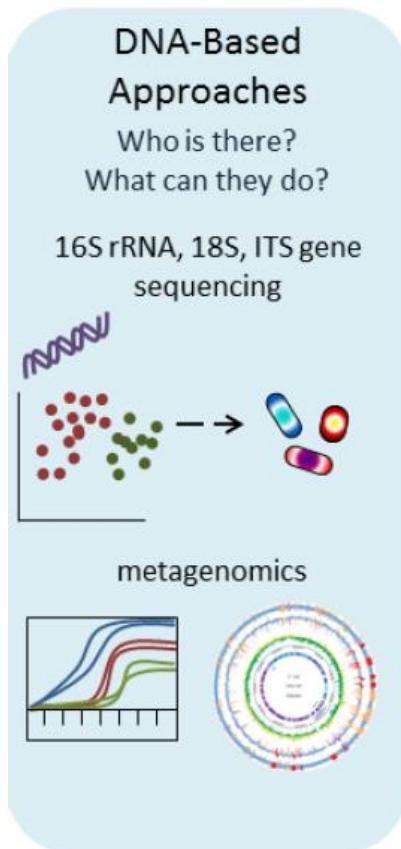
What are the chemical outcomes of their activity?

metabolomics



How do we detect/study a microbiome

Current approach

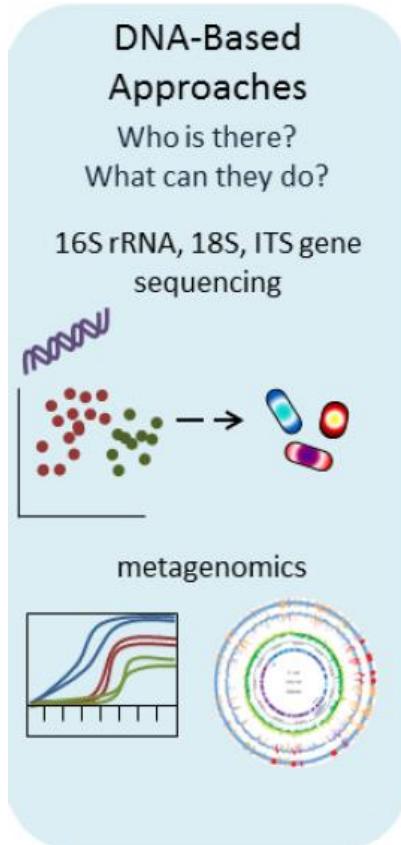


DNA Sequence information

- Is a universal "language"
- Provided in standardized format
- Share across labs and disciplines
- Serves as an archive, available for reanalysis

How do we detect/study a microbiome

Current approach



Microbial Genomics

16S rRNA gene profiling



Metagenomics



Whole Genome Sequencing (WGS)



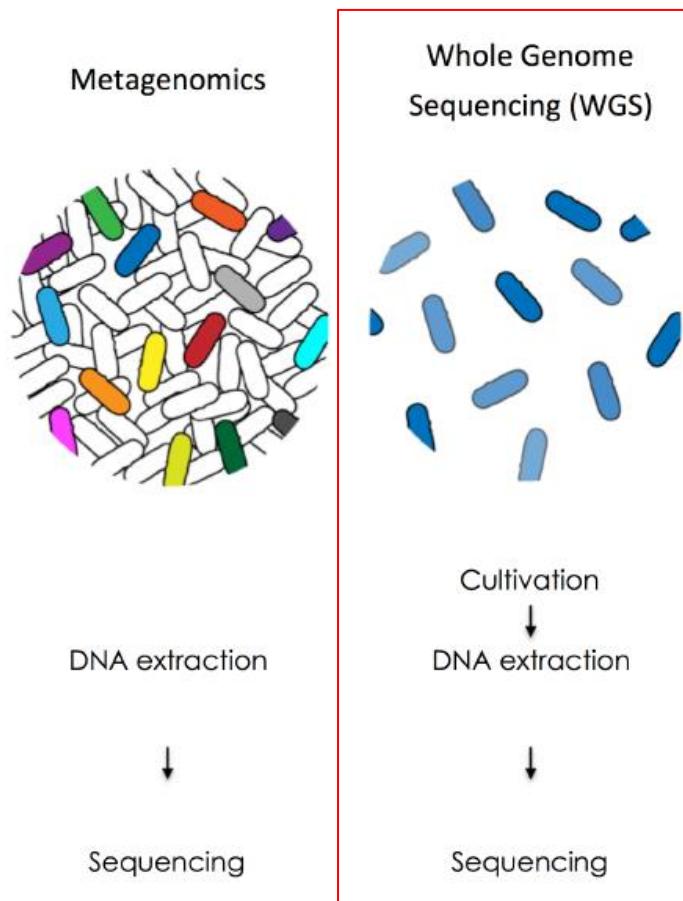
Single Cell Sequencing



Critically choose sample suitable for sequencing

Metagenomics (e.g., soil, meat, faeces)

Whole-Genome sequencing (pure culture)



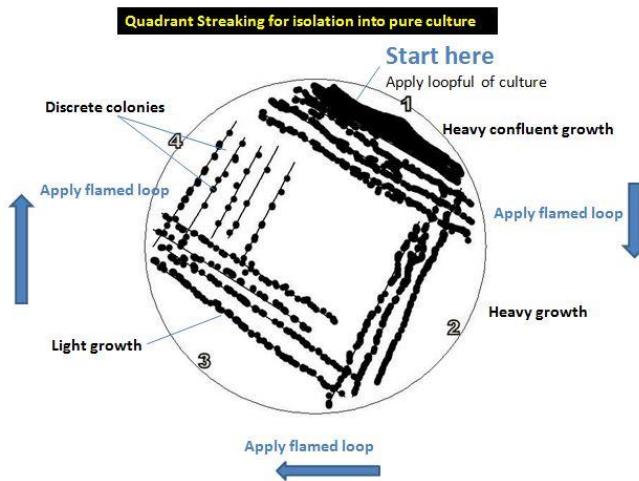
Critically choose sample suitable for sequencing

Metagenomics (*e.g.*, soil, meat, faeces)

Whole-Genome sequencing (pure culture)

Critically choose bacterial cultures suitable for sequencing (pure)

Streak plate method.



Critically choose sample suitable for sequencing

Metagenomics (*e.g.*, soil, meat, faeces)

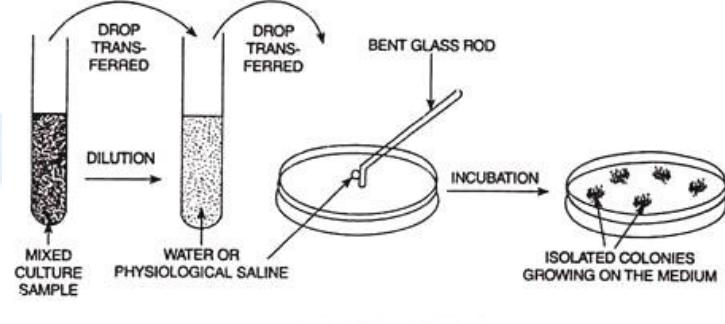
Whole-Genome sequencing (pure culture)

Critically choose bacterial cultures suitable for sequencing (pure)

Streak plate method.



Suspension dilutions.



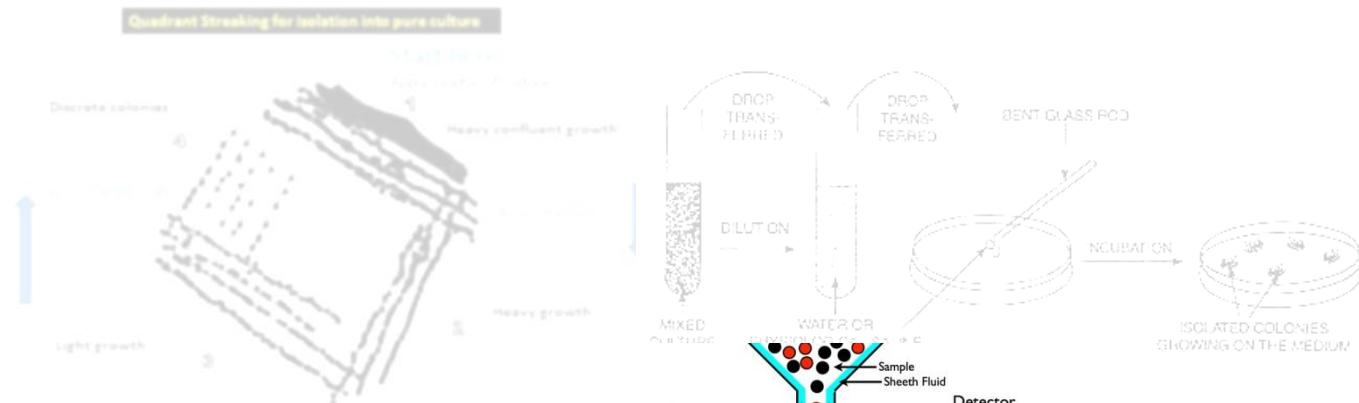
Critically choose sample suitable for sequencing

Metagenomics (*e.g.*, soil, meat, faeces)

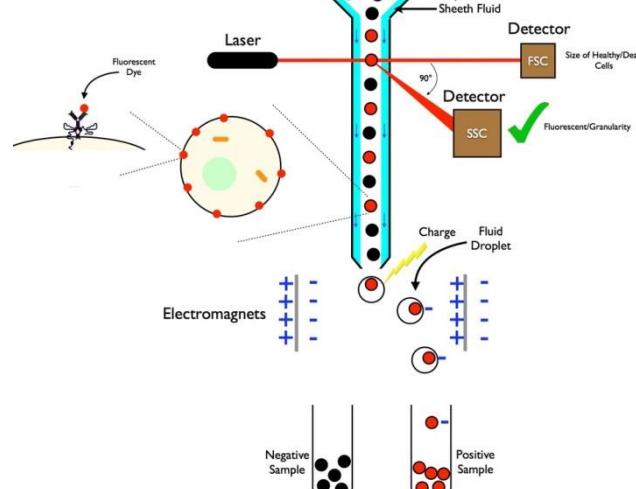
Whole-Genome sequencing (pure culture)

Critically choose bacterial cultures suitable for sequencing (pure)

Streak plate method.



Suspension dilutions.



FACS (fluorescence-activated cell sorting).

Critically choose bacterial cultures suitable for sequencing

1



2



3



4

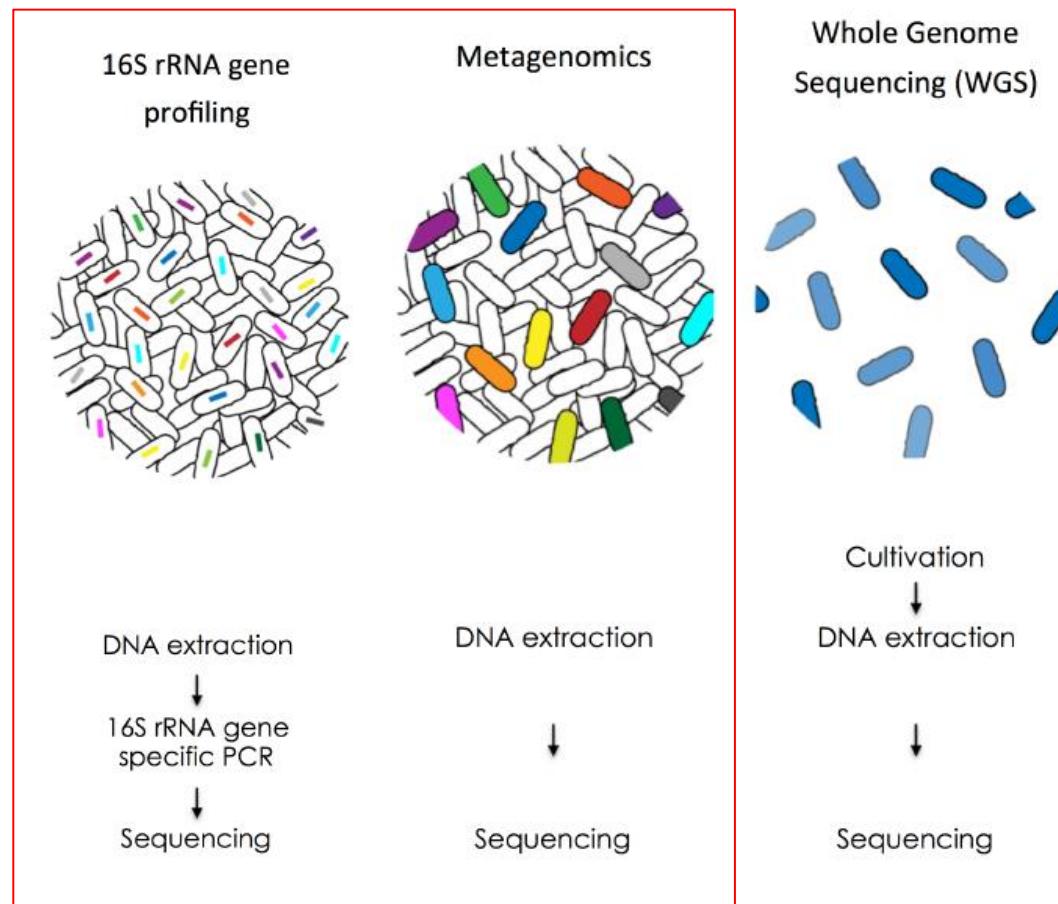


Critically choose sample suitable for sequencing

Metagenomics (e.g., soil, meat, faeces)

Whole-Genome sequencing (pure culture)

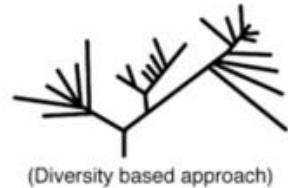
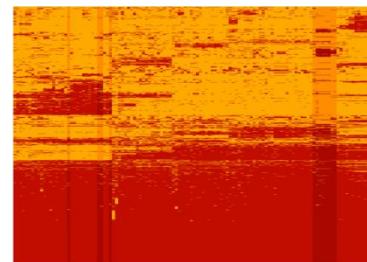
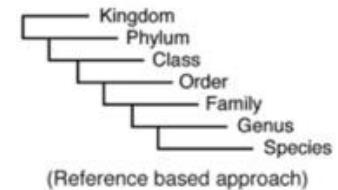
Different samples need different treatments and will have different microbiomes



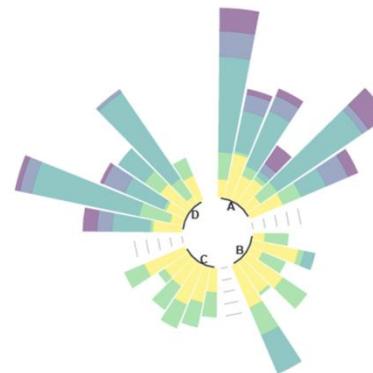
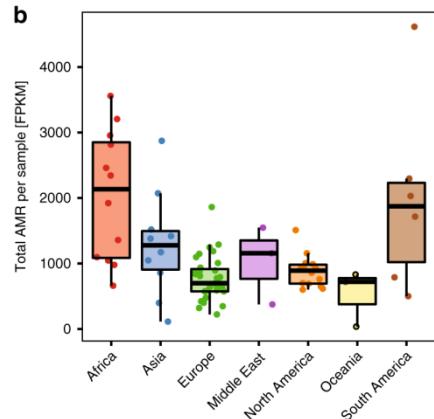
From sample to analysis

What do we want to achieve?

Bacterial profiling, microbiome.



Functional analyses: gene identifications, AMR, virulence, diet-decomposition, immunity-related, evolutionary traits.

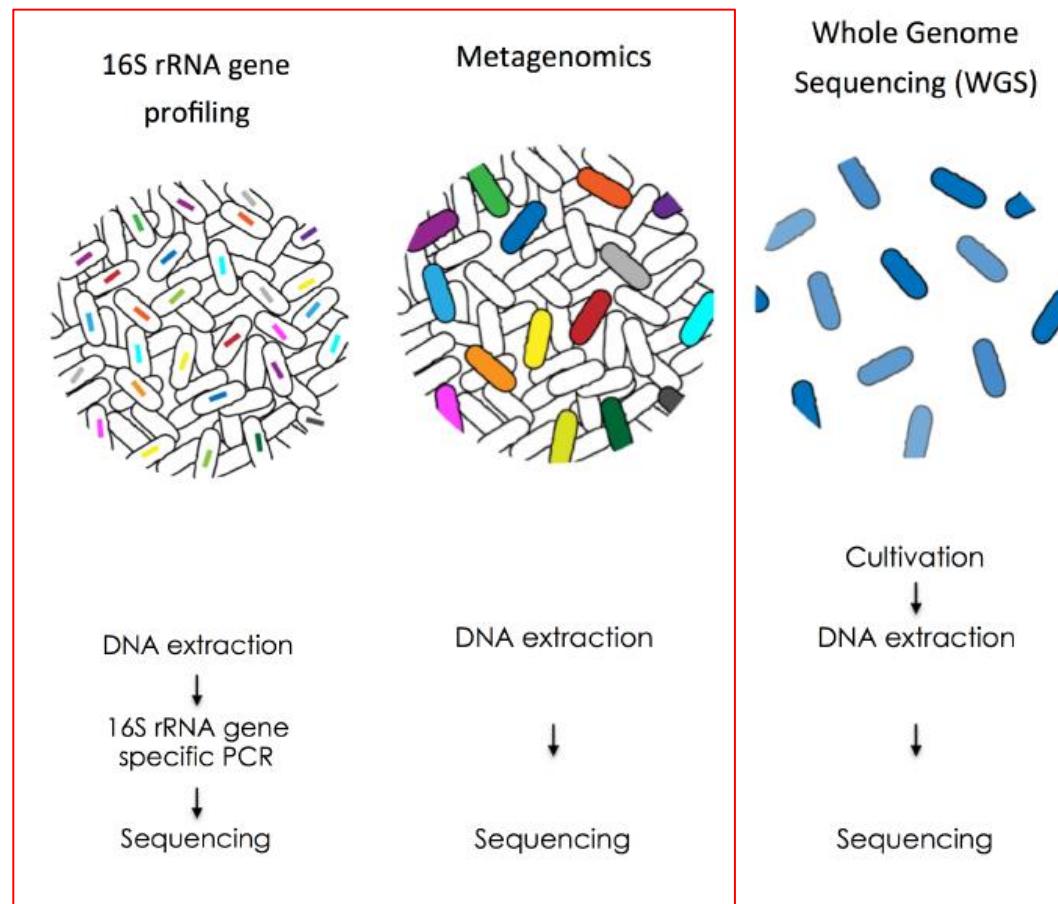


Critically choose sample suitable for sequencing

Metagenomics (e.g., soil, meat, faeces)

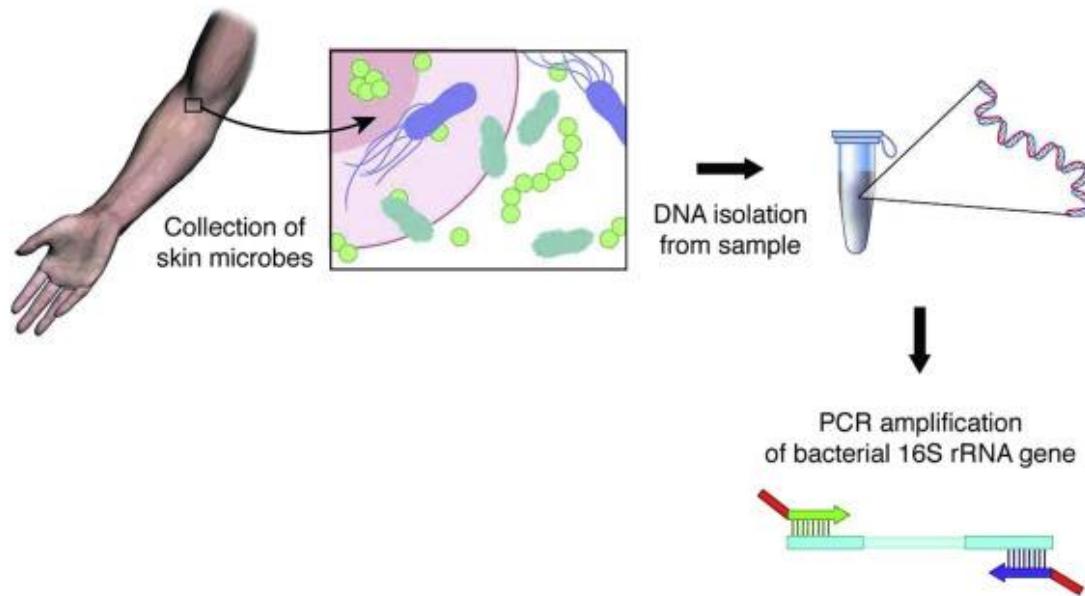
Whole-Genome sequencing (pure culture)

Different samples need different treatments and will have different microbiomes



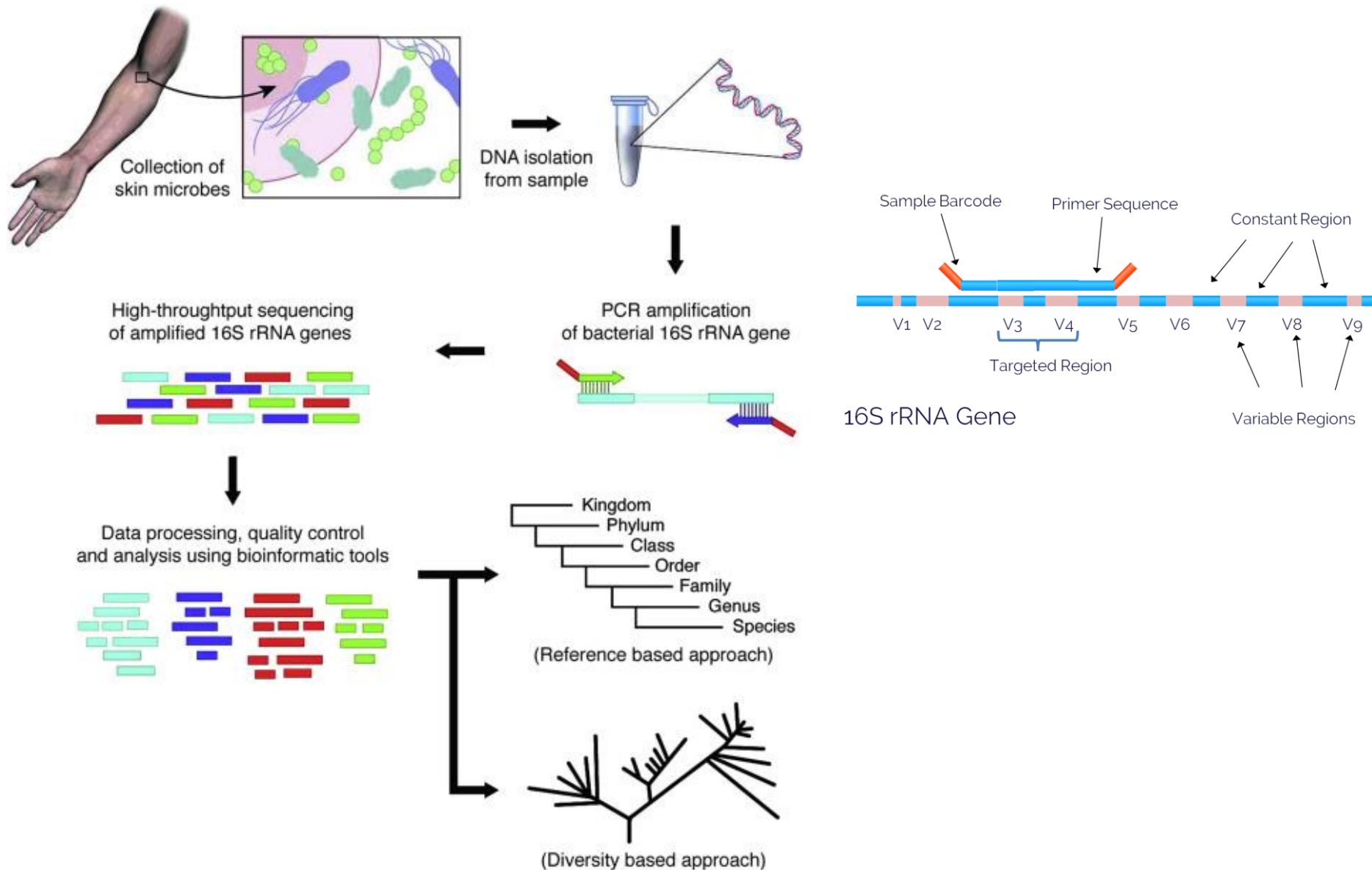
How do we detect/study a microbiome

16S rRNA profiling



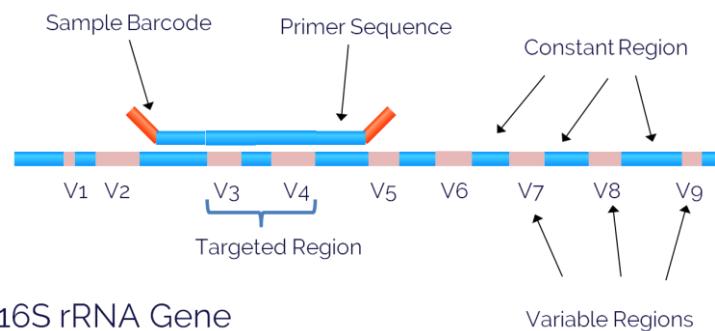
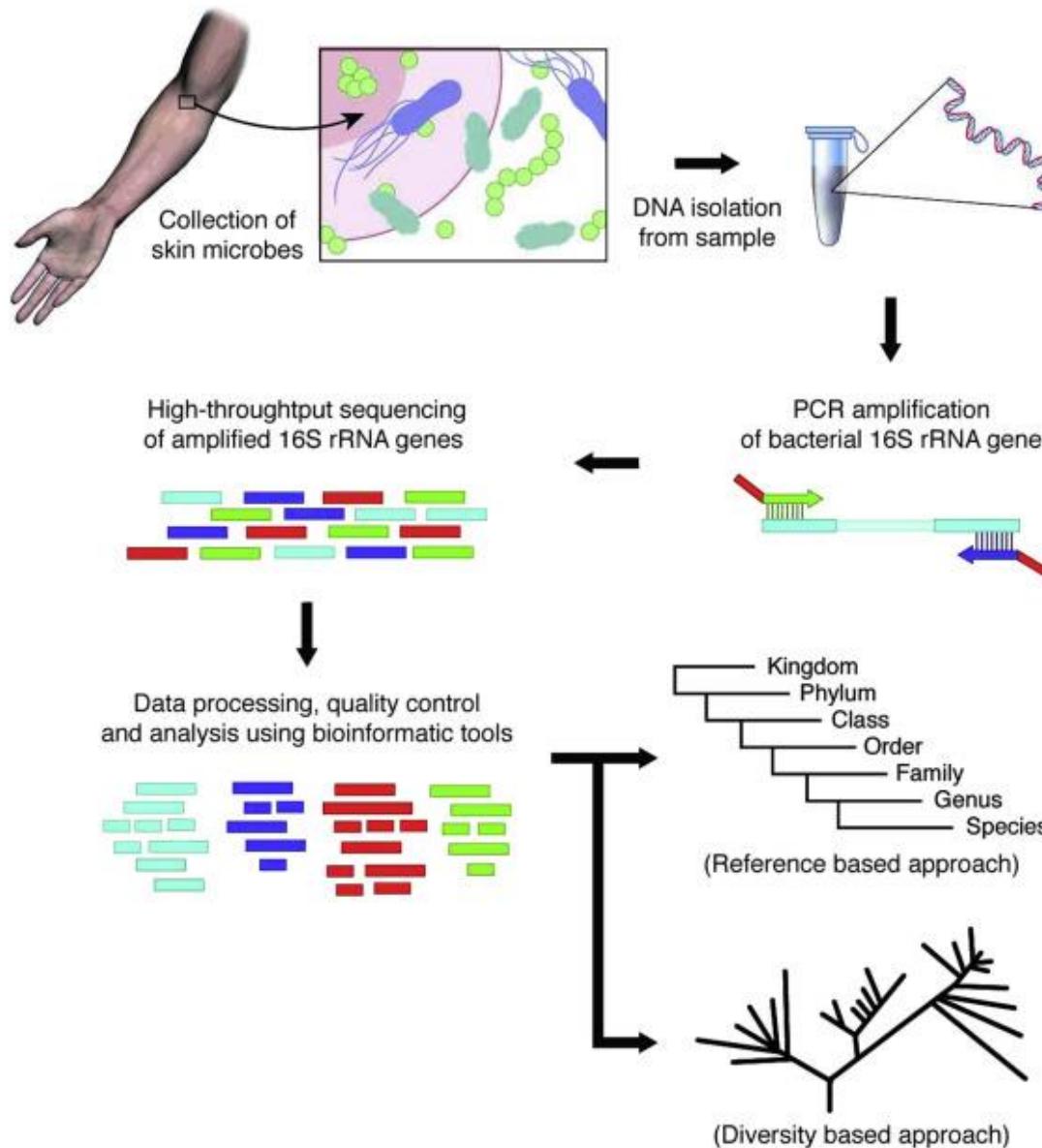
How do we detect/study a microbiome

16S rRNA profiling



How do we detect/study a microbiome

16S rRNA profiling



End product:

A profile of all bacterial taxa
in the studied niche.

Composition, who is there!

How do we detect/study a microbiome

16S rRNA profiling,

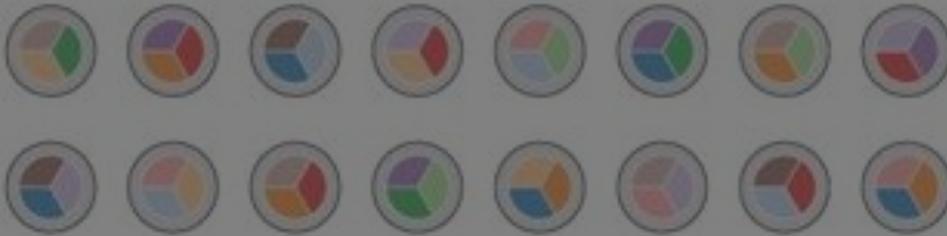
Example:

A microbiome
soil, plant...



What does 16S rRNA sequencing lack?

Sequence only the 16S gene, each colour corresponds to a bacterium species (variable regions)



End product:

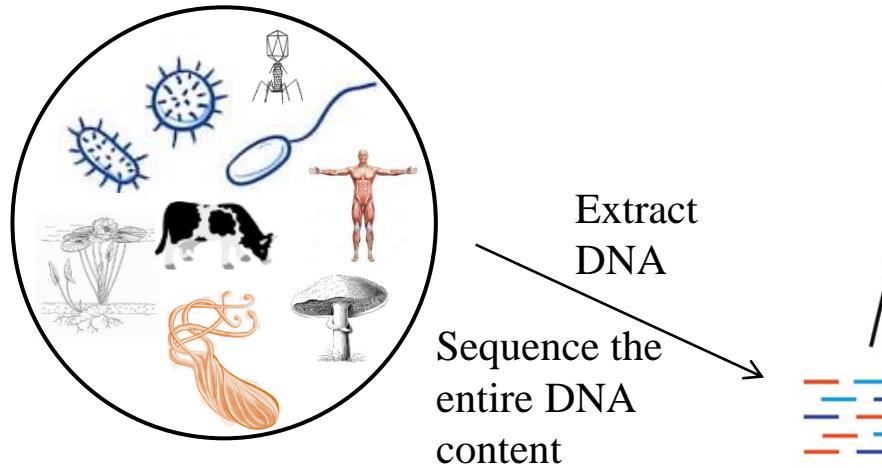
A profile of all bacterial taxa in the studied niche. Each colour

Composition, who is there!

How do we detect/study a microbiome

Metagenomics

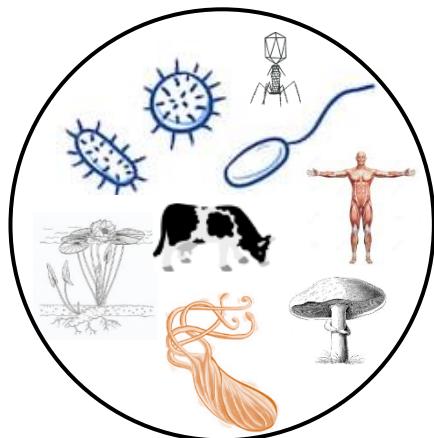
sequencing-based analysis of the entire genomic content in an environmental sample.



How do we detect/study a microbiome

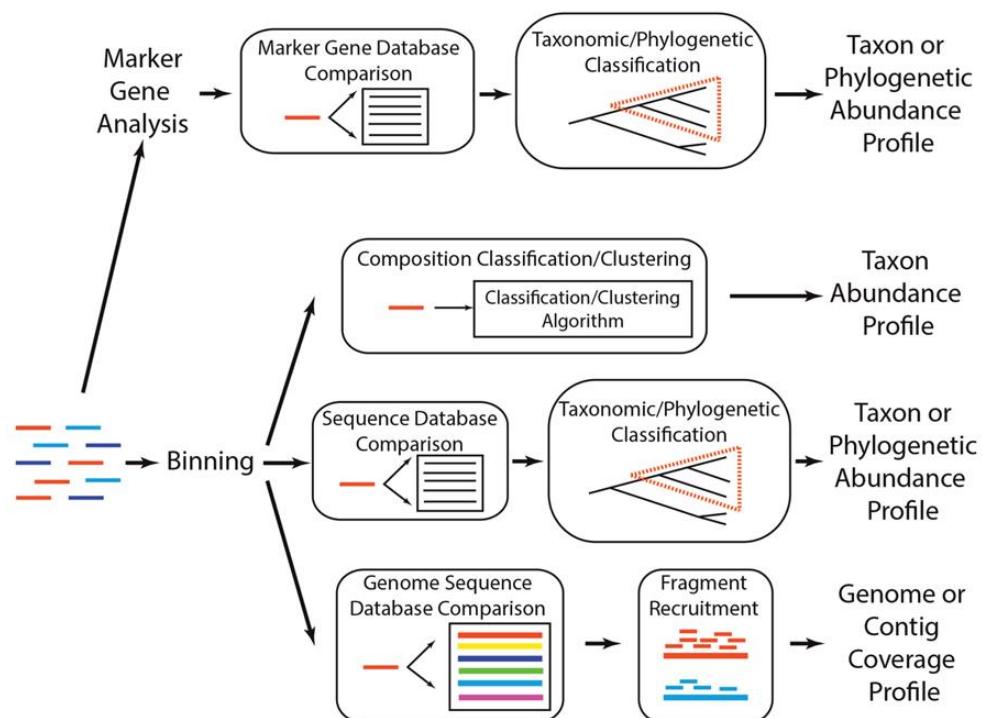
Metagenomics

sequencing-based analysis of the entire genomic content in an environmental sample.



Extract
DNA

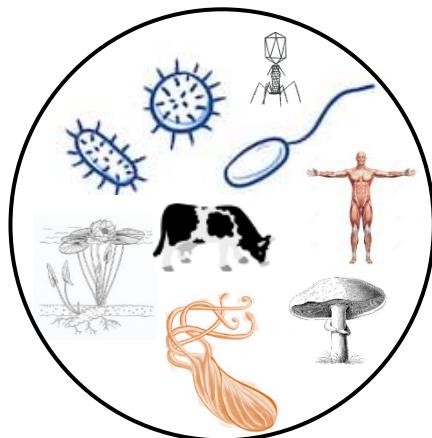
Sequence the
entire DNA
content



How do we detect/study a microbiome

Metagenomics

sequencing-based analysis of the entire genomic content in an environmental sample.



Extract
DNA

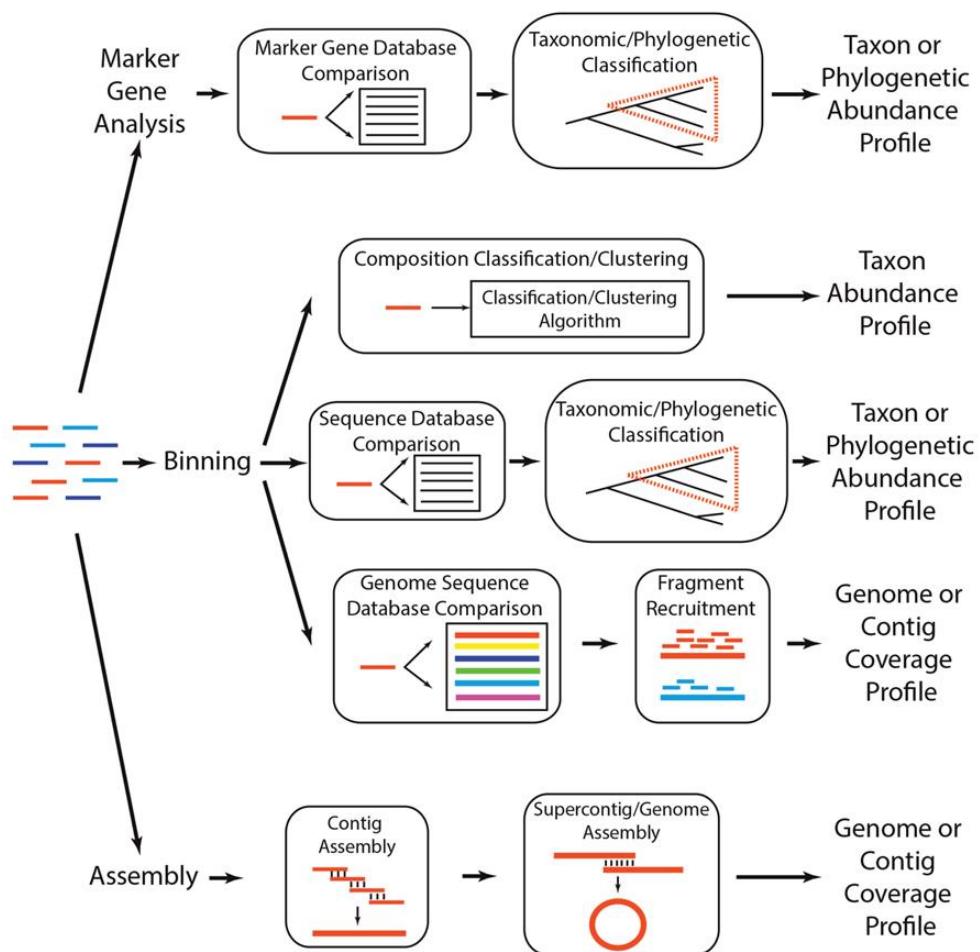
Sequence the
entire DNA
content

End product:

Information on the diversity
and function of all organisms
in the sample

Composition, who is there!

Roles, what do they do!



Analyse a metagenome:

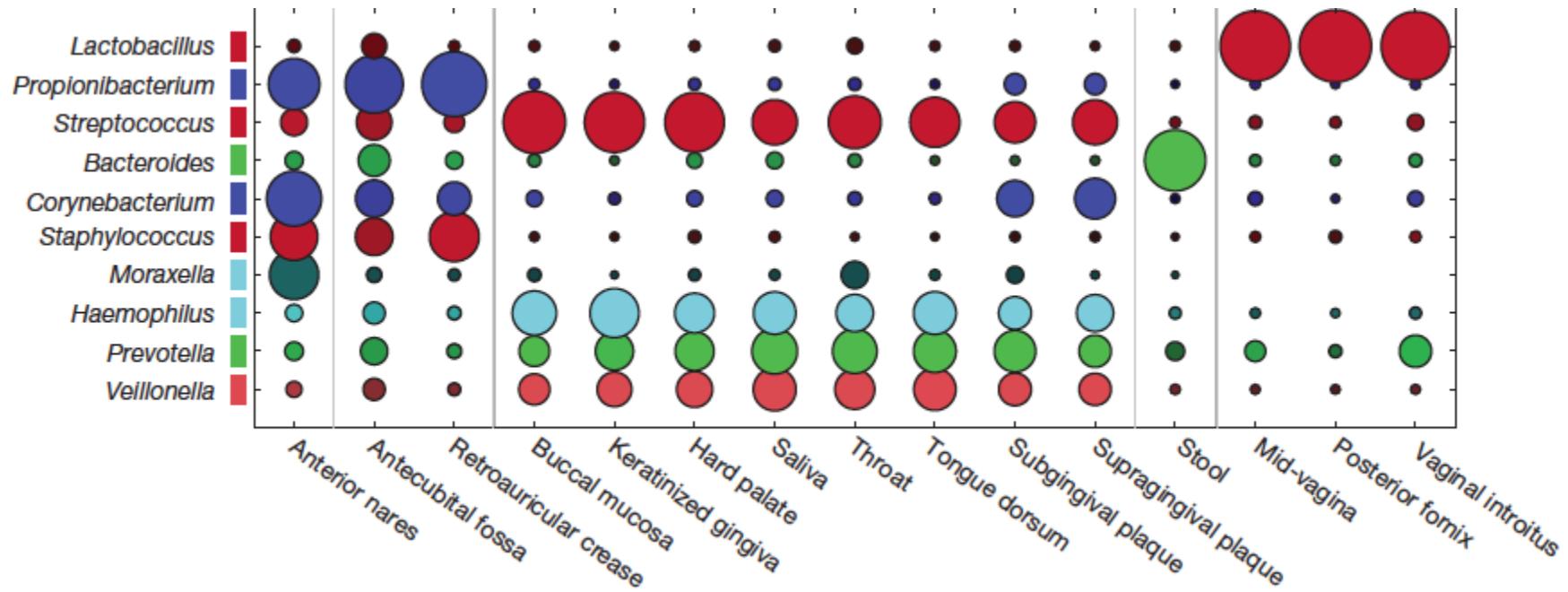
Table 1 from <https://learn.inside.dtu.dk/d2l/le/content/126041/Home>

Table 2 from <https://learn.inside.dtu.dk/d2l/le/content/126041/Home>

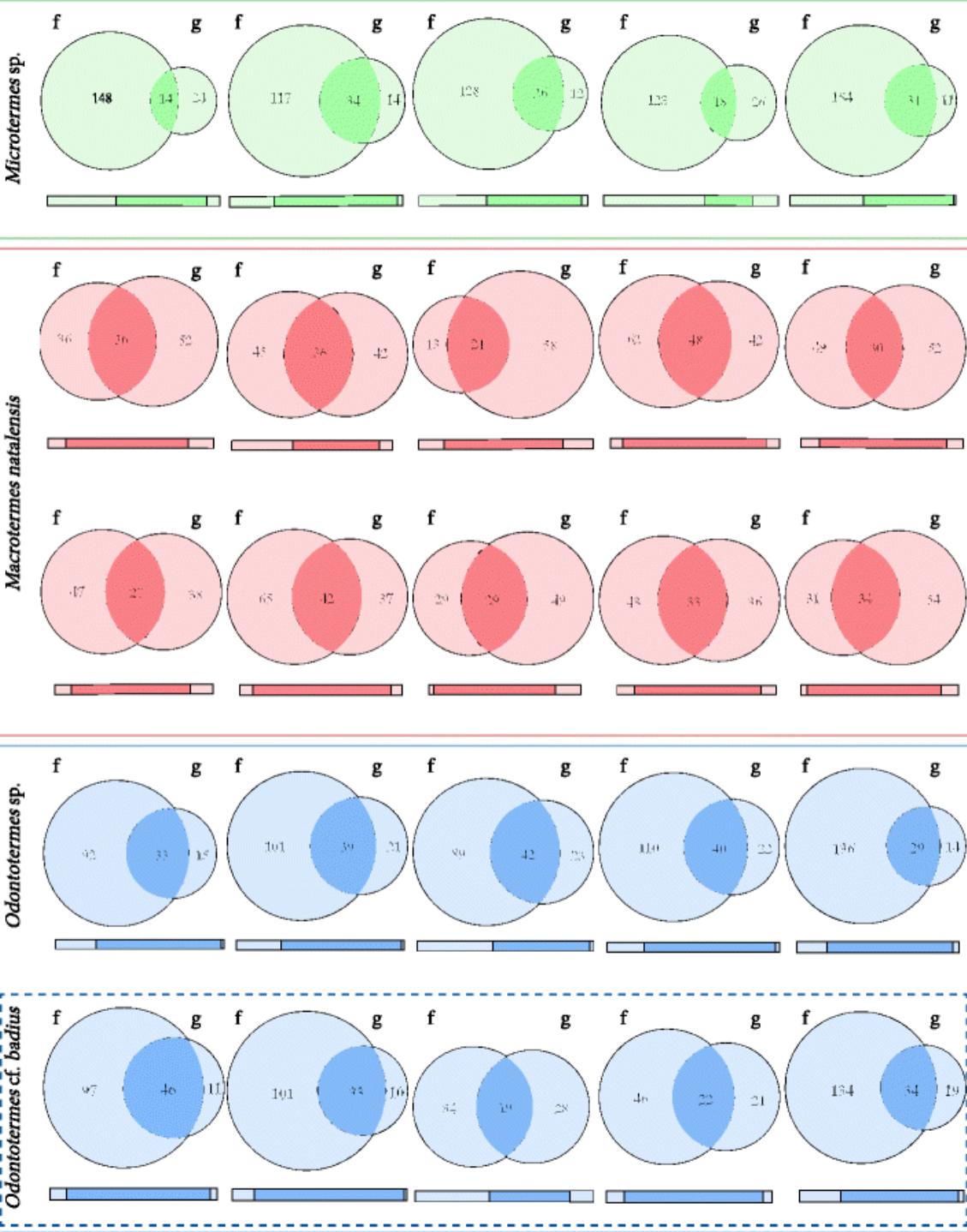
Table 3 from <https://learn.inside.dtu.dk/d2l/le/content/126041/Home>

Different microbiome composition in various body sites

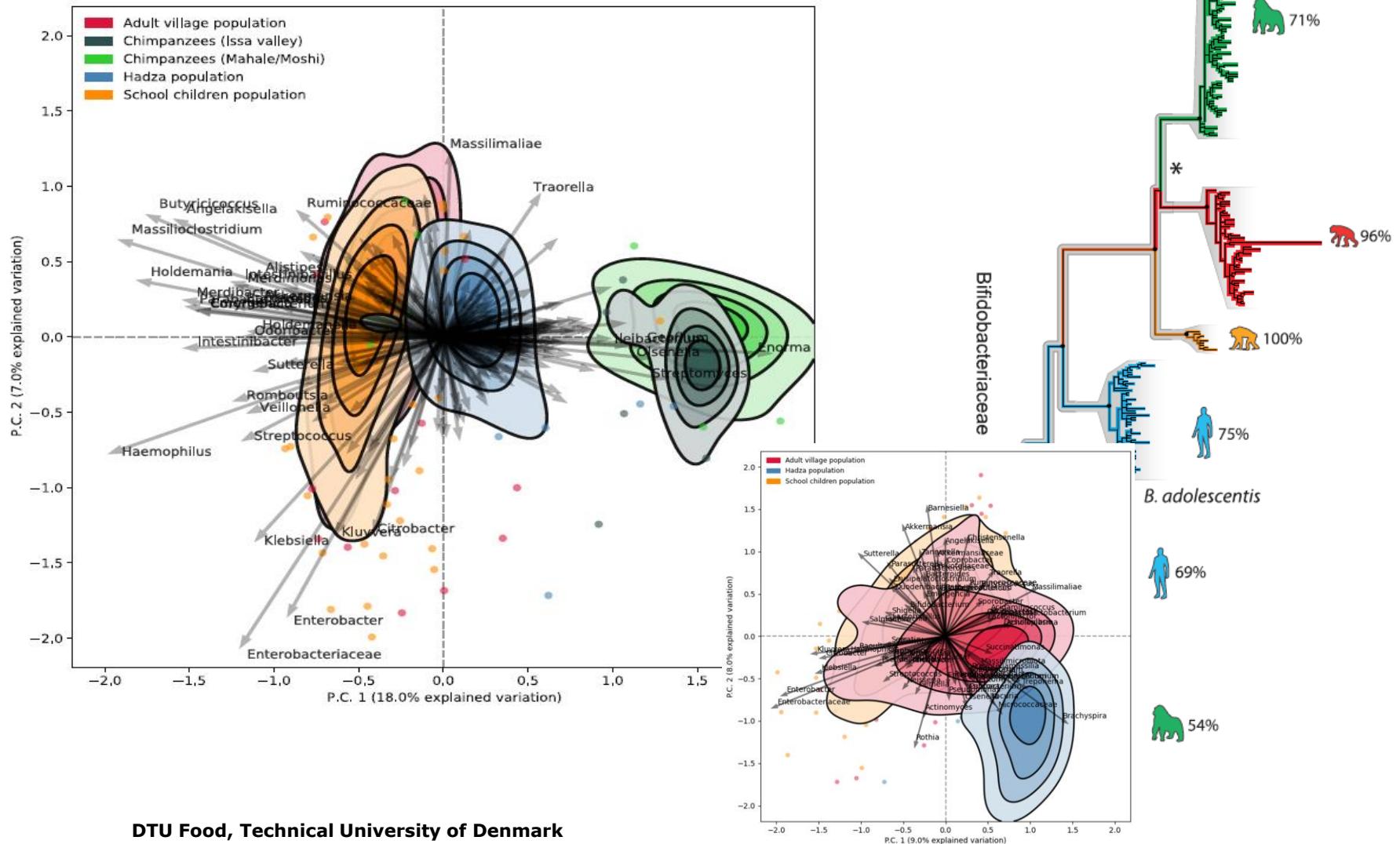
example 1:



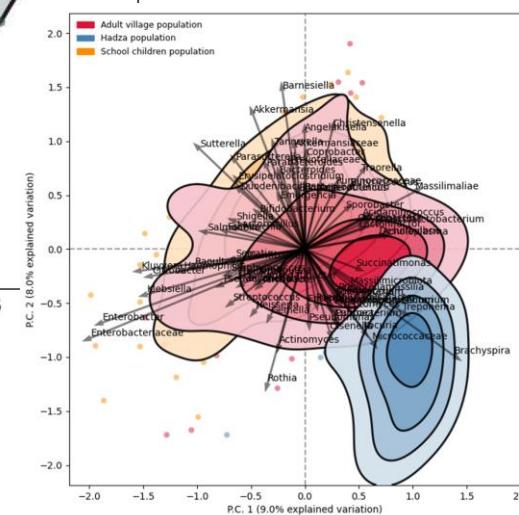
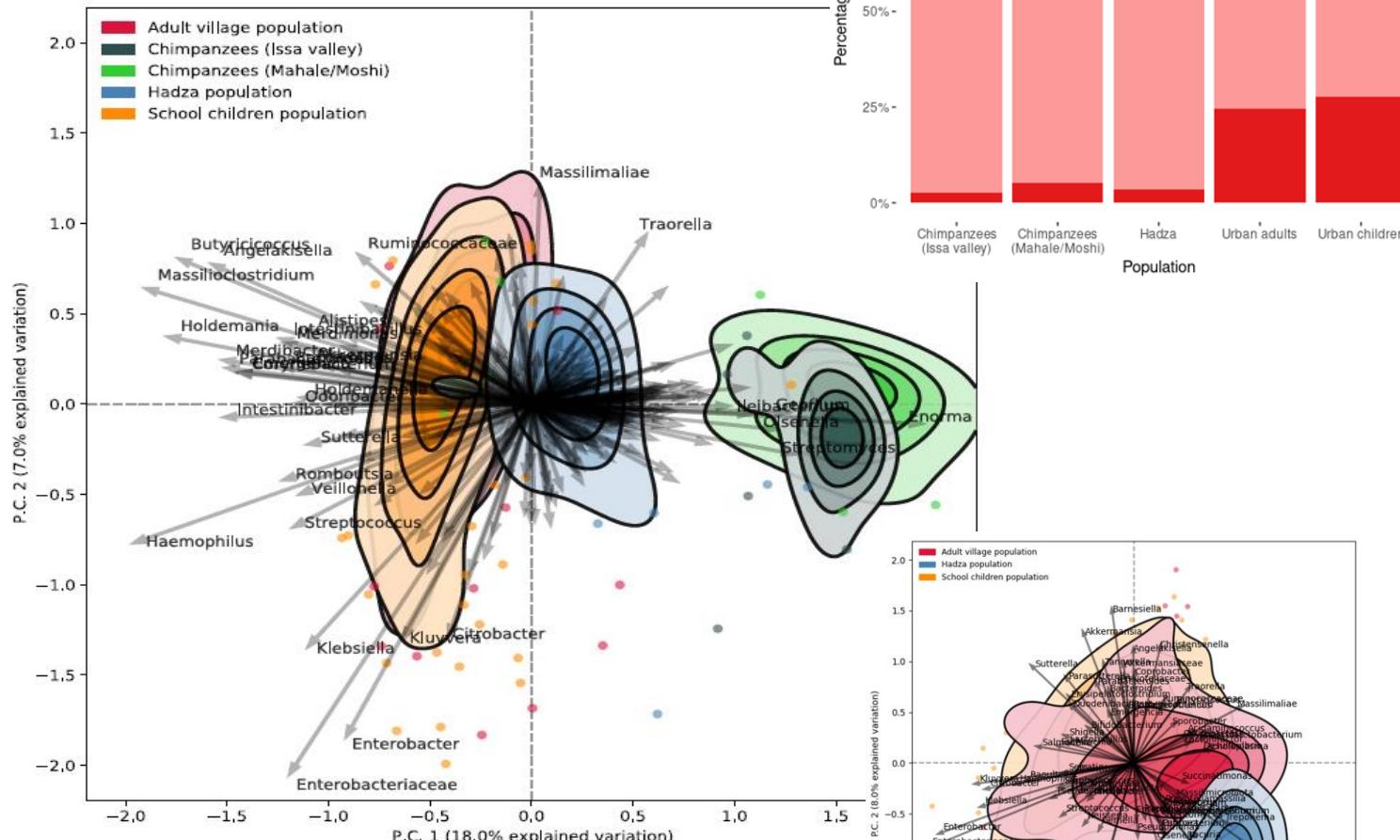
Soil - gut microbiome comparisons example 2:



Host-microbiome co-speciation example 3:



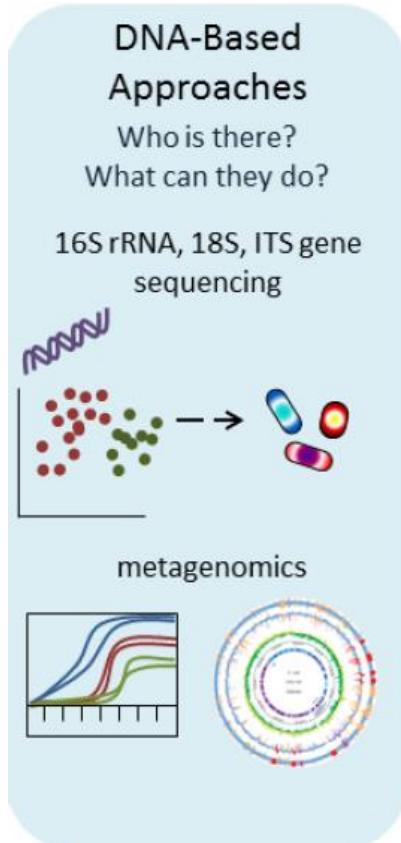
Host-microbiome co-speciation example 3:



DTU Food, Technical University of Denmark

How do we detect/study a microbiome

Current approach



Microbial Genomics

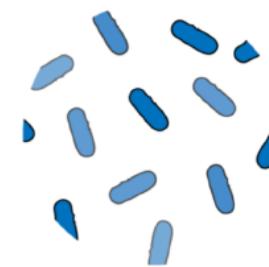
16S rRNA gene profiling



Metagenomics



Whole Genome Sequencing (WGS)



DNA extraction
↓
16S rRNA gene specific PCR
↓
Sequencing

DNA extraction
↓
Sequencing

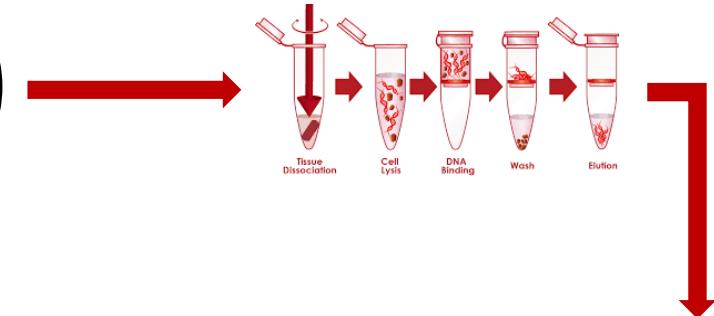
Cultivation
↓
DNA extraction
↓
Sequencing



A project workflow in metagenomics

Plan ahead: what exactly are you looking for? design the experiment accordingly!

Sample.



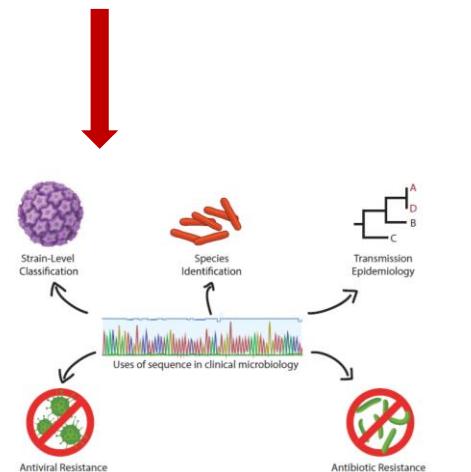
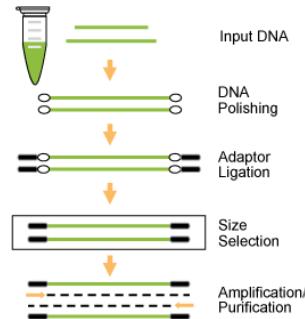
DNA extraction.

Library preparation.



Sequencing platforms.

Analyses: *e.g.*, diagnostics



A project workflow in metagenomics

Plan ahead: what exactly are you looking for? design the experiment accordingly!

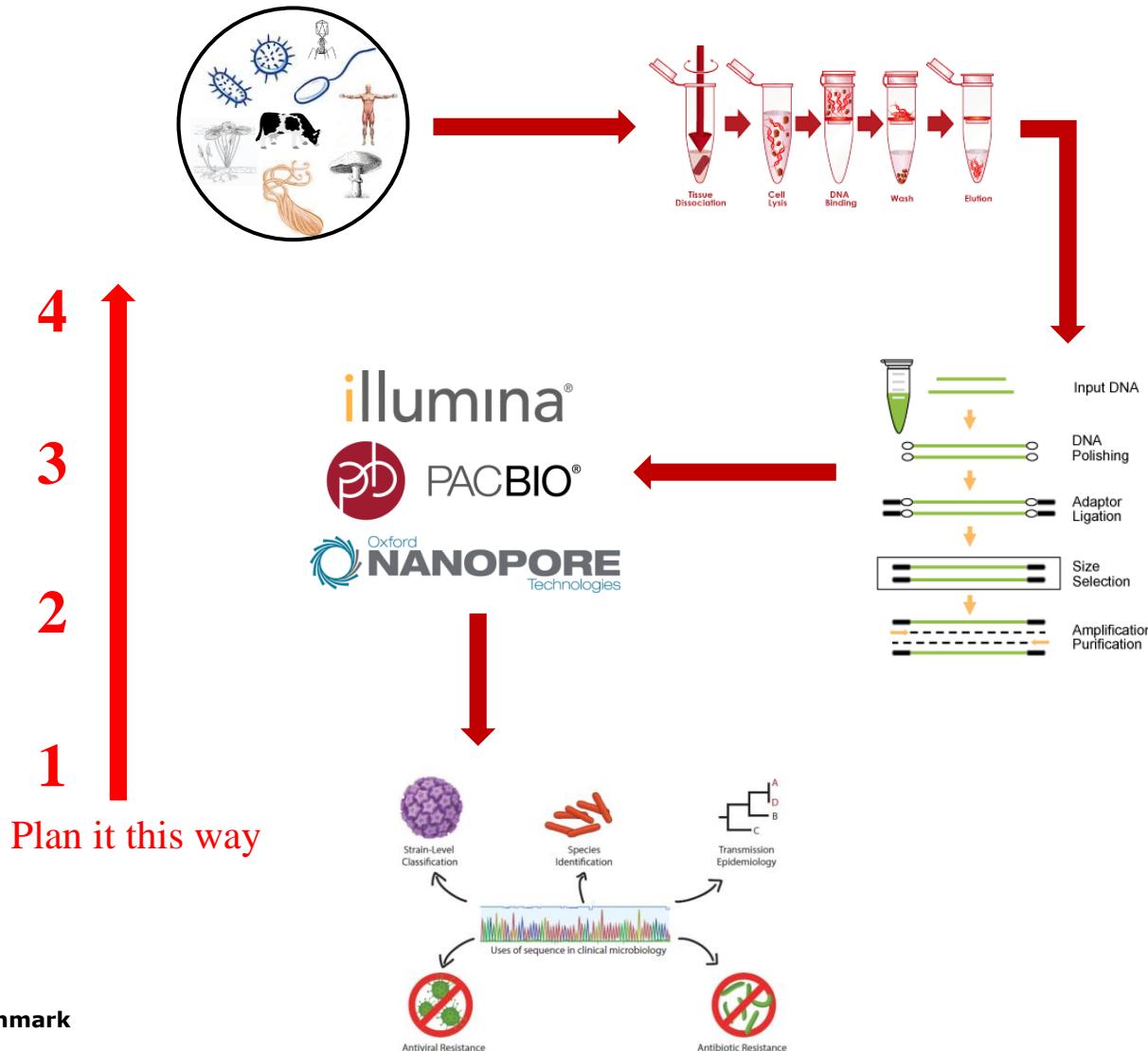
Sample.

DNA extraction.

Library preparation.

Sequencing platforms.

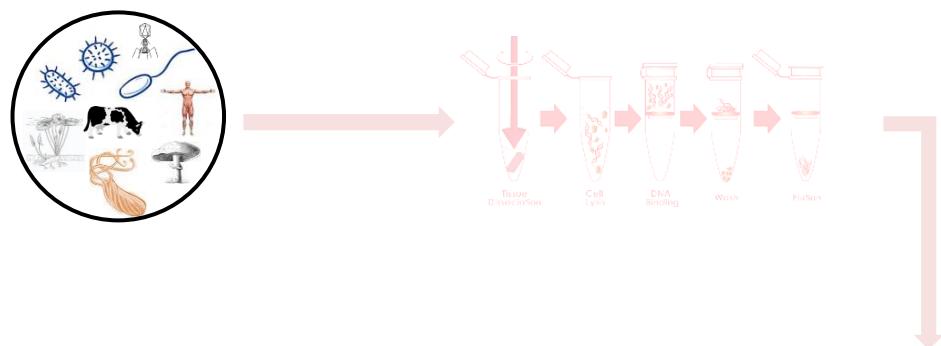
Analyses: *e.g.*, diagnostics



1. The workflow from alive material to DNA:

-Starting materials and sample selection

-DNA extraction and challenges



2. DNA preparation for sequencing:

-DNA library preparation

-Sequencing technology selection

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Oxford
NANOPORE
technologies

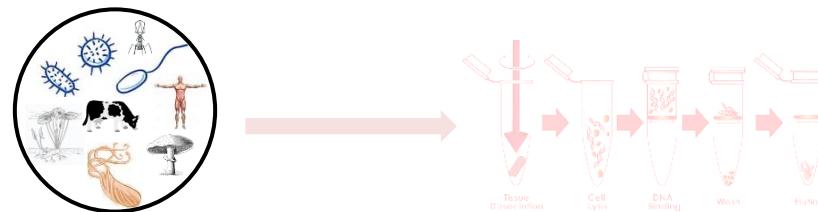


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IA TGGTGAACCCAT
IA GGTGAAACCCAT
IA TGGTGAACCCAT
IACACATGGTGAAACCCAT
IACACATGGTGAAACCCAT
```

1. The workflow from alive material to DNA:

-Starting materials and sample selection (know your sample nature)

-DNA extraction and challenges



2. DNA preparation for sequencing:

-DNA library preparation

-Sequencing technology selection

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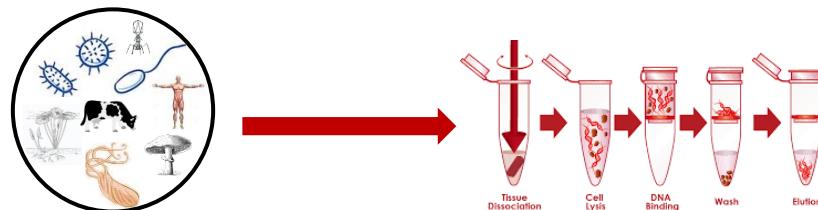
Oxford
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technologies



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ACATGGTGAAACCCAT
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TGGTGAACCCAT
IA TGGTGAACCCAT
IA GGTGAAACCCAT
IA TGGTGAACCCAT
IACACATGGTGAAACCCAT
IACACATGGTGAAACCCAT
```

1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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Oxford
NANOPORE
technologies



```
CACATGGTGAAACCCAT
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IA GGTGAAACCCAT
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IACACATGGTGAAACCCAT
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Genomic DNA extraction

Must know:

The starting material.

The downstream application.

Genomic DNA extraction

Must know:

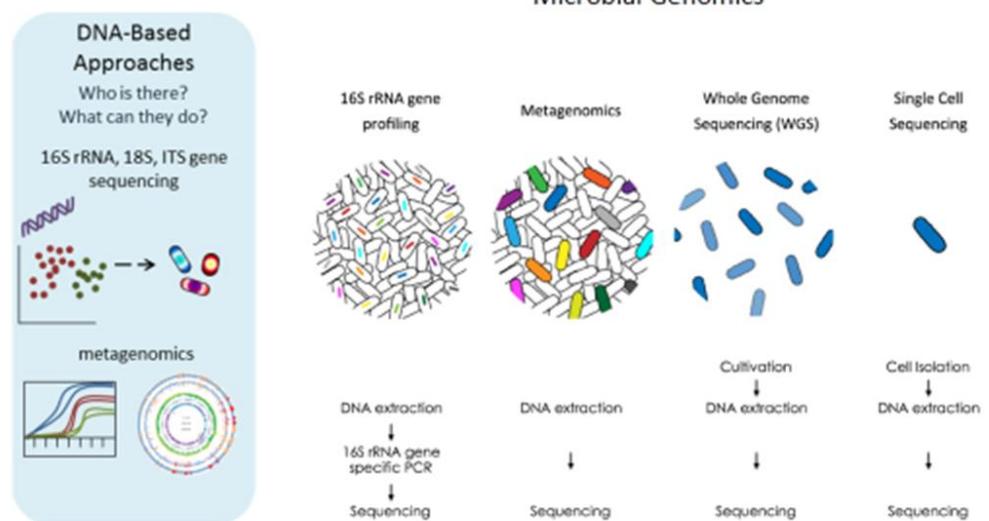
The starting material.

The downstream application.

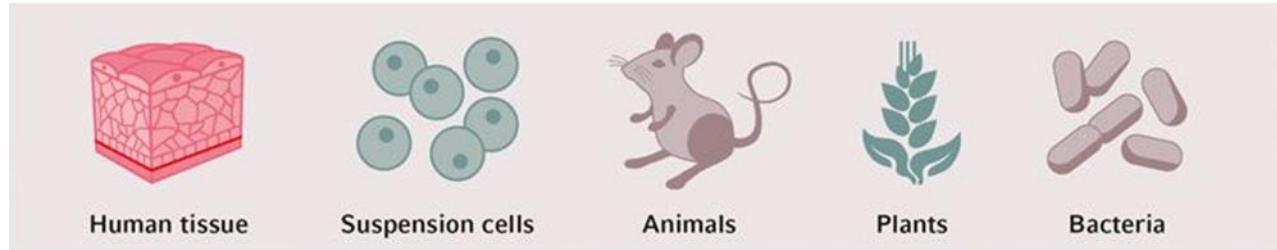
Please keep in mind:

No commercially available kit is optimal for all.

A method that works for all, yes.



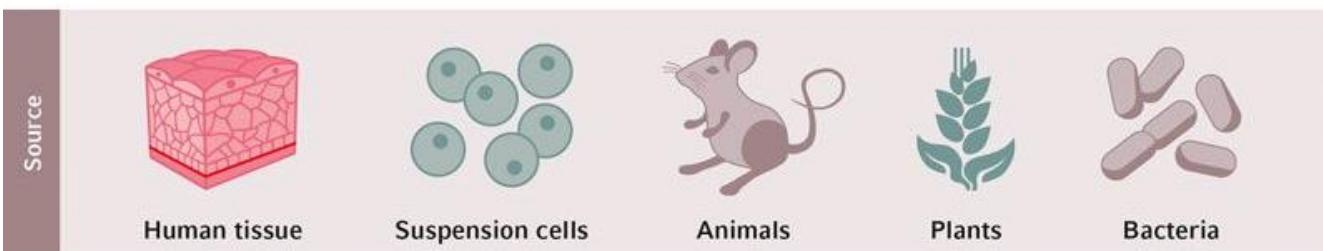
Tissues



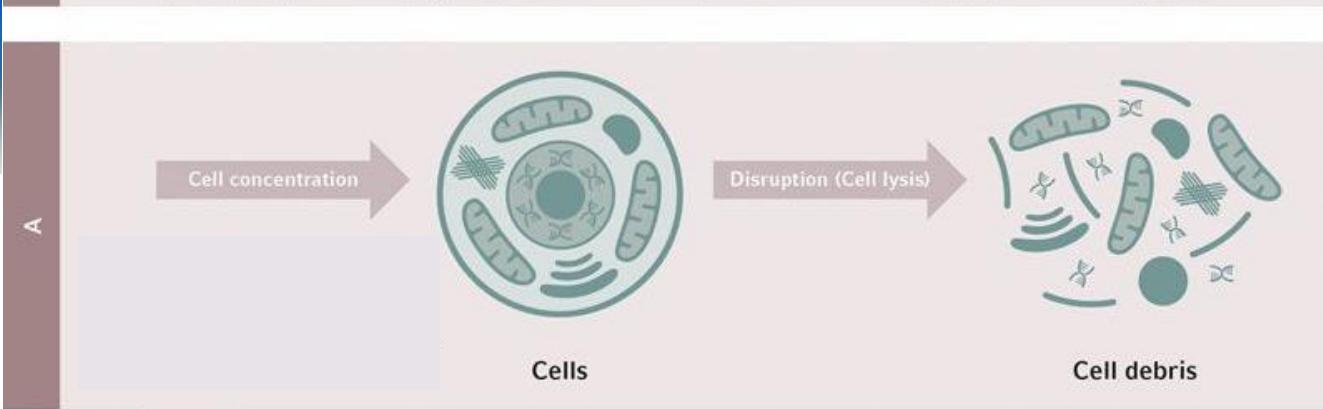
Break down the tissue
and the cells

Genomic DNA extraction – kit example

Starting material

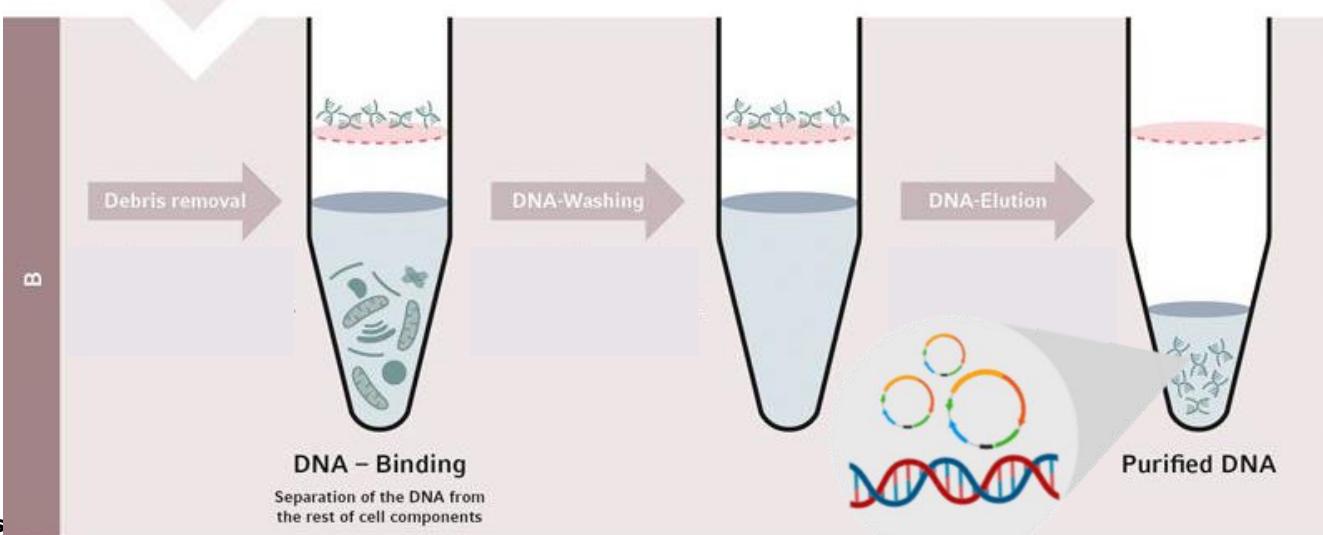


Cell lysis



DNA, RNA, protein

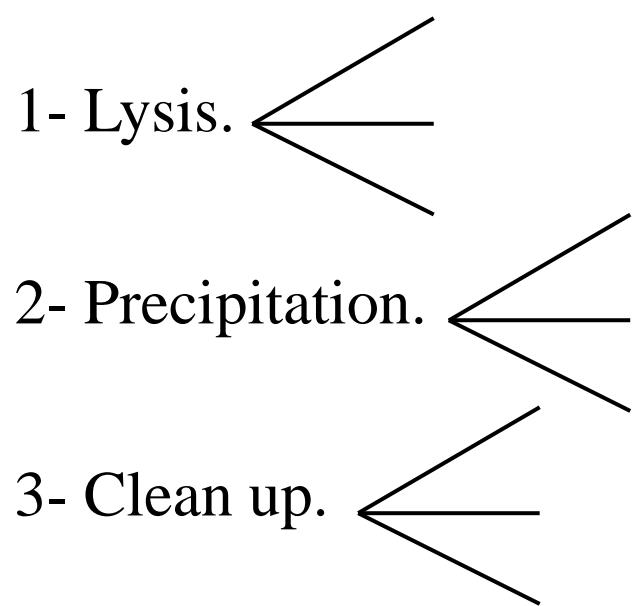
Precipitation



Clean up

Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:



Differences in how these 3 steps are carried out.



Because

Starting material.

Downstream application.

Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:

1- Lysis.

Tissues:

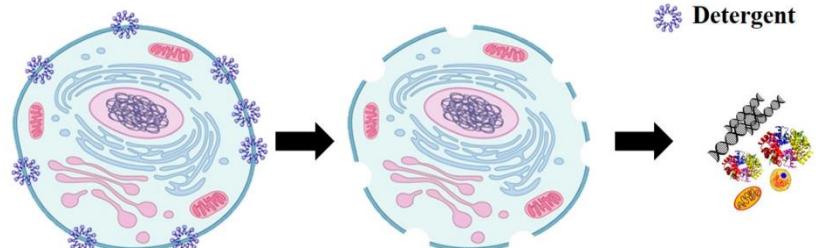
Homogenization: mechanical or chemical.



Cells: 60-70 C degree temperature treatment

Alkaline/Detergents: (*e.g.*, SDS, triton X-100, CTAB) breaks down cell membrane

Proteinases: *e.g.*, Proteinase K



Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:

Left after the lysis:

1- Lysis. **DNA, protein (denatured and Tissues: not)**

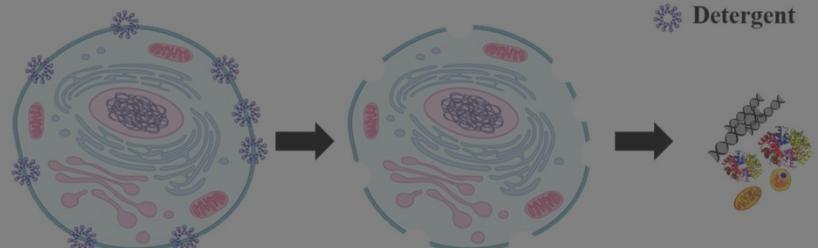
Homogenization: mechanical or chemical.



Cells:

Alkaline/Detergents: (*e.g.*, SDS, triton X-100, CTAB) breaks down cell membrane

Proteinases: *e.g.*, Proteinase K



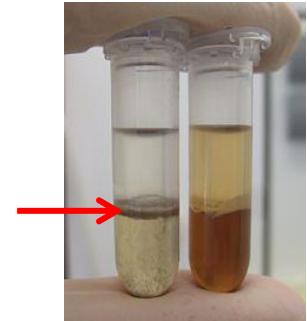
Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:

2- Precipitation: separates DNA from the remaining cellular components.

Organic: (*e.g.*, Phenol-chloroform, Trizol) denatures and dissolves proteins

Salt: (*e.g.*, NaCl, ammonium acetate) releases proteins



Both allow the protein to precipitate with centrifugation.

Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:

2- Precipitation: separates DNA from the remaining cellular components.

DNA is left. Precipitated with, alcohol (isopropanol or ethanol), collect pellet by centrifugation.

Genomic DNA extraction

Any DNA extraction (*e.g.*, commercial kit, in-house protocol, paper methods) is 3 steps:

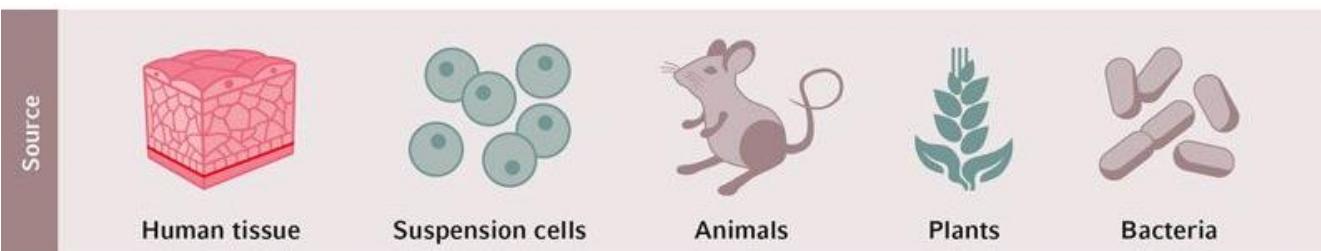
3- Clean up.

Alcohol: wash with 70% ethanol to remove salts and other impurities.

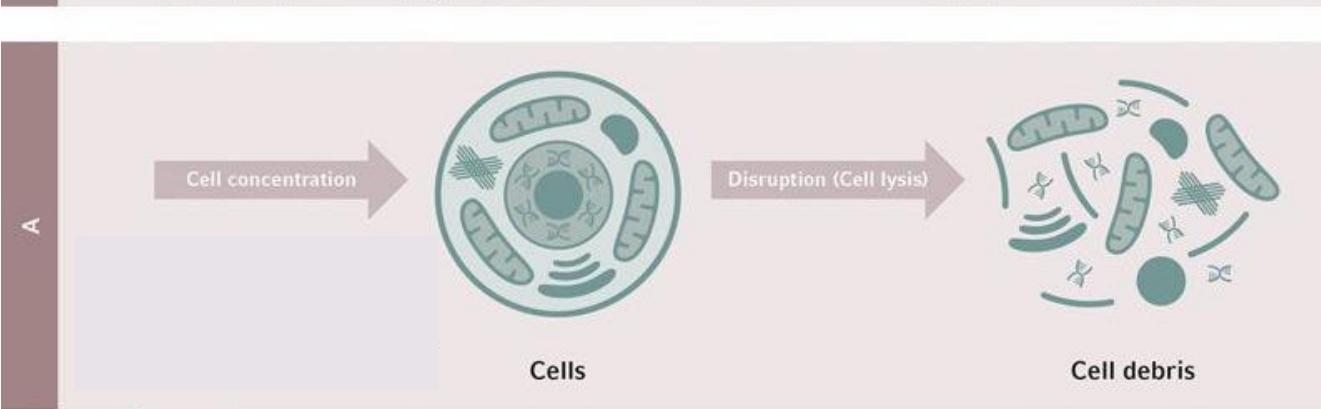
Clean DNA.

Genomic DNA extraction – kit example

Starting material

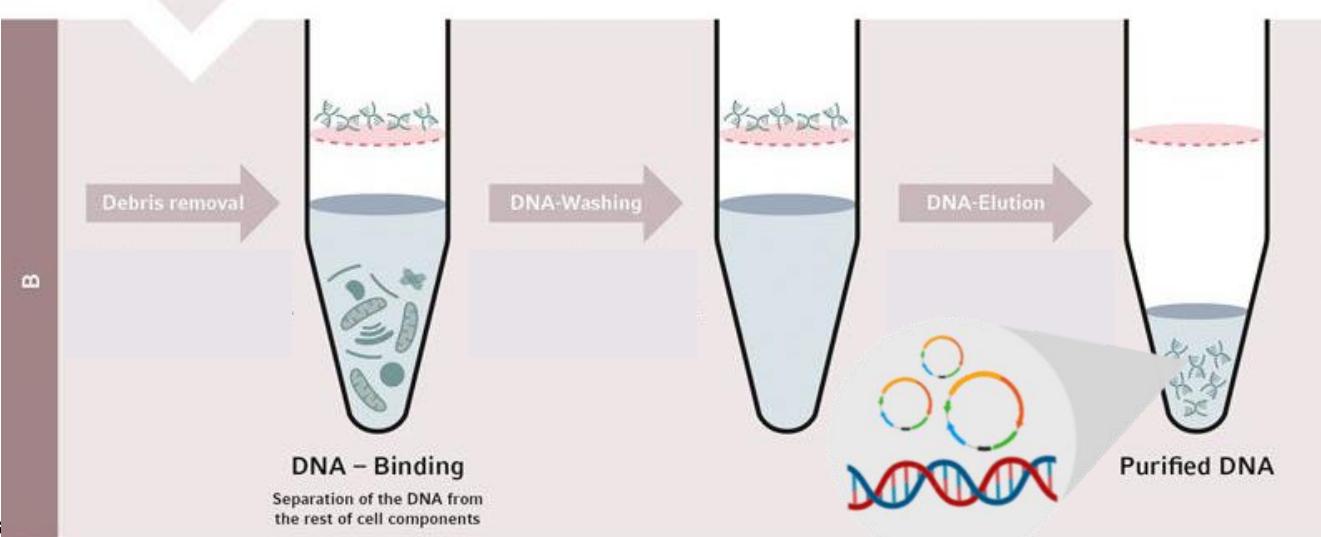


Cell lysis



Left after the lysis:
DNA, RNA, protein

Precipitation



Clean up

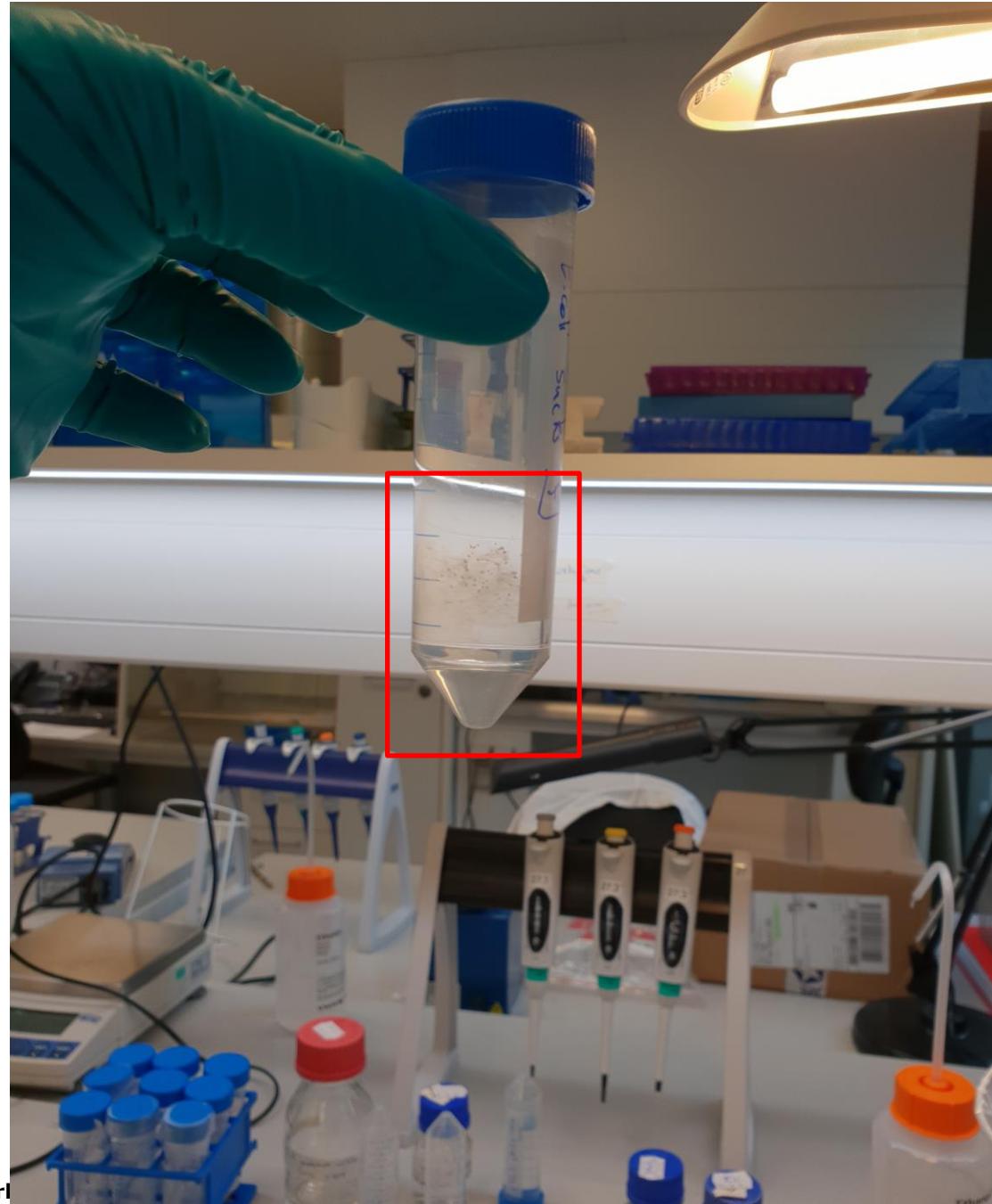
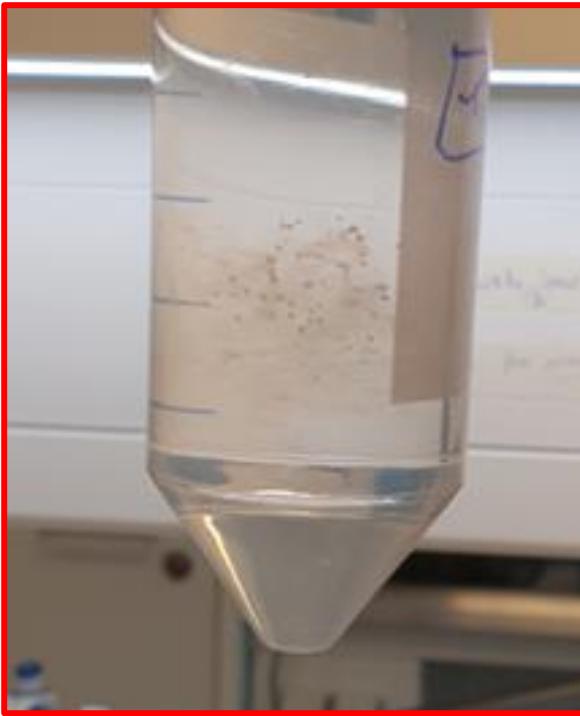
Genomic DNA extraction, example:

1- Lysis.

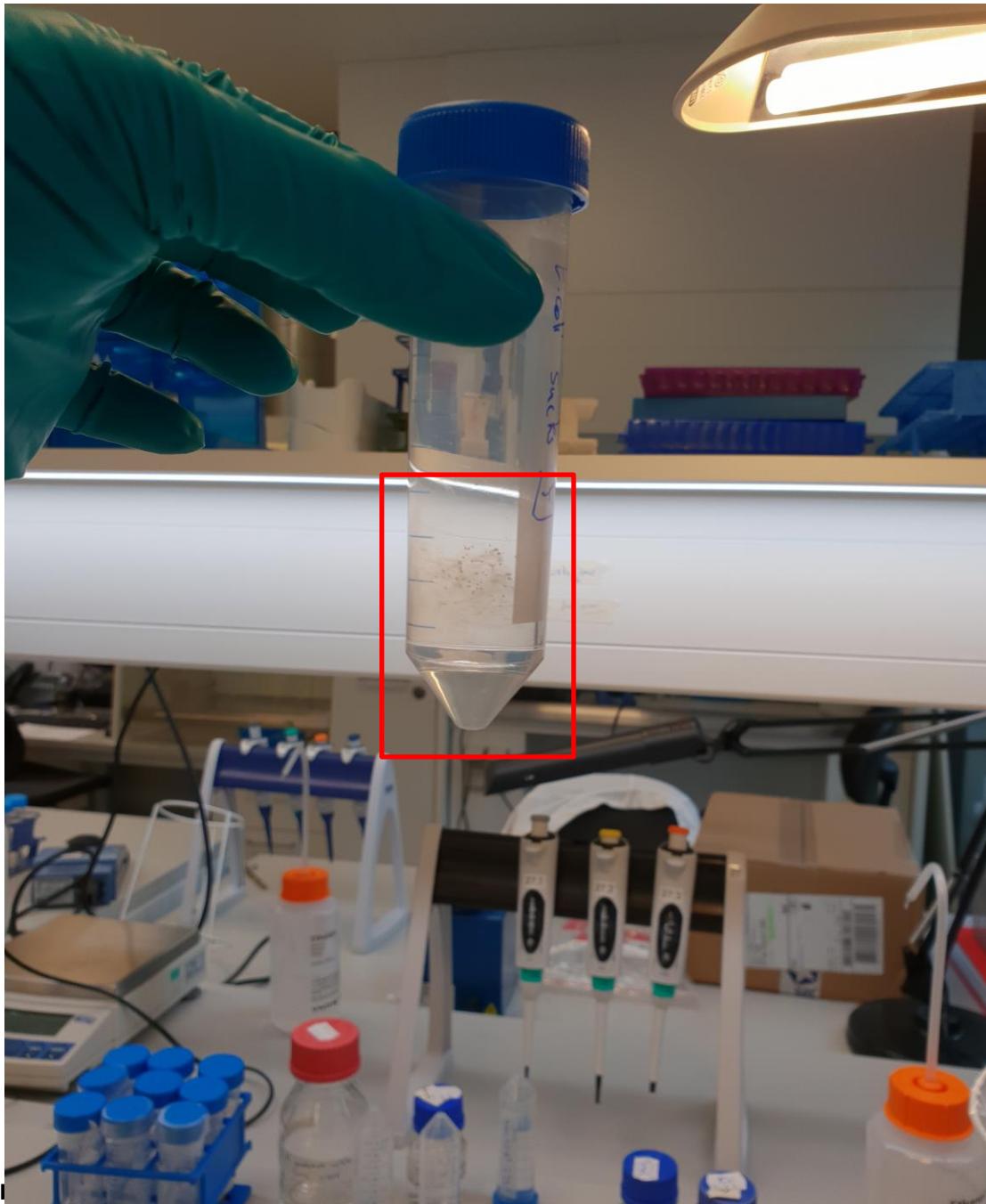
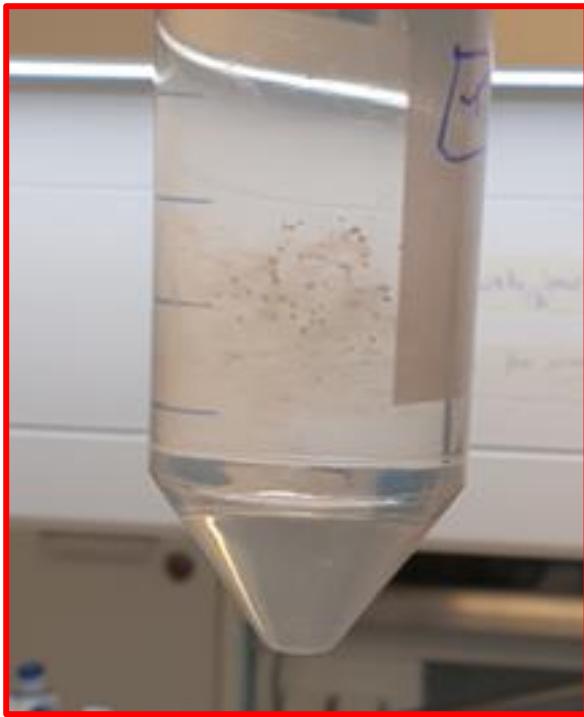
2- Precipitation.

3- Clean up.





Vid.mp4



DNA extraction

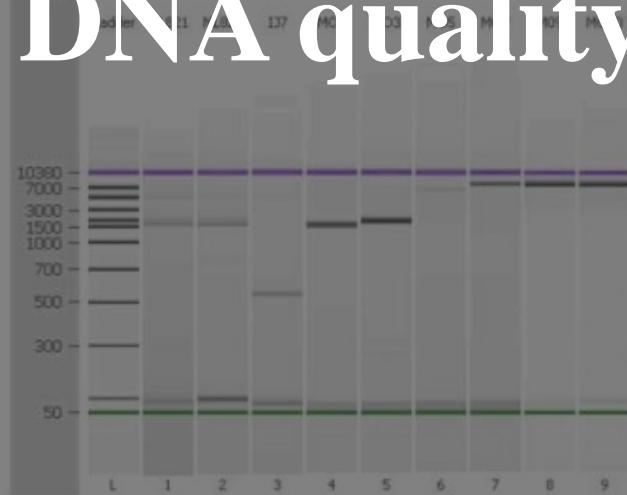
Quality/integrity of DNA

- Qubit

- Nanodrop

Why is it important to check DNA quality?

- Bioanalyzer



What is it the object you are looking at

The dress colour “enigma”



e.g., the light exposure, the observer, the device.

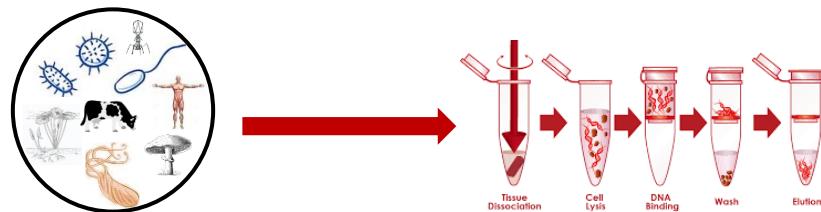
What is it the bacterium you are looking at



e.g., the light exposure, the observer, the device (ID'ing).

1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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CACATGGTGAAACCCAT
ACATGGTGAAACCCAT
ACATGGTGAAACCCAT
TGGTGAAACCCAT
IA TGGTGAAACCCAT
IA GGTGAAACCCAT
IA TGGTGAAACCCAT
IACACATGGTGAAACCCAT
IACACATGGTGAAACCCAT
```

Input DNA

DNA Polishing

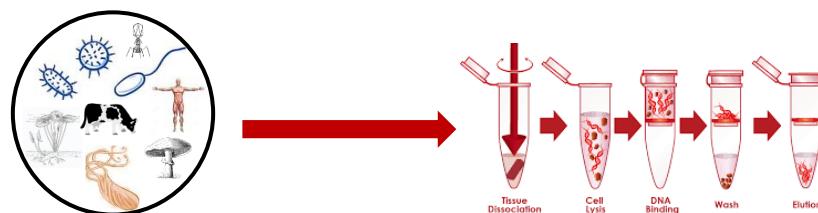
Adaptor Ligation

Size Selection

Amplification/Purification

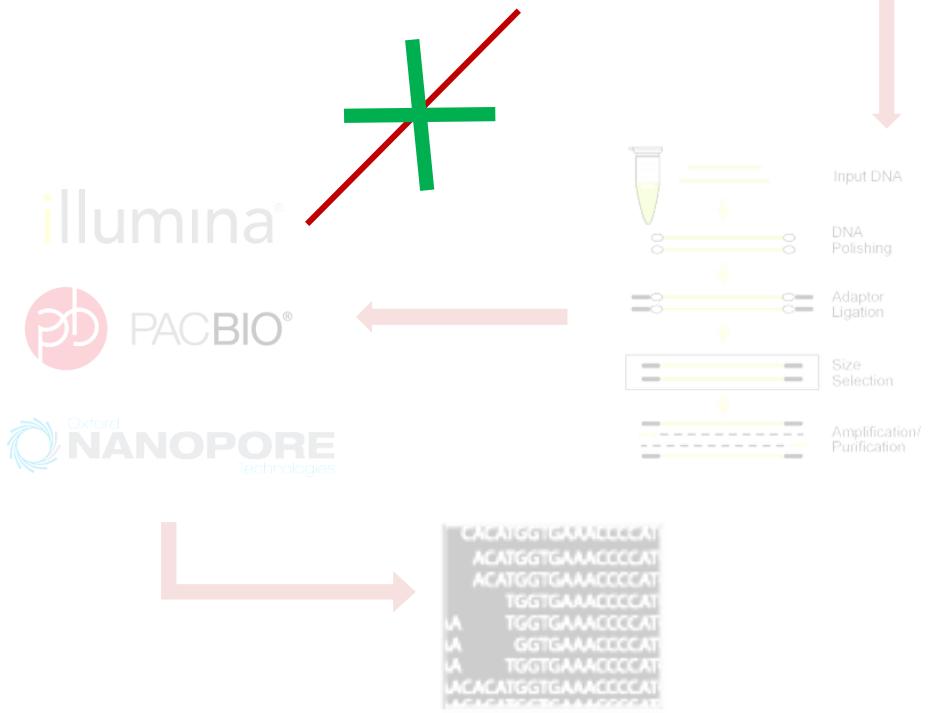
1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



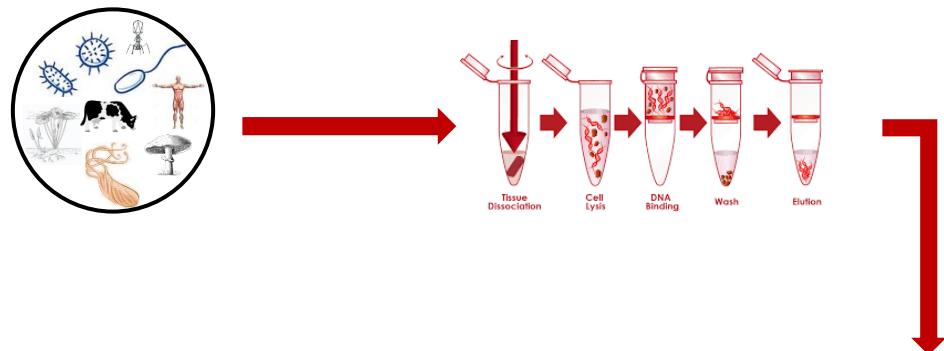
2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection



1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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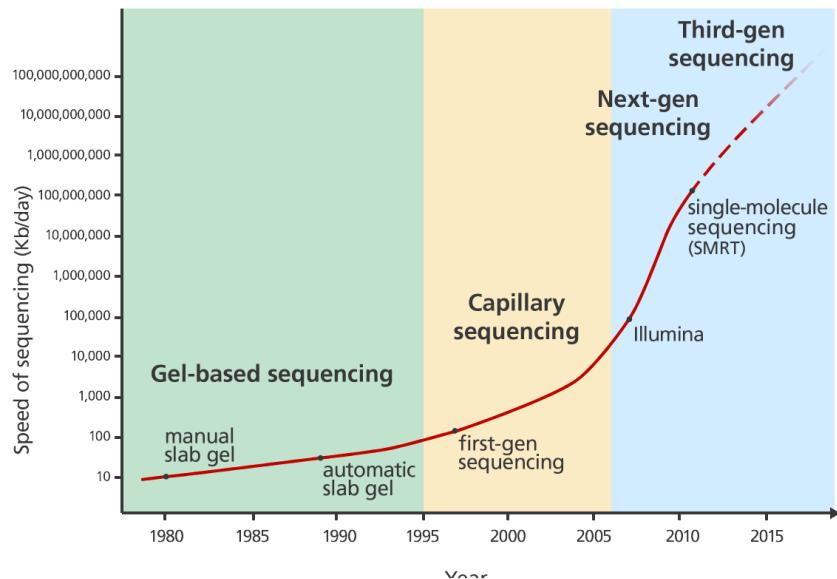
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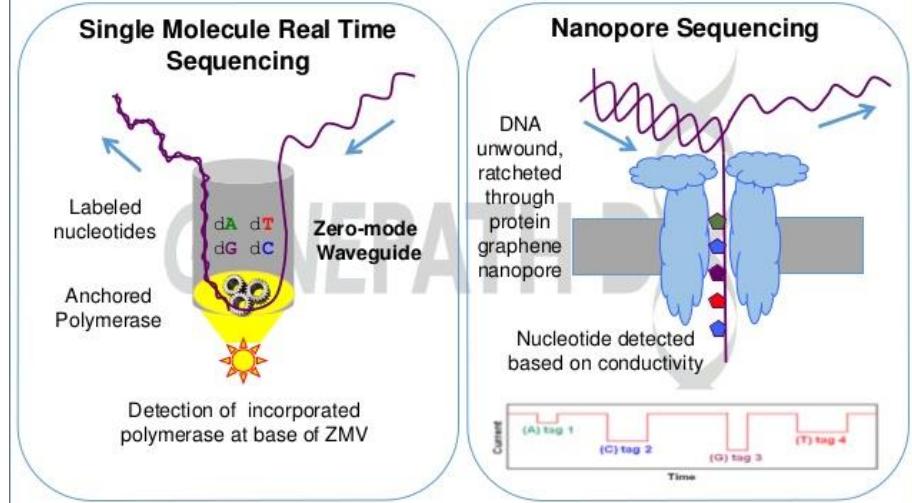
```
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ACATGGTGAAACCCAT
ACATGGTGAAACCCAT
TGTTGAAACCCAT
IA TGTTGAAACCCAT
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IACACATGGTGAAACCCAT
IACACATGGTGAAACCCAT
```

Sequencing technology selection

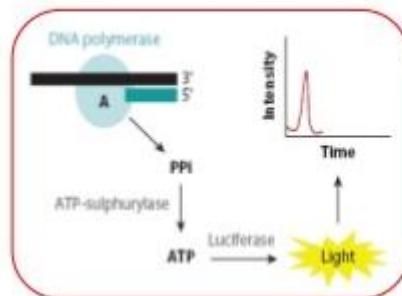
Many classifications for the different methods:



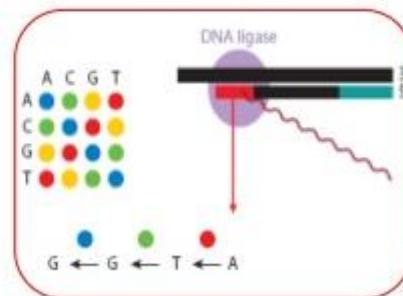
Single molecule (3rd gen) sequencing



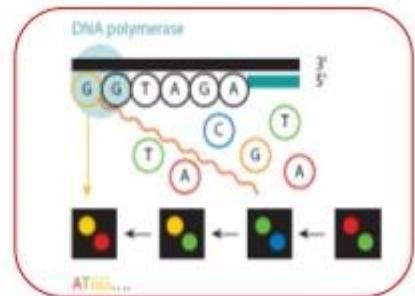
Pyrosequencing



Sequencing-by-ligation

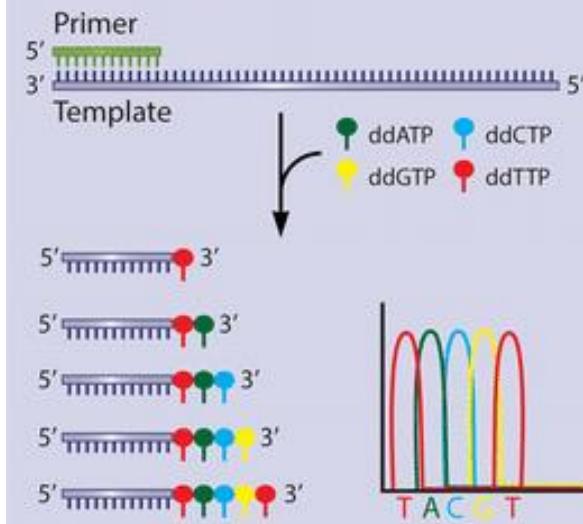


Sequencing-by-synthesis



First Generation

Shotgun Sequencing



- Sequencing by synthesis
- High accuracy
- Long read lengths
- Relatively small amount of data generated

e.g., ABI capillary sequencer (ABI)

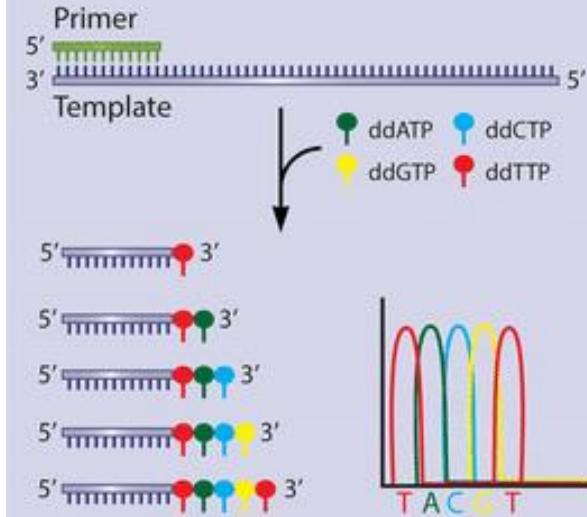
Sequencing technology selection

This is commonly known
as NGS



First Generation

Shotgun Sequencing

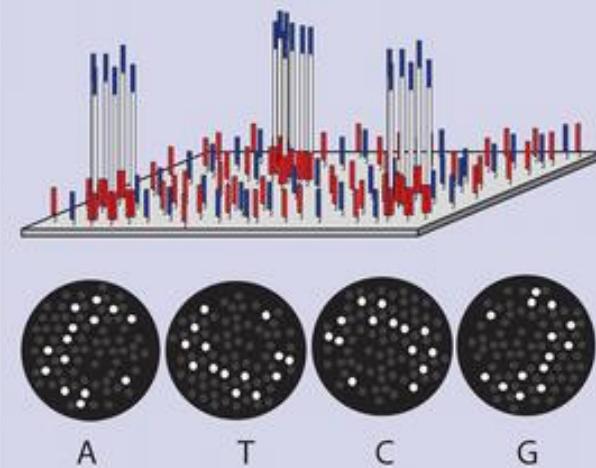


- Sequencing by synthesis
- High accuracy
- Long read lengths
- Relatively small amount of data generated

e.g., ABI capillary sequencer (ABI)

Second Generation

Massively Parallel Sequencing



- Sequencing by synthesis
- Amplified templates are generated during sequencing, reducing the requirements for starting material
- High accuracy
- Short read lengths

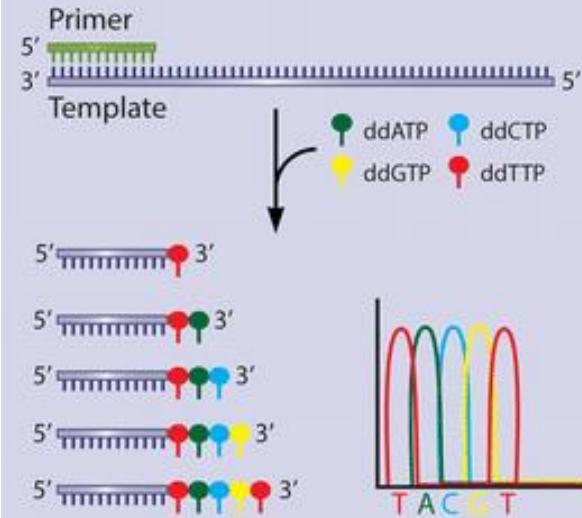
e.g., MiSeq (Illumina), Ion Torrent (Thermo Fisher Scientific)

Sequencing technology selection

This is commonly known
as NGS


First Generation

Shotgun Sequencing

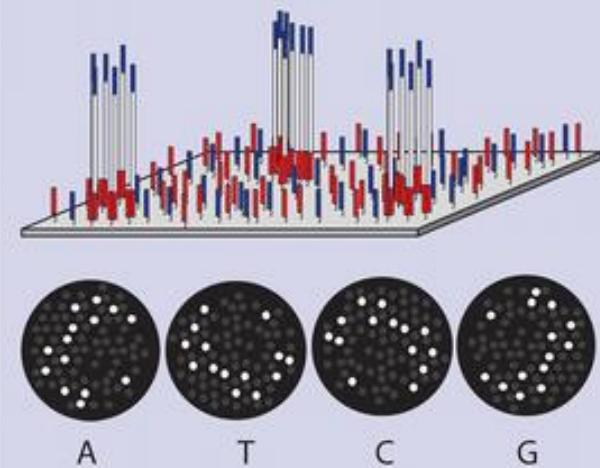


- Sequencing by synthesis
- High accuracy
- Long read lengths
- Relatively small amount of data generated

e.g., ABI capillary sequencer (ABI)

Second Generation

Massively Parallel Sequencing

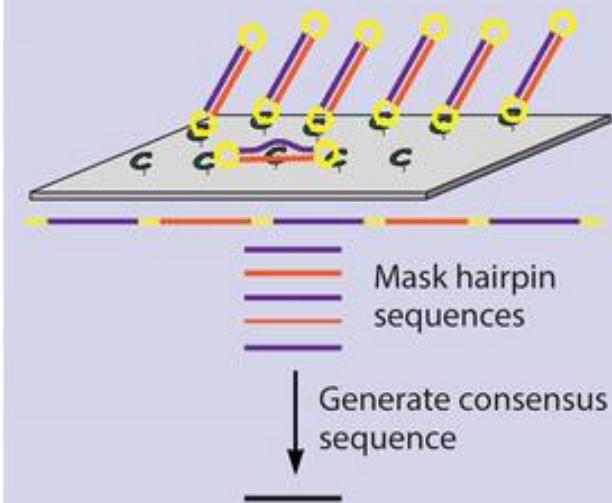


- Sequencing by synthesis
- Amplified templates are generated during sequencing, reducing the requirements for starting material
- High accuracy
- Short read lengths

e.g., MiSeq (Illumina), Ion Torrent (Thermo Fisher Scientific)

Third Generation

Single-molecule Sequencing



- Single-molecule templates
- Low accuracy
- Long read lengths

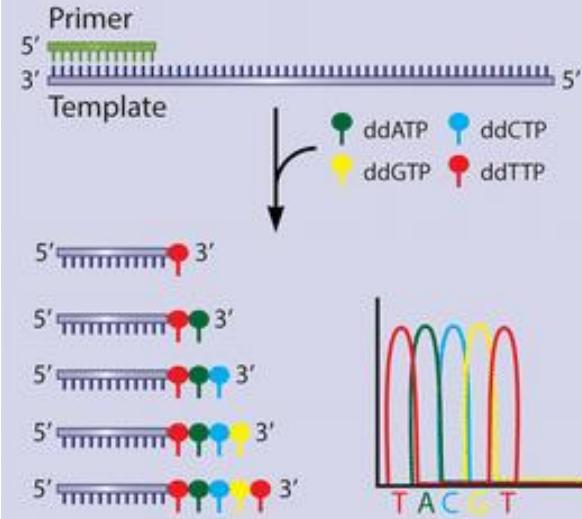
e.g., Single-Molecule Real-Time (SMRT) — Sequencing (Pacific Biosciences), MinION (Oxford Nanopore Technologies)

Sequencing technology selection

This is commonly known
as NGS


First Generation

Shotgun Sequencing

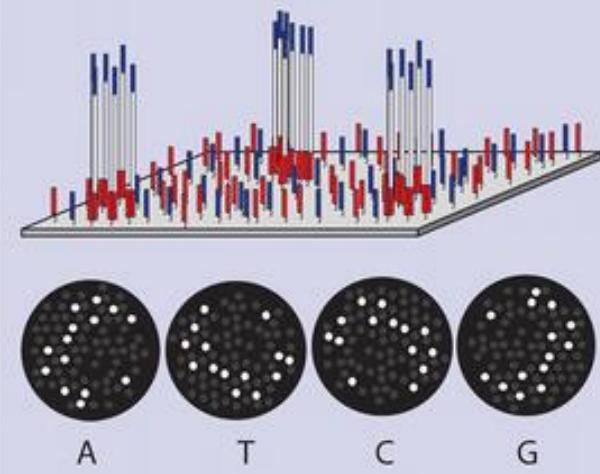


- Sequencing by synthesis
- High accuracy
- Long read lengths
- Relatively small amount of data generated

e.g., ABI capillary sequencer (ABI)

Second Generation

Massively Parallel Sequencing

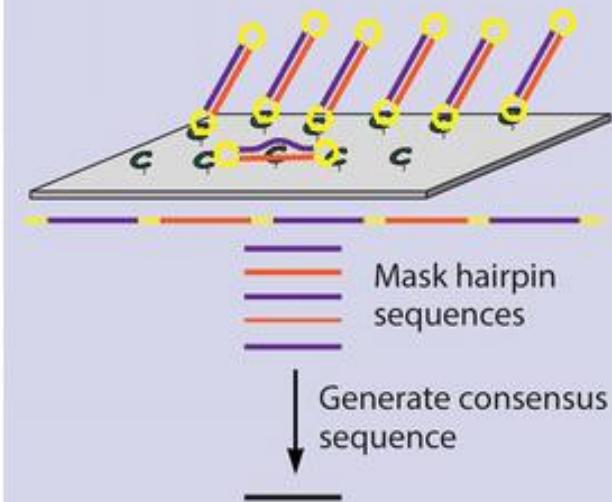


- Sequencing by synthesis
- Amplified templates are generated during sequencing, reducing the requirements for starting material
- High accuracy
- Short read lengths

e.g., MiSeq (Illumina), Ion Torrent (Thermo Fisher Scientific)

Third Generation

Single-molecule Sequencing



- Single-molecule templates
- Low accuracy
- Long read lengths

e.g., Single-Molecule Real-Time (SMRT) — Sequencing (Pacific Biosciences), MinION (Oxford Nanopore Technologies)

Sequencing technology selection

A bit confusing!

What do I want it for? Gene detection (AMR)? Plasmids?
Evolutionary analyses? Microbiome?

Sequencing technology selection

- **Short read technologies**
 - Illumina (MiSeq, NextSeq, HiSeq, NovaSeq...)
 - Ion Torrent
- **Long read technologies**
 - Pacific Biosciences (PacBio)
 - Oxford Nanopore Technologies (MinION)

Sequencing technology selection

- **Short read technologies**

- Illumina (MiSeq, NextSeq, HiSeq, NovaSeq...):

- Average 300 bp reads

- Good accuracy

- Error rate ~0.1%

- Ion Torrent: Fastest runtime (until 2018) and work-flow in this category

- Average 400 bp reads

- Error rate ~1%

Sequencing technology selection

- **Long read technologies**

- Pacific Biosciences (PacBio):

- Long reads. (Max: 50 kb. Avg.: 10-15 kb – more in 2022)

- Low throughput

- Oxford Nanopore Technologies (MinION):

- Very long reads (up to 900 kb. – more 2022)

- Fast turnaround time (2 hrs)

- Portable and real-time sequencing

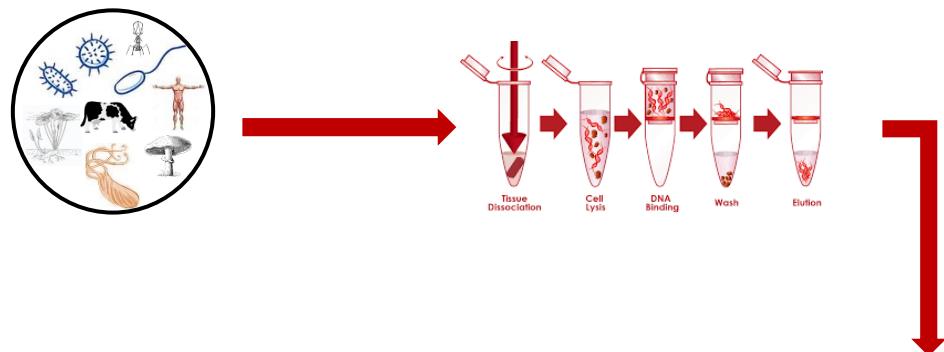
- Large error rates (3-8%: 2019)

- Reduced error rate (<1%: 2022)



1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



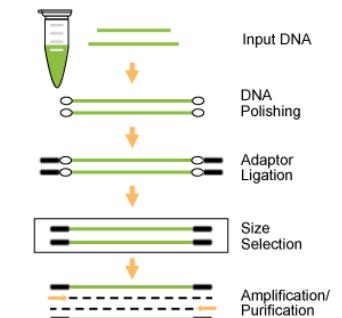
2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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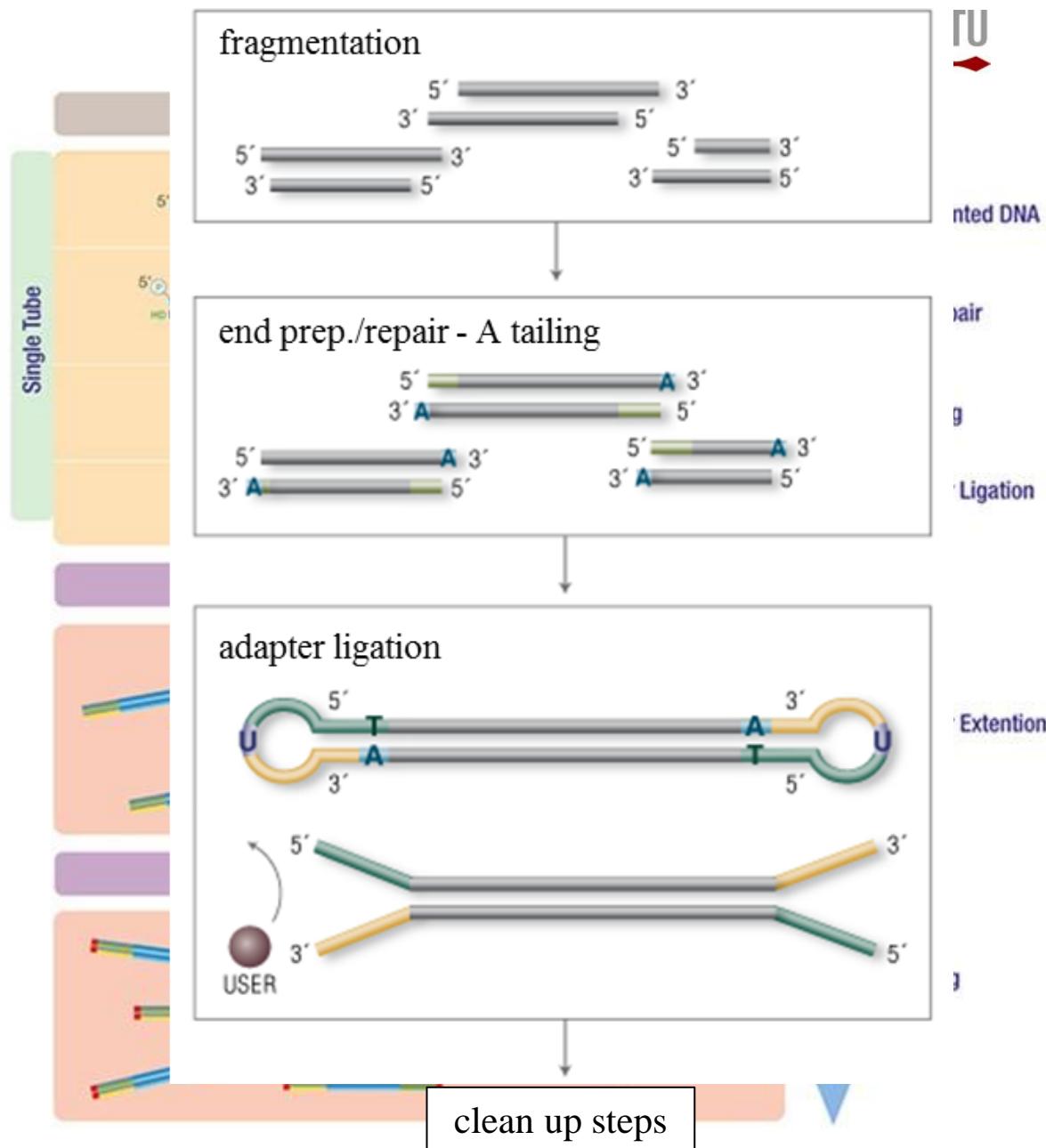
Oxford
NANOPORE
technologies



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CACATGGTGAAACCCAT
ACATGGTGAAACCCAT
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IACACATGGTGAAACCCAT
```

DNA library preparation:

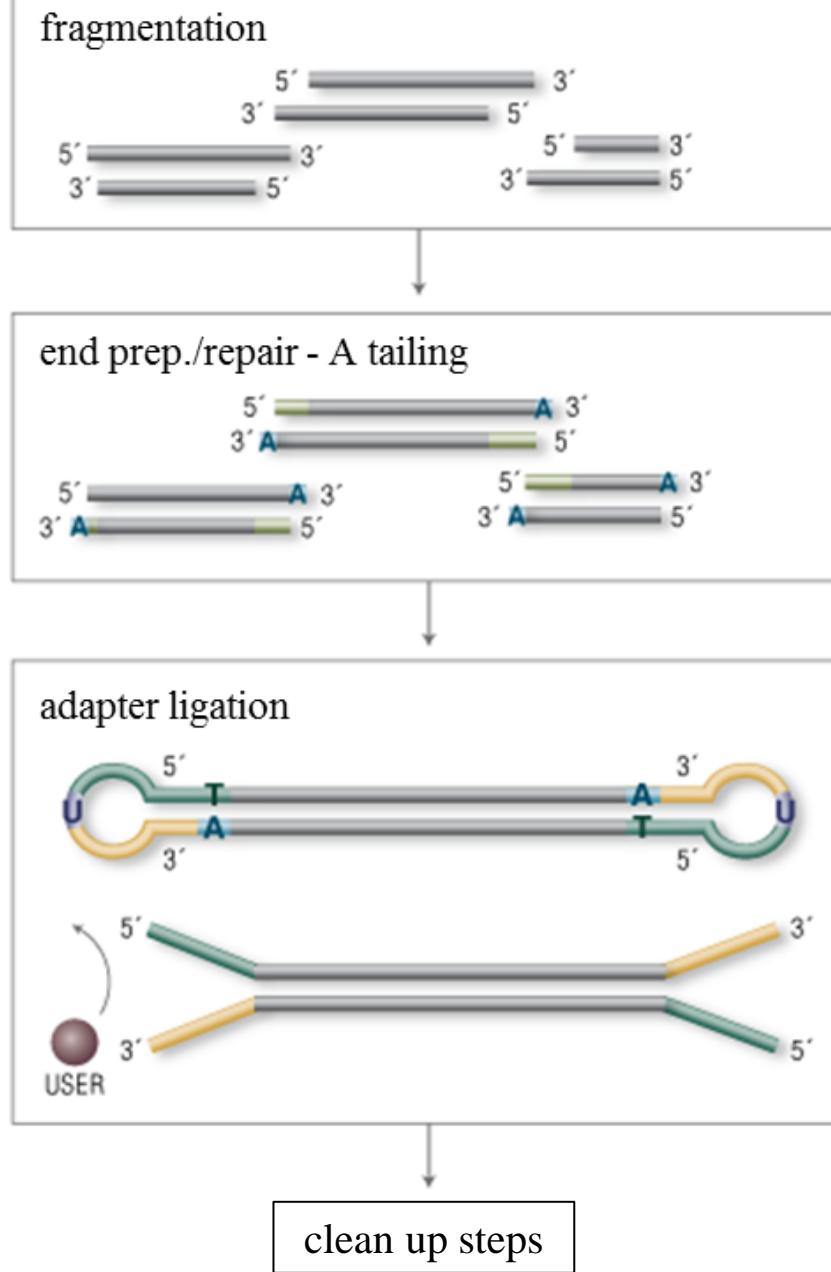
Collection of steps to prepare DNA to be read by a sequencing machine.



DNA library preparation:

Collection of steps to prepare DNA to be read by a sequencing machine.

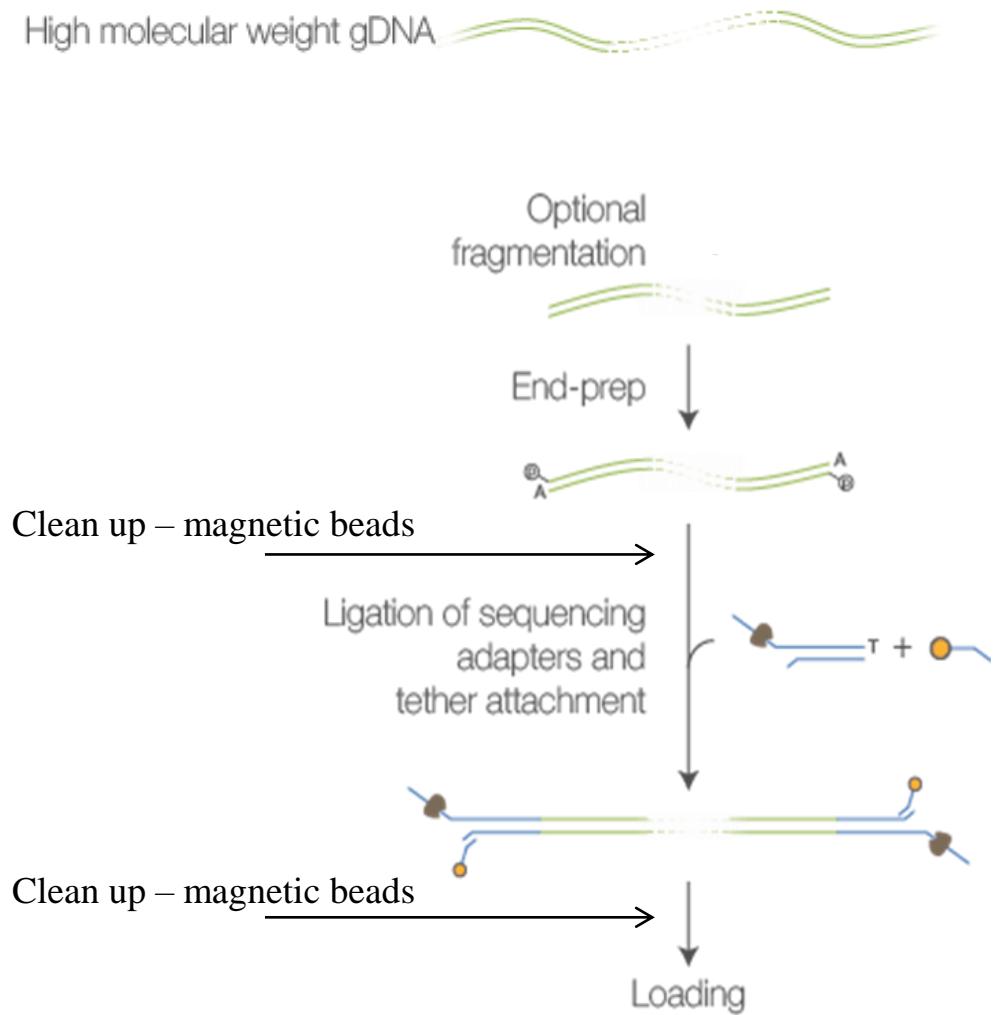
It is the same for all platforms.



DNA library preparation:

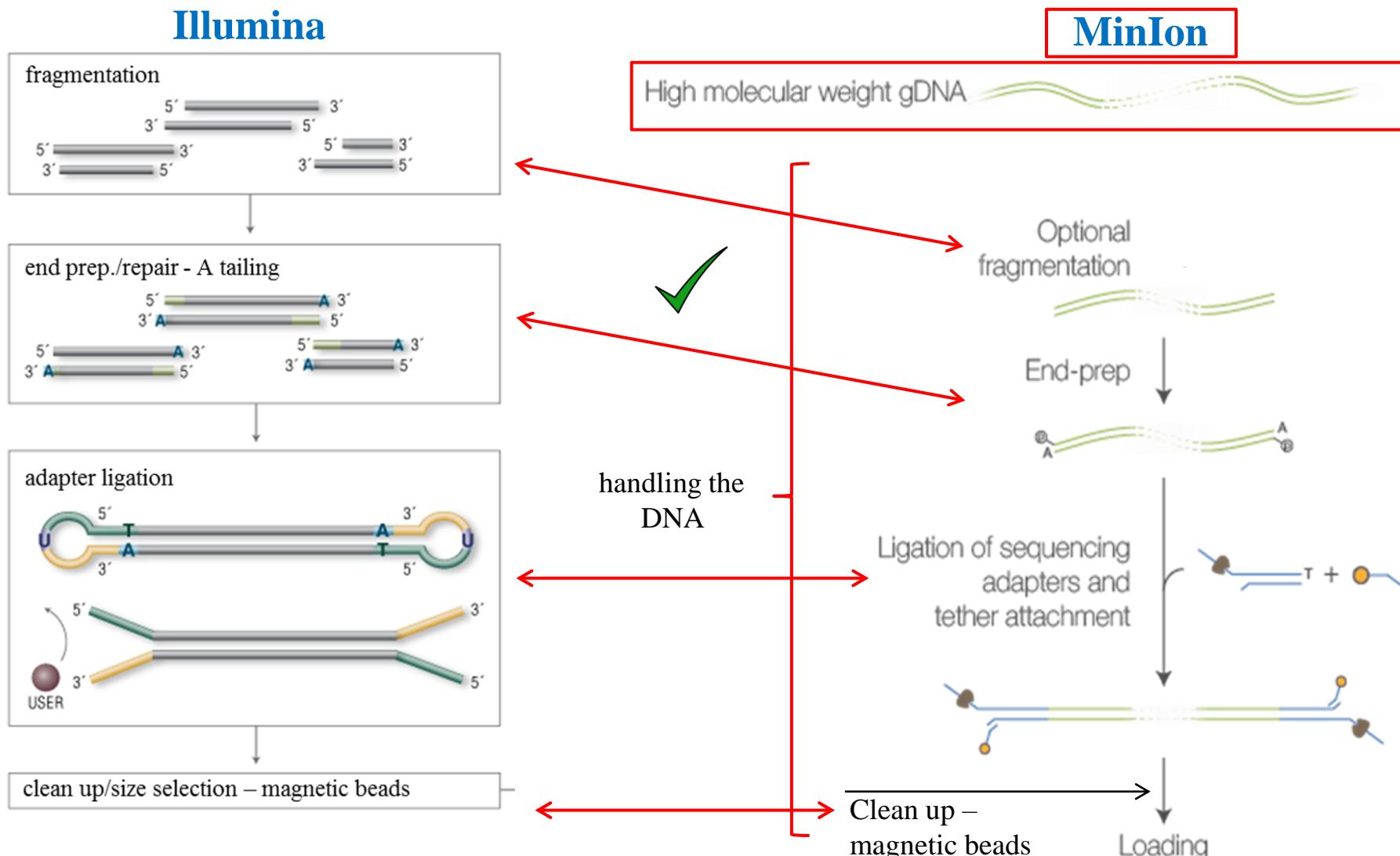
Collection of steps to prepare DNA to be read by a sequencing machine.

This example is for Oxford Nanopore machines.



DNA library preparation Illumina vs MinIon:

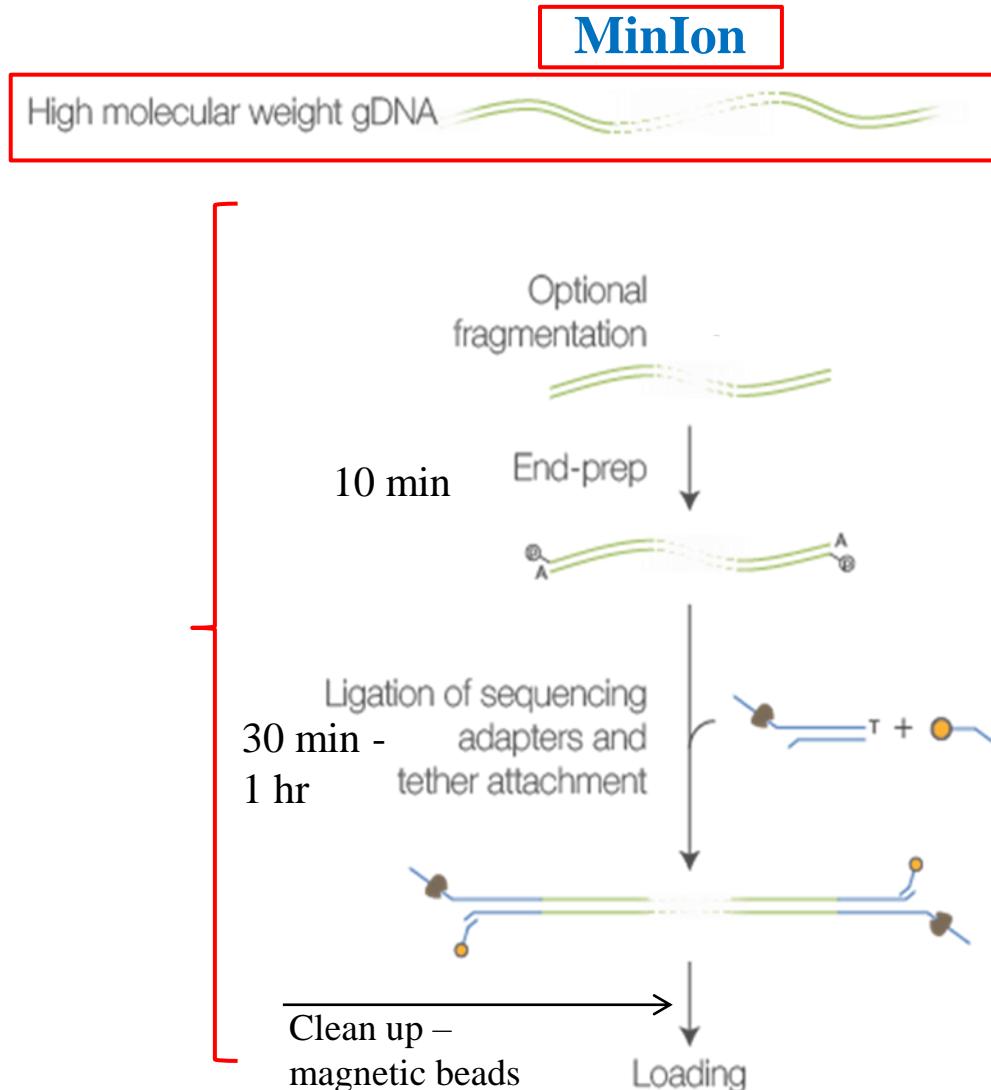
Same steps, yet different ingredients and handling!



DNA library preparation MinIon:

Same steps, yet different ingredients and handling!

- Keep in the fridge.
- Wide bore tips whenever handling the DNA.
- Flick tubes, no vortex.
- Increase all incubation times in lib. prep. protocol.



DNA library preparation MinIon:

Genomic DNA by Ligation (SQK-LSK109)

Version: GDE_9063_v109_revL_14Aug2019
Last update: 20/09/2019

Flow Cell Number:



DNA Samples:

Before start checklist

Materials

1 µg (or 100-200 fmol) high molecular weight genomic DNA

Ligation Sequencing Kit (SQK-LSK109)

Flow Cell Priming Kit (EXP-FLP002)

Consumables

Agencourt AMPure XP beads

NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:

NEBNext FFPE Repair Mix (M6630)

NEBNext End repair / dA-tailing Module (E7546)

NEBNext Quick Ligation Module (E6056)

1.5 ml Eppendorf DNA LoBind tubes

0.2 ml thin-walled PCR tubes

Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

Freshly prepared 70% ethanol in nuclease-free water

Equipment

Hula mixer (gentle rotator mixer)

Magnetic separator, suitable for 1.5 ml Eppendorf tubes

Microfuge

Vortex mixer

Thermal cycler

Ice bucket with ice

Timer

Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS

DNA fragmentation (optional, for lower inputs)

OPTIONAL

Covaris g-TUBE

Prepare the DNA in nuclease-free water.

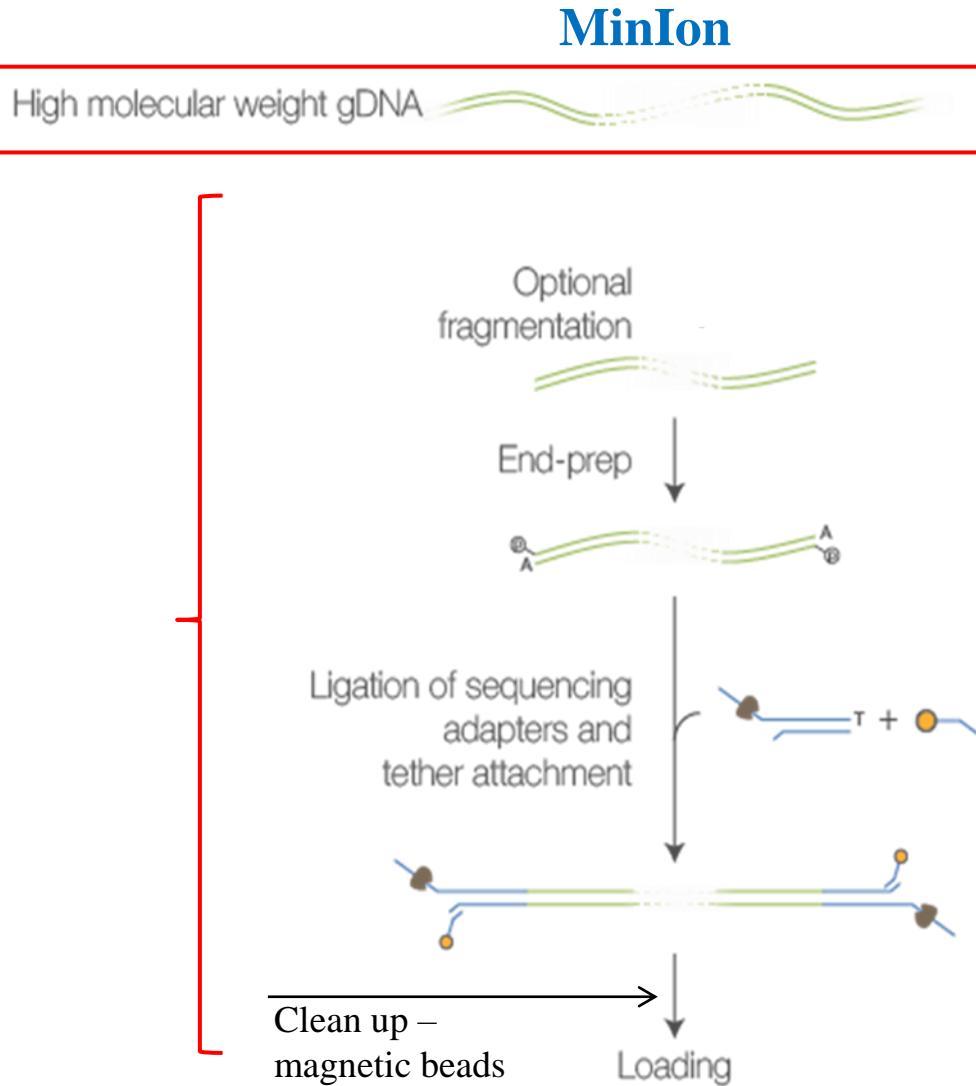
- Transfer 1 µg (or 100-200 fmol) genomic DNA into a DNA LoBind tube
- Adjust the volume to 19 µl with Nuclease-free water
- Mix thoroughly by inversion avoiding unwanted shearing
- Spin down briefly in a microfuge

Transfer the genomic DNA sample in 49 µl to the Covaris g-TUBE.

Spin the g-TUBE for 1 minute at RT at the speed for the fragment size required.

- Spin the g-TUBE for 1 minute
- Remove and check all the DNA has passed through the g-TUBE
- If DNA remains in the inner chamber spin again for 1 minute at the same speed

NOTES/OBSERVATIONS



DNA library preparation head ups - Illumina:

Low sequencing output:
low DNA input - library prep failure.

Failed clustering:
Failed library prep.

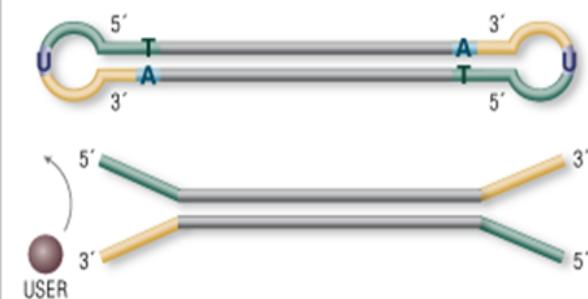
fragmentation



end prep./repair - A tailing



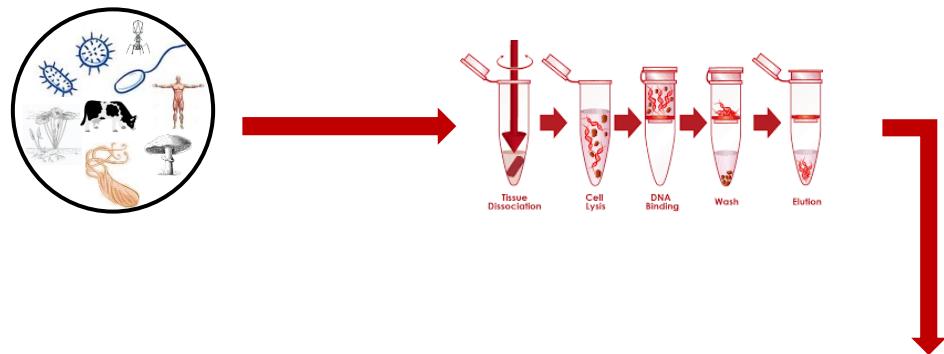
adapter ligation



clean up steps

1. The workflow from alive material to DNA:

- Starting materials and sample selection
- DNA extraction and challenges



2. DNA preparation for sequencing:

- DNA library preparation
- Sequencing technology selection

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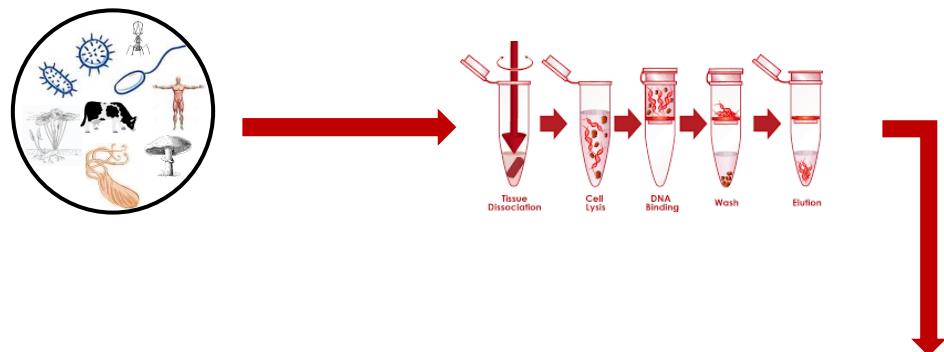
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NANOPORE
technologies



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CACATGGTGAAACCCAT
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IA TGTTGAAACCCAT
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IACACATGGTGAAACCCAT
```

1. The workflow from alive material to DNA:

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CALATGGTGAAACCCAT
ACATGGTGAAACCCAT
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TGTTGAAACCCAT
IA TGGTGAAACCCAT
IA GGTGAAACCCAT
IA TGTTGAAACCCAT
MACACATGGTGAAACCCAT
MACACATGGTGAAACCCAT

```

"Now, here, you see, it takes all the running you can do just to keep in the same place. If you want to get somewhere else, you must run at least twice as fast!"

- Red Queen from *Through the Looking Glass*



Thank you ☺

Twitter: @SariaOtani

LinkedIn: Saria Otani

Email: saot@food.dtu.dk

Questions ☺

From Organisms to Genomics: From Alive to Live (cont'd)

Saria Otani, MRes., MSc., PhD

Applied Methods in Metagenomics - 23260
Denmark, 6/09/2022

$$f(x+\Delta x) = \sum_{i=0}^{\infty} \frac{(\Delta x)^i}{i!} f^{(i)}(x)$$
