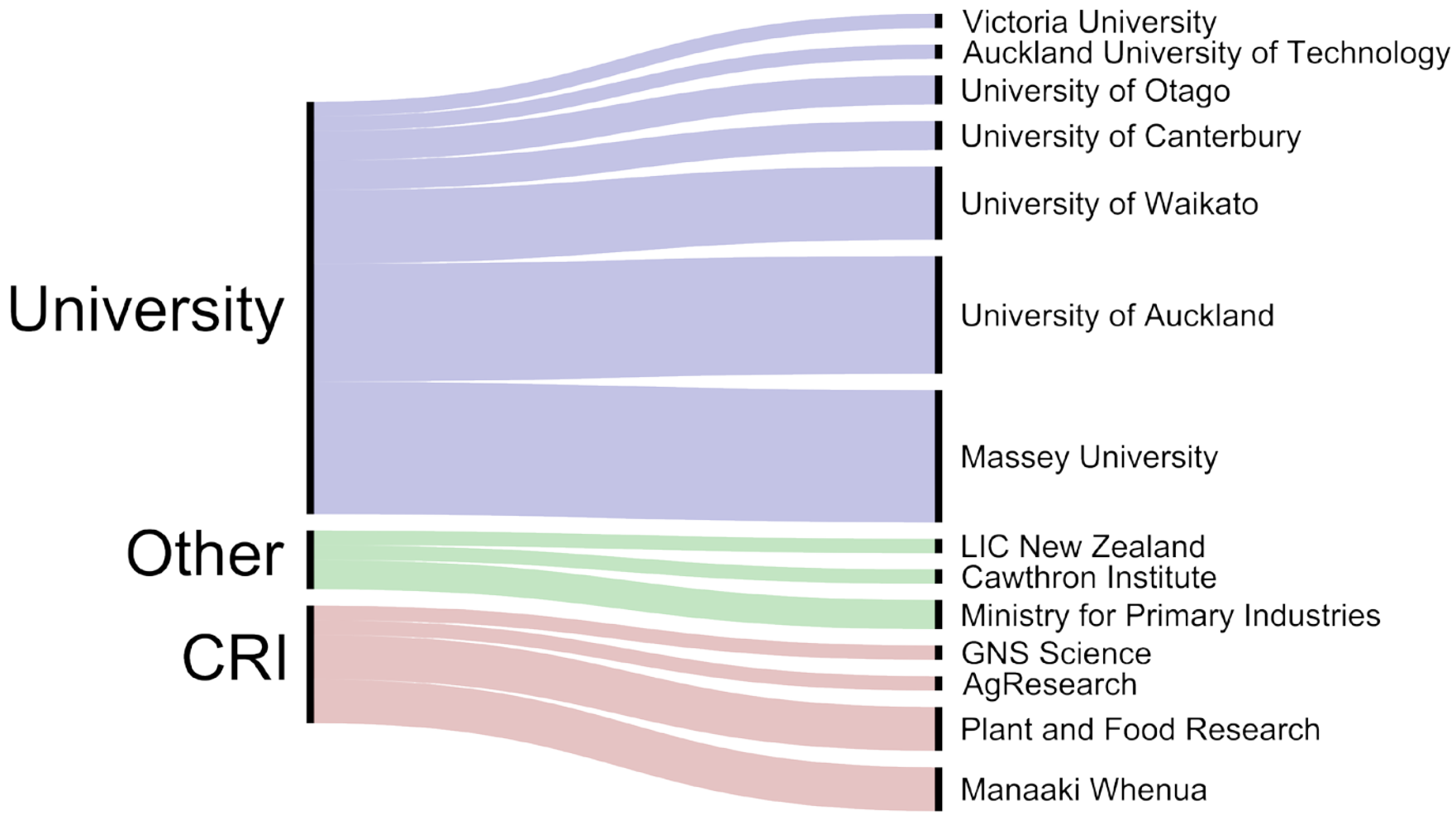
Day 1

Introduction to Bash scripting
Decision tree
Quality filtering WGS data
Genome assembly

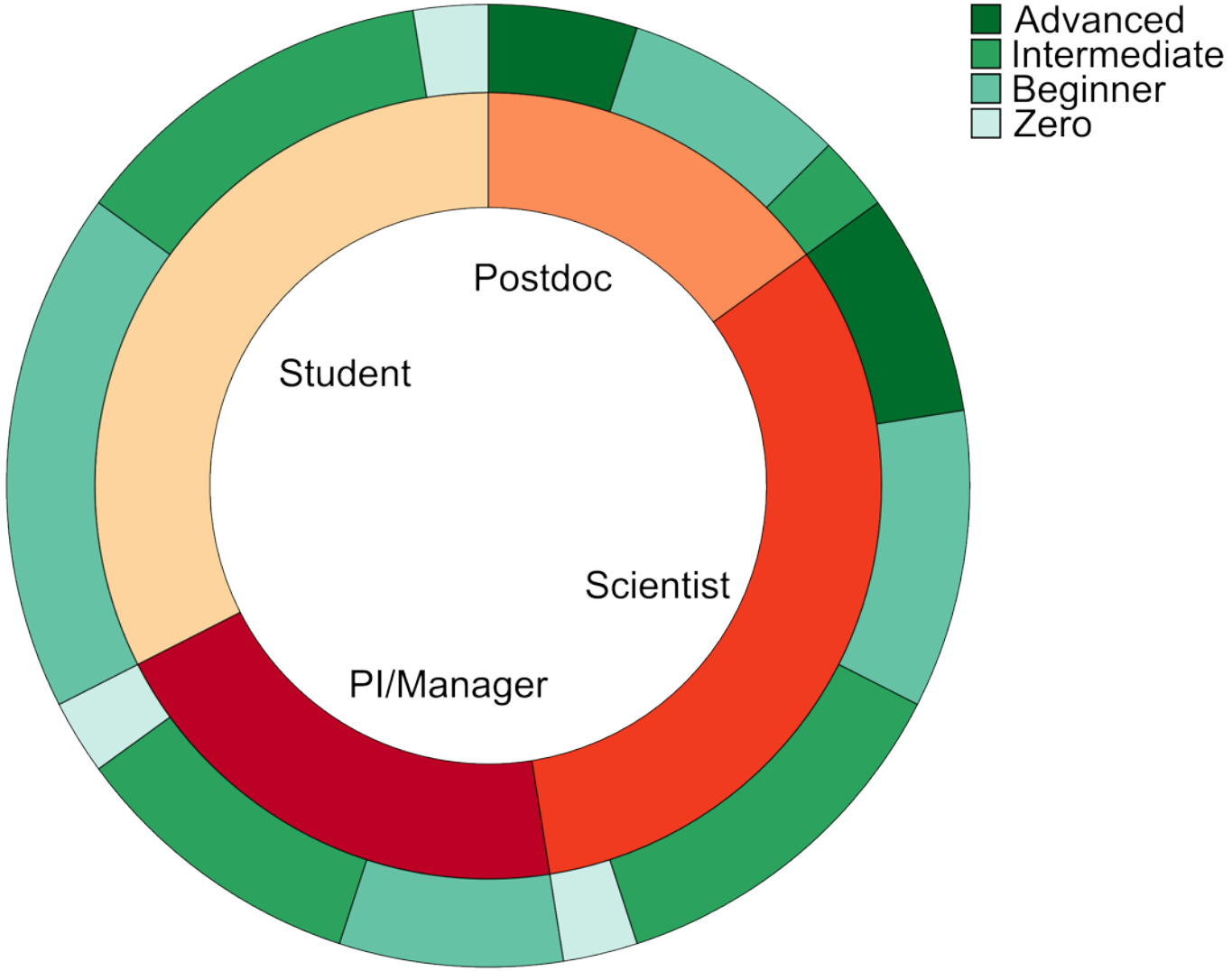
Welcome!

- **Housekeeping**
- **Overview of attendees**
  - Where are we from?
  - How experienced are we?
- **Any questions?**

Where are we from?



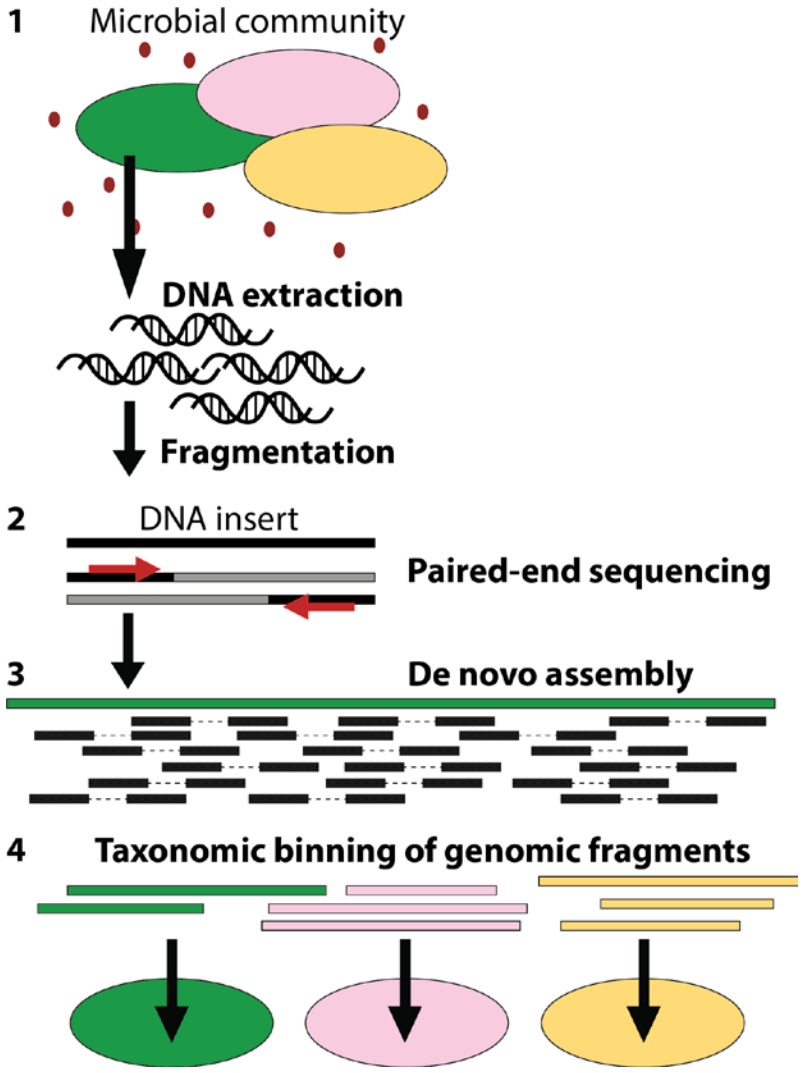
How experienced are we?



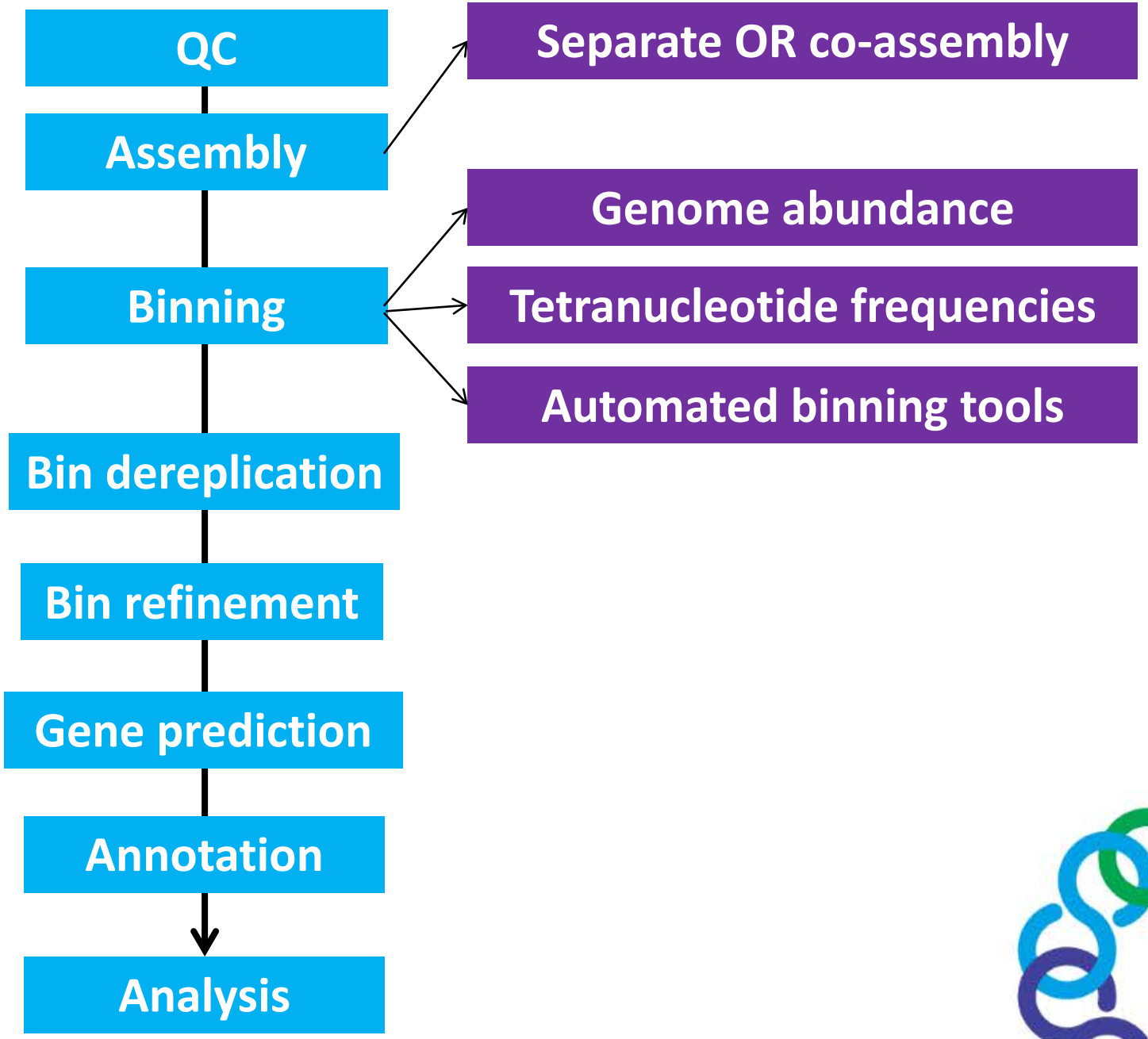
Bash scripting

Metagenomic decision tree(s)

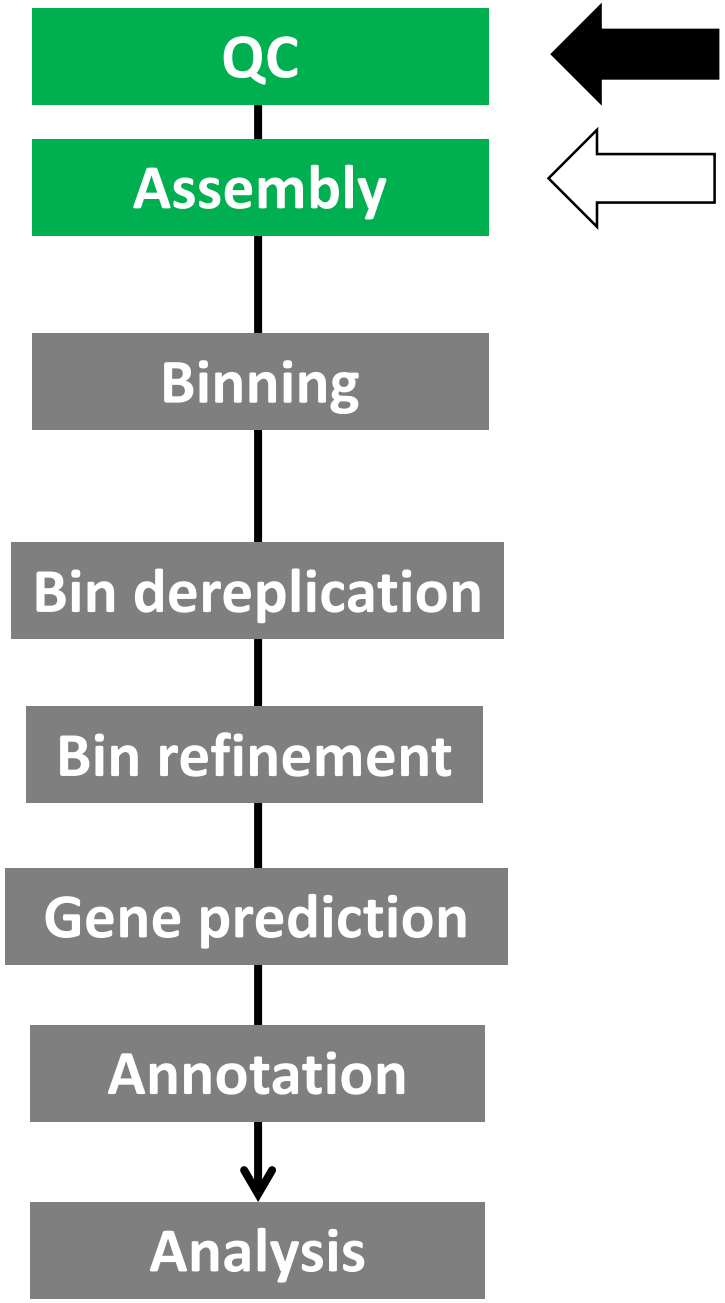
Our goal: genome recovery



Overview


QC
Assembly
Binning
Bin dereplication
Bin refinement
Gene prediction
Annotation
Analysis
Separate OR co-assembly
Genome abundance
Tetranucleotide frequencies
Automated binning tools

Overview


QC
Assembly
Binning
Bin dereplication
Bin refinement
Gene prediction
Annotation
Analysis

Decision tree

- **Starts with experimental design**
- **DNA extraction**
- **WGS library prep**
- **Amount of sequencing**

Samples/$$$

Many samples/Limited $$$

Few samples/Ample $$$

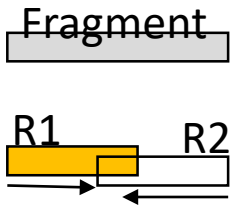
Aim:
Read analysis

Aim:
Genome recovery

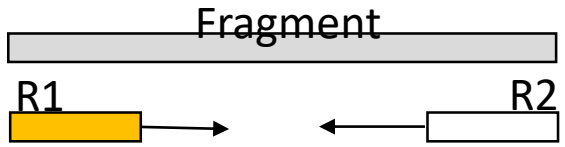
Libraries:
Short overlapping PE inserts

Libraries:
Longer gapped PE inserts

(e.g. 200 bp DNA fragments
for 2x125 bp reads)

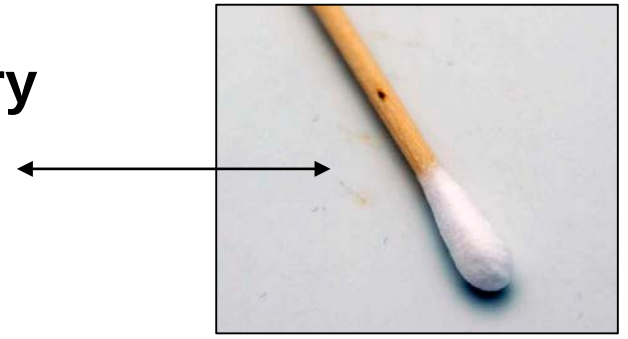


(e.g. >=550 bp DNA fragments)



DNA input

- **Very low inputs (e.g. nanograms) for Nextera library prep = enzymatic fragmentation with broad size distributions**



- **High inputs (e.g. 100s ng) for TruSeq = physical fragmentation with defined size selection**

Tends to yield sequences of larger inserts

Samples/$$$

Many samples/Limited $$$

Few samples/Ample $$$

Aim:
Read analysis

Aim:
Genome recovery

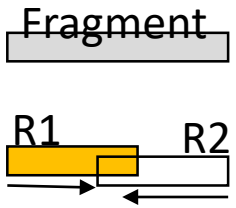
Libraries:
Short overlapping PE inserts

Libraries:
Longer gapped PE inserts

Sequencing depth:
Shallow (<10 Gbp)

Sequencing depth:
Deep (e.g. >=10s Gbp)

(e.g. 200 bp DNA fragments
for 2x125 bp reads)



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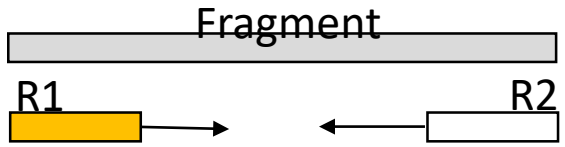
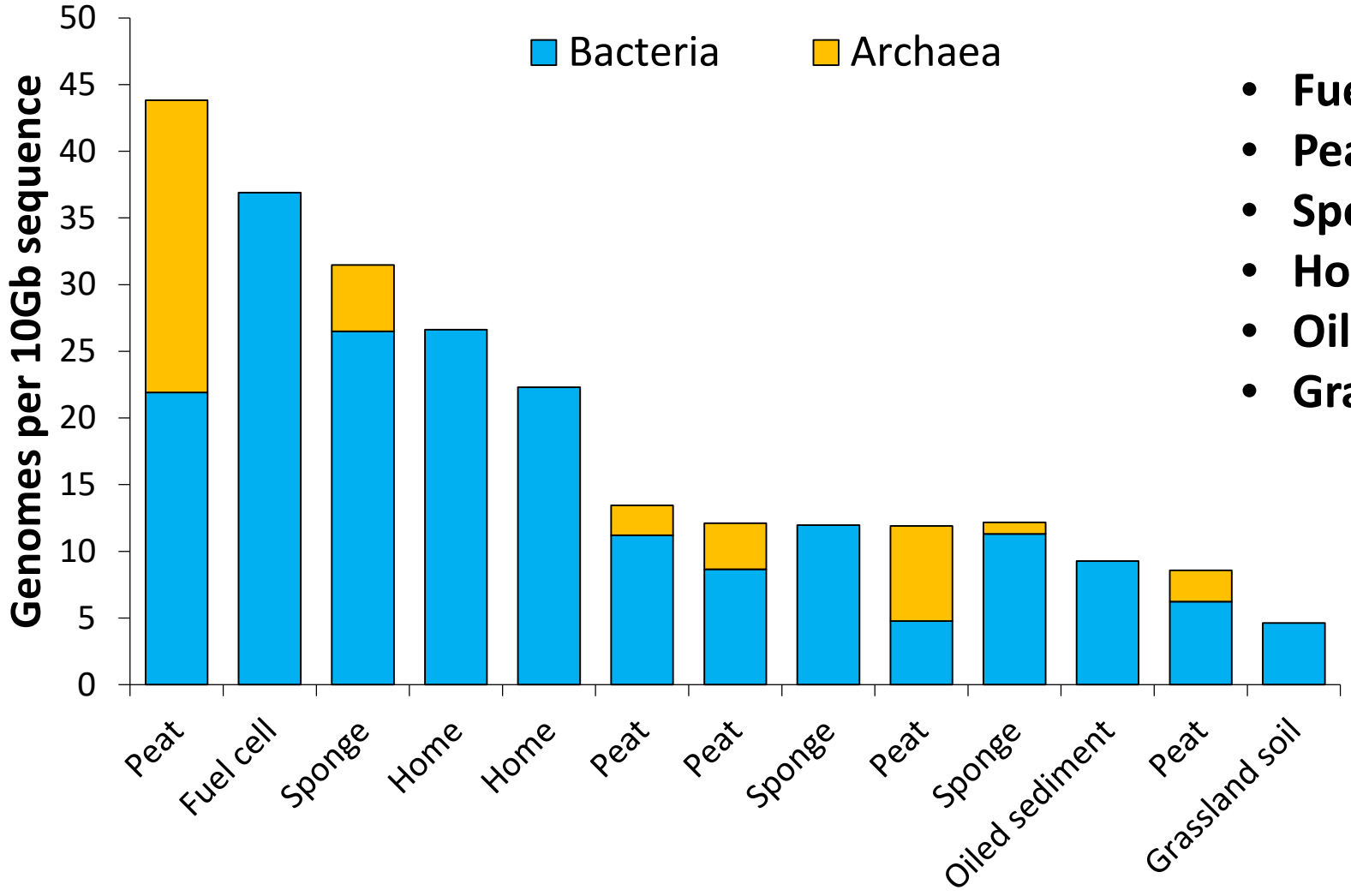
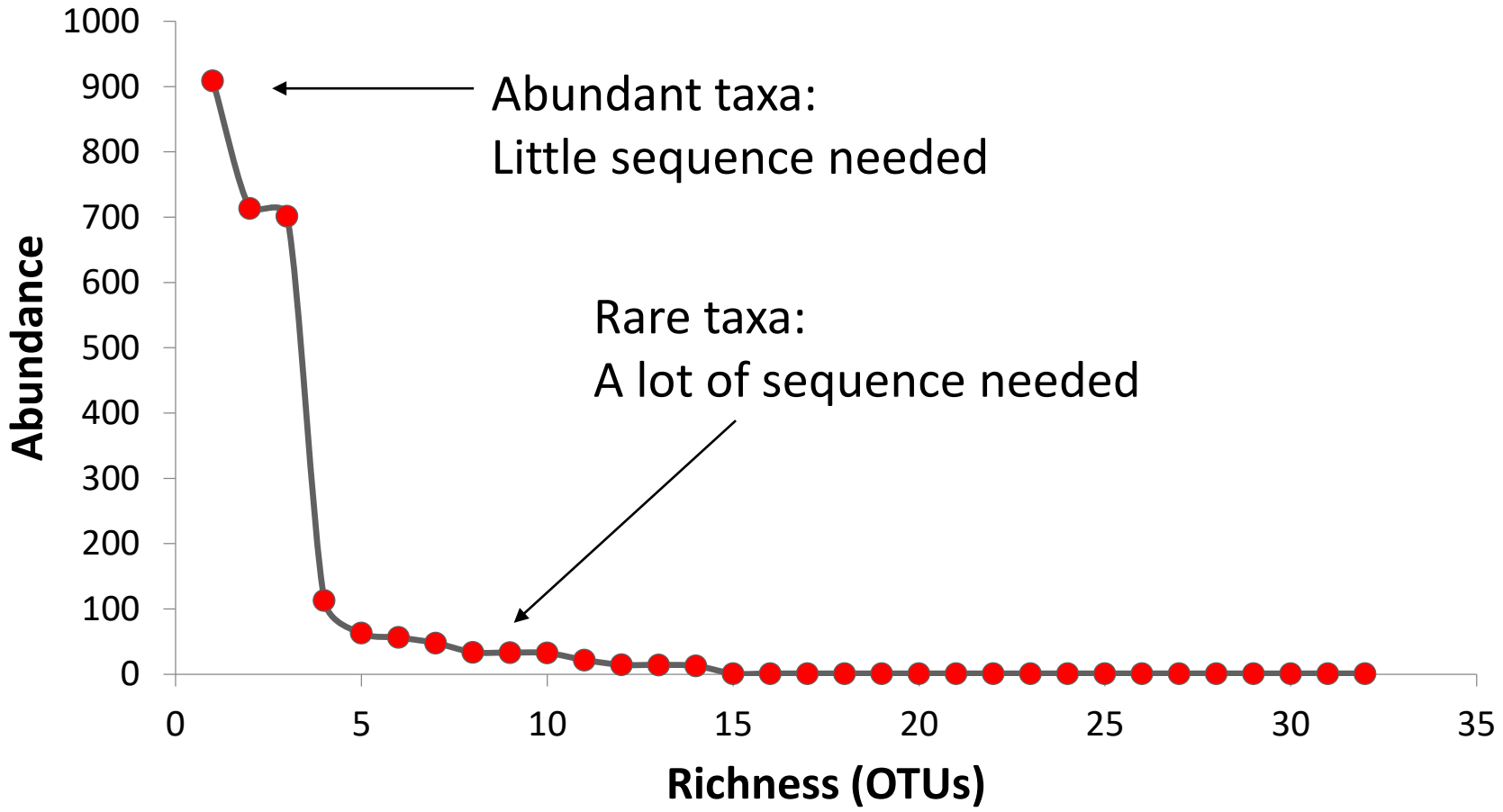


Genome recovery per environment

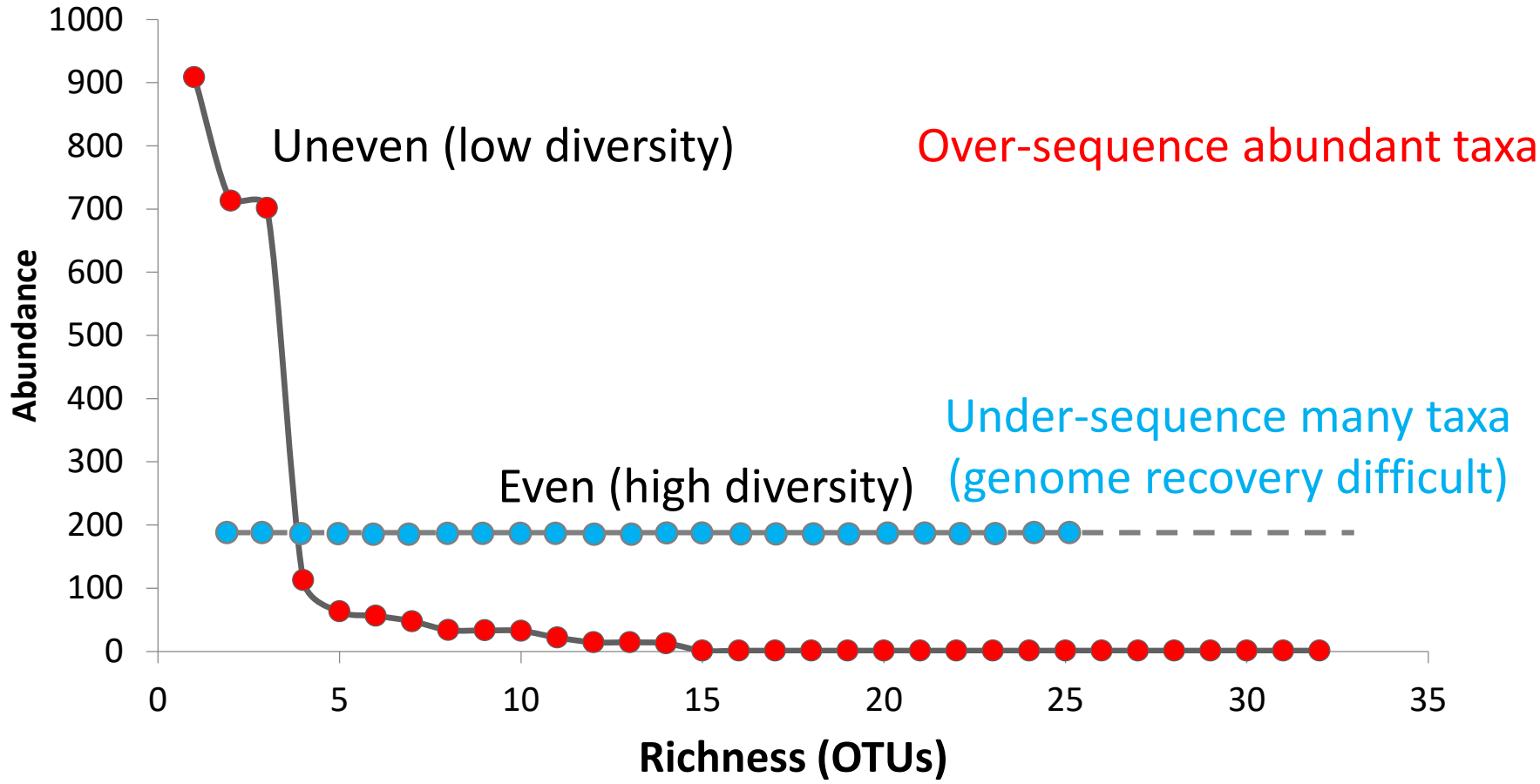


- **Fuel cell microbiome**
- **Peat (boreal)**
- **Sponge microbiome**
- **Home microbiome**
- **Oiled sediment (seafloor)**
- **Grassland soil**

Estimate sequencing depth

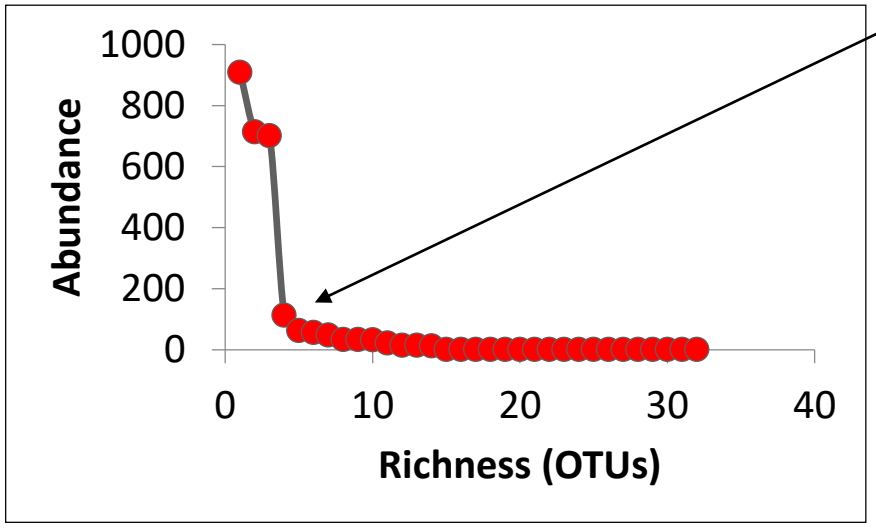


Community structure matters



Estimate sequencing depth

- **Estimate generously**
- **Determine/guesstimate relative abundance of rarest target organism**
- **Determine/guesstimate the average genome size**
- **Factor in larger eukaryote genomes**
- **Decide the minimum desired coverage (e.g. 30x)**



e.g., 5% relative abundance = 5% of sequence data

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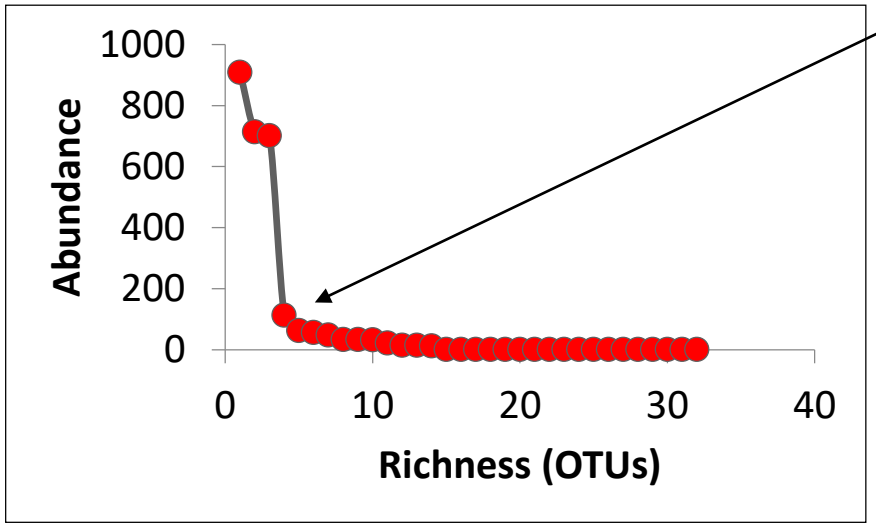


e.g., 5% relative abundance = 5% of sequence data

Mock parameters:

- Bacterial genome 5 Mbp long
- 5% abundance (need 100/5 or 20x a genome at 100% abundance)
- 30x coverage

5 Mbp x 20 x 30 = 3,000 Mbp (or 3 Gbp)

When you have so many genomes

You need a:

- **Clear goal**
- **Question**
- **Hypothesis to test**

Working groups of 4 people

- **Self organize into teams of 4 with mixed skillsets and skill levels**
- **This is your team for the next four days**
- **Pick one of ten defined goals**
- **Each group will have a different goal**
- **First in first served**

Group goals

Determine which genome(s) have the following attributes, and the genetic mechanisms used for these attributes:

1. **Denitrification** (Nitrate or nitrite to nitrogen)
2. **Ammonia oxidation** (Ammonia to nitrite or nitrate)
3. **Anammox** (Ammonia and nitrite to nitrogen)
4. **Sulfur oxidation** (SOX pathway, thiosulfate to sulfate)
5. **Sulfur reduction** (DSR pathway, sulfate to sulfide)
6. **Photosynthetic carbon fixation**
7. **Non-photosynthetic carbon fixation** (Reverse TCA or Wood-Ljundahl)
8. **Non-polar flagella expression due to a chromosomal deletion**
9. **Plasmid-encoded antibiotic resistance**
10. **Aerobic (versus anaerobic) metabolism**

Quality filtering

Quality filtering WGS data

- Remove barcode and adapter regions
- Remove low-quality regions of reads
- Identify potential problems during sequencing
  - Adapter read-through
  - Deciphering ‘aberrant’ metrics in FastQC

The FastQ data format

```
@SEQUENCE_1
ATCGATCGATCG
+
4:<AIIIFIIII
@SEQUENCE_2
AATGATCCATG
+
IIIIIIIIIII
@SEQUENCE_3
TGTGTGACATG
+
BBGBBCIFIII
```

Each sequence is represent by four lines

1. Sequence name
2. Sequence content
3. Spacer line (+, or +Sequence name)
4. Quality information

The FastQ data format

- **What does the quality score even mean?**
  - **It represents the probability of a nucleotide position being *<u>incorrectly</u>* called**

$$Q = -10\ log_{10}p$$

Q	p	Prob. correct
0	1	0
10	0.1	0.9
20	0.01	0.99
30	0.001	0.999
40	0.0001	0.9999

How each Q value is encoded varies between sequencing platforms

<u>Generally</u> we work with the **Illumina 1.8+** (Phred+33) standard

Task: Quality filtering

Visualising data with FastQC

1. Inspecting *fastq* files
2. Identifying regions of concern

Quality filtering with trimmomatic

1. Removing adapter sequences
2. Removing low-quality regions

Common issues with WGS data

Do I need to remove adapters?	→	Yes.
I don't know if adapters have been removed or not	→	Check the per-nucleotide distributions You will see 100% skews if they remain.
What's the lowest Q to allow when trimming?	→	Assembly is a self-correcting process, so you can be surprisingly lenient
What if my GC skew is outside of the expected range?	→	FastQC is calibrated to genome data where you expect GC conservation. Metagenomes do not adhere to this assumption

How to interpret over-represented kmers

What do high sequence duplication levels mean?

Issues when working with low-input DNA libraries

Task: Quality filtering

- Visualisation with FASTQC
- Read trimming and adapter removal

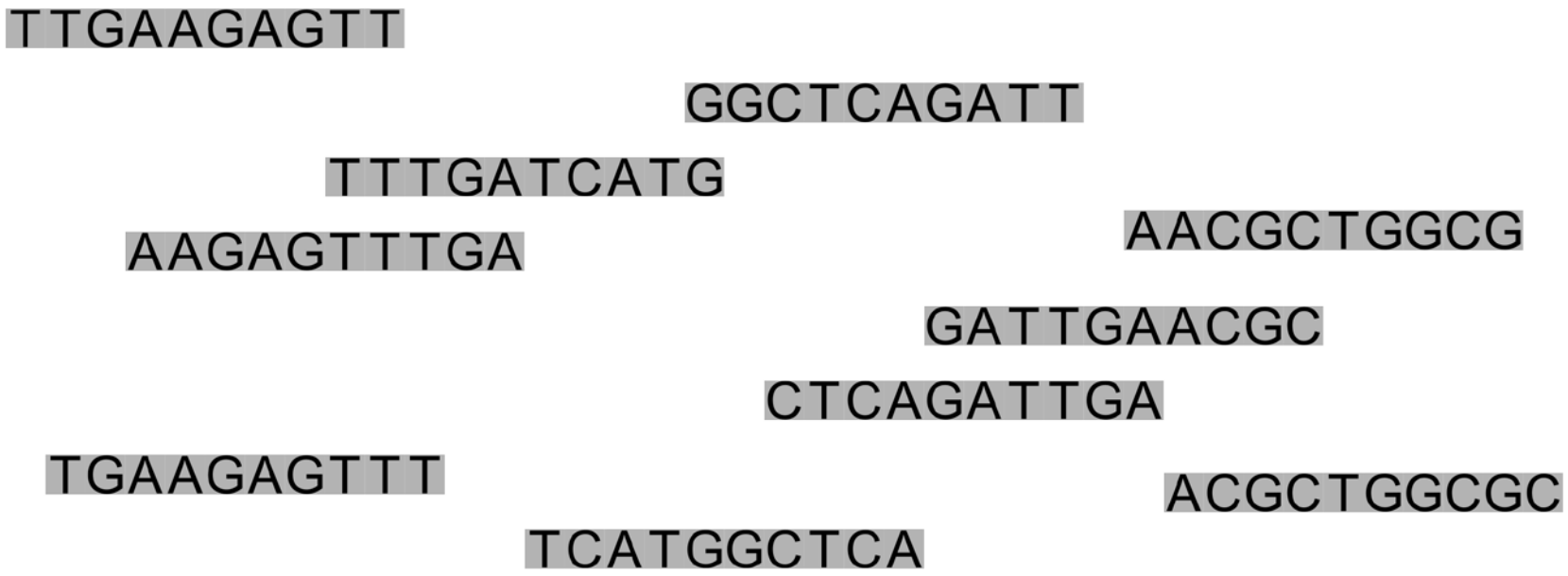
Assembly

Genome assembly

Overlap-Consensus-Layout (OCL) assembly

Genome assembly

Overlap-Consensus-Layout (OCL) assembly



Genome assembly

Overlap-Consensus-Layout (OCL) assembly

The problem for *de novo* assembly?

$$N.comparisons = \frac{(n)(n-1)}{2} = \frac{(10)(10-1)}{2} = 45$$

Genome assembly

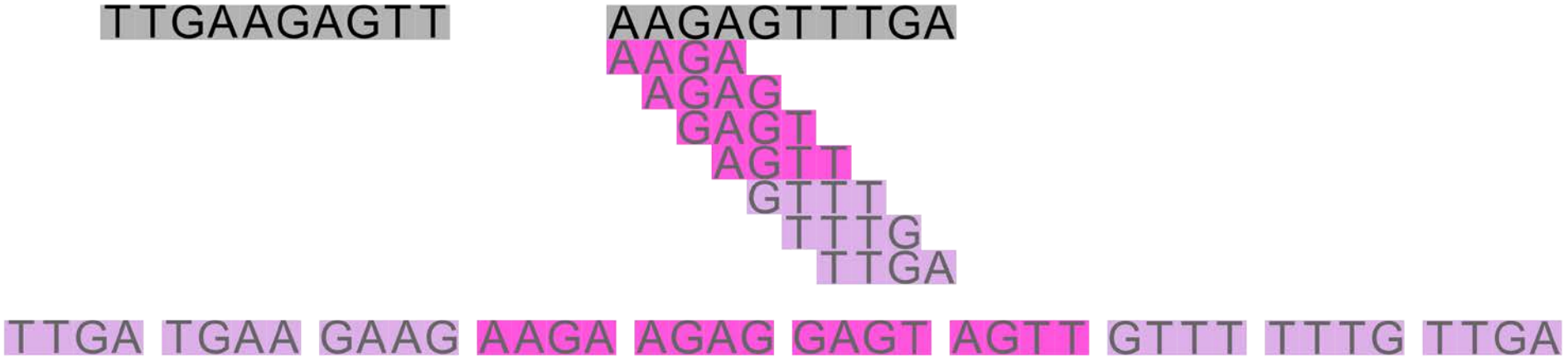
De Bruijn graph assembly

Break reads into shorter *k*-mers

Genome assembly

De Bruijn graph assembly

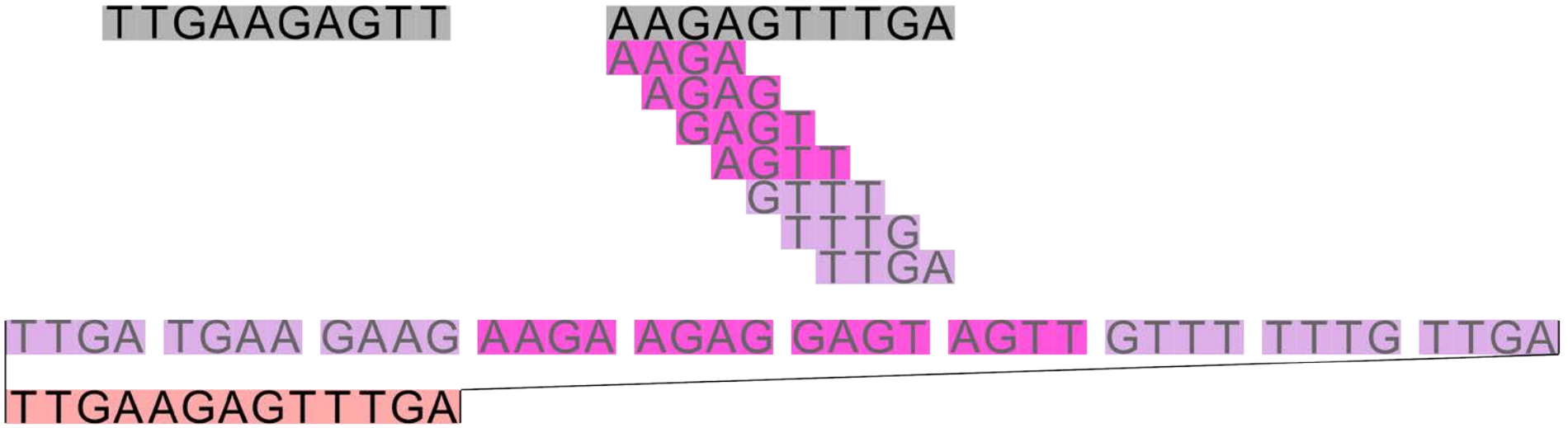
Identify sequences of shared *k*-mers



Genome assembly

De Bruijn graph assembly

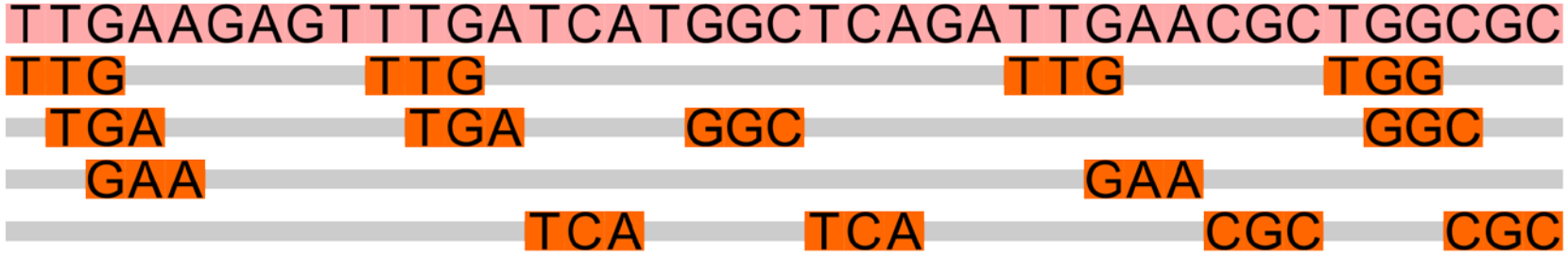
Identify sequences of shared *k*-mers



Genome assembly

De Bruijn graph assembly

Problem #1 – *k*-mers are short?



Genome assembly

De Bruijn graph assembly

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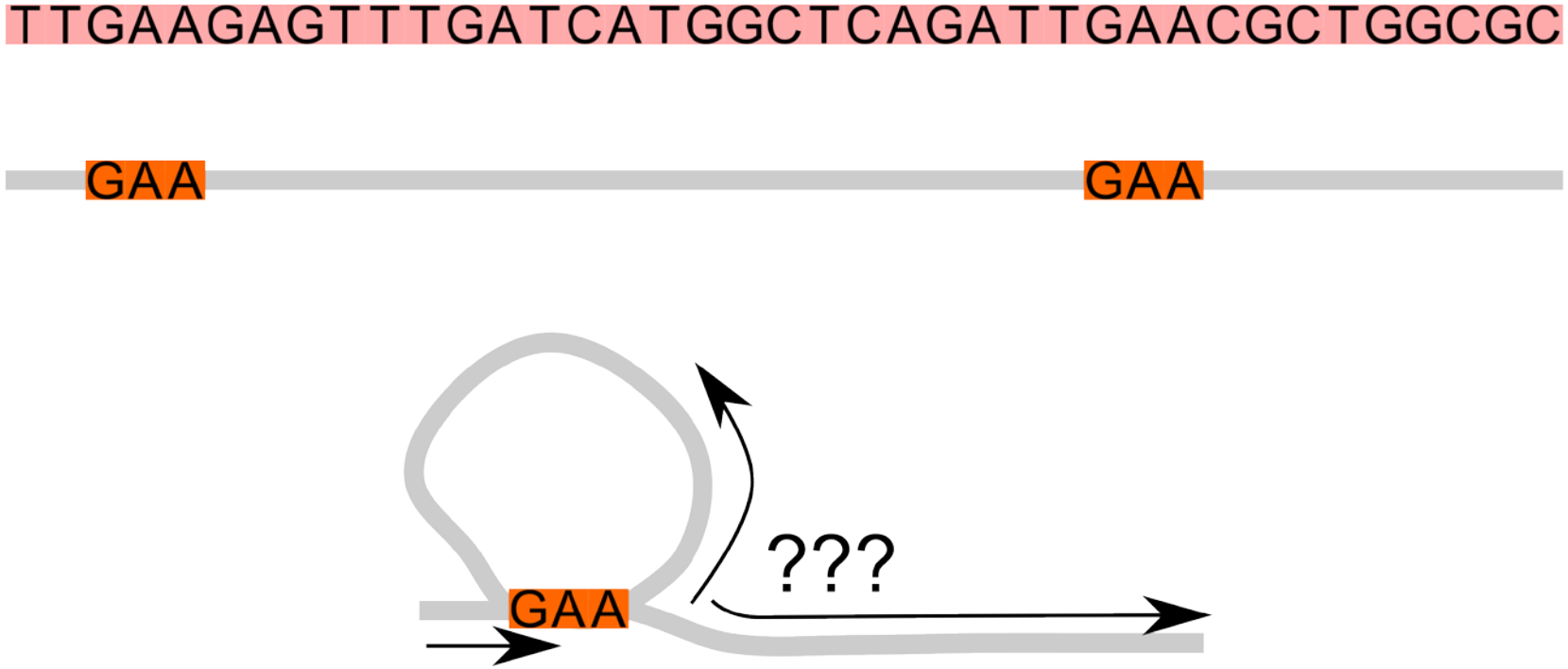
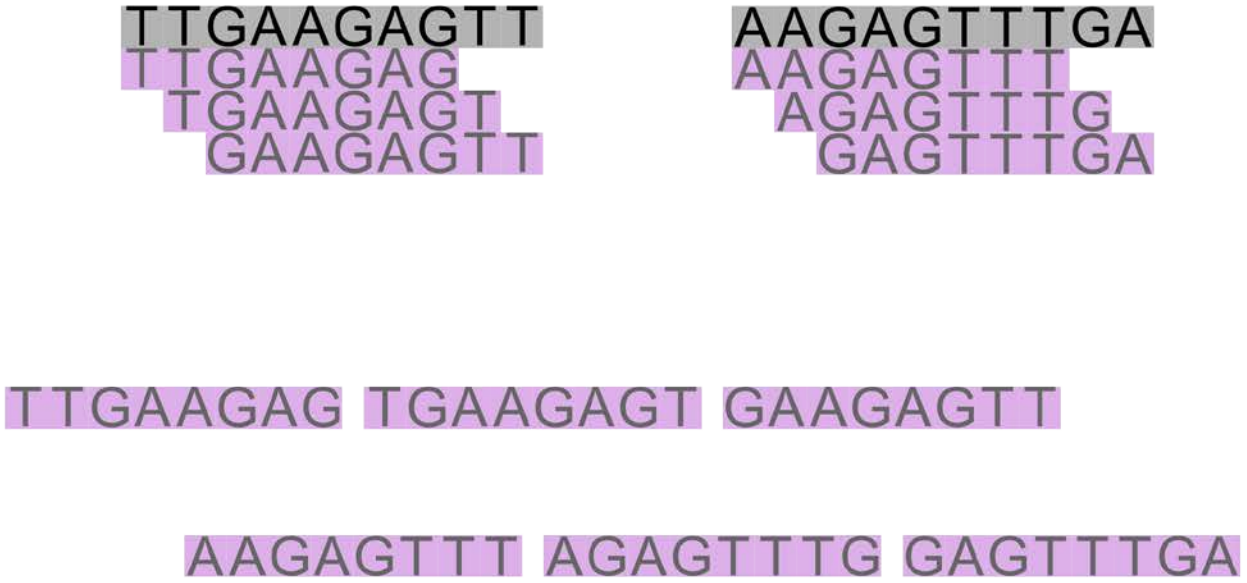


Genome assembly

De Bruijn graph assembly

Problem #2 – *k*-mers are long?



De Bruijn graph assembly

We want a range of *k*-mer sizes

- Short *k*-mers yield higher coverage
- Long *k*-mers assemble longer contigs (jump repeat regions)

Other considerations for picking *k*-mer sizes

- Size cannot be longer than read length
- Always pick odd *k*-mer sizes
- The more sizes you use, the longer assembly will take

K-mers	N. contigs	Longest contig	N50 >2kbp	L50 >2kbp
21, 33, 55	4,239,806	660,812	6,782	12,906
43, 55, 77, 99, 121	2,519,669	1,022,083	7,990	12,673
21, 43, 55, 77, 99, 121	3,388,682	1,022,083	7,789	13,327

Which assembler is best?

There are three good options

- SPAdes
- IDBA-UD
- MegaHIT

In conclusion, it can be said that the choice of assembler should depend on the data at hand and on the exact research question asked. Generally, the best assembly is performed by multi k-mer assemblers such as metaSPAdes, Megahit and IDBA-UD. If micro diversity is not a major issue, and the primary research goal is to bin and reconstruct representative bacterial genomes from a given environment, metaSPAdes should clearly be the assembler of choice. This assembler yields the best contig size statistics while capturing a high degree of community diversity, even at high complexity and low read coverage. If mico diversity is however an issue, or if the degree of captured diversity is far more important than contig lengths, then IDBA-UD or Megahit should be preferred.

Vollmers *et al.* 2017 (https://doi.org/ 10.1371/journal.pone.0169662)

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What are some key considerations?

Biological

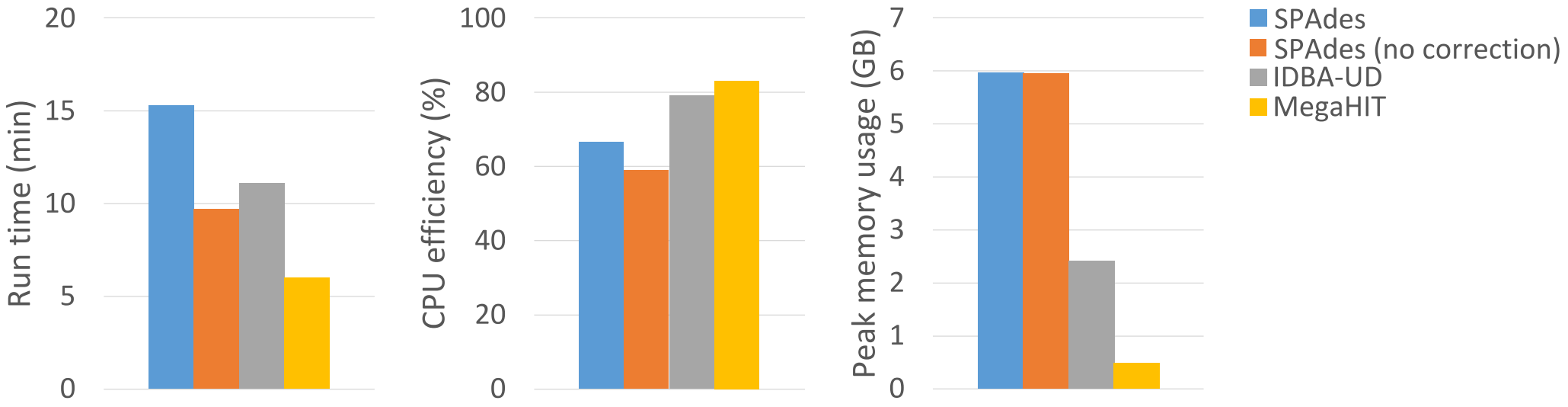
1. What is your hypothesis?
2. What do you want from the data?

Computational and resource

1. How much data do you have?
2. What are your computational resources?
3. What are your ***time*** resources?

Genome assembly

What are some key considerations?



Too much data?

- Consider testing sub-samples when coverage is very high, e.g. 100s or 1000s
- Example: abundant groundwater genome at 2000x coverage in full dataset
- Empirical testing of subsample sizes identified assembly sweet spot

(Fig. S1, Handley et al., 2014, Environ. Microbiol.)

Task: Assembly

Preparing data for assembly

1. Learn to prepare input files for SPAdes and IDBA-UD
2. Configure the basic parameters for assembly

Perform assembly (assemblies)

1. Prepare an assembly job to run under slurm
2. Submit several jobs with varying parameters

Optional: work with own data