(First) steps in NGS data analysis

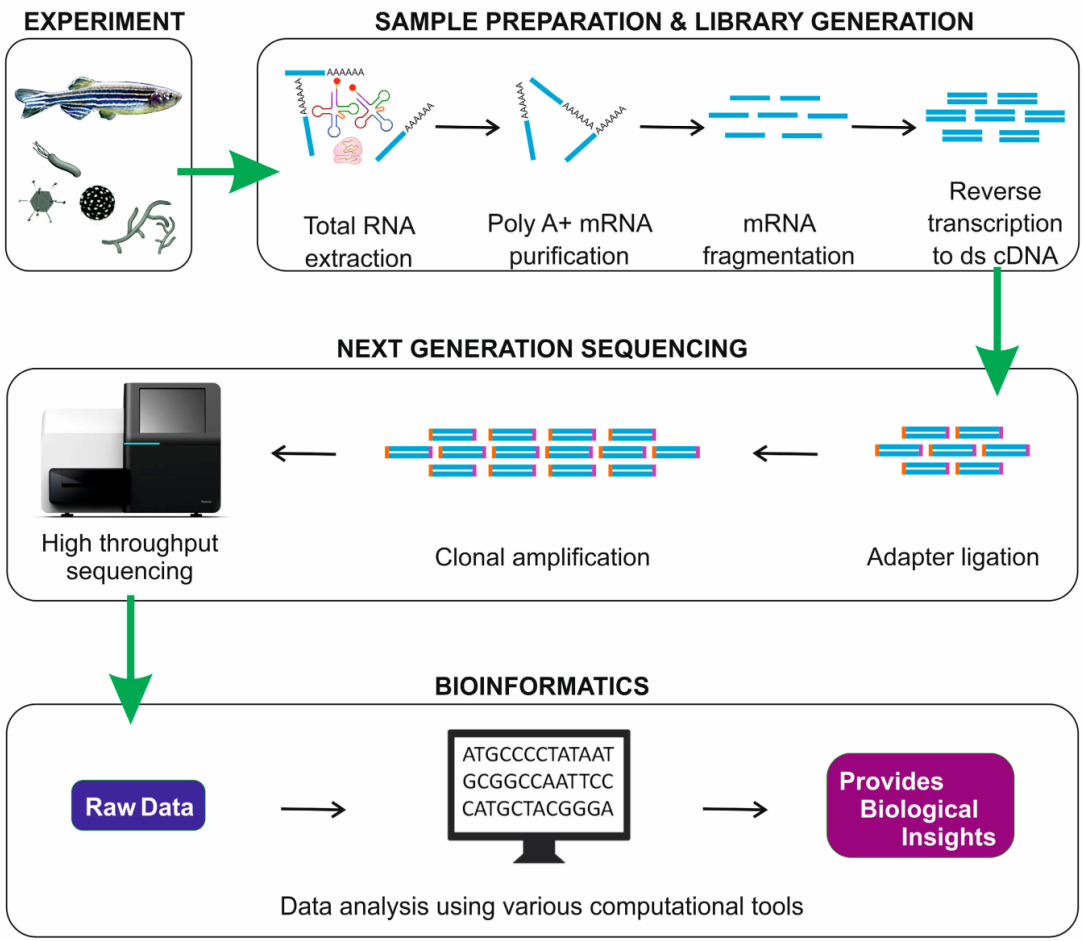
Bioinformatics Course UEB-VHIR
June 2022

Mireia Ferrer[1], Álex Sánchez[1,2], Esther Camacho[1], Angel Blanco[1,2], Berta Miró[1]

[1] Unitat d'Estadística i Bioinformàtica (UEB) VHIR
[2] Departament de Genètica Microbiologia i Estadística, UB

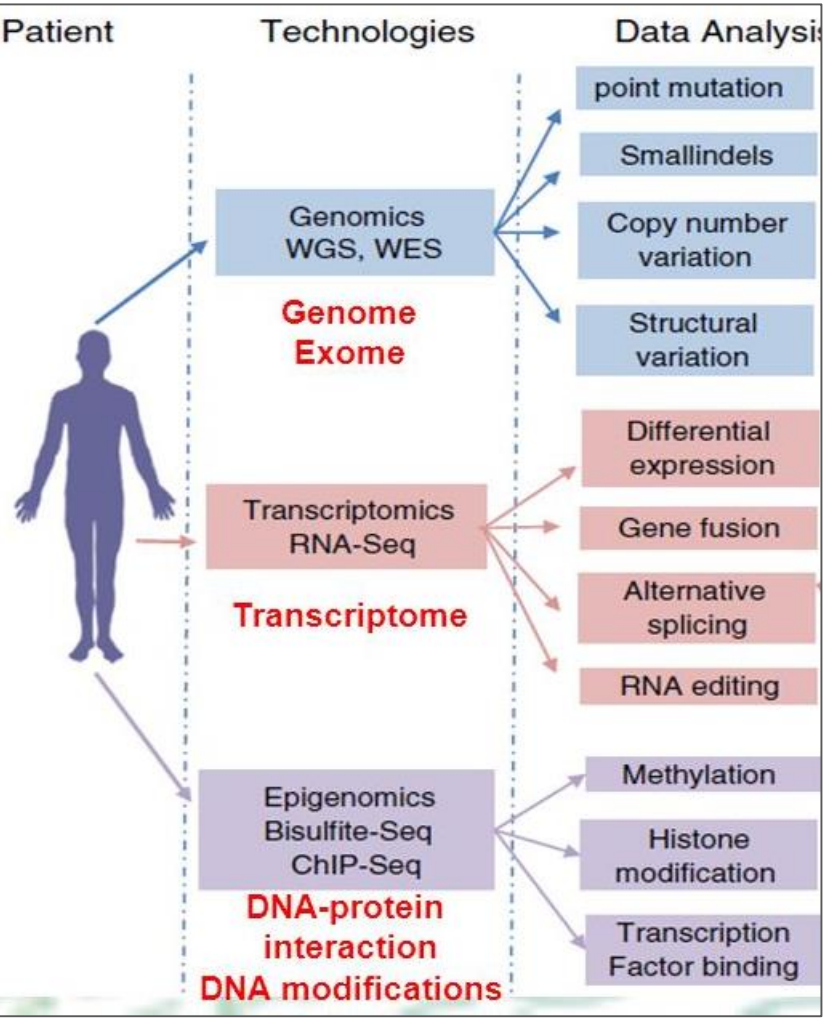
Steps in NGS analysis

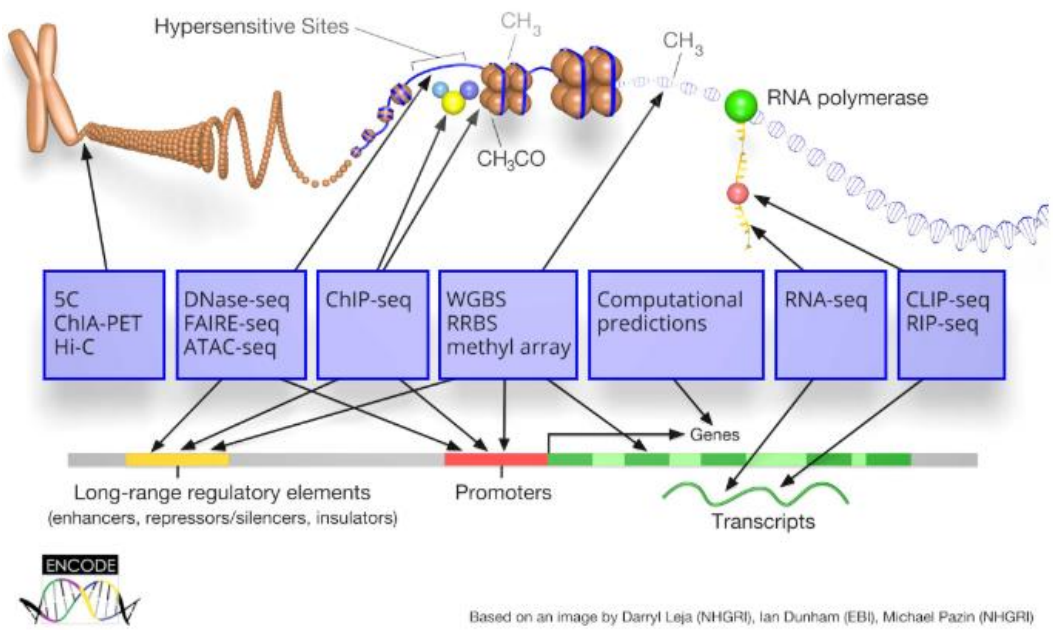
General workflow



Steps in NGS analysis

Applications



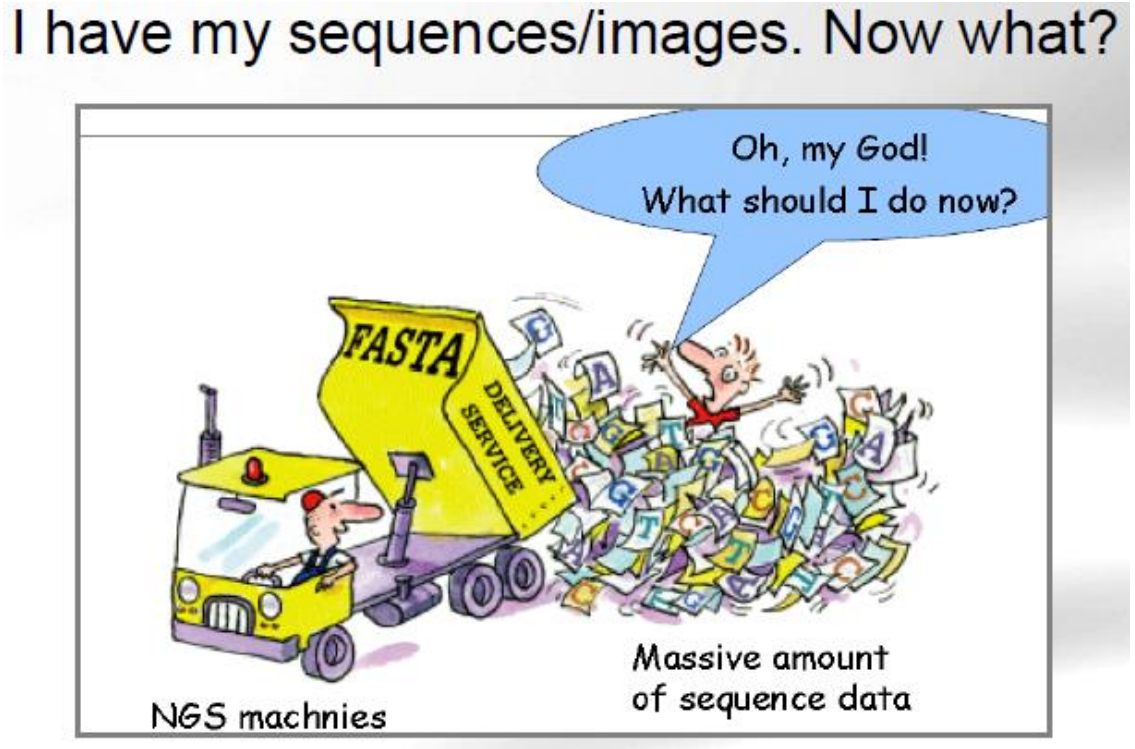


- But also...
  - Metagenomics
  - *De novo* genome assembly

More info: http://allseq.com/kb-category/applications/

Steps in NGS analysis

Bioinformatics challenges of NGS



Steps in NGS analysis

Bioinformatics challenges of NGS

A single sequencing experiment can generate 100's of millions of reads, 10's to 100's gigabytes of data.

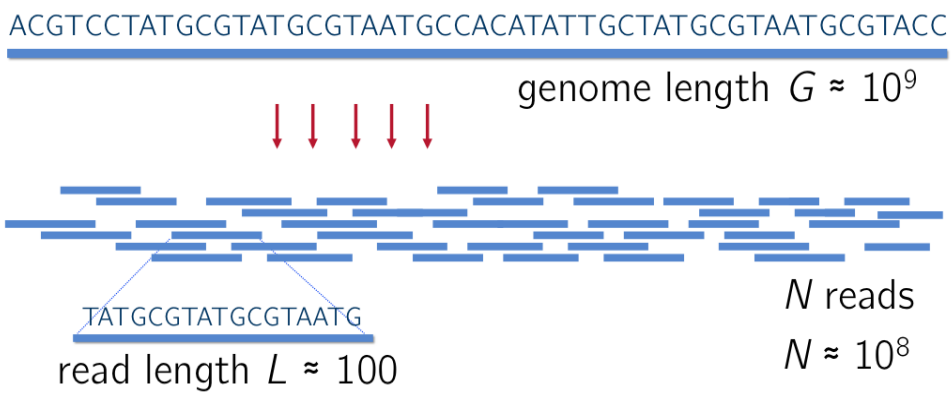
We need:

- Huge data storage and transfer technology
- Algorithms for managing, analyzing and visualizing data
- Reproducible workflows and standards for analysis
- Specialized tools for integrating various data types

Steps in NGS analysis

Terminology

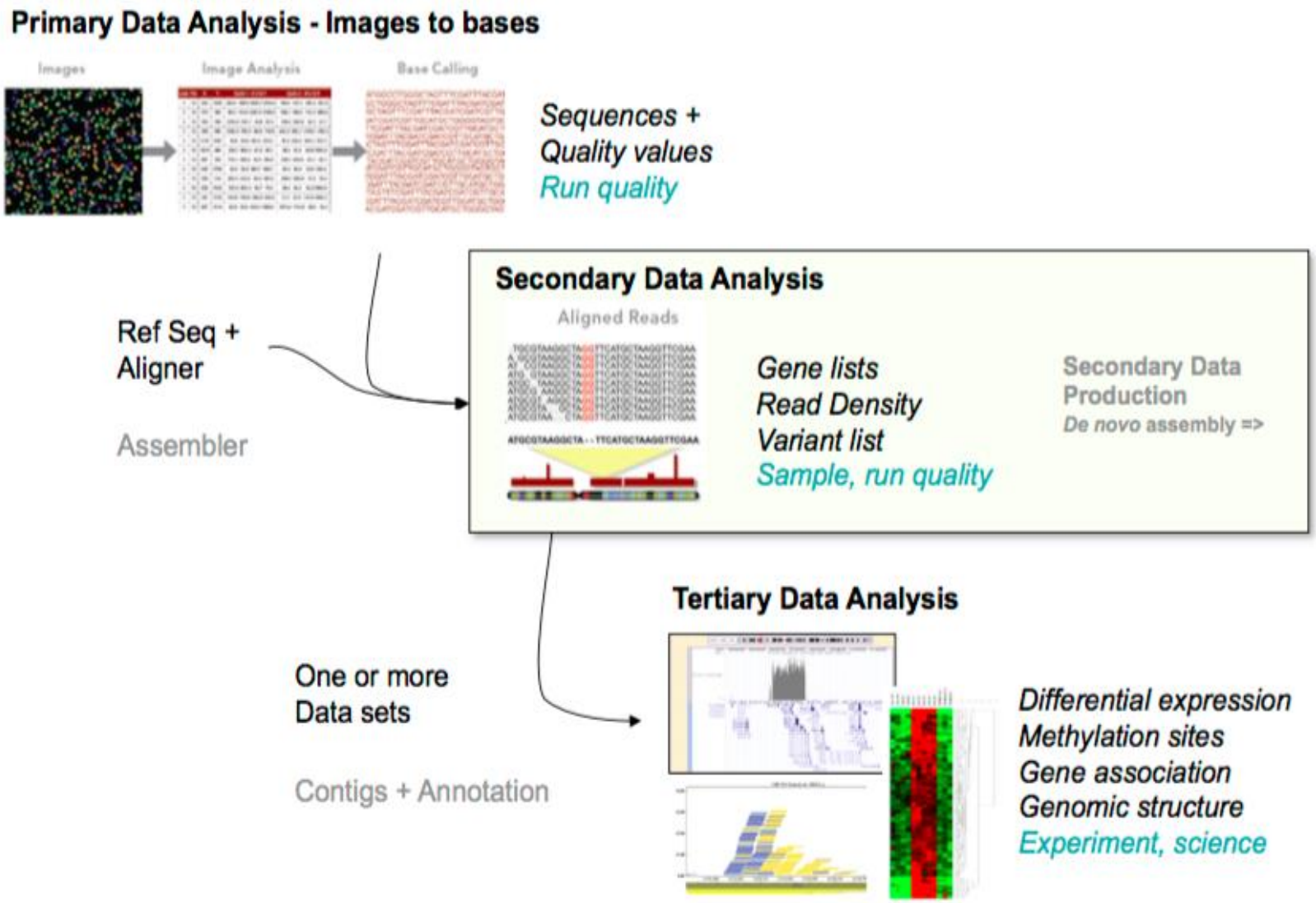
- **Library –** collection of DNA fragments for sequencing
- **Read –** a sequenced fragment
- **Read length** - the average number of contiguous nucleotide bases in a polynucleotide sequence that are produced by a particular sequencing instrument (14-400)



- **Contig –** set of overlapping reads
- **Sequencing depth/Library size –** total number of usable reads from the sequencing machine
- **Coverage –** Number of times a nucleotide base is read (# followed by X: 300X)
- **Single/Paired end** – in paired end sequencing each fragment is sequenced from the two ends and so generates two reads/fragment.
- **Call –** determination of a given base or base sequence by a sequencing instrument

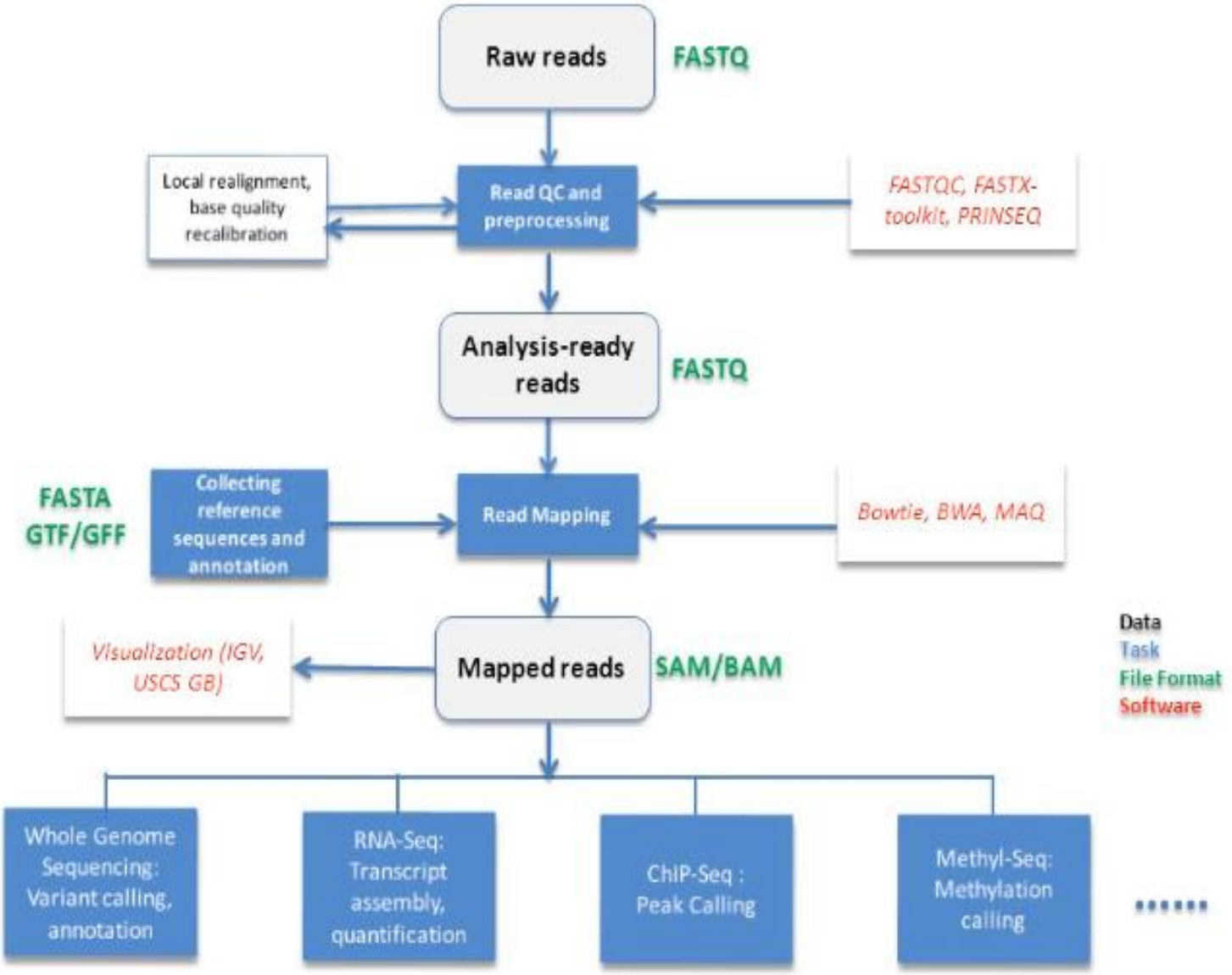
Steps in NGS analysis

- NGS data is analyzed in three stages



Steps in NGS analysis

- We will have different data (file) formats and tools for each step

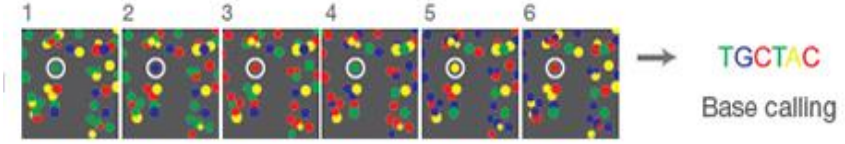


Steps in NGS analysis

Base calling: obtaining the raw read sequences (FASTQ files)

Sanger

Illumina (NGS)



- Base calling accuracy often measured by the Phred Quality Score (Q score) which assesses the accuracy of a sequencing platform.
- It indicates the probability that a given base is called incorrectly by the sequencer.

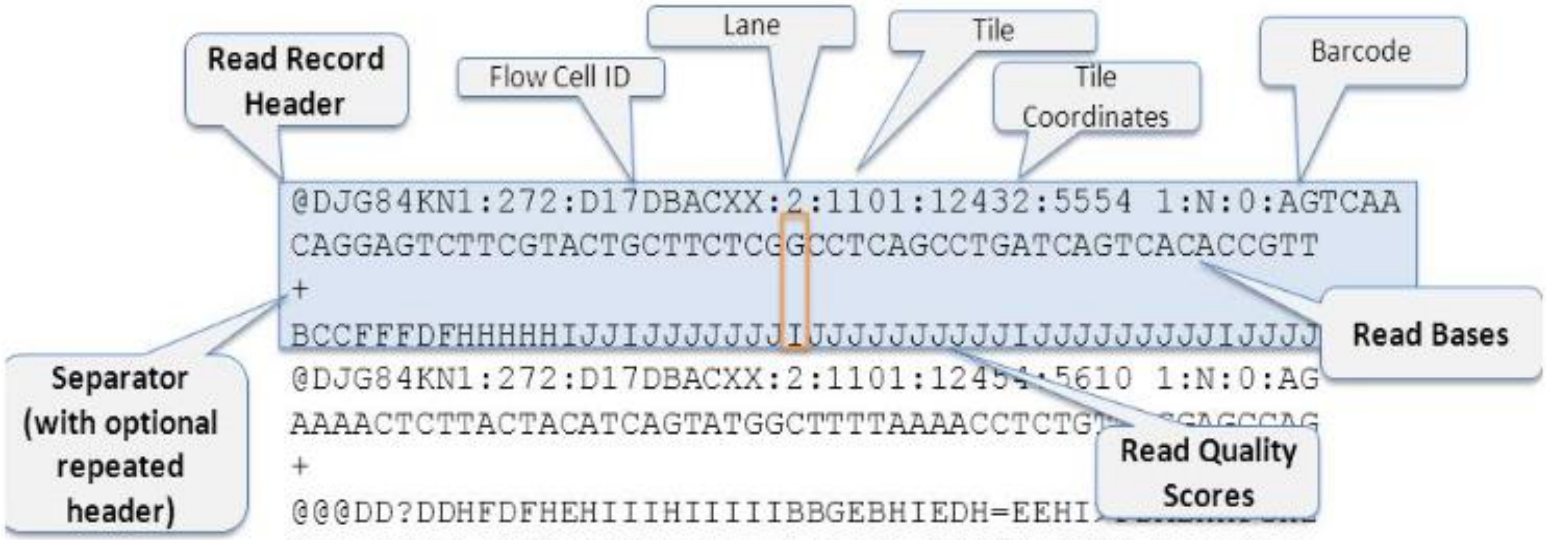
$$Q_{\text{PHRED}} = -10 \times \log_{10}(P_e)$$

Phred quality score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%

- Ambiguous positions with Phred scores <= 20 are labeled with N.
- To assign each base a unique score identifier (instead of numbers of varying character length), Phred scores are typically represented as ASCII characters.

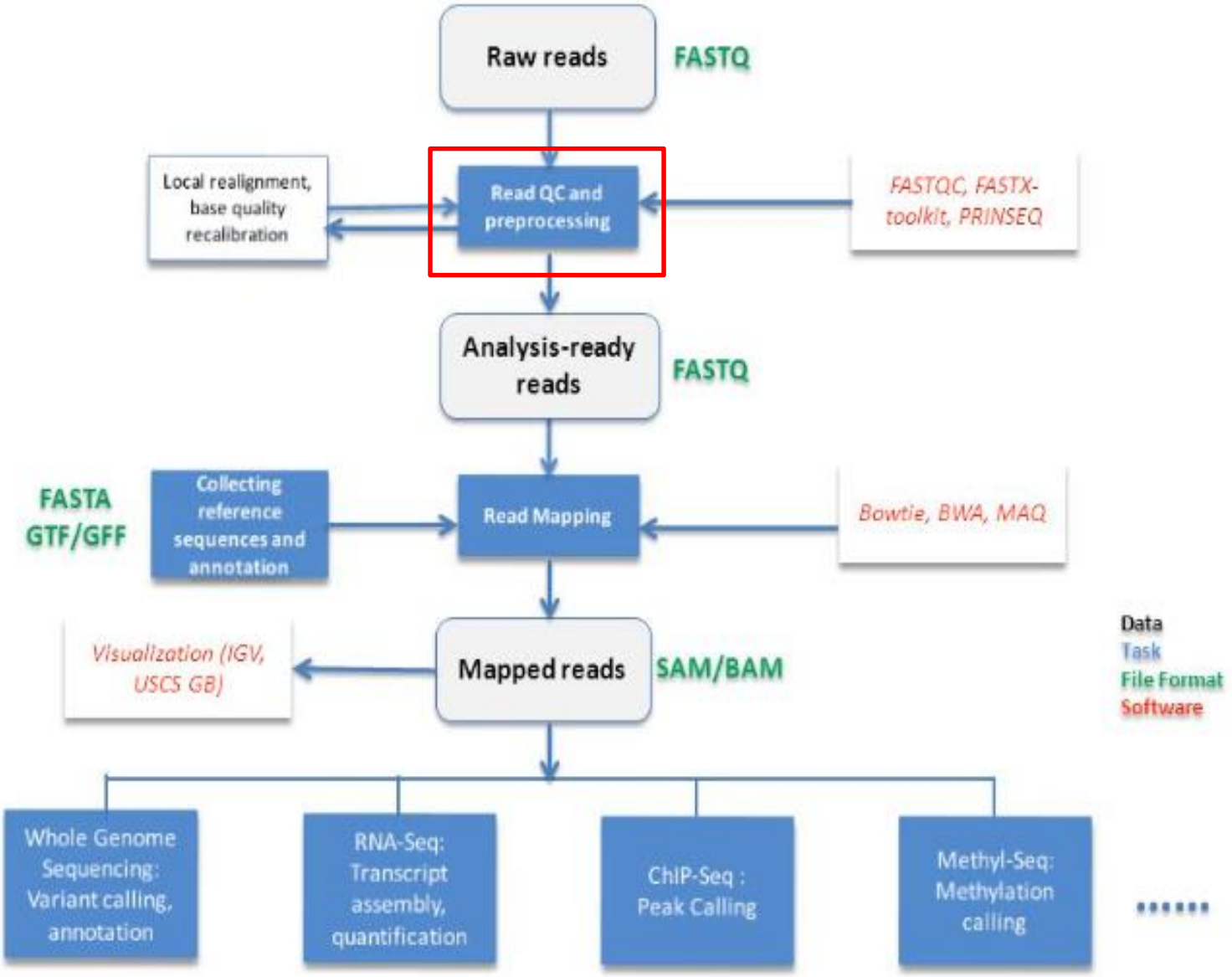
Steps in NGS analysis

FASTQ format = DNA sequence data + Phred quality scores of each base



NOTE: for paired-end runs, there is a second file with one-to-one corresponding headers and reads.

Steps in NGS analysis

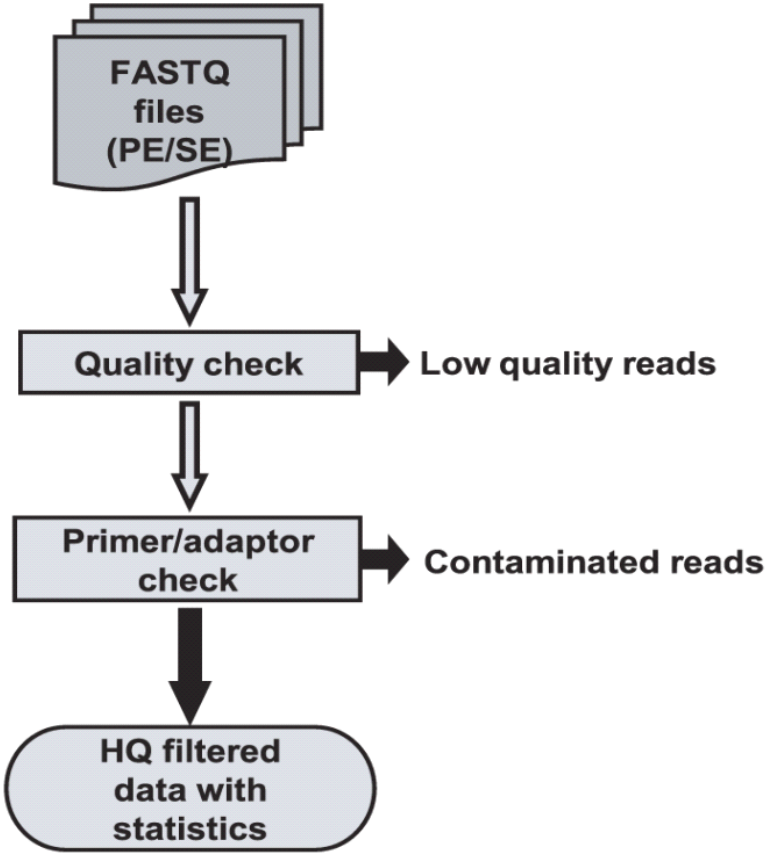


Steps in NGS analysis

Quality Control and Preprocessing

• Quality Control analysis of sequence data is extremely important for meaningful downstream analysis

- To analyze problems in quality scores/ statistics of sequencing data
- To check whether further analysis with sequence is possible
- To remove redundancy (filtering)
- To remove low quality reads from analysis
- To remove adapter contamination



Quality Control

FastQC tool

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

- Basic statistics
- Quality- Per base position
- Per Sequence Quality Distribution
- Nucleotide content per position
- Per sequence GC distribution
- Per base GC distribution
- Per base N content
- Length Distribution
- Overrepresented/ duplicated sequences
- K-mer content

Quality Control

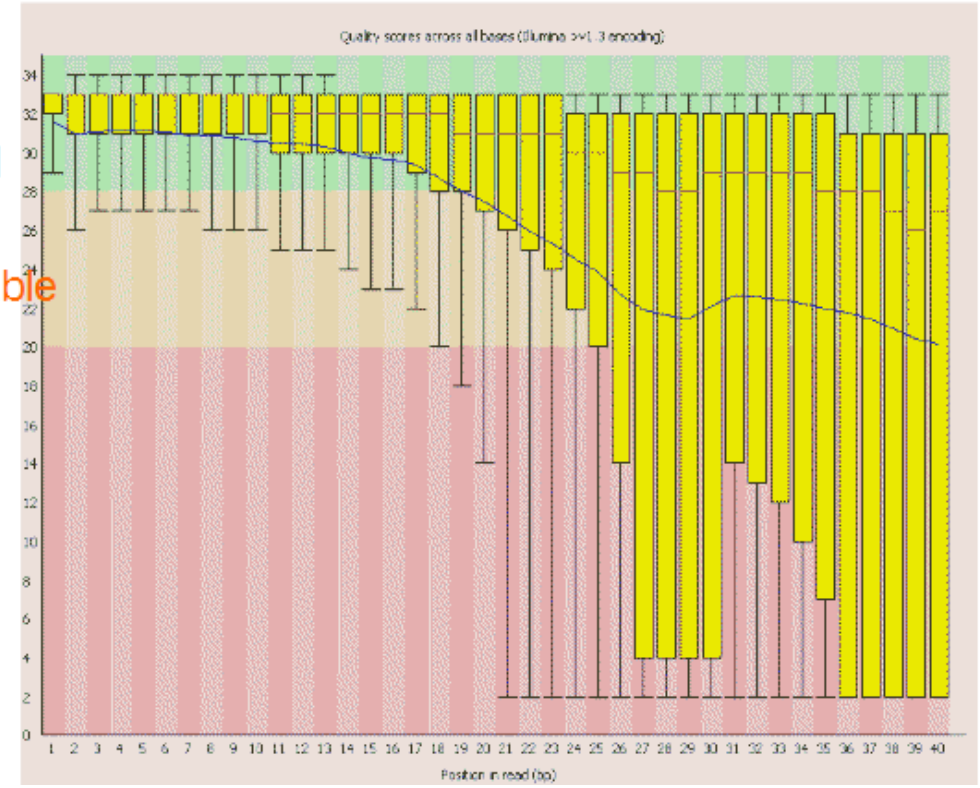
FastQC

Per base sequence quality (Boxplot)

Good

Acceptable

Bad



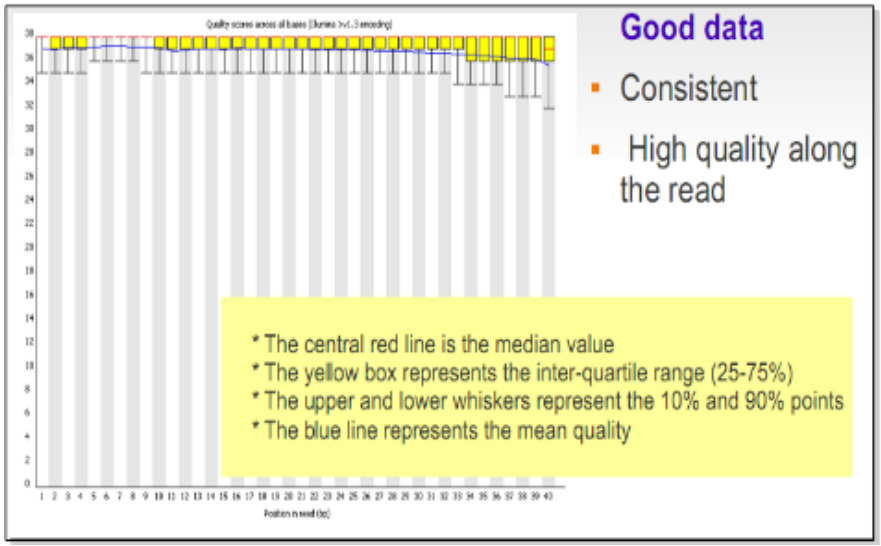
shows an overview of the range of quality values across all bases at each position in the FastQ file

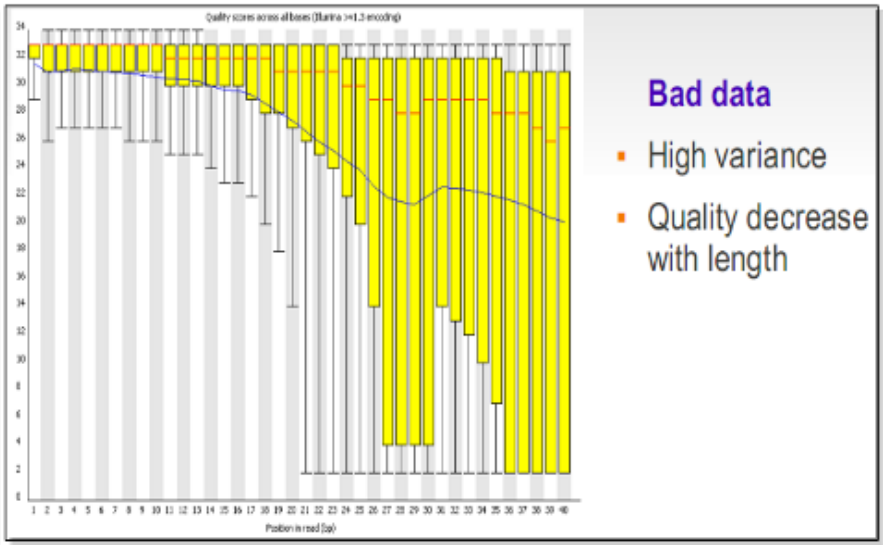
Y axis- Quality Score
X axis- Base position

Quality Control

FastQC

Per base sequence quality (Boxplot)



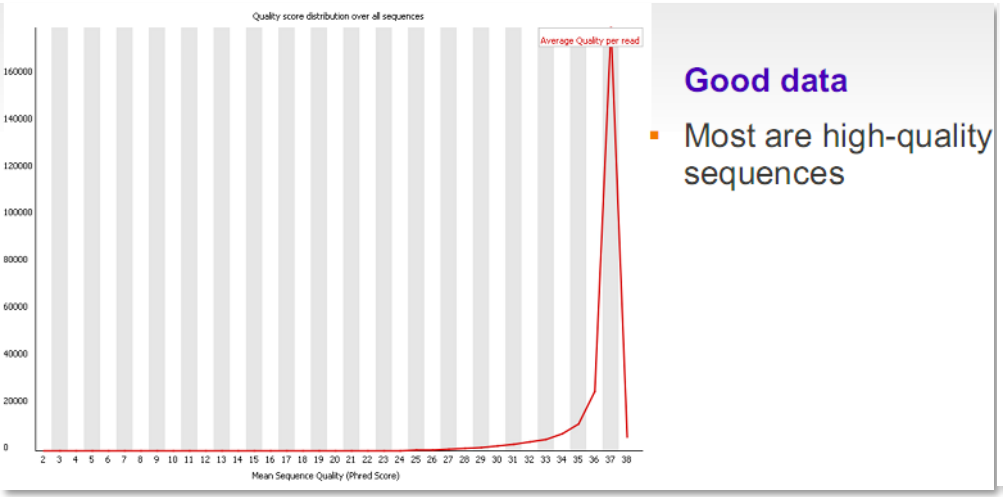


Quality Control

FastQC

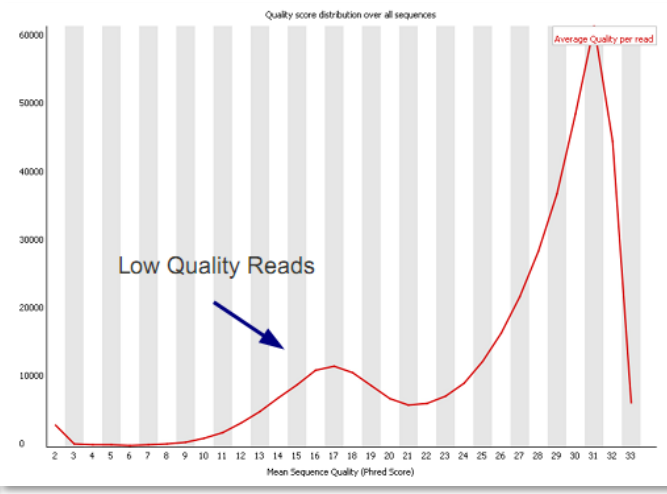
Per sequence quality scores

allows you to see if a subset of your sequences have universally low quality values.



Good data

- Most are high-quality sequences



Bad data

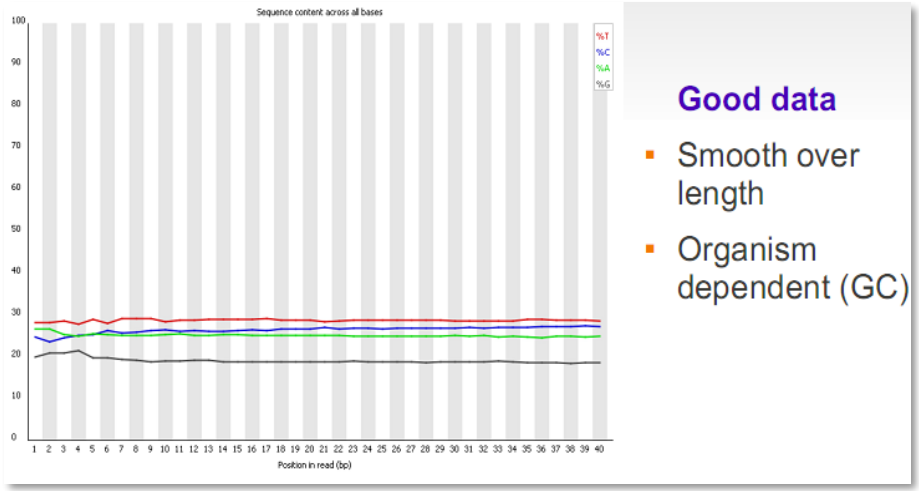
- Not uniform distribution

Quality Control

FastQC

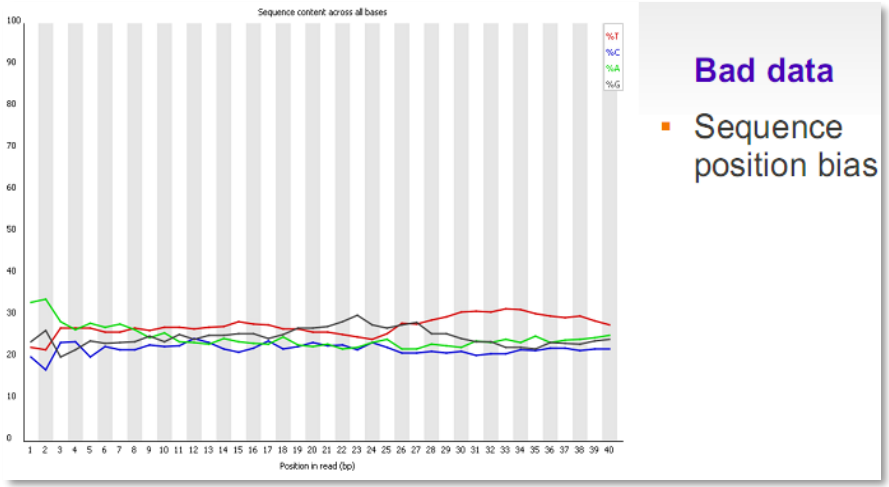
Per base sequence content

proportion of each base position in a file for which each of the four normal DNA bases has been called



Good data

- Smooth over length
- Organism dependent (GC)



Bad data

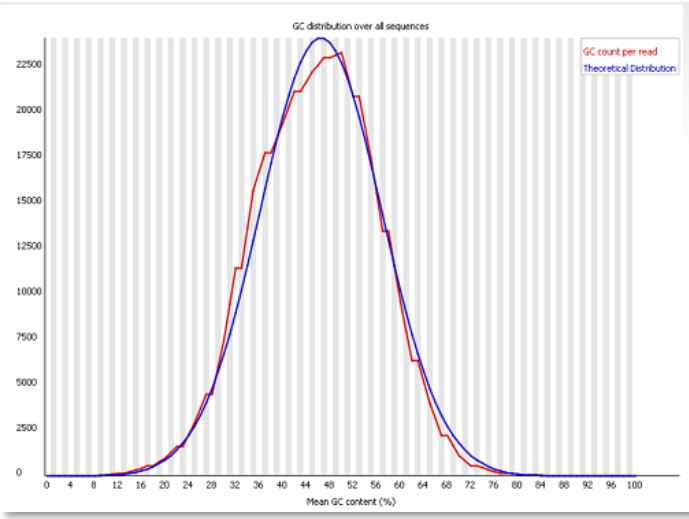
- Sequence position bias

Quality Control

FastQC

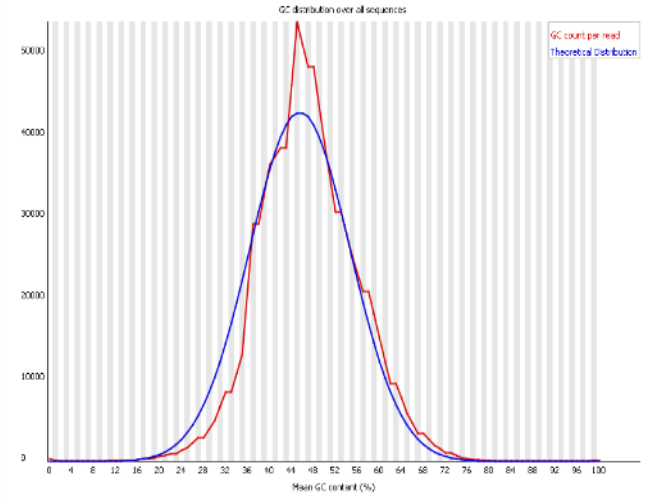
Per sequence GC content

measures the GC content across the whole length of each sequence in a file and compares it to a modelled normal distribution of GC content



Good data

- Fits with the expected
- Organism dependent



Bad data

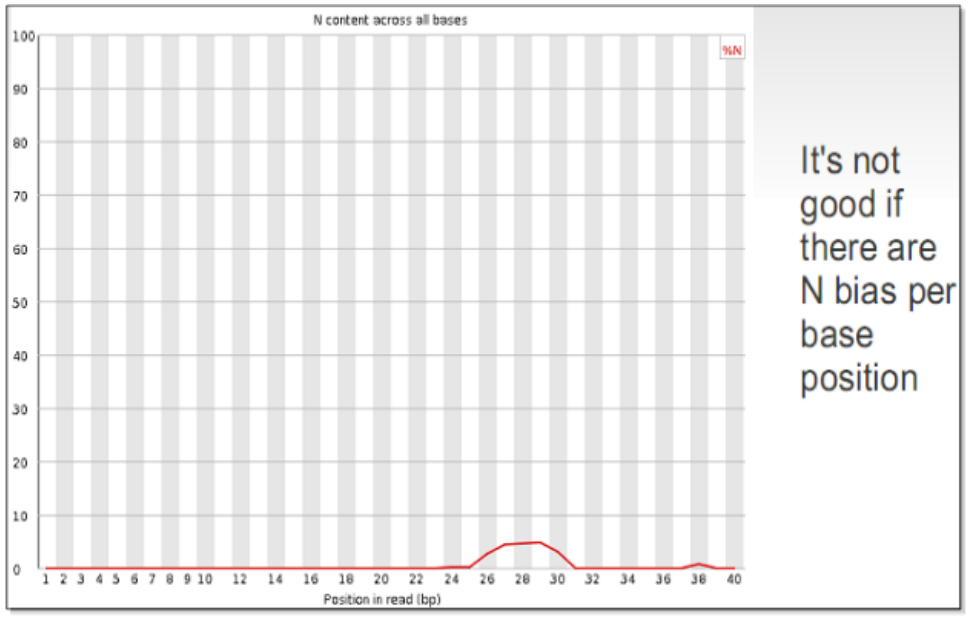
- It does not fit with expected
- Organism dependent

  Library contamination?

Quality Control

FastQC

Per base N content

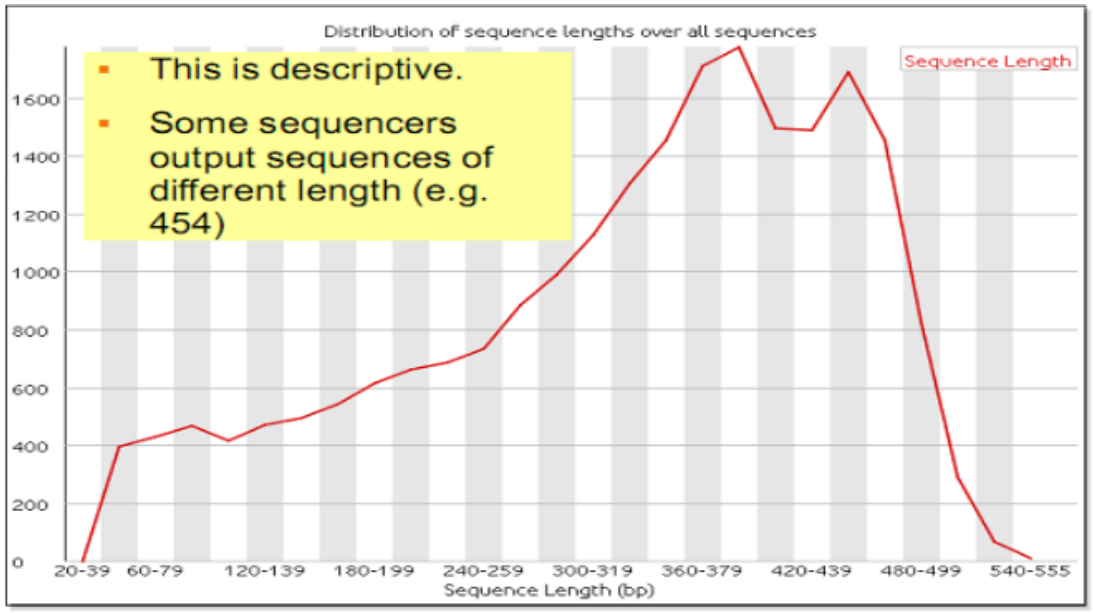


If a sequencer is unable to make a base call with sufficient confidence then it will normally substitute an N rather than a conventional base call. It plots out the percentage of base calls at each position for which an N was called.

Quality Control

FastQC

Sequence length distribution

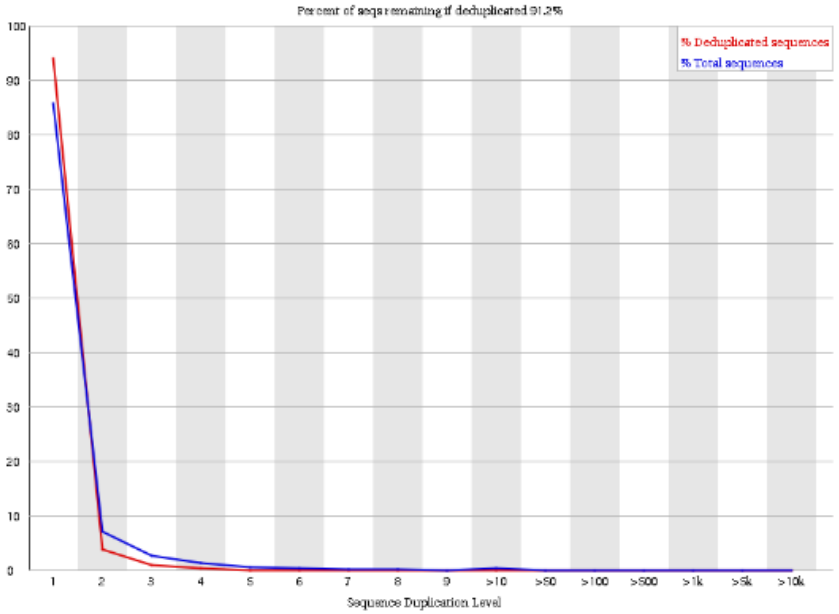


In many cases it will produce a simple graph showing a peak only at one size, but for variable length FASTQ files, it will show the relative amounts of each different size of sequence fragment.

Quality Control

FastQC

Sequence duplication level



Counts the degree of duplication for every sequence. Too many duplicate regions in the sequence may indicate contamination or technical problems

Quality Control

FastQC

Overrepresented sequences

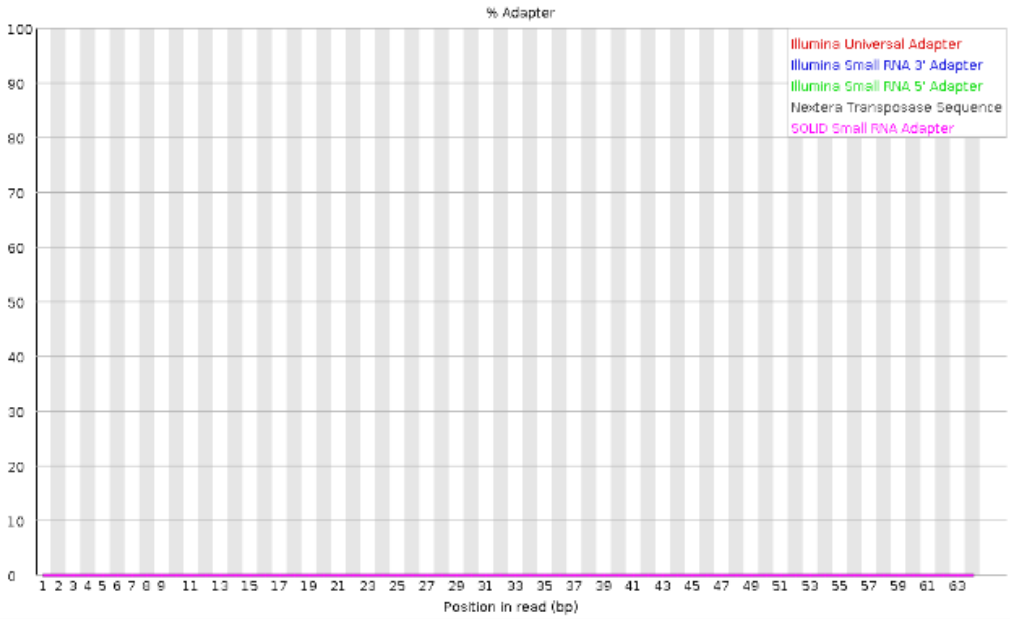
Sequence	Count	Percentage	Possible Source
AAGATCCGAGTCGTCCGGAAATCCATTGCCCGTGTTCTCACAGTTATTAA	432	0.43585733743631133	No Hit
AGATCCGAGTCGTCCGGAAATCCATTGCCCGTGTTCTCACAGTTATTAAC	335	0.33799122231750994	No Hit
TGGCAGAAGTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTAC	250	0.25223225546082834	No Hit
CAGAAGTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTACCGC	237	0.23911617817686526	No Hit
GTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTACCGCGGCGT	223	0.22499117187105888	No Hit
AAGAAATCTGACCCGGTCGTCTCGTACCGCGAGACGGTCAGTGAAGAGTC	204	0.2058215204560359	No Hit
AAGTAGAGCAGAAGAAGAAGCGGACCTTCCGCAAGTTCACCTACCGCGGC	151	0.1523482822983403	No Hit
CACCTGGAGATCTGCCTGAAGGACCTGGAGGAGGACCACGCCTGCATCCC	147	0.14831256621096706	No Hit
TCTGCCTGAAGGACCTGGAGGAGGACCACGCCTGCATCCCCATCAAGAAA	146	0.14730363718912376	No Hit

Lists all of the sequence which make up more than 0.1% of the total. Finding that a single sequence is very overrepresented in the set either means that is highly biologically significant, or that the library is contaminated. For each overrepresented sequence it will look for matches in a database of common contaminants.

Quality Control

FastQC

Adapter content



Does a generic analysis of all the Kmers in the library to find those that don't have even coverage through the length of the reads.

Quality Control

FastQC

- Good (Illumina) quality data:

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc.html

- Bad (Illumina) quality data:

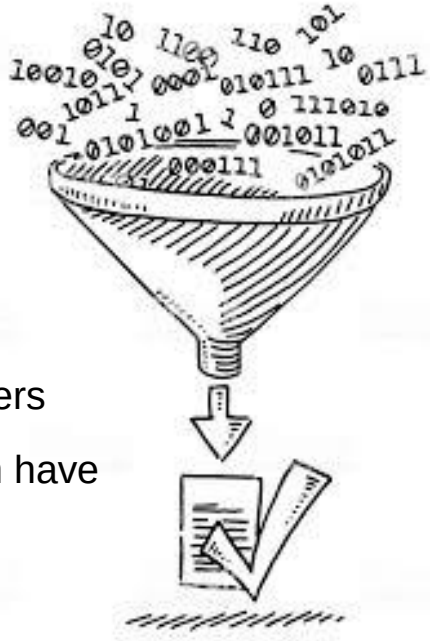
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc.html

Quality Control

Preprocessing of raw data

Based on the information provided by the QC graphs, the sequences may be treated to reduce bias in downstream analysis:

- **Filtering sequences**
  - with low mean quality score
  - too short
  - with too many ambiguous (N) bases
  - based on their GC content
  - Biological contamination: polyA-tails, rRNA or mtDNA sequences,…
  - Technical contamination: PhiX internal control sequences, adapters/primers
  - Removing duplicate reads is not advised since high expressed genes can have genuine duplicate reads that are not due to the PCR amplification step.
- **Cutting/Trimming sequences**
  - from low quality score regions
  - beginning/end of sequence
  - removing adapters, primers



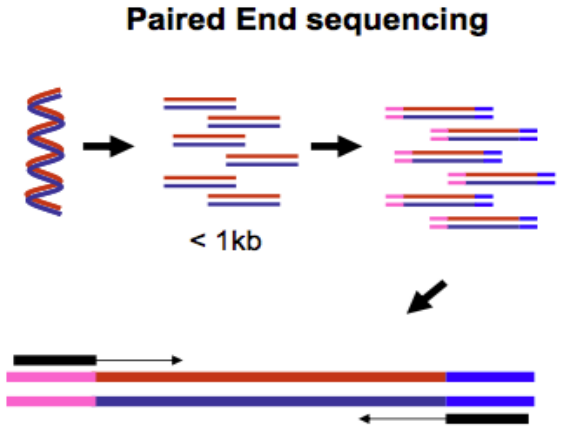
Quality Control

Your turn!

- We will analyze exome sequencing data from a study that aimed to identify genetic variants associated to a disease. The data comes from paired-end sequencing, each file corresponding to the forward or reverse, respectively:

https://zenodo.org/record/3243160/files/proband_R1.fq.gz
https://zenodo.org/record/3243160/files/proband_R2.fq.gz

Paired-end data: a single physical piece of DNA/RNA is sequenced from two ends and so generates two reads. These can be represented as separate files (two fastq files with first and second reads) or a single file were reads for each end are interleaved.



Introduction to Galaxy

Training Infrastructure as a Service

We want to help you conduct your training seminars. You provide the training, we provide you training infrastructure *at no cost*.

Why use UseGalaxy.eu training infrastructure?

- Free
- Private queue, no wait times
- No Galaxy Maintenance
- No Galaxy Administration
- Official Galaxy Training Materials guaranteed to work

Simply fill out the infrastructure request form and we'll get back to you shortly.

Find out more

After registration in [European Galaxy server]

https://usegalaxy.eu/join-training/ueb_bi2022

Quality Control

Your turn!

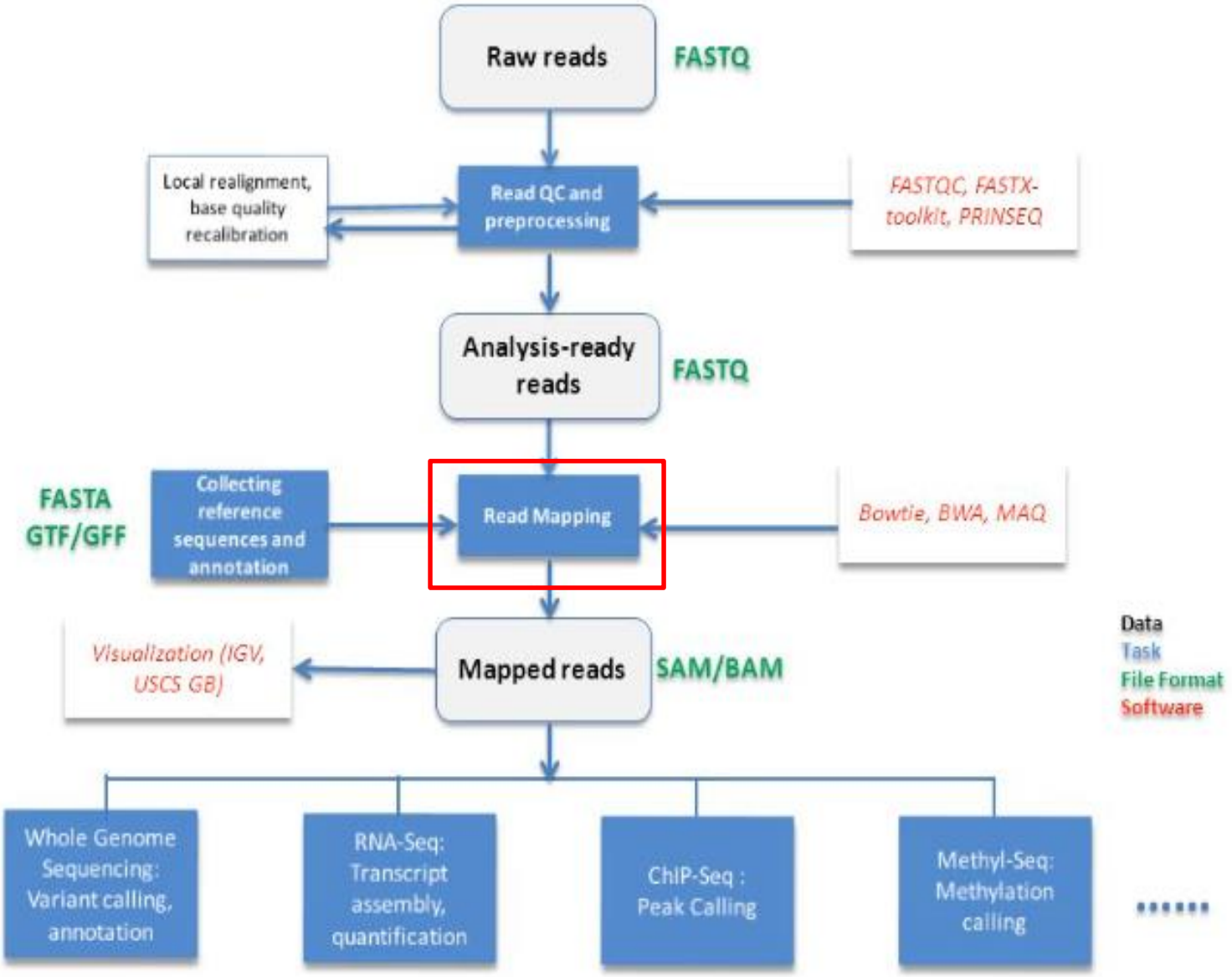
1. Create a new history and name it as you want (eg. Practica1)
2. Upload the fastq files into Galaxy from the urls copied above
3. Update the attributes of the two datasets (pencil icon):
   a) Rename the datasets to “sample-f.fq.gz” and “sample-r.fq.gz”, respectively.
   b) Check data type is set to “fastqsanger”
   c) Associate the dataset with the human hg38 genome in the Database/Build field.
4. Run a quality control on each dataset using the FastQC tool.
   a) What is the length of reads?
   b) Are sequences of good quality? Any adapter that should be removed?
5. What would be the next step in the analysis workflow?

Quality Control

Your turn!

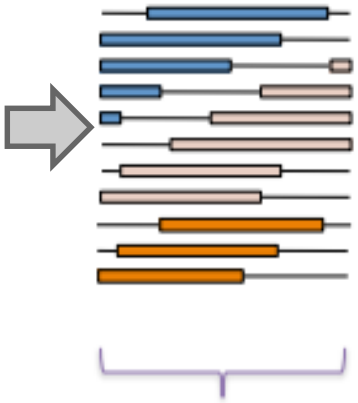
6. Trim the reads in each dataset using **Cutadapt** tool. Set the parameters:
    a) Paired-end data
        File 1: sample-f (forward)
        File 2: sample-r (reverse)
    b) Determine from the FastQC boxplot where the quality of the reads begins to drop off sharply. Calculate how many bases have to be trimmed from the end and use that number as the Offset from 3' end.
    c) Output options: Report=yes
7. Inspect the results:
    a) How many datasets do we get? Rename them to sample-f-trim / sample-r-trim, respectively. What is their format?
    b) Do they have the same number of reads?
8. Re-run FastQC on the trimmed data, and inspect the new FastQC report. Has the sequence quality been improved?
9. Convert your analysis history into a workflow
10. What would be the next step in the analysis workflow?

Steps in NGS analysis

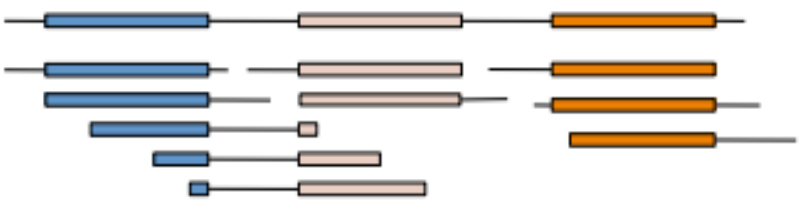


Steps in NGS analysis

Mapping reads to the genome



Mapping and alignment algorithms

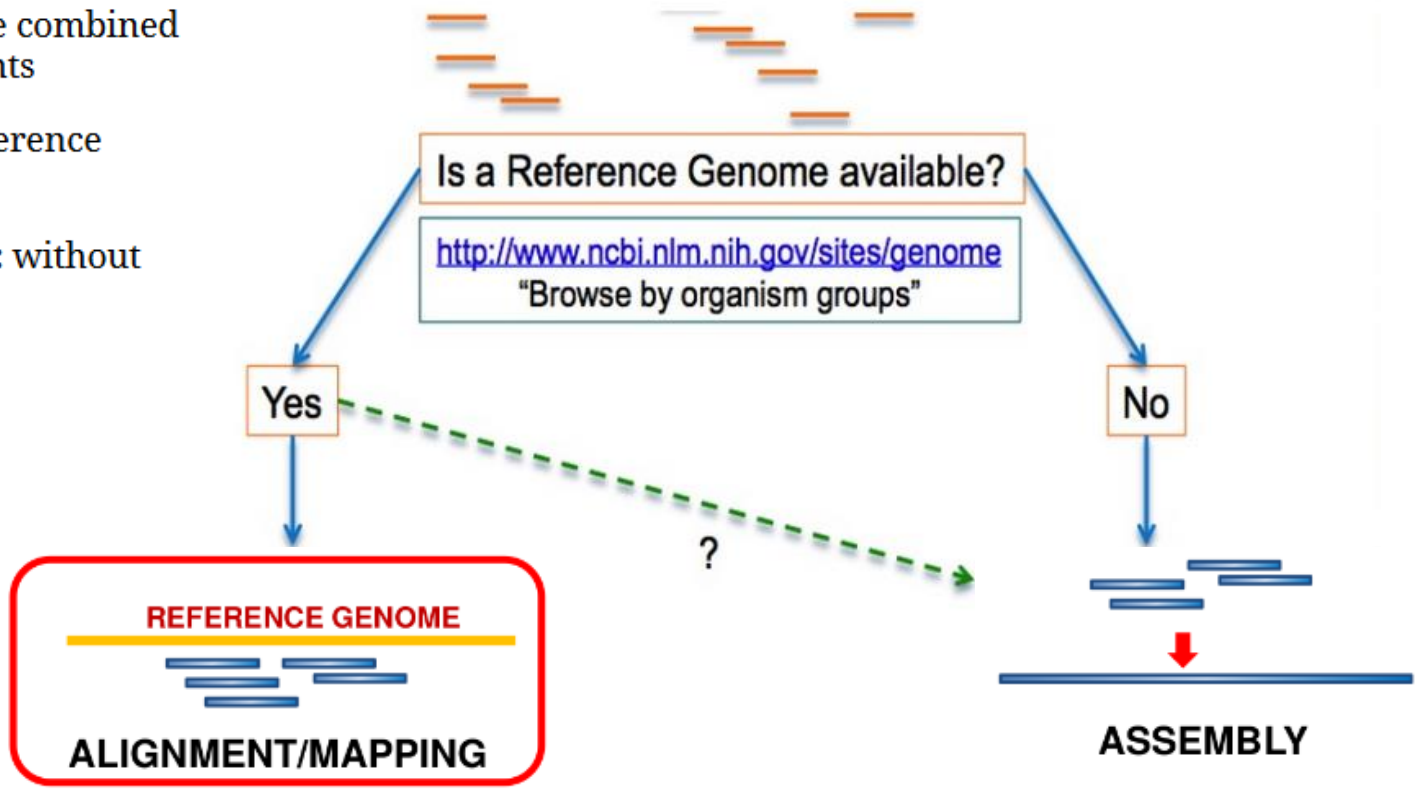


Steps in NGS analysis

Mapping reads to the genome

Mapping/Alignment vs Assembly

- Short reads must be combined into longer fragments
- **Mapping:** use a reference genome as a guide
- **De-novo assembly:** without reference genome



Steps in NGS analysis

Mapping reads to the genome

- Determine position of short read on the reference genome

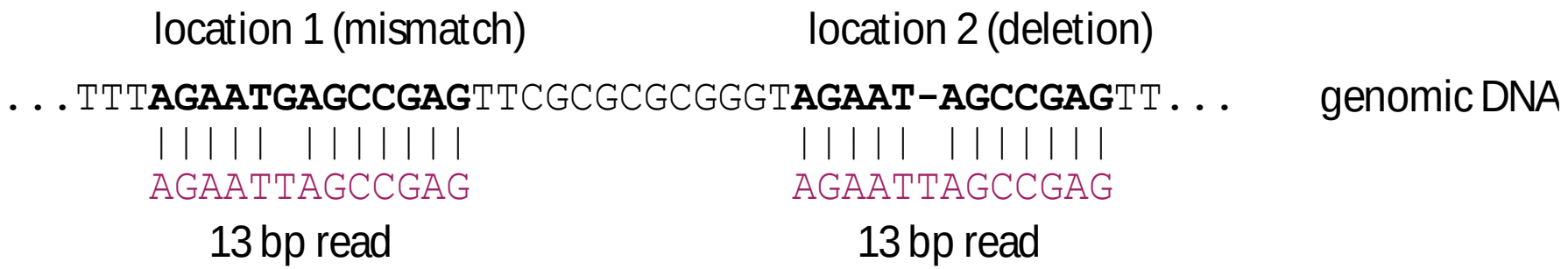
```
Reference: . . . A A - C G C C T T . . .          | = match
.                  | : - : | | | | |             : = mismatch
Read:              A G G G G C C T T             - = gap
```

Steps in NGS analysis

Mapping reads to the genome

Challenging!

- There is ambiguity mapping a read with a mismatch versus a deletion



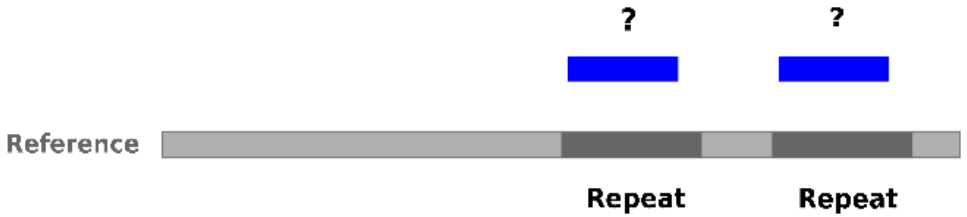
Steps in NGS analysis

Mapping reads to the genome

Challenging!

- There is ambiguity mapping a read with a mismatch versus a deletion

- A read could align to multiple places (repeats)

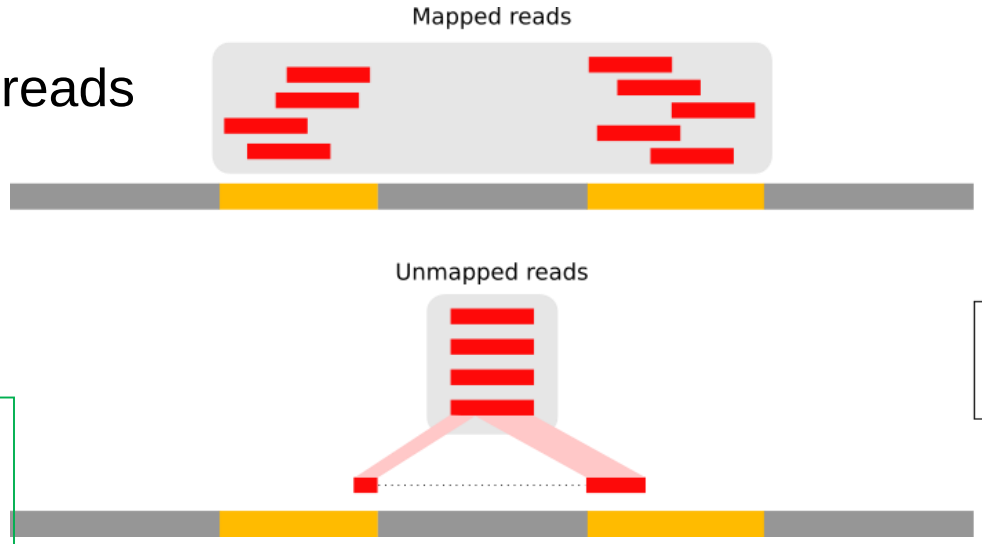


Steps in NGS analysis

Mapping reads to the genome

Challenging!

- There is ambiguity mapping a read with a mismatch versus a deletion
- A read could align to multiple places (repeats)
- In RNA-seq, splicing may split reads



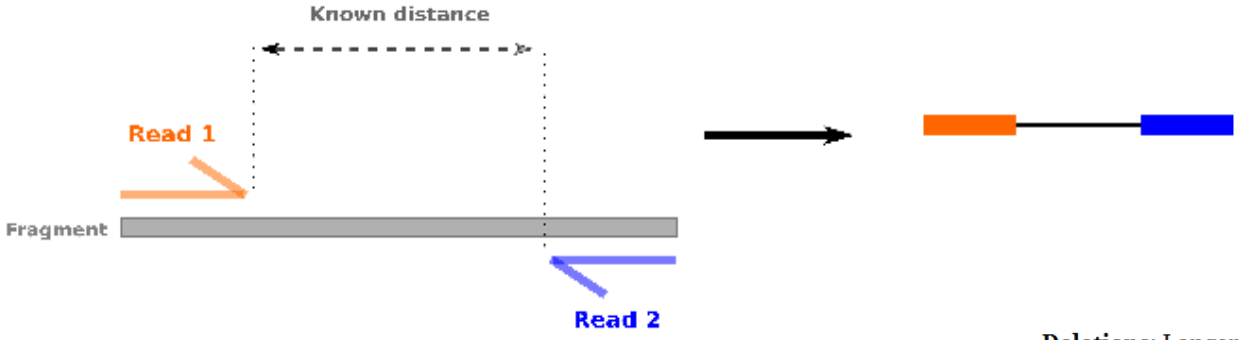
- Complex algorithms have been developped
- Choose appropriate tool/parameters

Steps in NGS analysis

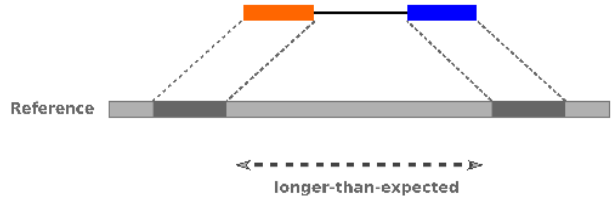
Mapping reads to the genome

- Paired-end sequencing improves accuracy of mapping

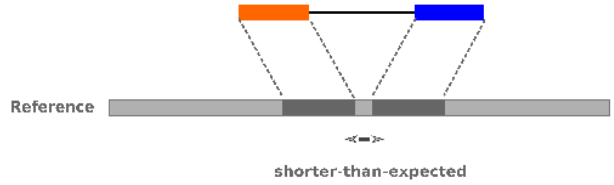
- **Sequencing:** Cut longer fragments of DNA, sequence only the ends

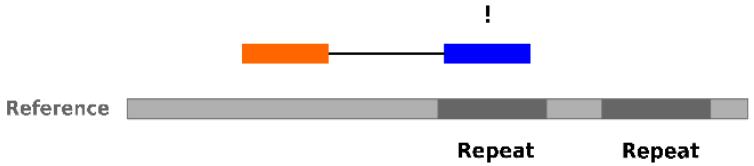


- **Deletions:** Longer mapping distance than expected



- **Insertions:** Shorter mapping distance than expected

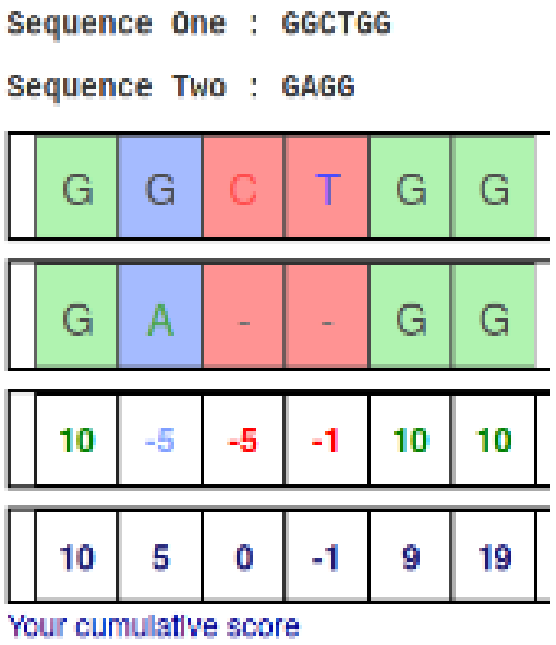




Steps in NGS analysis

Mapping reads to the genome

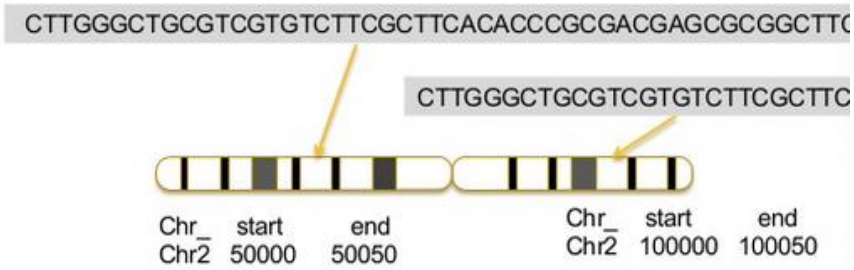
- Quality scores to assess mapping accuracy
  - quantify the probability that a read is misplaced.
  - Function of factors such as:
    - uniqueness (ie not a multi-mapper)
    - number of mismatches in read
    - number of insertions/deletions in read
    - quality of bases in read



Steps in NGS analysis

Mapping reads to the genome

SAM/BAM format = Aligned read sequence + Mapping info (position, quality score...)



- SAM files typically contain a short header section with information about the genomic loci of each read and a very long alignment section where each row represents a single read alignment. For each read, there are 11 mandatory fields that always appear in the same order:

header

```
@HD    VN:1.0
@SQ    SN:chr20 LN:62435964
@RG    ID:L1 PU:SC_1_10 LB:SC_1 SM:NA12891
@RG    ID:L2 PU:SC_2_12 LB:SC_2 SM:NA12891
```

Read name | Alignment gap information | Quality scores (fastq format)

SLX1:1:127:63:4 ... 1 10052169 ... 23M6N10M ... GAAGATACTGGTTTTTTTCTTATGAGACGGAGT 768832'48:::::::;;:/78$88818099897 SM:Z:JPTGBMN01 ...

Locus | Read sequence | Meta data

Steps in NGS analysis