institutCurie

GALAXY INITIATION

A. Lermine

U900 Institut Curie, INSERM, Mines ParisTech

institutCurie

How does Next-Gen sequencing work?

DNA fragmentation

Size selection and clonal amplification

Massive <u>parallel</u> sequencing

ACCGTTTGCCG…
10^6-10^9 <u>short reads</u>
<u>stocked in FASTQ format</u>

institutCurie

Hi-seq, SOLiD, PGM, ..., What does it mean ?

Platform	Provider	Reads Number (M)	Max Reads Size (bp)	Throughput (Gb)	Time	Space
GS Flex	Roche	1	700	0.7	8d	Base
Hiseq 2000/2500 Normal mode	Illumina	3000	2x100	600	11d	Base
Hiseq 2500 rapid mode	Illumina	600	2x150	120	40h	Base
MiSeq	Illumina	15	2x250	8.5	40h	Base
SOLiD	LifeTech	1400	75-35	150	25d	Color
PGM 314	Ion Torrent	0.5	400	>0.01	2-4h	Base
PGM 316	Ion Torrent	2	400	>0.1	2-4h	Base
PGM 318	Ion Torrent	4	400	>1	2-4h	Base
Proton	Ion Torrent	60-80 M	200	>12	2-4h	Base

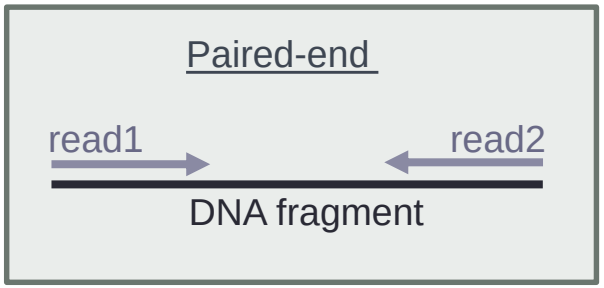
institutCurie

Let's start after sequencing …

- A **raw** data file containing **millions** of reads (A/C/G/T/N sequence + base qualities):
  - **IonTorrent PGM**: FASTQ, SFF, Unmapped BAM of reads of different sizes

    → SFF and Unmapped BAM contain specific IonTorrent flowspace information (helps improve the accuracy during the mapping and variant calling steps)
  - **Illumina**: FASTQ of same size reads

- Reads can be **single-end** (one end of the fragment sequenced) or **paired-end** (both ends sequenced)



ACTGATTAGTCTGAATTAGANNGATAGGAT

ACTAGGCATCGGCATCACGGACNNNNNNNN

GATCGATGCATAGCGATCAGCATCGATACG

ACTAGCTATCGAGCTATCAGCGAGCATCTATC

CGGCGCTCCGCTCTCGAAACTAGCACTGAC

ACTAGCTACTATCGAGCGAGCGATCATCGAC

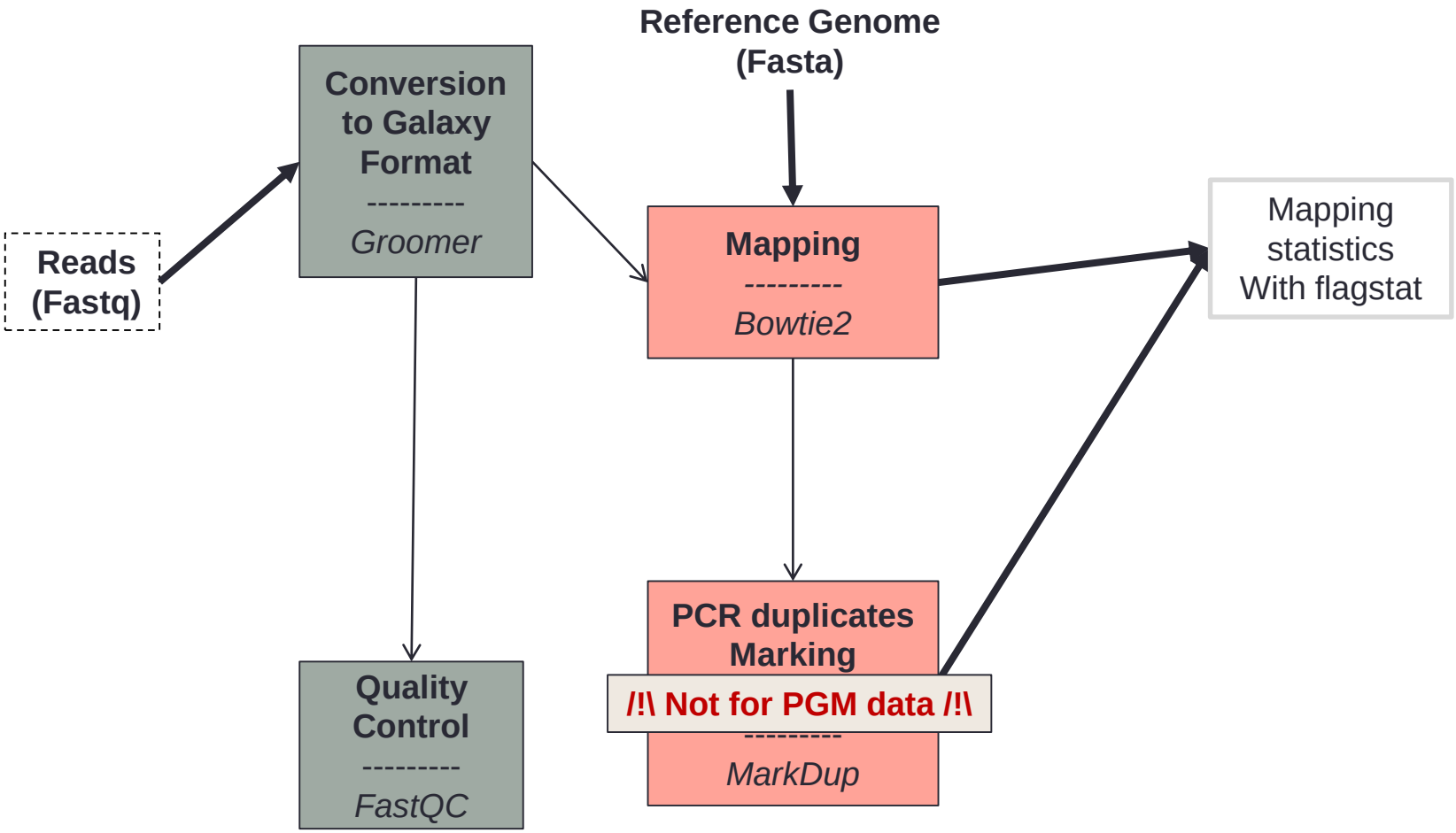
AGCATCAGGATCTACGATCTAGCGAACTGAC

CTGACTACTATCGAGCGAGCTACTAACTGAC

NNNNNNNNNNNNNNNNNNNNNNNNNNNN

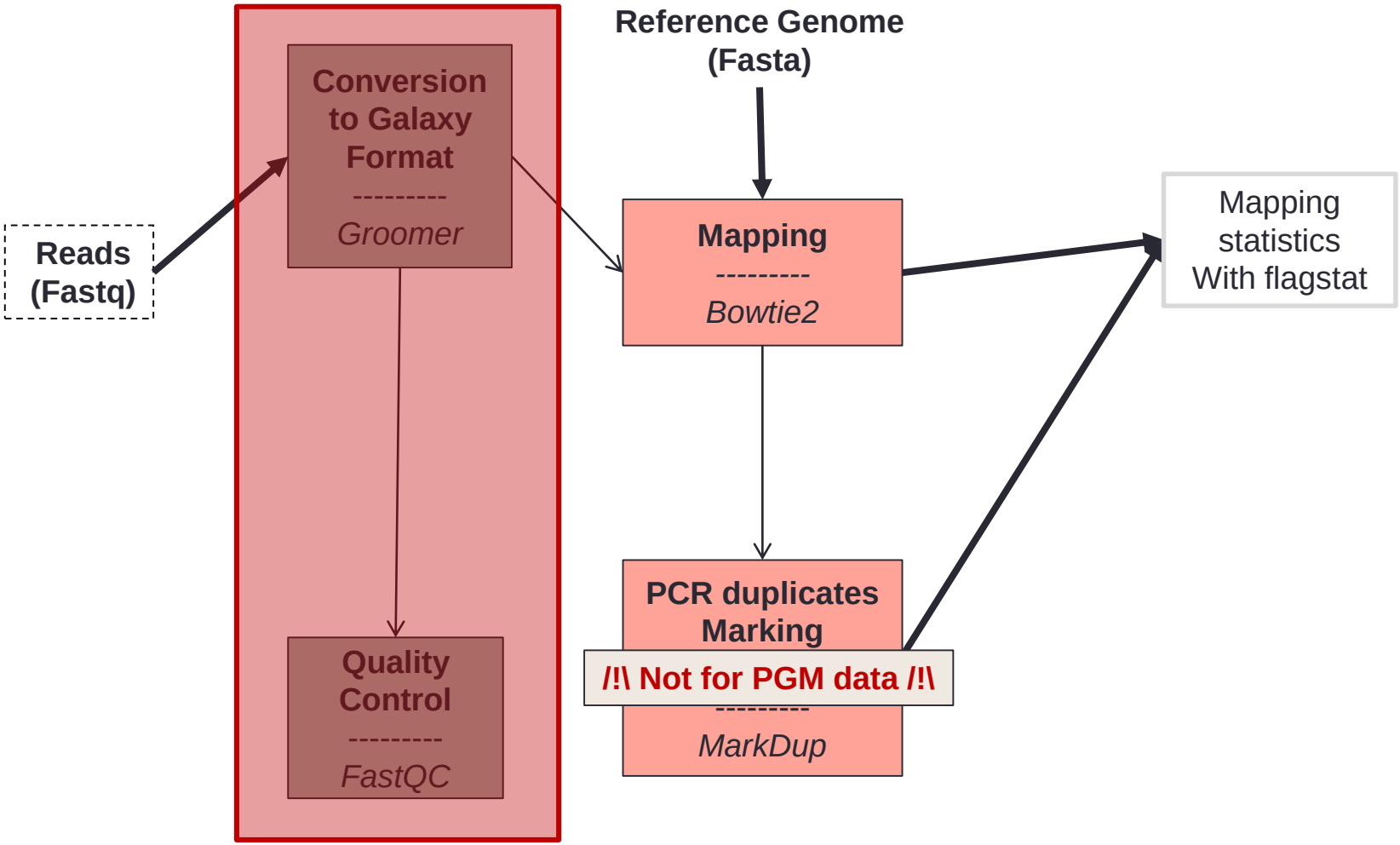
institutCurie

Galaxy Workflow



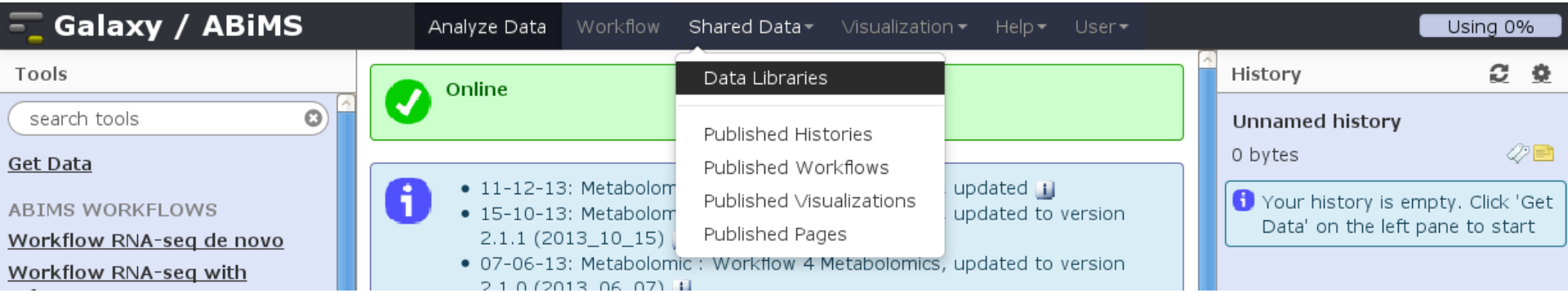
institutCurie

Galaxy Workflow



Two available datasets on Galaxy

1. Open your web browser and go to your IFB Cloud Galaxy instance
2. In the top menu, click on « Shared Data » then « Data librairies »



3. Click on «**TP-INITIATION** »:

→ **Chr4.fastq:** raw reads file

→ **Chr4.fasta:** reference sequence for alignement

institutCurie

Sequence Quality Encoding (FASTQ)

- Extension from traditional FASTA format

- Each block has 4 elements (in 4 lines):
  - Sequence name (read name, group, etc…)
  - Sequence
  - + (optional: sequence name again)
  - Associated quality scores (phred-scaled) : different encoding possible

- Example record:
  - @FCD19MJACXX:2:1101:1735:1993#GTTCGACA/1
  - NGAGGCTGAGGCGGGCAGAGGTCAGGAGATCGAGACCATC
  - +
  - BP\ccccc]ceecheheeZbe_cZbd_dbbdd\aXab_`b

institutCurie

Sequence Quality Encoding (FASTQ)

- The base calling (A, T, G or C) is performed based on Phred Scores.

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%
50	1 in 100,000	99.999%

→ 1% error rate

- Phred scores provide $\log_{10}$-transformed error probability values

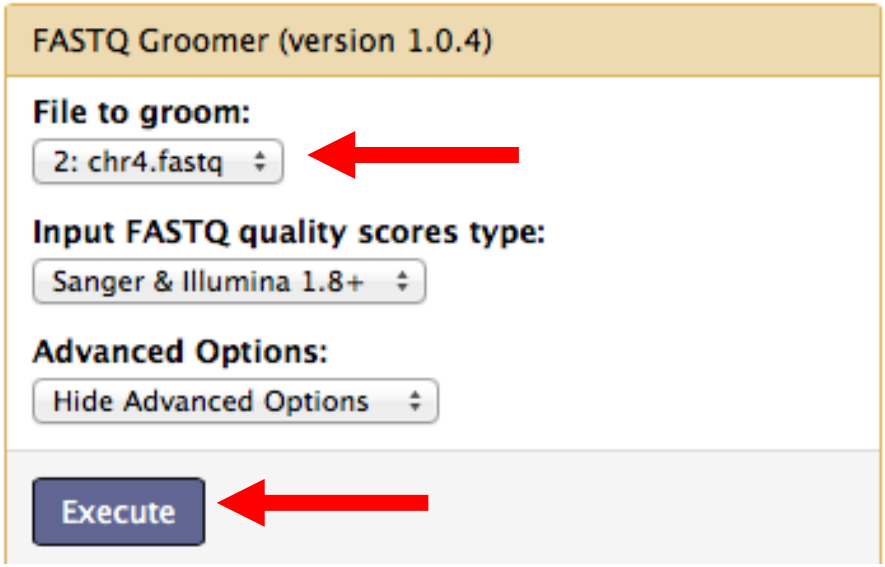
→ If p is probability that the base call is wrong the Phred score is

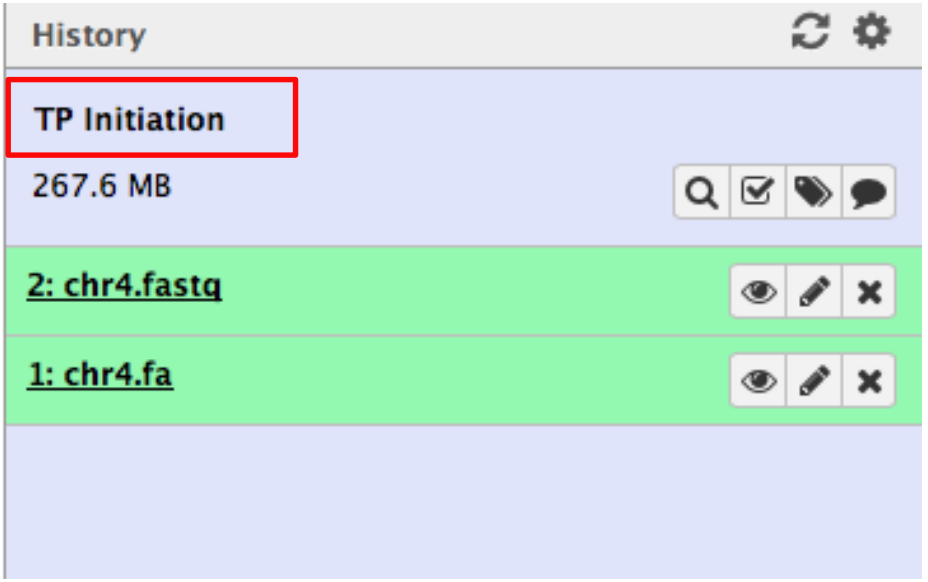
$$Q = -10 \log_{10}(P) \Leftrightarrow P = 10^{-Q/10}$$

institutCurie

FASTQ format conversion

1. Rename your history to « **TP Initiation** » by clicking on « **Unnamed history** »
2. In the left panel, click on « FASTQ Groomer » under the NGS: QC and manipulation section to convert **your FASTQ** into FASTQ Sanger Format
3. Click on « Execute » to launch the conversion





institutCurie

FASTQC: Quality Control on raw data

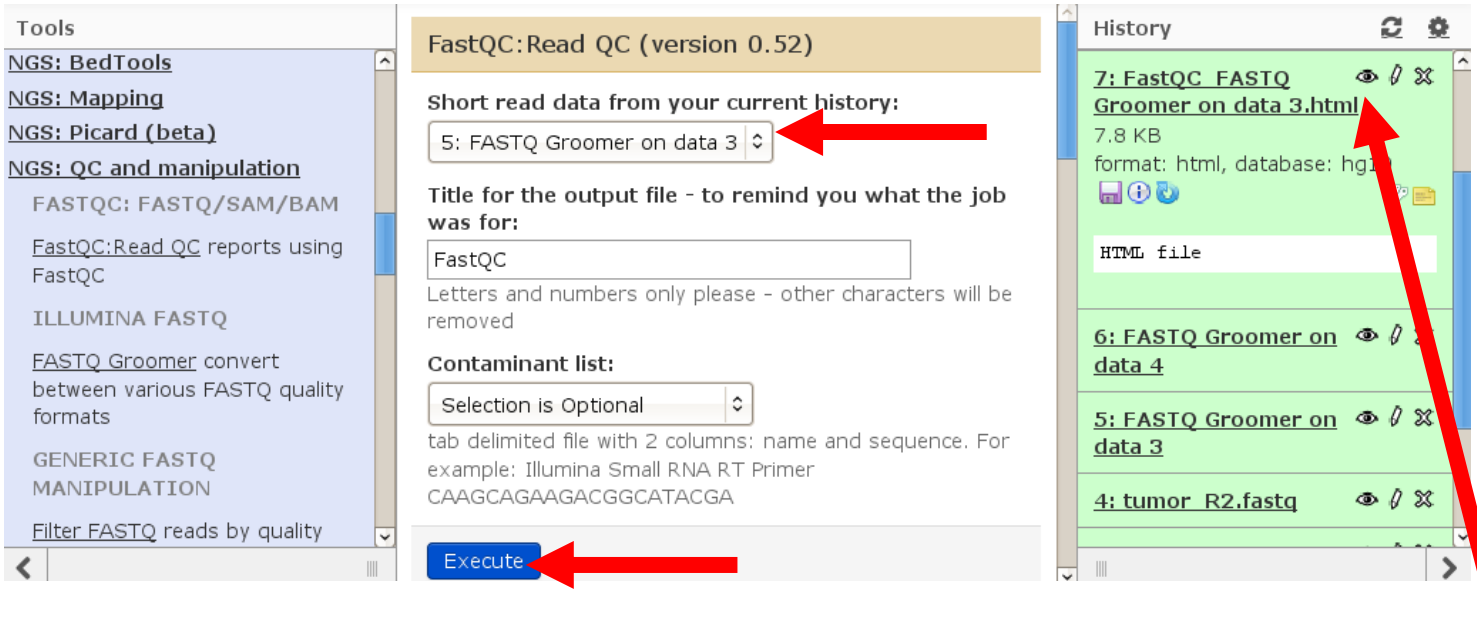
- **Sequence Length Distribution**: sequencers produce same (Illumina) or different (PGM) read length. This metric helps identify abnormal read length
- **Sequence content**: % A/C/G/T ; %GC; %N at each position in the read

  → Proportion biased for targeted sequencing
- **Quality score**:

  1. **Per base**: identify base calls with low quality (commonly towards the end of a read)
  2. **Per sequence**: to see if a subset of your sequences have universally low quality values
- **K-mers content**: a k-mer is a motif of length k observed more than once in a sequence (repeats : ACACAC ; spaced occurences : tccGAGGaaggGAGGaag)
- **Over-represented sequences**: highly duplicated sequence in your library (primer, adapter..)

FASTQC : FASTQ Quality Control

1. In the left panel, click on « FASTQC: Read QC »
2. Select the FASTQ Groomer dataset and click on « Execute »



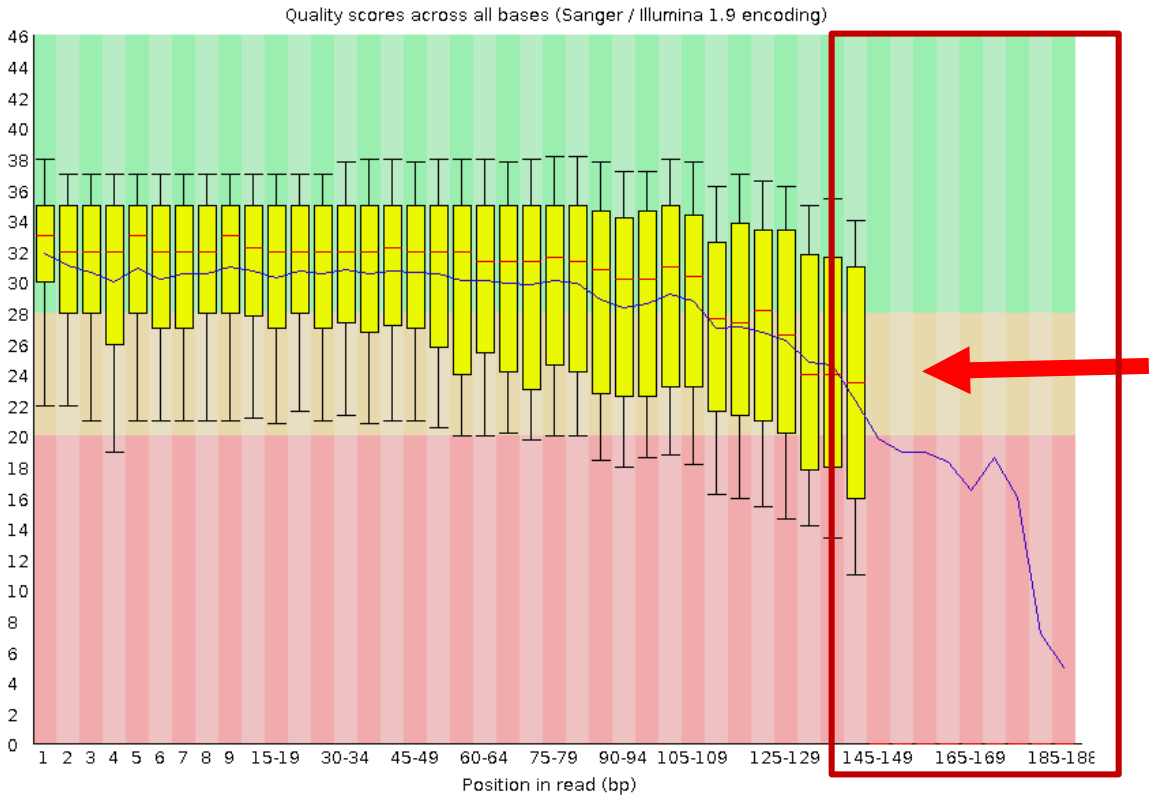
→The result of FASTQC is an html page that you can view by clicking on the **eye**

institutCurie

FASTQC Metrics

- Look at the different metrics for reads
- **Problem**: the per base sequence quality are quite low towards the end

Per base sequence quality



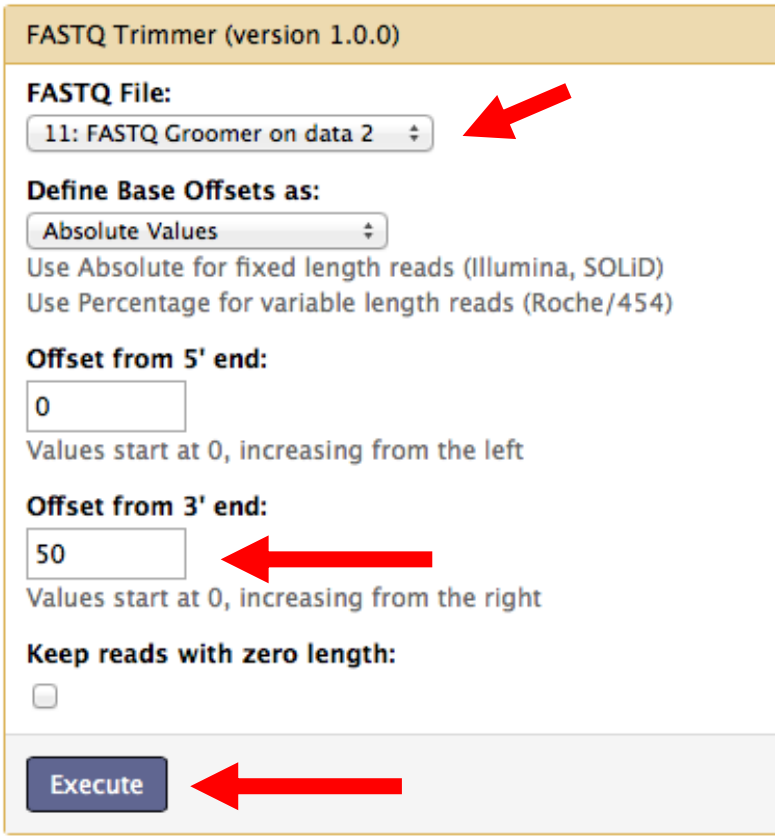
Solution:

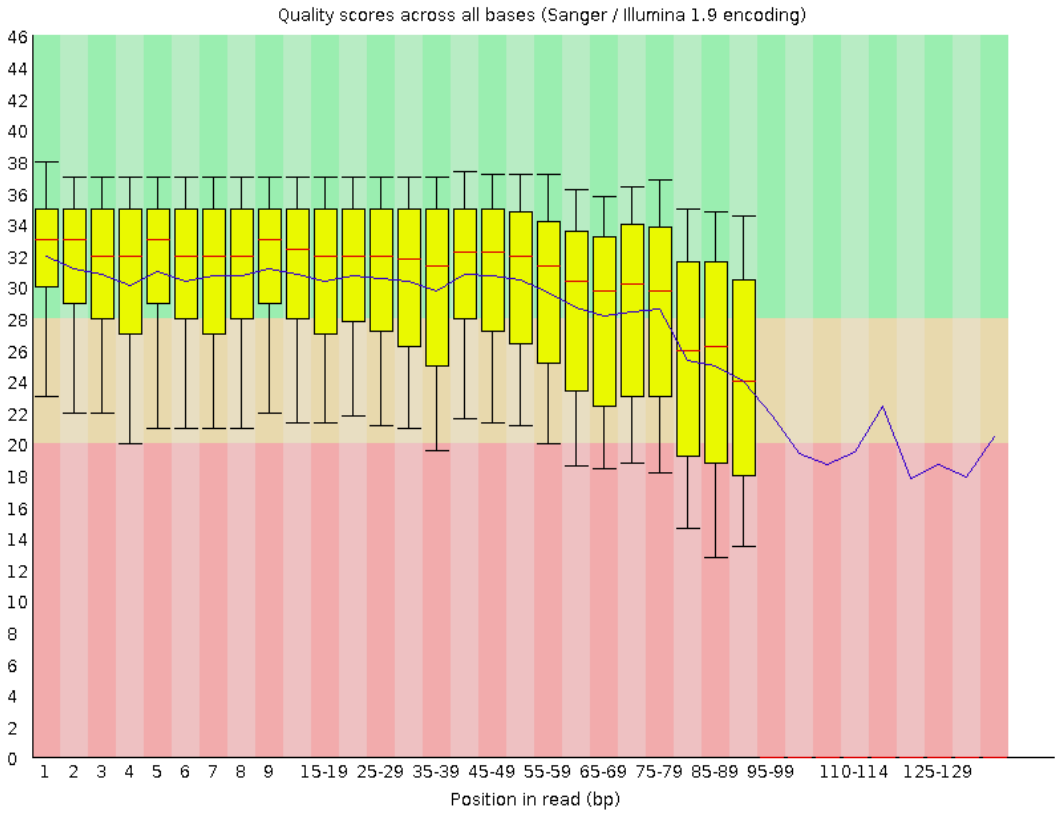
Trim the 50bp from the 3' end of the reads

→ Higher confidence in the sequenced information

FASTQ Trimmer to improve reads quality

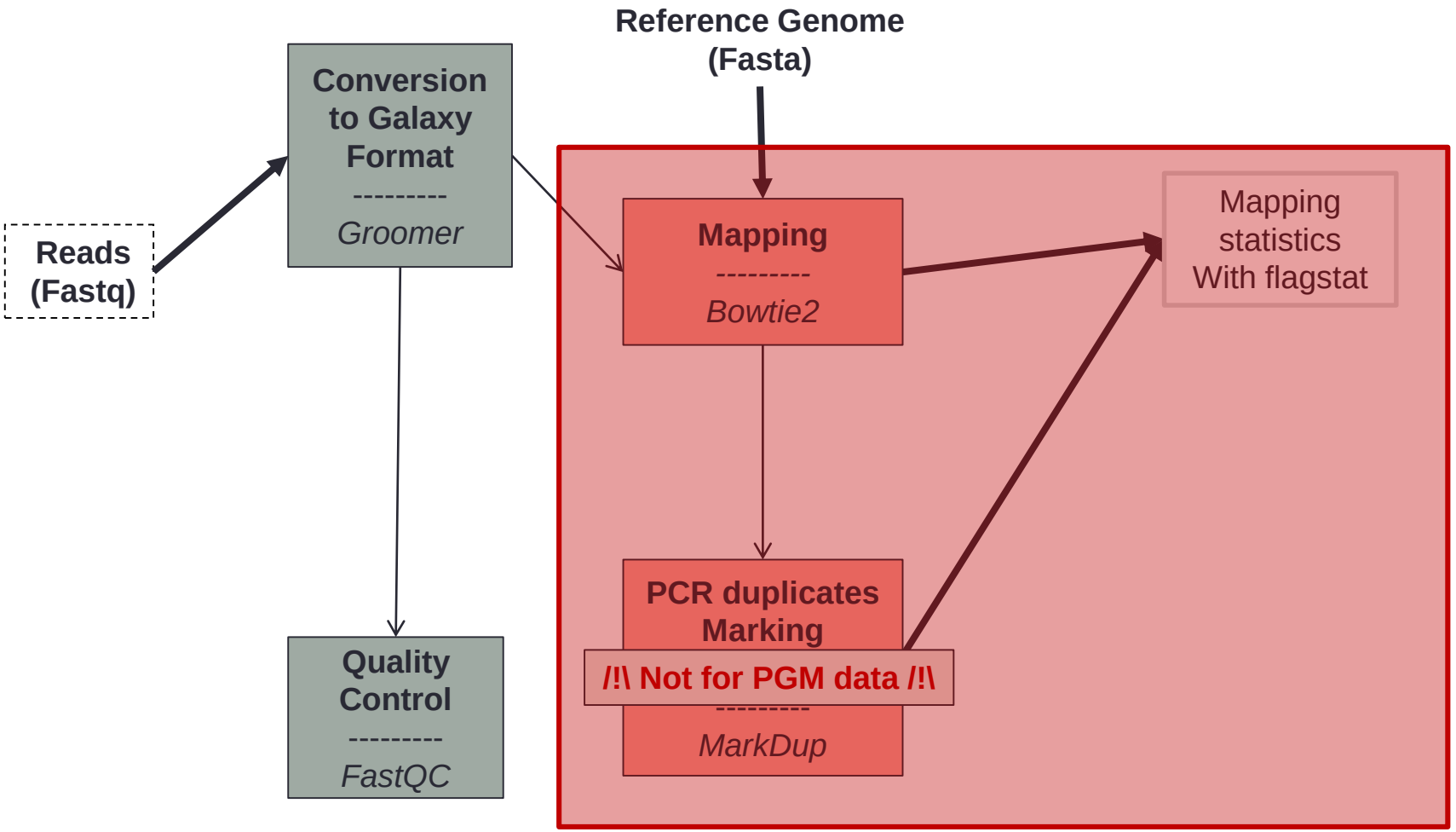
1. Use « FASTQ Trimmer » to cut off 50bp from 3' (use the « search tools » object to find the tool)
2. Run « FASTQC» on the trimmed reads





institutCurie

Galaxy Workflow

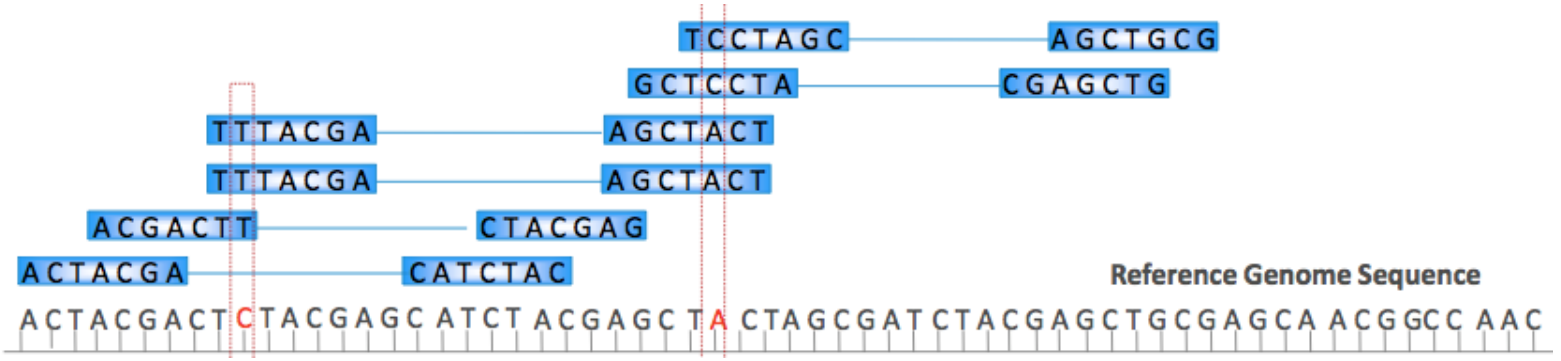


Mapping on a reference Genome

- Reads are aligned **>=1** times on the reference genome
- A **mapping quality** is associated to each alignment:

  → Quantify the probability that the alignment is correct

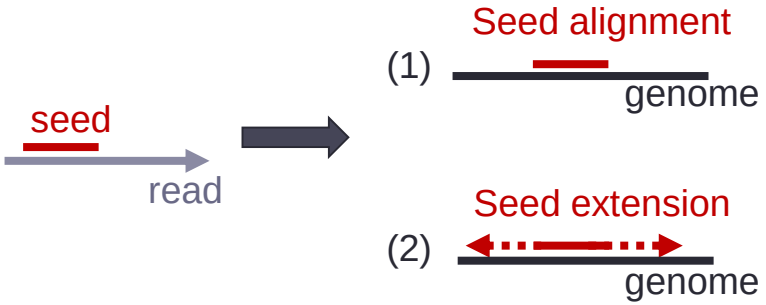
  → Decreases with the number of mismatches (wrong nucleotide) & gaps (small insertions/deletions) & the number of alignments



Reads Alignment - Vocabulary

Mapping method: seed & extend

1. Aligning the seed (small part of the read)
2. Extending the seed to align the whole read



Mismatch: Incoherence between two nucleotides

Indels: Insertion/Deletion into the reference genome

Gap: Bridge within the read alignment (*i.e.* small indels)

Mappability: Uniqueness of a region

- repeated region = low mappability
- unique region = good mappability

Multiple Alignments

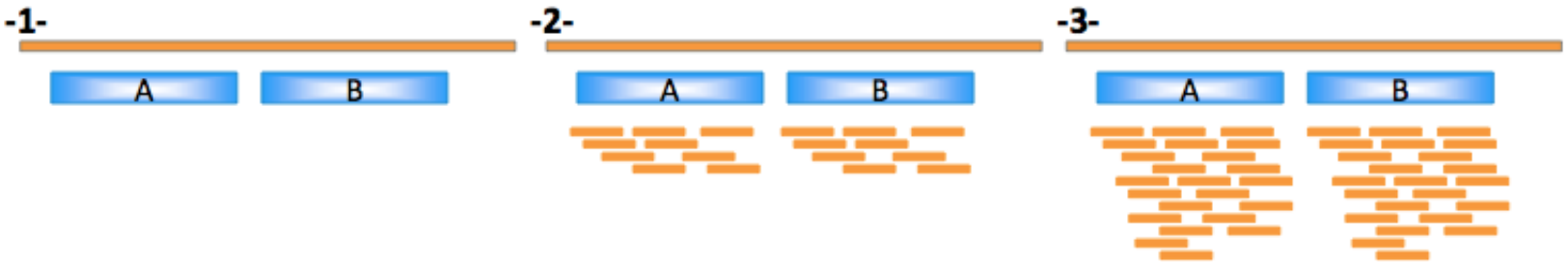
- A read can align **multiple times** on the genome (repeated elements…)
- How to deal with these multiple alignments reads ?

Three strategies:

-1- Report only unique alignment

-2- Report best alignments & randomly assign reads across equally good loci

-3- Report all (best) alignments



- **Mapping Quality:** quantify the probability that a read is misplaced
  → Low if a read has multiple alignments ; several mismatches/gaps

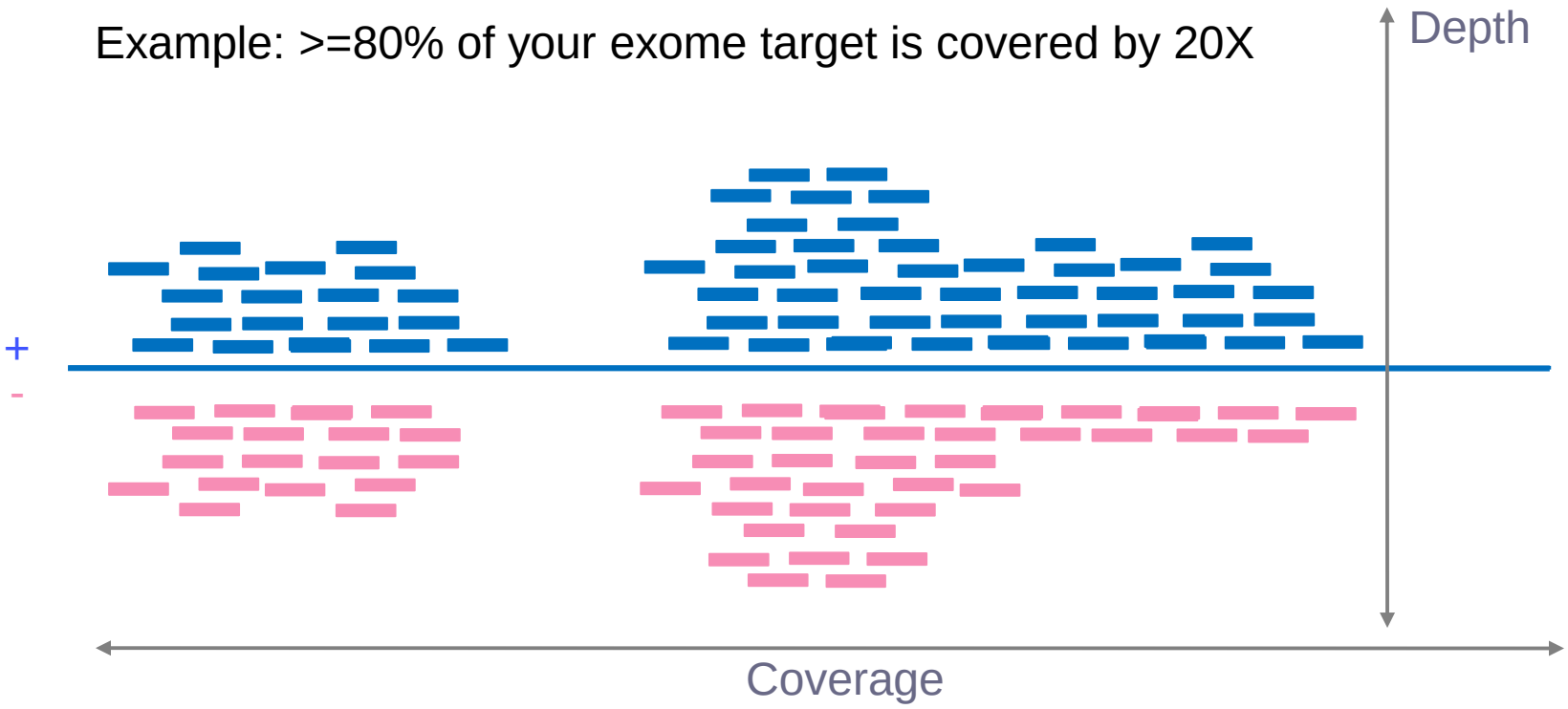
Statistics used as Quality Control

- **Depth of coverage** = mean number of reads covering a base (X)

  Example: 30X for normal sample, 100X for tumor sample

- **Coverage** = part of the reference with at least one read

  Example: >=80% of your exome target is covered by 20X

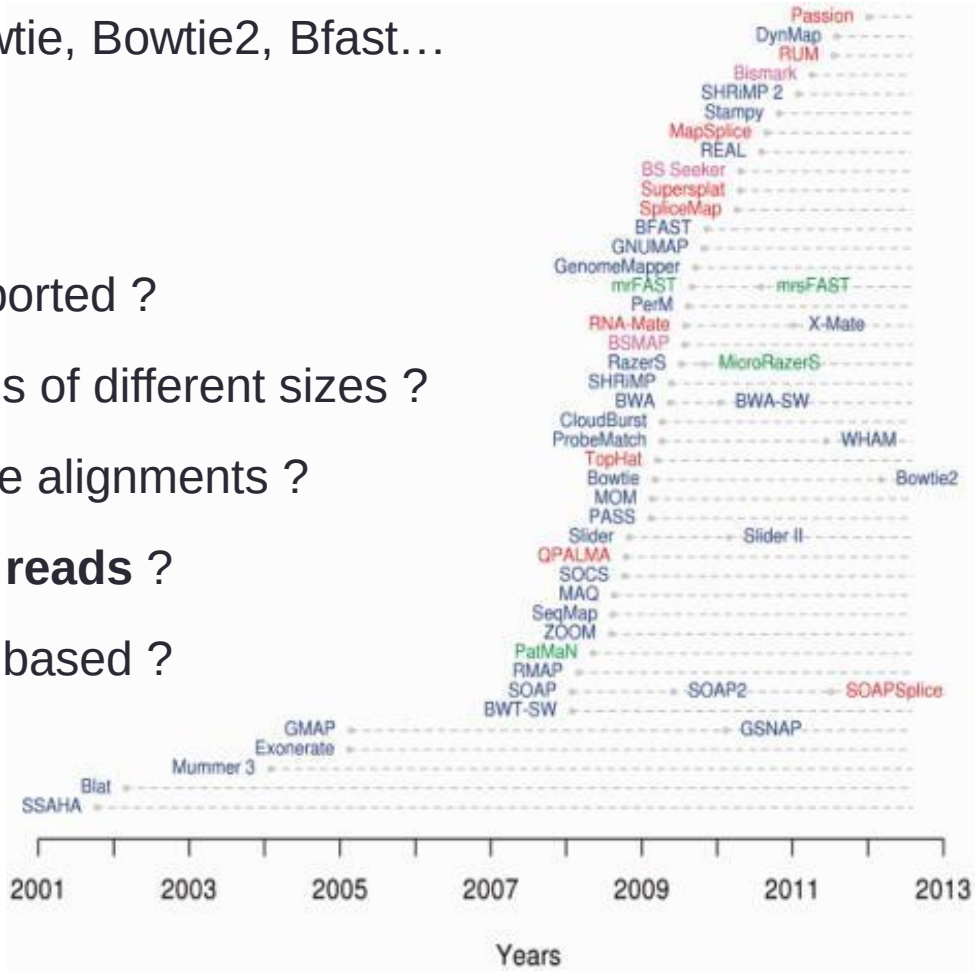


institutCurie

Alignment tools

- Multitude of alignment tools: BWA, Bowtie, Bowtie2, Bfast…

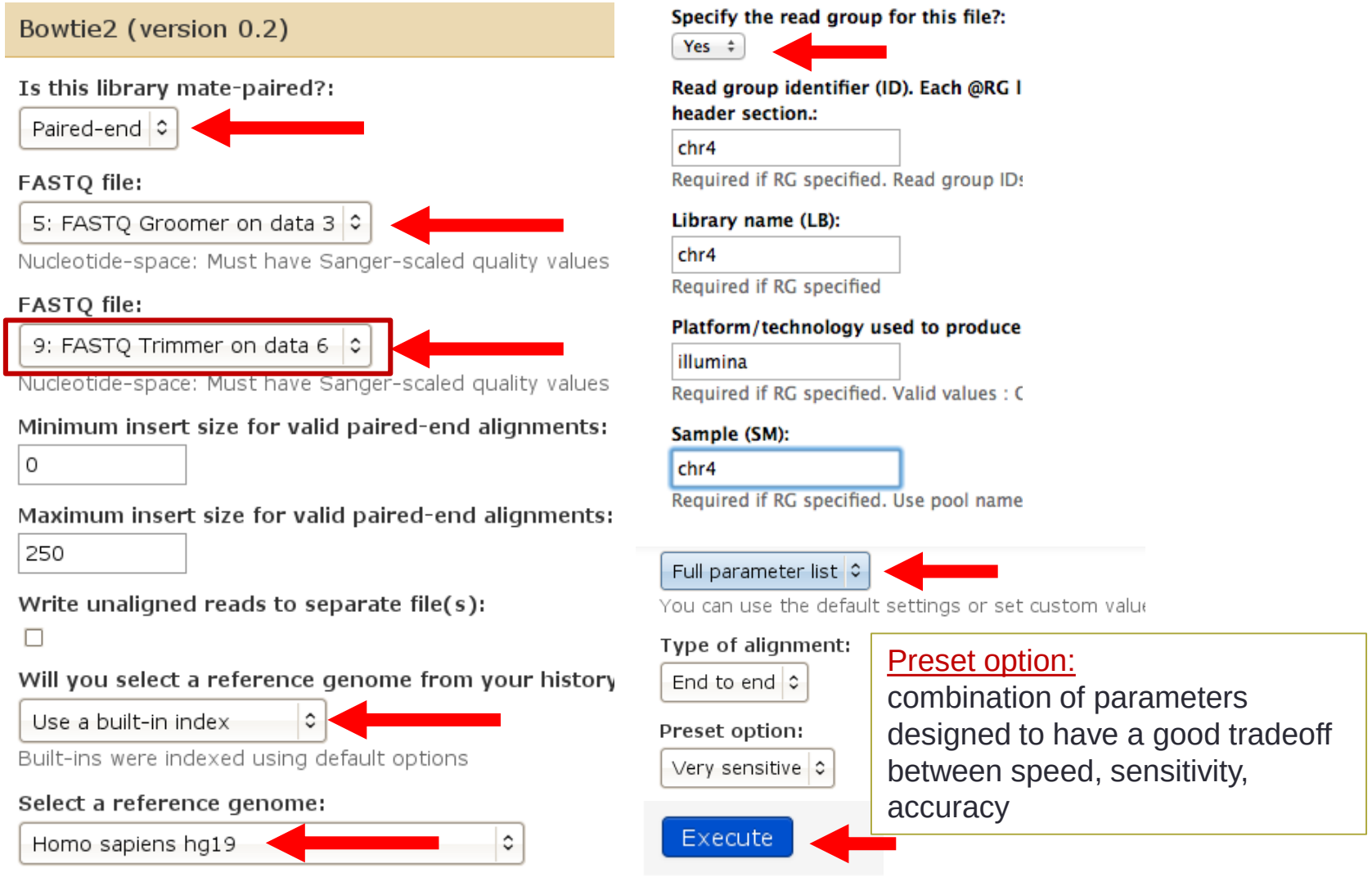
- How to choose the best tool ?
  - Is my sequencing technology supported ?
  - Do I have short/long reads ? Reads of different sizes ?
  - **Do I want to allow gaps ?** Multiple alignments ?
  - Does it support single/**paired-end reads** ?
  - On which alignment algorithm is it based ?
  - Computational issues ?
  - Is it used by the community ?



institutCurie

Mapping with Bowtie2

1. Use « Bowtie2 to align reads on the hg19 genome



Preset option:
combination of parameters designed to have a good tradeoff between speed, sensitivity, accuracy

institutCurie

SAM/BAM aligned format

- SAM Format: aligned format, human readable

@SQ SN:chr12 LN:133851895

@RG ID:Sample_ID LB:Sample_Library PL:ILLUMINA SM:Sample_Name PU:Platform_Unit

Read name	Flag	Chr	5' pos	MAPQ	Cigar	paired	5' pos of the mate	Insert size
ERR166338.1	99	chr12	82670685	23	101M	=	82670850	266

GCCCCTGGGGATGTTTTGCACCAAGCCACTGTCTCCAGCTGG sequence

BBC@GIIHGCFCIEHEAIEIFFGEONDNJFINIONHNGJNNNNKNJN Base quality

RG:Z:Sample_ID XT:A:U NM:i:0 X0:i:1 X1:i:1 XM:i:0 XO:i:0 XG:i:0 MD:Z:100 XA:Z tags

Group affiliation

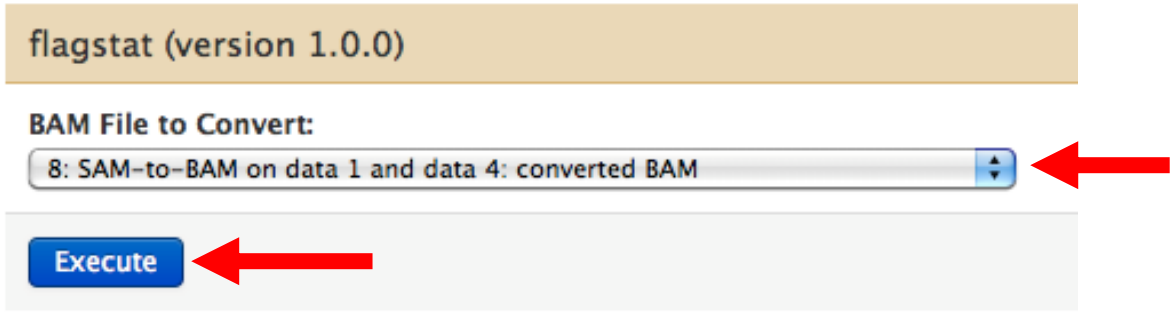
- BAM Format: Binary SAM Format (not human readable but compressed = smaller)

Official specification at http://samtools.sourceforge.net

institutCurie

Mapping Statistics

- Use « Flagstat » from « Samtools » to see some mapping statistics



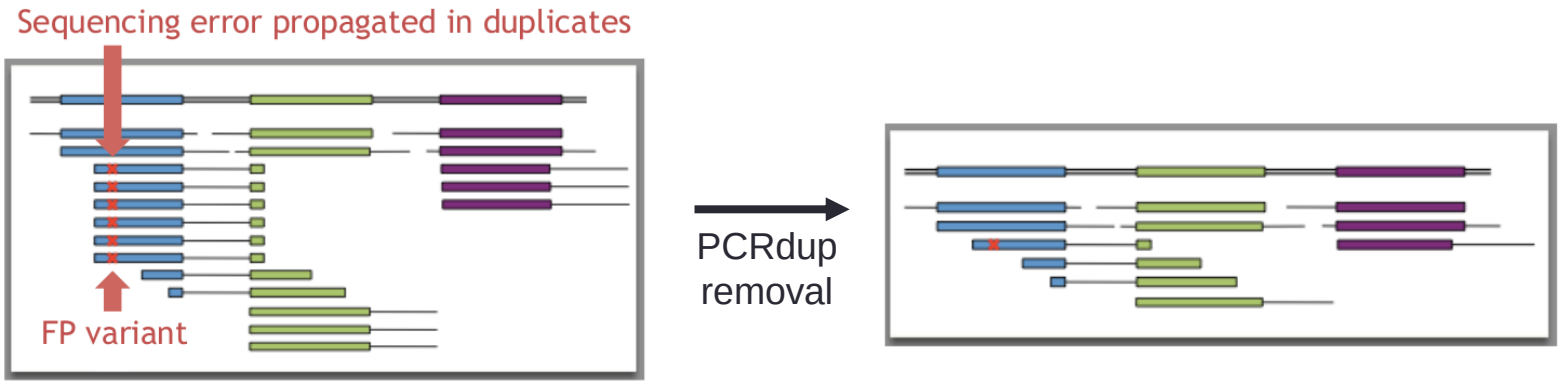

```
109073 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 duplicates
107254 + 0 mapped (98.33%:-nan%)
0 + 0 paired in sequencing
0 + 0 read1
0 + 0 read2
0 + 0 properly paired (-nan%:-nan%)
0 + 0 with itself and mate mapped
0 + 0 singletons (-nan%:-nan%)
0 + 0 with mate mapped to a different chr
0 + 0 with mate mapped to a different chr (mapQ>=5)
```

% of mapped reads

Removing Duplicates

• **Duplicates reads**: different reads having the same sequence caused by PCR amplication during sequencing library preparation

• The removal of the duplicates depends on the application (not suitable for sequencing on small target)



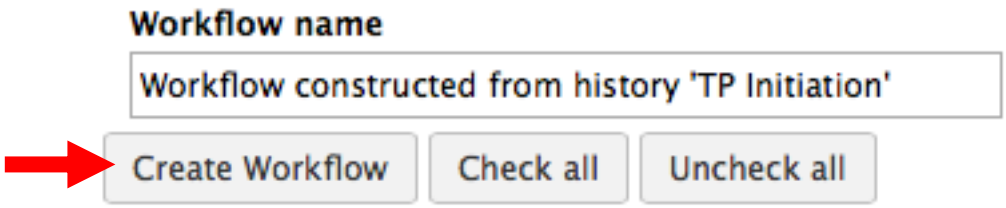
- **Galaxy:** Use “Mark Duplicates reads” from “NGS:Picard” to **mark** duplicates (don’t remove them)

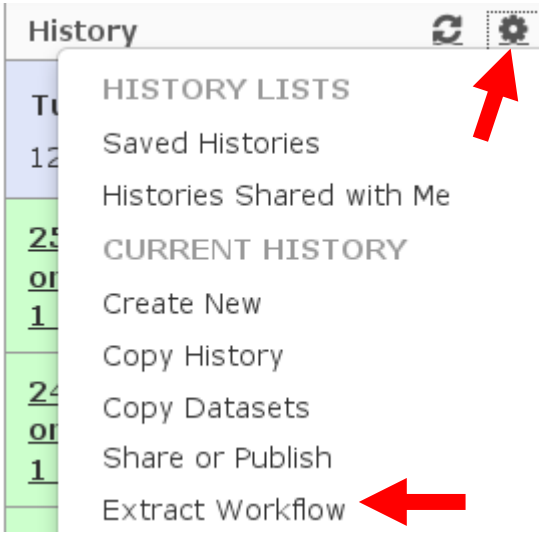
  → If duplicates are marked, samtools and GATK tools will ignore them

- **Galaxy:** Run “Flagstat” on the output BAM to see the number of PCR duplicates

Extract workflow from history

- In the « history » panel, click on the topside wheel then
- on « Extract Workflow »
- Write a name for your workflow then click on



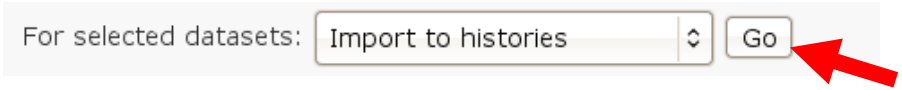


- Select the steps you want to see
- Check every steps!

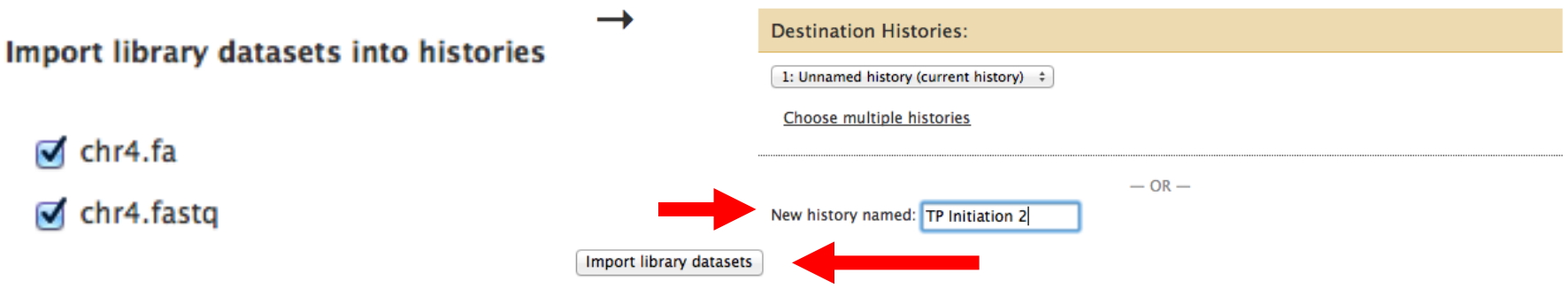
Select Librairies on Galaxy

You can only run tools on data that are present in your current history

1. In the Data Libraries
2. Select « **chr4.fastq** » ; « **chr4.fa** »
3. Select « Import to Histories » then click on Go



4. Write a new history name and click on « import library datasets »



institutCurie

Let Galaxy work for you!

Successfully ran workflow "Workflow constructed from history 'TP Initiation'". The following datasets have been added to the queue:

1: chr4.fa
2: chr4.fastq
22: FASTQ Groomer on data 2
23: FastQC_FASTQ Groomer on data 2.html
24: FASTQ Trimmer on data 22
25: FastQC_FASTQ Trimmer on data 22.html
26: Bowtie2 on data 1 and data 24: aligned reads
27: flagstat on data 26
28: MarkDups_Dupes Marked.bam
29: MarkDups_Dupes Marked.html