Preliminary Processing of NGS Data

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Practical setup

Go to one of following Galaxy servers

- Surnames: A-K
  - https://bioinf-galaxian.erasmusmc.nl/galaxy/
- Surnames: L-N
  - https://galaxy.hidelab.org/
- Surnames: O-Z
  - http://services.cbib.u-bordeaux.fr/galaxy/

Data Upload

- Go *Get Data*
  - *Upload File* (may be a different place on the menu depending which server you connect to!)

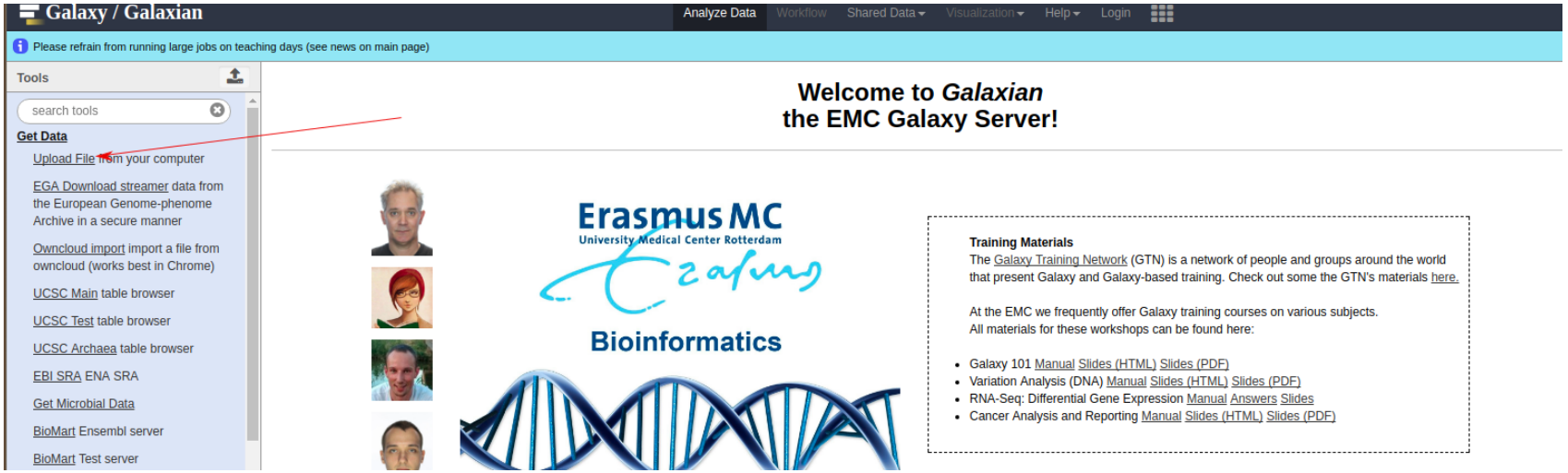
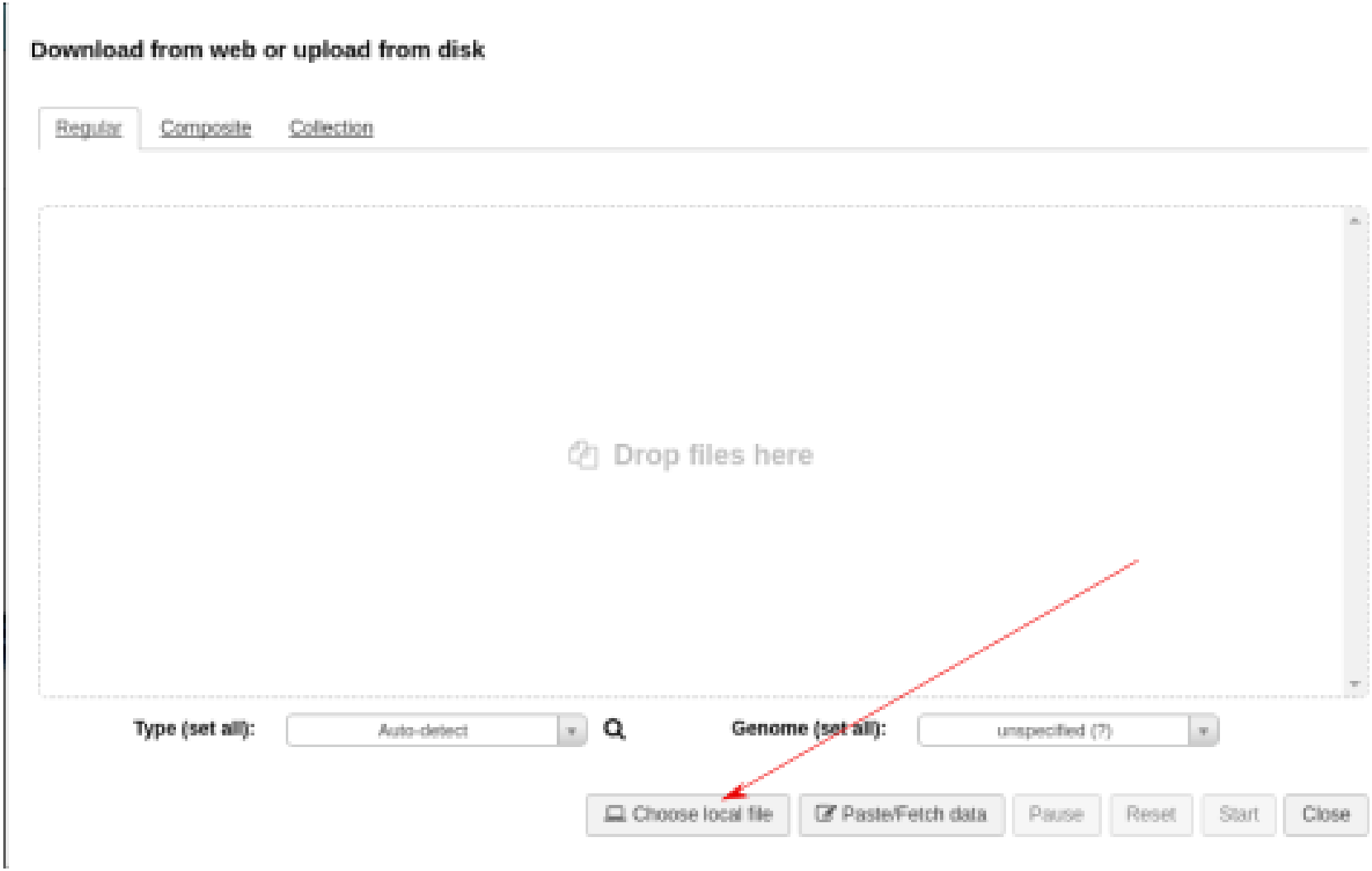


Figure 1

Data Upload

- *Choose local file*
  - Select `JoeBlogsBRCAPanel_R1.fastq`, `JoeBlogsBRCAPanel_R2.fastq` and click **Start**



Outline

- The *Fastq* format for sequencing reads
- Quality assessment of *fastq* files
- The *bam* format for representing *aligned* reads
- Stage 1 of an analysis *pipeline*

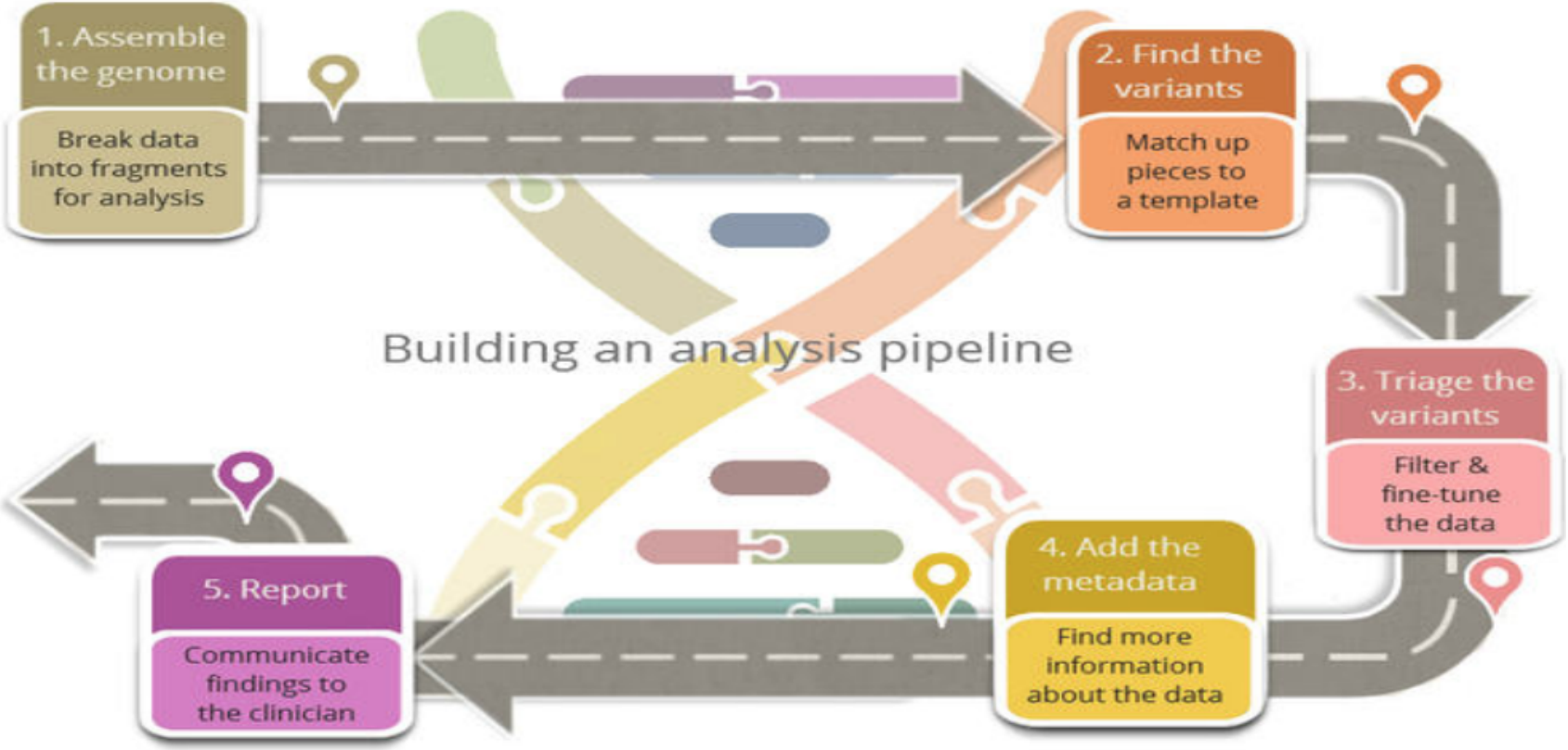
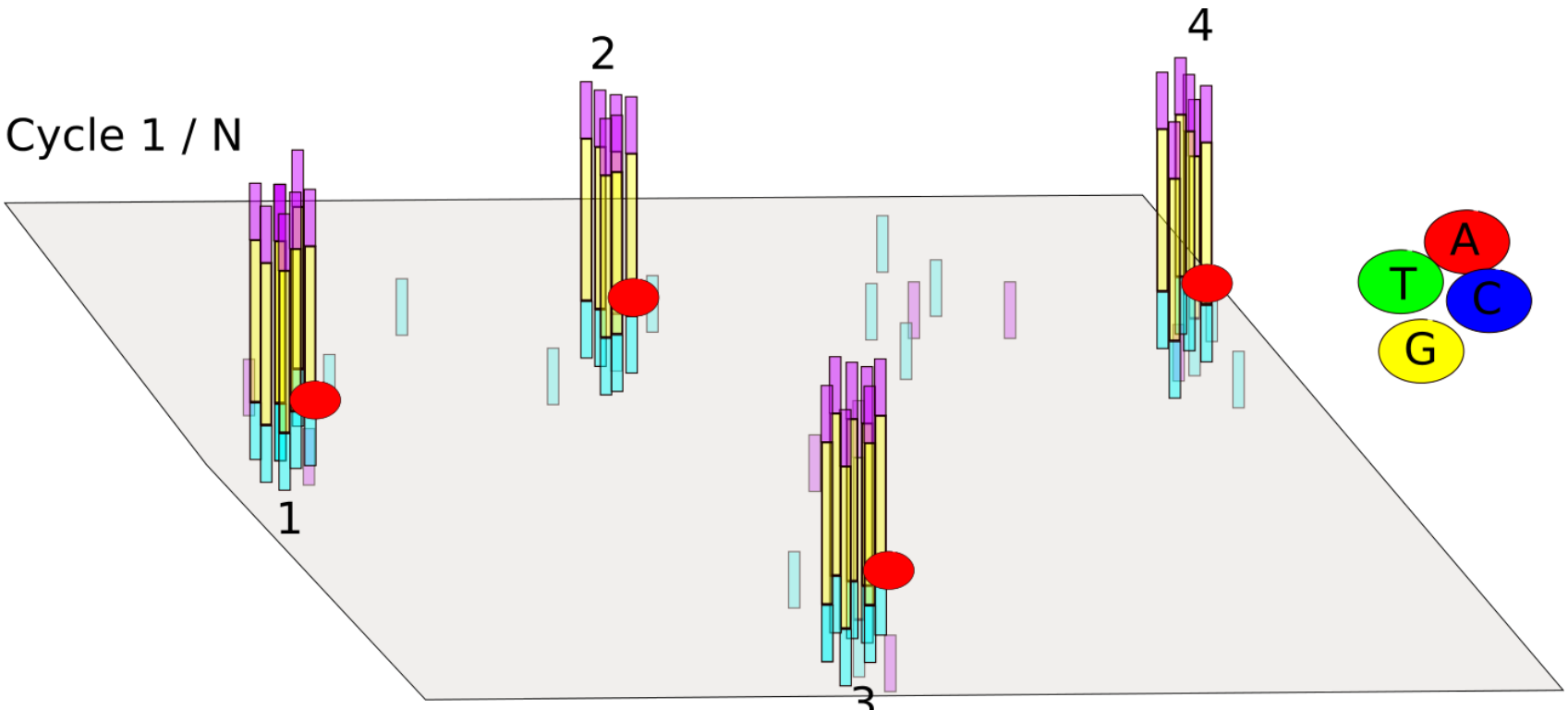


Figure 3

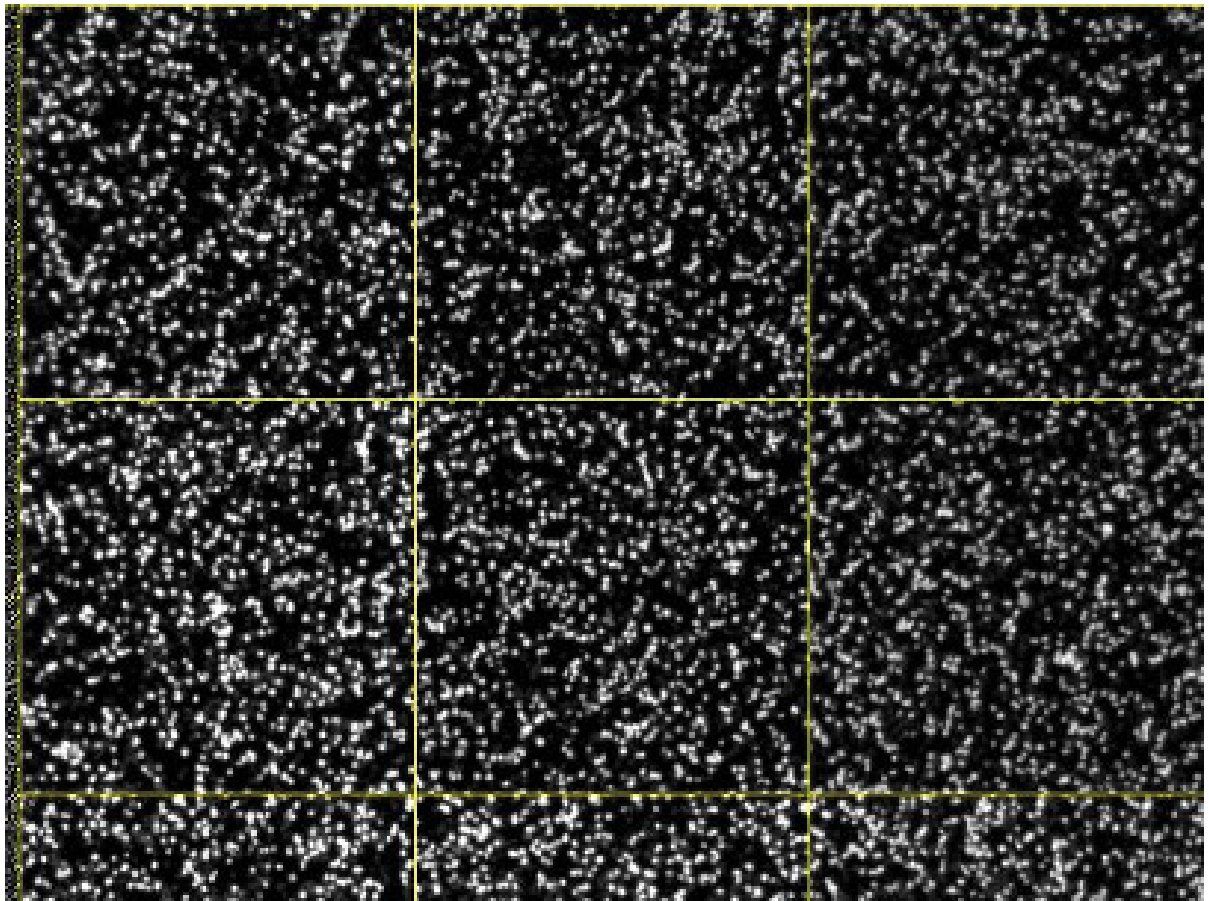
The sequencing process

- https://www.youtube.com/embed/HMyCqWhwB8E for a nice video
- sequencing consists of a series of *cycles* (e.g. 100), at each cycle we try and incorporate different bases (A, T, C, G)
- the base that is successfully added will illuminate brightly in a particular colour



Imaging

- much of the sequening time is spent taking images of the flowcell
- it is these images that are used to discover what fragments of DNA were sequenced
- this process is not perfect and can introduce *uncertainty*



Scale of data

Instrument	No. of Reads	Size
Ion Torrent PGM	5 million reads	1Gb
MiSeq	25 million reads	6GB
HiSeq rapid run	600 million	150GB (*)
HiSeq high-output	4 billion	1 TB

- Equivalent to **40** HD movies
- File sizes are for 100 bp reads, unzipped
- Number of reads from thermofisher.com and illumina.com

Fastq format

- a text file
- can be *compressed* as a gz file
- four lines per read
- the sequenced is most interesting, there are two other lines that we potentially investigate

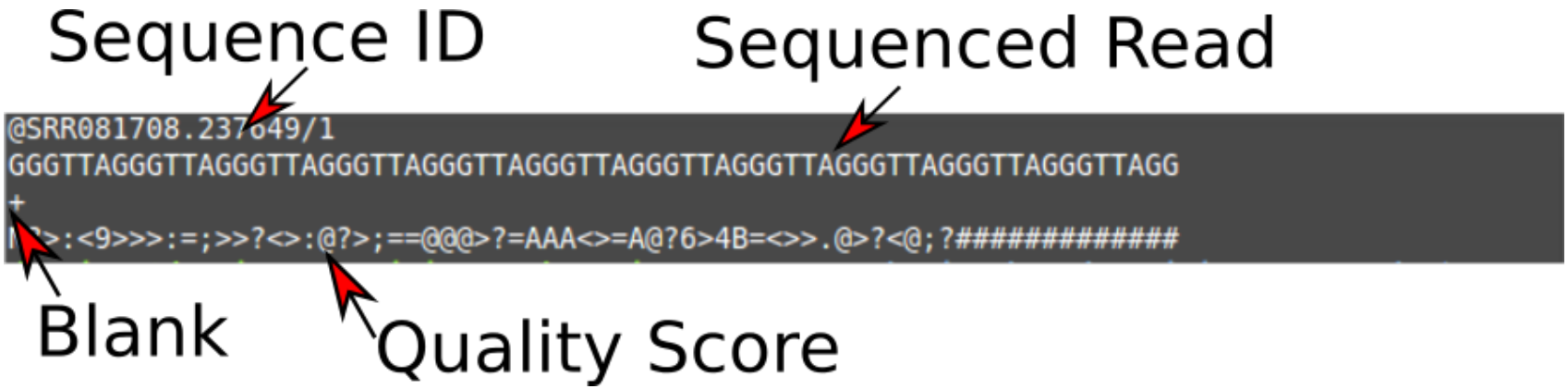


Figure 6

Sequence ID

Can contain the following

- name of sequencer
- flow cell *lane*
- coordinates of the read on the flow cell
- whether this is a *paired* read and whether it is read 1, or 2
- which biological sample the read came from (if multiple samples were sequenced on the same lane)

Quality scores

- base-calling has some probability (p) that we make a mistake.
- the quality score expresses our *confidence* in a particular base-call; higher quality score, higher confidence
- one such score for each base of sequencing. i.e. 100 scores for 100 bases of sequencing
- these are of importance if we want to call SNVs etc.
  - need to be sure that differences detected from the reference genome are real, and not caused by sequencing error

```
N?>:<9>>>:=;>>?<>:@?>;==@@@>?=AAA<>=A@?6>4B=<>>.@>?<@;?####
```

Deriving the Quality Score

First of all, we convert the base-calling probability (p) into a `Q` score using the formula

- Quality scores

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

  - Q = 30, p=0.001
  - Q = 20, p=0.01
  - Q = 10, p=0.1

- These numeric quanties are *encoded* as **ASCII** code
  - At least 33 to get to meaningful characters (https://en.wikipedia.org/wiki/FASTQ_format)

```
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
|                           |    |        |                               |                     |
33                         59   64       73                             104                   126
```

Figure 7

Quality Scores to probabilities

- look-up the ASCII code for each character
- subtract the offset to get the Q score
- convert to a probability using the formula:-

$$p = 10^{-Q/10}$$

Worked Example

for our particular example:

```
N?>:<9>>>:=;>>?<>:@?>;==@@@>?=AAA<>=A@?6>4B=<>>.@>?<@;?####
```

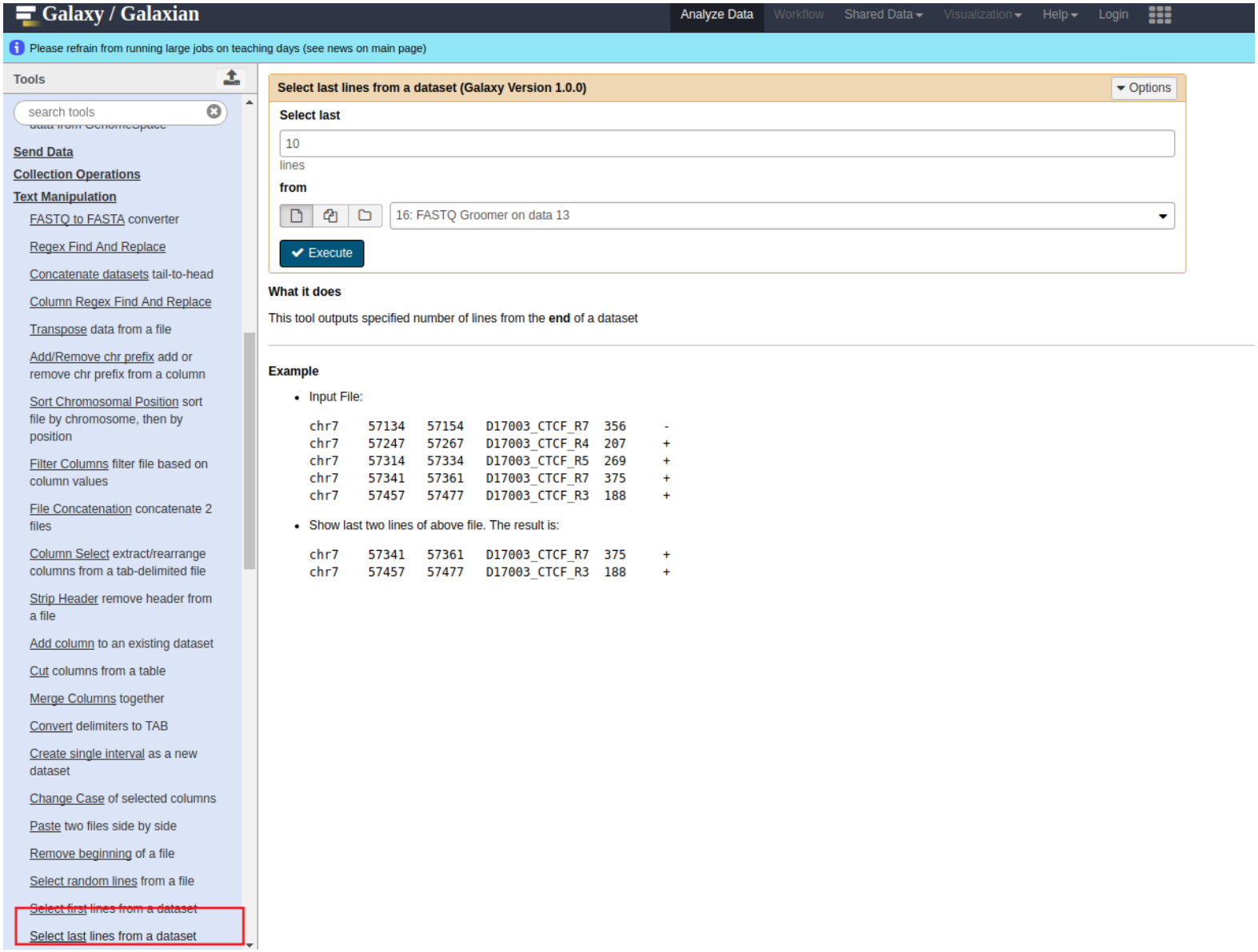
it works out as follows:-

	Character	Code	Minus.Offset..33..	Probability
1	N	78	45	0.00003
2	?	63	30	0.00100
3	>	62	29	0.00126
4	:	58	25	0.00316
5	<	60	27	0.00200
6	9	57	24	0.00398
7	>	62	29	0.00126
8	>	62	29	0.00126
9	>	62	29	0.00126
10	:	58	25	0.00316

...

Exercise

- Use the Galaxy tool *Text Manipulation -> Select last*

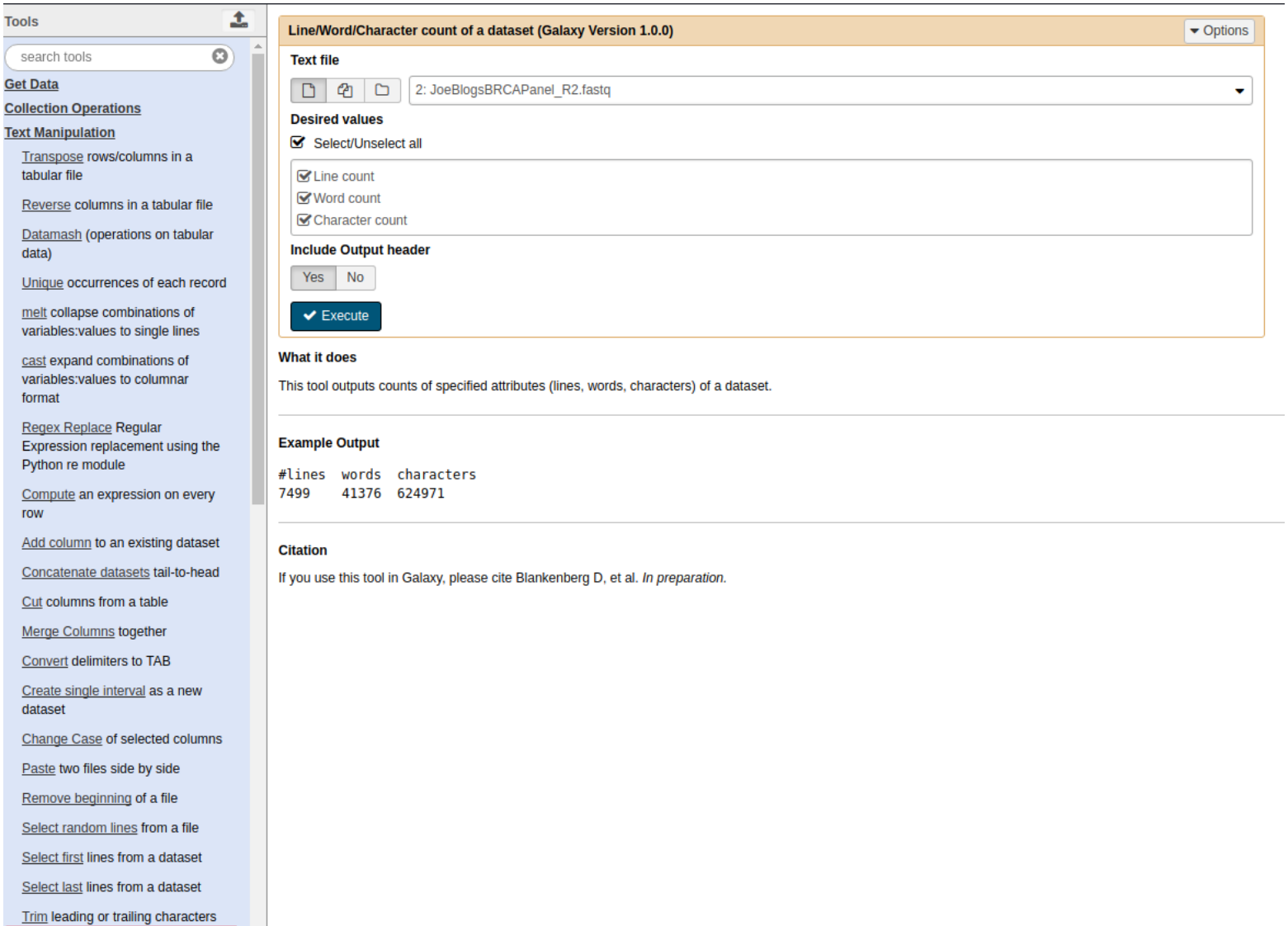


Exercise

- print the last *12* lines from the file `JoeBlogsBRCAPanel_R2.fastq`
- how many reads are shown in the result?
- Look at the last read and write down the first five and last five ASCII characters
  - is the quality greater at the start, or the end of the read?

Exercise

- Use the Galaxy tool *Text Manipulation -> Line/Word/Character count*



Exercise

- count how many lines are in the file `JoeBlogsBRCAPanel_R2.fastq` in total
- how many reads does this correspond to?

FastQC: Quality Assessment of fastq files

- FastQC from the Babraham Institute Bioinformatics Core has emerged as the standard tool for performing quality assessment on sequencing reads; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
- The manual for `FastQC` is available online and is very comprehensive
- A *"traffic light"* system is used to draw your attention to sections of the report that require further investigation.
- `fastqc` will not actually *do* anything to your data. If you decide to trim or remove contamination for your samples, you will need to use another tool.
- it doesn't know what type of sequencing has been performed (WGS, exome, RNA-seq), which can affect interpretation of some of the plots

Example sections of a `fastqc` report

1. Basic Statistics

Basic Statistics

Measure	Value
Filename	sample.fastq
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	9053
Sequences flagged as poor quality	0
Sequence length	36
%GC	50

Figure 10

Example sections of a `fastqc` report

2. Per-base sequence quality

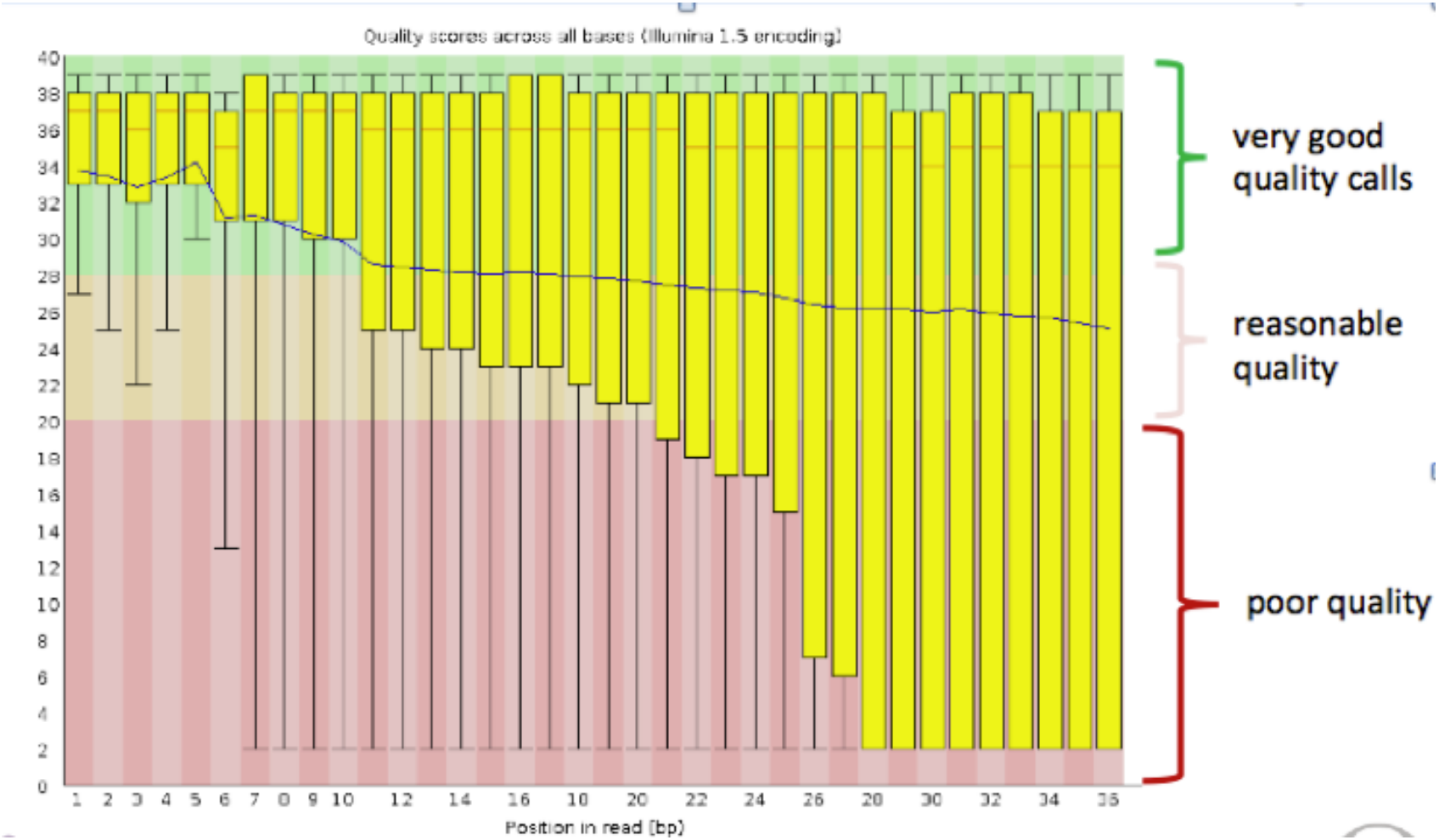
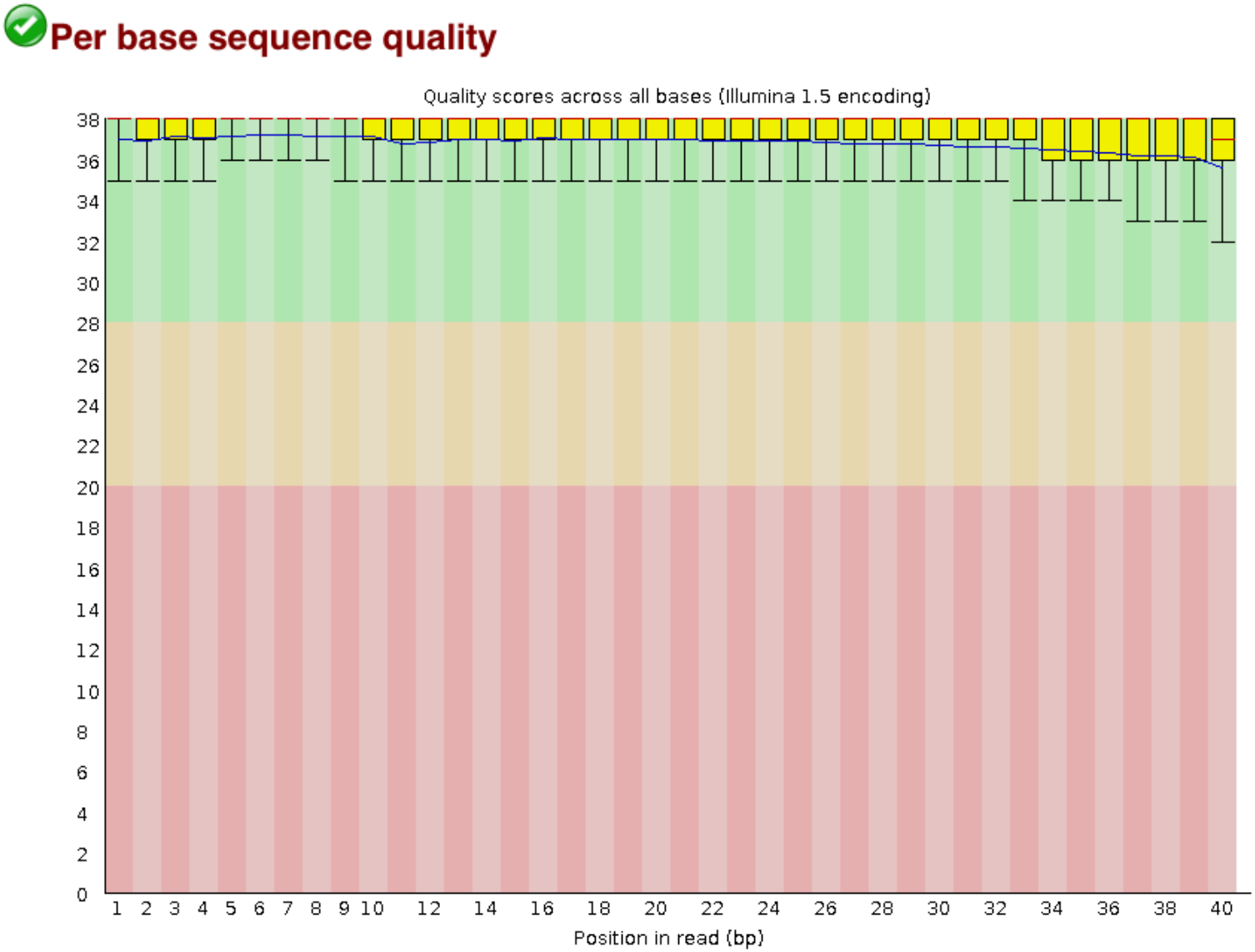


Figure 11

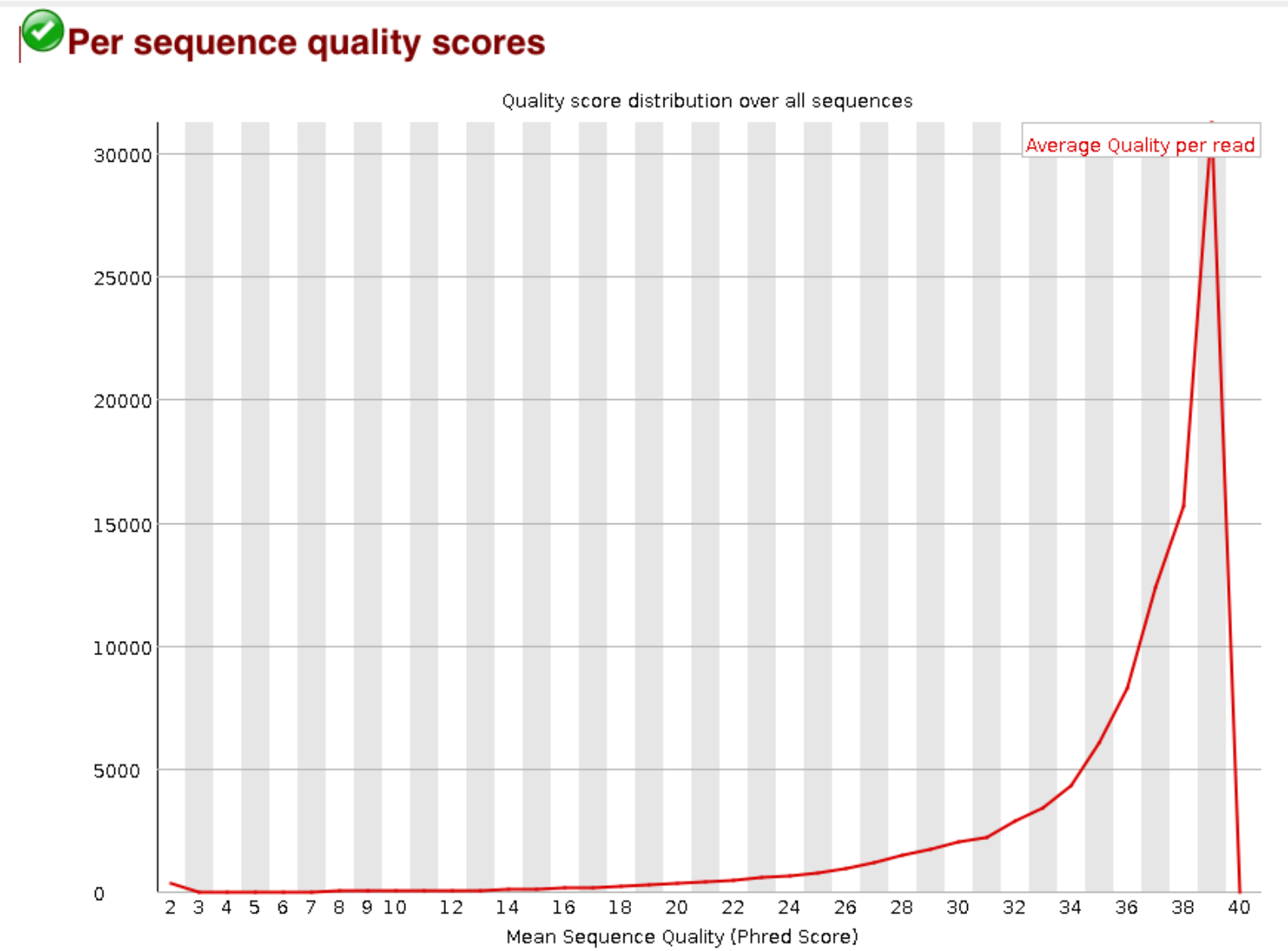
Example sections of a `fastqc` report

Ideally, the plot should look *something* like following:-



Example sections of a `fastqc` report

3. Per-sequence quality scores



Example sections of a `fastqc` report

[Per-base sequence content]

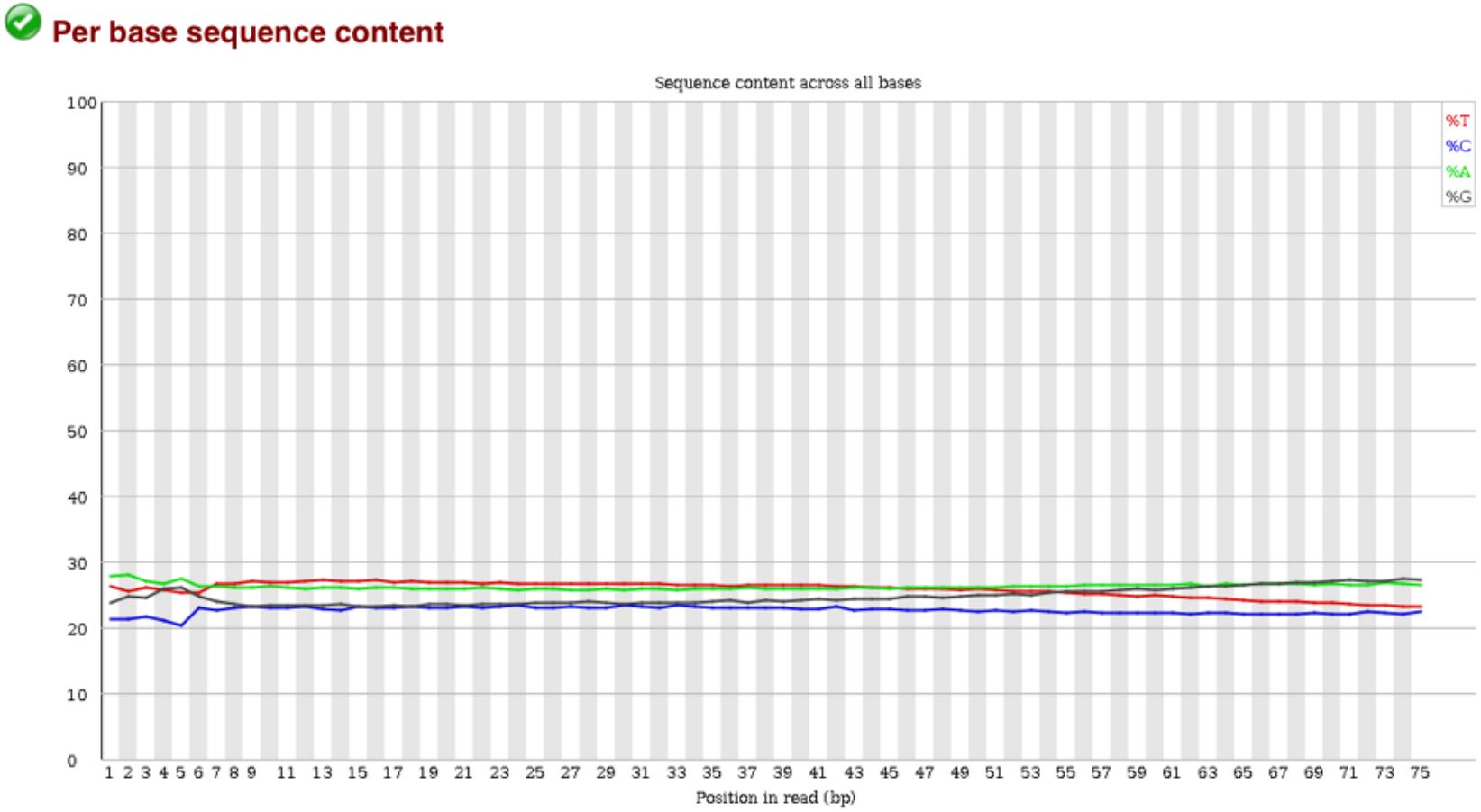


Figure 14

Exercise

- using the Galaxy Tool *NGS: QC and manipulation -> FastQC Read Quality reports*

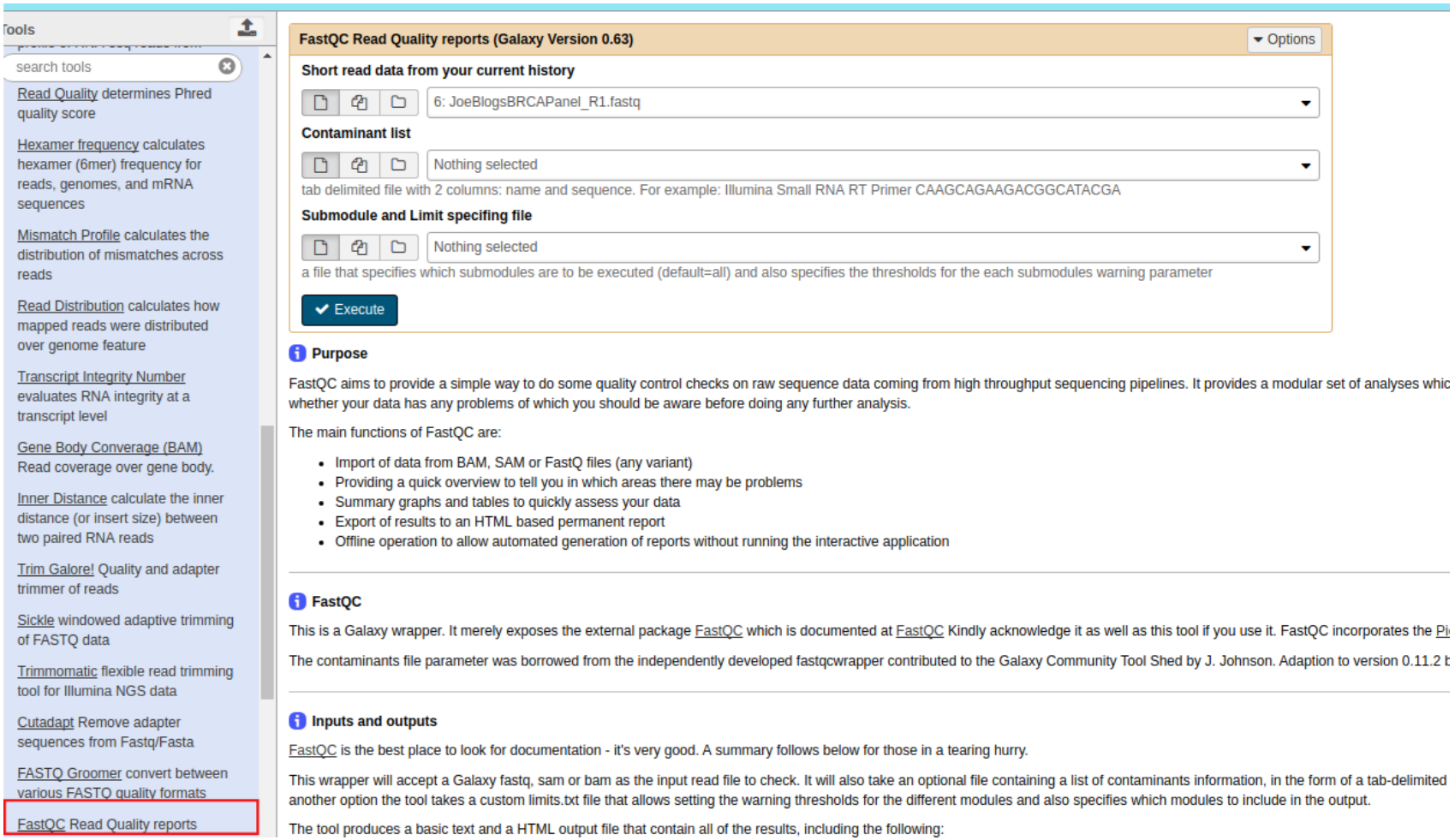


Figure 15

Exercise

- generate a QC report for the file `JoeBlogsBRCAPanel_R2.fastq`
- look at the basic statistics for the file
  - does the number of reads agree with your previous answer?
- is there any evidence for a decrease in quality as the read length increases?
- See https://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/ for descriptions of various sections of report

The SAM / BAM format

- we don't really spend much time look at *fastq* files
- most of our time is spent with *aligned* reads
  - i.e. we have used some software to tell us whereabouts in the genome each read belongs to
  - we will have a go at this in the practical

The `.sam` file

- **S**equence **A**lignment/**M**ap (sam)
- The output from an aligner such as `bwa` or `bowtie`
- Same format regardless of sequencing protocol (i.e. RNA-seq, ChIP-seq, DNA-seq etc)
- May contain un-mapped reads
- Official specification can be found online http://samtools.github.io/hts-specs/SAMv1.pdf
- We normally work on a compressed version called a `.bam` file. See later.

The .sam file

Comprises a *tab-delimited* section that describes the alignment of each sequence in detail.

```
1                 2   3      4   5      6   7      8   9                 11
SRR081708.237649  10 163    1      10003  6      1S67M  =      10041  105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13     RG:Z:SRR081708  NM:i:6  OQ:Z:GEGFFFEGGGDGDGGGDGA?
DCDD:GGGDGDCFGFDDFFFCCCBEBFDABDD-D:EEEE=D=DDDDC:
```

Figure 16

- 1:- Sequence ID
- 2:- Sequence quality expressed as a bitwise *flag*
- 3:- Chromosome that the read aligned to

Fun with flags!

The *"flags"* in the sam file can represent useful QC information

- Read is unmapped
- Read is paired / unpaired
- Read failed QC
- Read is a PCR duplicate (see later)

```
SRR081708.237649        163     1       10003   6       1S67M   =       10041   105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13     RG:Z:SRR081708  NM:i:6  OQ:Z:GEGFFFEGGGDGDGGGDGA?
DCDD:GGGDGDCFGFDDFFFCCCBEBFDABDD-D:EEEE=D=DDDDC:
```

Figure 17

Derivation

	ReadHasProperty	Binary	MultiplyBy
Paired?	TRUE	1	1
Properly Paired?	TRUE	1	2
Unmapped?	FALSE	0	4
Unmapped Mate?	FALSE	0	8
On Minus Strand?	FALSE	0	16
Mate on Minus Strand?	TRUE	1	32
Is First Read?	FALSE	0	64
Is Second Read?	TRUE	1	128
Is Secondary Alignment?	FALSE	0	256
Is Not Passing QC?	FALSE	0	512
Is Duplicate Read?	FALSE	0	1024

1x1 + 1x2 + 0x4 + 0x8 + 0x16 + 1x32 + 0x64 + 1x128 + 0x256 + 0x512 + 0x1024 = 163

https://broadinstitute.github.io/picard/explain-flags.html

The .sam file

```
1                2   3     4   5     6  7     8   9                11
SRR081708.237649 10 163    1     10003  6     1S67M  =     10041  105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13     RG:Z:SRR081708  NM:i:6  OQ:Z:GEGFFFEGGGDGDGGGDGA?
DCDD:GGGDGDCFGFDDFFFCCCBEBFDABDD-D:EEEE=D=DDDDC:
```

Figure 18

- 4:- Start Position
- 5:- Mapping Quality; Confidence that an alignment is correct
  - higher is more confident
  - 0 means read maps to multiple locations
- 6:- CIGAR; Describes number of matches (M), insertions (I), deletions (D)

CIGAR string

Compact **I**diosyncratic **G**apped **Alignment** **R**eport

- Value before **M** is number of consecutive mapping bases (can be mismatches)
- Value before **I** is number of bases inserted relative to reference
- Value before **D** is number of bases deleted relative to reference
  - e.g. 142**M**2**I**7**M** 2 bp insertion after 142 bases then 7 aligned bases

The .sam file

```
SRR081708.237649        163     1       10003   6       1S67M   =       10041   105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>   MD:Z:5A11A5A11A5A11A13  RG:Z:SRR081708  NM:i:6  OQ:Z:GEGFFFEGGGDGDGGGDGA?
DCDD:GGGDGDCFGFDDFFFCCCBEBFDABDD-D:EEEE=D=DDDDC:
....
....
SRR081708.237649        83      1       10041   18      68M     =       10003   -105
CCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCC    #############?;@<?>@.>><=B4>6?@A=><AAA=?>@@@==;>?@:><?
>>;=:>>>9<:>?N  ZA:Z:<@;0;0;;308;;><&;0;0;;27;1S67M;5A11A5A11A5A11A13>  MD:Z:68 RG:Z:SRR081708  NM:i:0  OQ:Z:#############?<?8@?
@,?:88;165@AA?@?BEB@EBBBA@@@AB@?B?AAB?B>CCCAC>CCC
```

Figure 19

- 7, 8, 9: Alignment information for the paired read (if available)
  - whether they align to the same chromosome
  - where the position of the paired read is
  - how far apart did they map?

The .sam file

```
1              2   3     4   5     6   7     8   9
SRR081708.237649  10 163   1     10003 6     1S67M =     10041 105           11
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13     RG:Z:SRR081708  NM:i:6  OQ:Z:GEGFFFEGGGDGDGGGDGA?
DCDD:GGGDGDCFGFDDFFFCCCBEBFDABDD-D:EEEE=D=DDDDC:
```

Figure 20

- 10:- Sequence
- 11:- Base Qualities
- This is the same as the `fastq` file; so if you have aligned data you can always go back and re-align
- The file may also have additional (optional) information recorded by the aligner or analysis tool

Sam and Bam

- `sam` is a human-readable file
  - which makes it quite large and unwieldy
- `bam` is the compressed binary version
  - needs special software to interrogate
  - better way of transferring data
- they contain *same* data
- the `bam` file needs to be *indexed* so we can access it more efficiently

Exercise: Alignment

- *NGS: QC and manipulation -> FASTQ Groomer*

Please refrain from running large jobs on teaching days (see news on main page)

Tools

search tools

quality score

Hexamer frequency calculates hexamer (6mer) frequency for reads, genomes, and mRNA sequences

Mismatch Profile calculates the distribution of mismatches across reads

Read Distribution calculates how mapped reads were distributed over genome feature

Transcript Integrity Number evaluates RNA integrity at a transcript level

Gene Body Converage (BAM) Read coverage over gene body.

Inner Distance calculate the inner distance (or insert size) between two paired RNA reads

Trim Galore! Quality and adapter trimmer of reads

Sickle windowed adaptive trimming of FASTQ data

Trimmomatic flexible read trimming tool for Illumina NGS data

Cutadapt Remove adapter sequences from Fastq/Fasta

FASTQ Groomer convert between various FASTQ quality formats

FastQC Read Quality reports

FASTQ Groomer convert between various FASTQ quality formats (Galaxy Version 1.0.4) Options

File to groom

1: JoeBlogsBRCAPanel_R1.fastq

Input FASTQ quality scores type

Sanger & Illumina 1.8+

Advanced Options

Hide Advanced Options

Execute

What it does

This tool offers several conversions options relating to the FASTQ format.

When using *Basic* options, the output will be *sanger* formatted or *cssanger* formatted (when the input is Color Space Sanger).

When converting, if a quality score falls outside of the target score range, it will be coerced to the closest available value (i.e. the minimum or maximum).

When converting between Solexa and the other formats, quality scores are mapped between Solexa and PHRED scales using the equations found in Cock PJ, Fields CJ, with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 2009 Dec 16.

When converting between color space (csSanger) and base/sequence space (Sanger, Illumina, Solexa) formats, adapter bases are lost or gained; if gained, the base 'G' i there is no adapter present in the color space sequence. Any masked or ambiguous nucleotides in base space will be converted to 'N's when determining color space en

Quality Score Comparison

```
 SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS
 ..............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
 ........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
 !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
 |                         |    |        |                              |                     |
33                        59   64       73                            104                   126

S - Sanger       Phred+33,  93 values  (0, 93) (0 to 60 expected in raw reads)
I - Illumina 1.3 Phred+64,  62 values  (0, 62) (0 to 40 expected in raw reads)
X - Solexa       Solexa+64, 67 values (-5, 62) (-5 to 40 expected in raw reads)
```

Diagram adapted from http://en.wikipedia.org/wiki/FASTQ_format

Figure 21

Exercise: Alignment

- Select file to groom as `JoeBlogsBRCAPanel_R1.fastq`
  - press **Execute**
- Repeat with `JoeBlogsBRCAPanel_R2.fastq`

Exercise: Alignment

- *NGS: Mapping -> Bowtie2*

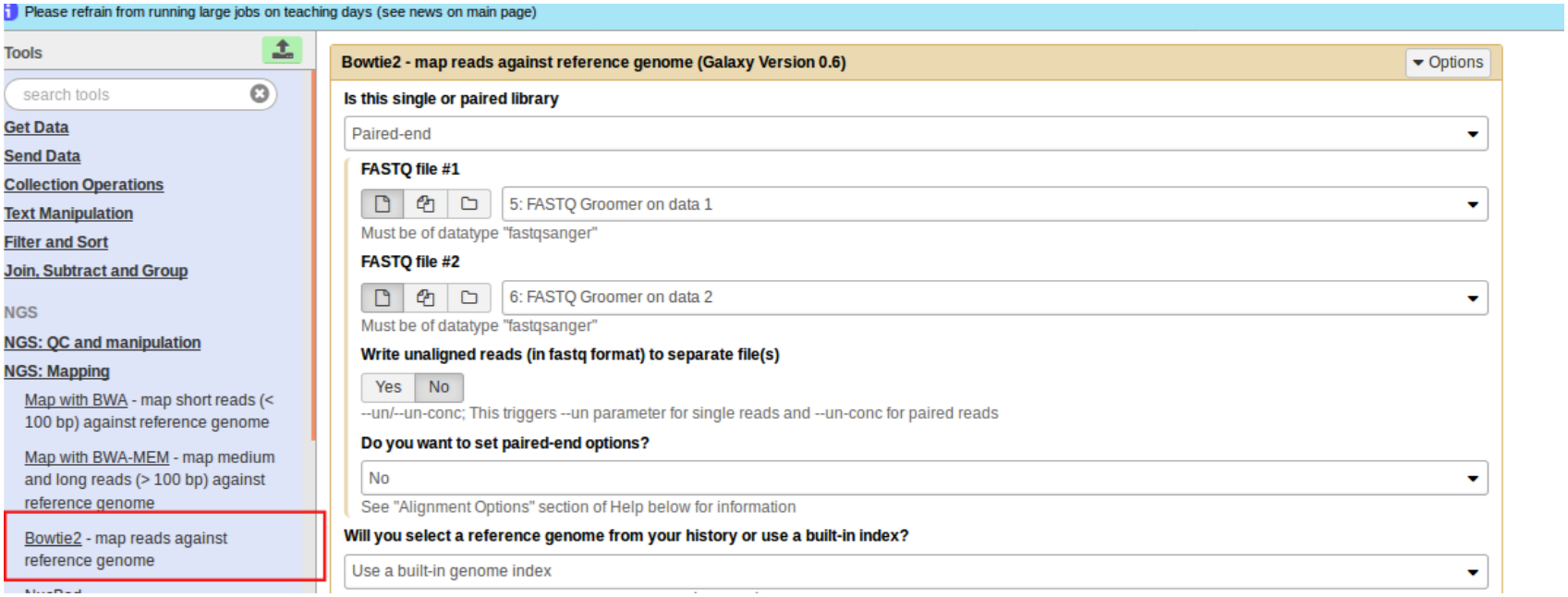


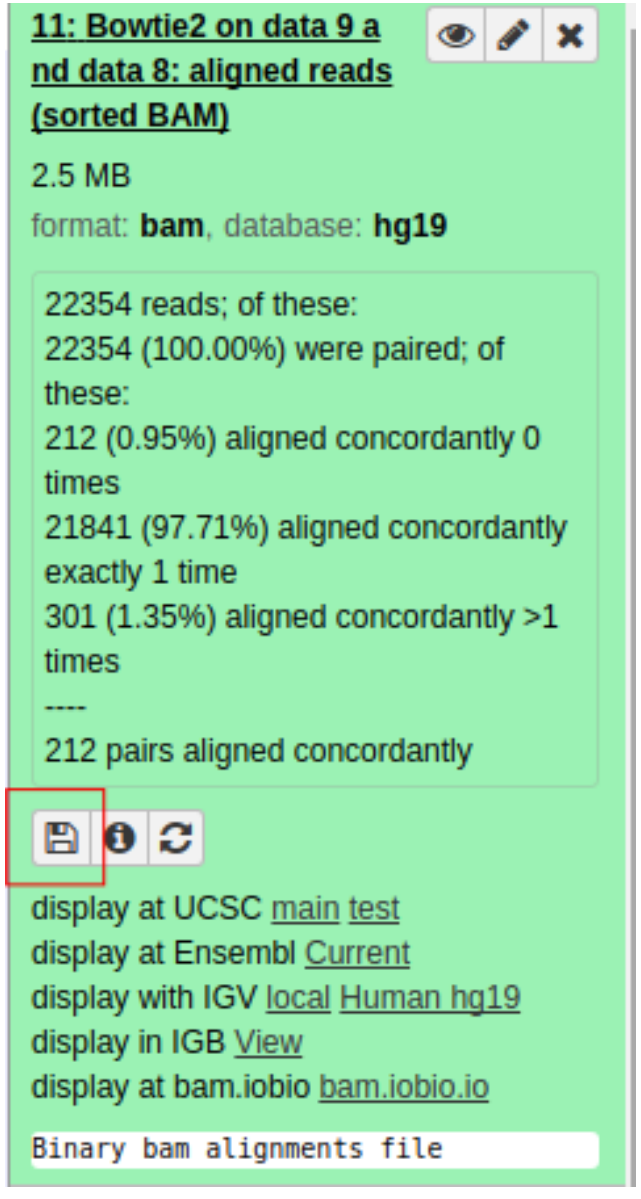
Figure 22

Exercise: Alignment

- In *Is this single-end or Paired-end?* Select **Paired-end**
- Set *FastQ file #1* and *FastQ file #2* to the two files you created in the previous step
- Make sure the reference genome is set to **Human (Homo sapiens)(b37):hg19**
- Press *Execute*
- Wait!

Exercise: Visualisation of reads

- Download the `bam` file you have just created, and it's index file



Exercise: Visualisation of reads

- Load into **IGV**
- Navigate to the BRCA1 gene and zoom-in to see the reads
- Can you see any possible mutations?
- *Hover* over particular reads to get information about the aligment of the read
- (if you didn't manage to align the data, the file `JoeBlogsBRCAPanel_bowtie2.bam` can be used)