Preliminary Processing of NGS Data

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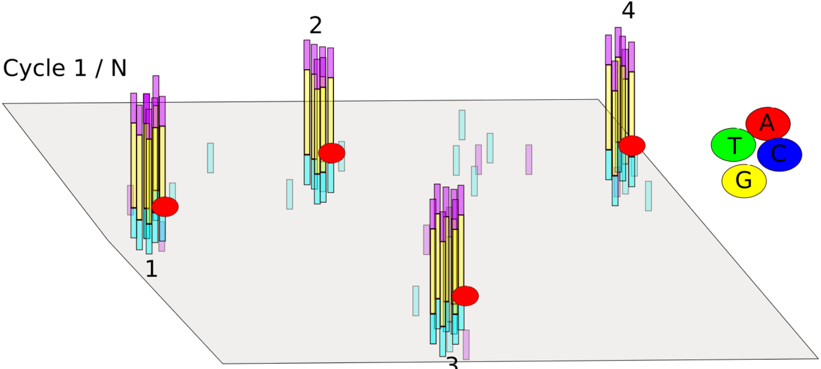
Outline

This afternoon we will cover

- The *Fastq* format for sequencing reads
- Quality assessment of *fastq* files
- The *bam* format for representing *aligned* reads
- Practice using *Galaxy*

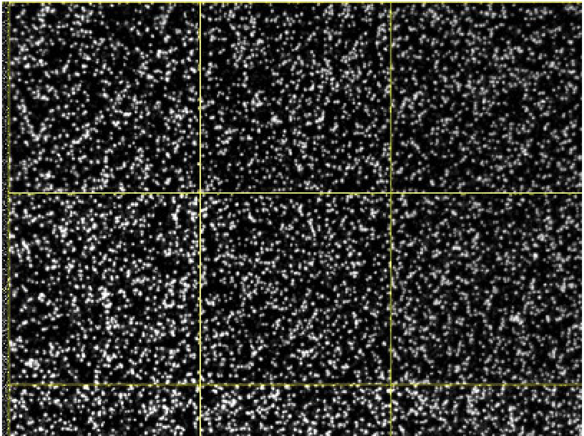
The sequencing process

- https://www.youtube.com/embed/HMyCqWhwB8E for a nice video
- Sequencing consists of a serices of *cycles* (e.g. 100), at each cycle we try and incorporate different bases (A, T, C, G)
- Base that is successfully added will illuminate 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*



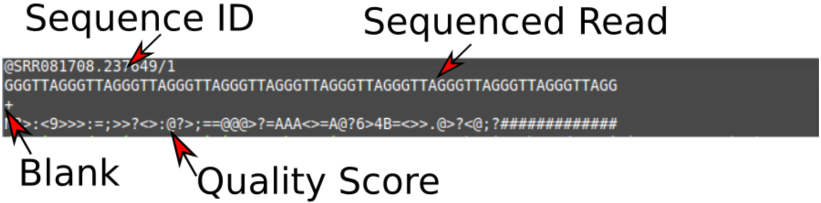
Scale of data

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



```
@SRR081708.237649/1
GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGG
+
N?>:<9>>>:=;>>?<>:@?>;==@@@>?=AAA<>=A@?6>4B=<>>.@>?<@;?#############
```

Figure 3

Sequence names

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

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 and legitimate, and not caused by sequencing error

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

Deriving the Quality Score

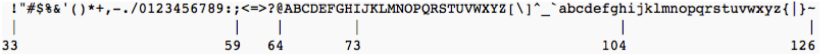
First of all, we convert the base-calling probability (p) into a `Q` score using the formula

- 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 4

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

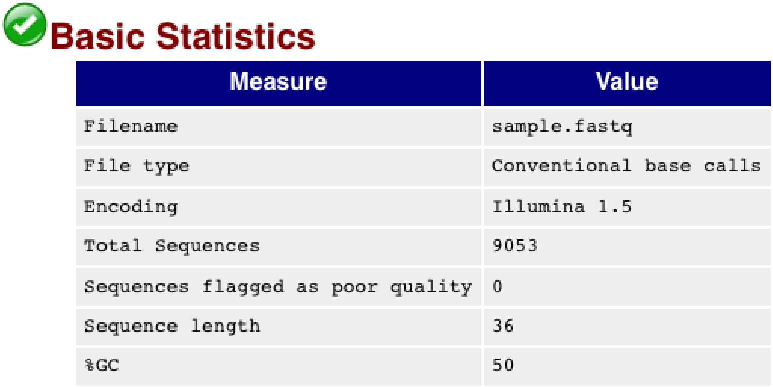
- Use the Galaxy tool *Text Manipulation* -> *Select last*
  - print the last *12* lines from the file `JoeBlogsBRCAPanel_R2.fastq`
- How many reads are shown in the result?
- Look at the last read and write down the first five and last five ASCII characters
  - is the quality greater at the start, or the end of the read?
- Use the Galaxy tool *Text Manipulation* -> *Line/Word/Character count*
  - count how many lines are in the file `JoeBlogsBRCAPanel_R2.fastq` in total
  - how many reads does this correspond to?

FastQC: Quality Assessment of fastqc

- 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



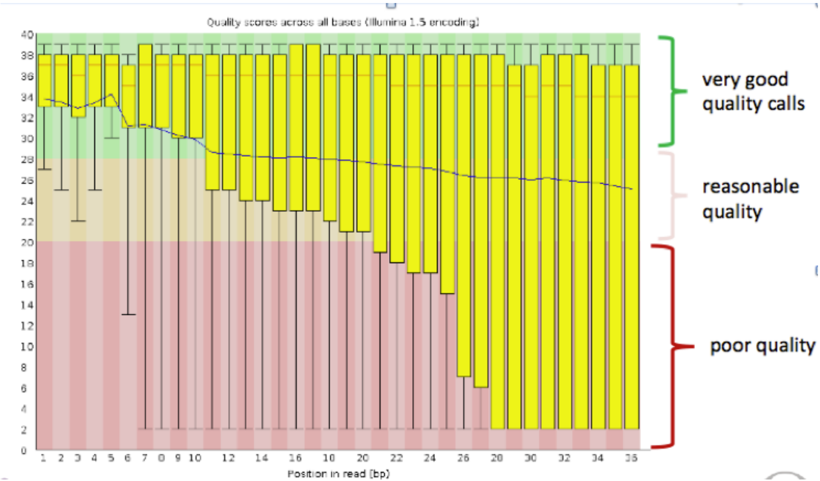
✓ Basic Statistics

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 5

Example sections of a `fastqc` report

2. Per-base sequence quality



very good quality calls

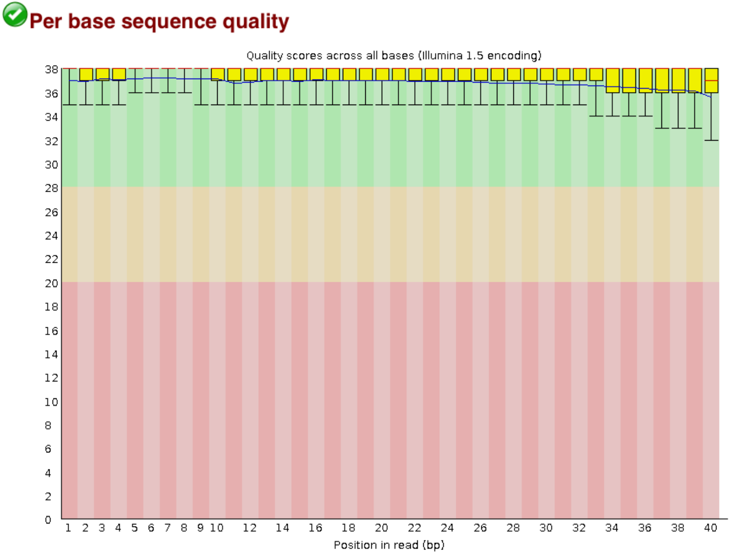
reasonable quality

poor quality

Figure 6

Example sections of a `fastqc` report

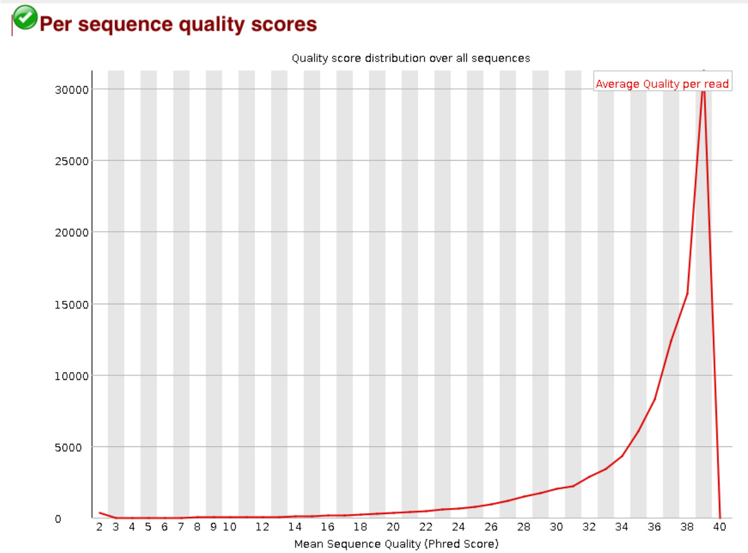
Ideally, the plot should look *something* like following:-



✓ Per base sequence quality

Example sections of a fastqc report

3. Per-sequence quality scores



✓ Per sequence quality scores

Exercise

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

The SAM / BAM format

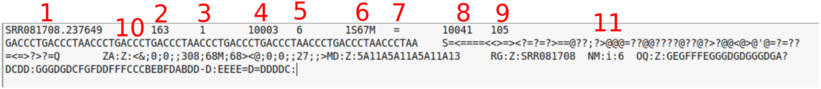
- we don't really spend much time look at *fastq* files
- most of our time is spent with *aligned* reads
  - i.e. we have used some software to tell us whereabouts in the genome each read belongs to
  - we will have a go at this in the practical

The `.sam` file

- **S**equence **A**lignment/**M**ap (sam)
- The output from an aligner such as `bwa`
- Same format regardless of sequencing protocol (i.e. RNA-seq, ChIP-seq, DNA-seq etc)
- May contain un-mapped reads
- Potentially large size on disk; ∼100s of Gb
- 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.



```
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:
```

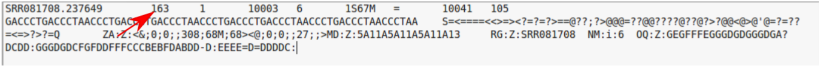
Figure 9

- 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 10

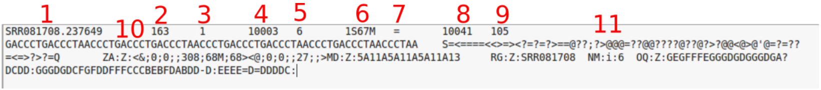
Derivation

	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

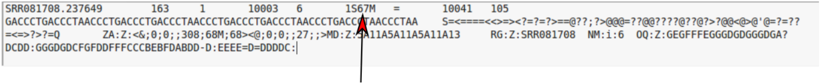


```
SRR081708.237649          163     1        10003   6       1S67M    =         10041   105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13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

Figure 11

- 4:- Start Position
- 5:- Mapping Quality; Confidence that an alignment is correct
- 6:- CIGAR; Describes positions of matches, insertions, deletions w.r.t reference

Have a CIGAR!



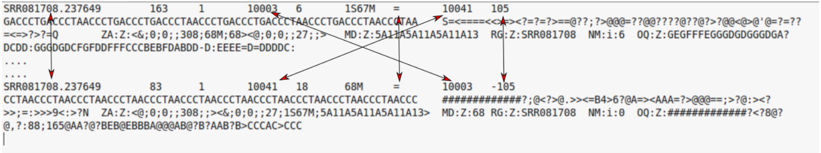
```
SRR081708.237649        163     1       10003   6       1S67M   =       10041   105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>MD:Z:5A11A5A11A5A11A13     RG:Z:SRR081708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

Figure 12

The ***CIGAR*** (**C**ompact **I**diosyncratic **G**apped **Alignment** **R**eport) string is a way of encoding the match between a given sequence and the position it has been assigned in the genome. It is comprised by a series of letters and numbers to indicate how many consecutive bases have that mapping.

- `68M`
  - 68 bases matching the reference
- `1S67M`
  - 1 soft-clipped read followed by 67 matches
- `15M87N70M90N16M`
  - 15 matches following by 87 bases skipped followed by 70 matches etc.

The .sam file

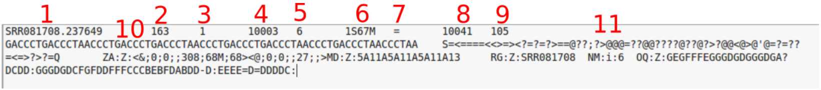


```
SRR081708.237649        163     1       10003   6       1S67M   =       10041   105
GACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTGACCCTAACCCTGACCCTAACCCTAA    S=<====<<>=><?=?=?>==@??;?>@@@=??@@????@??@?>?@@<@>@'@=?=??
=<=>?>?=Q       ZA:Z:<&;0;0;;308;68M;68><@;0;0;;27;;>   MD:Z:5A11A5A11A5A11A13  RG:Z:SRR081708  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 13

7, 8, 9:- Alignment information for the paired read

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:
```

Figure 14

- 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