Train MALTA Horizon 2020

**HTS Summer School**
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# DATA FORMATS AND QUALITY CONTROL

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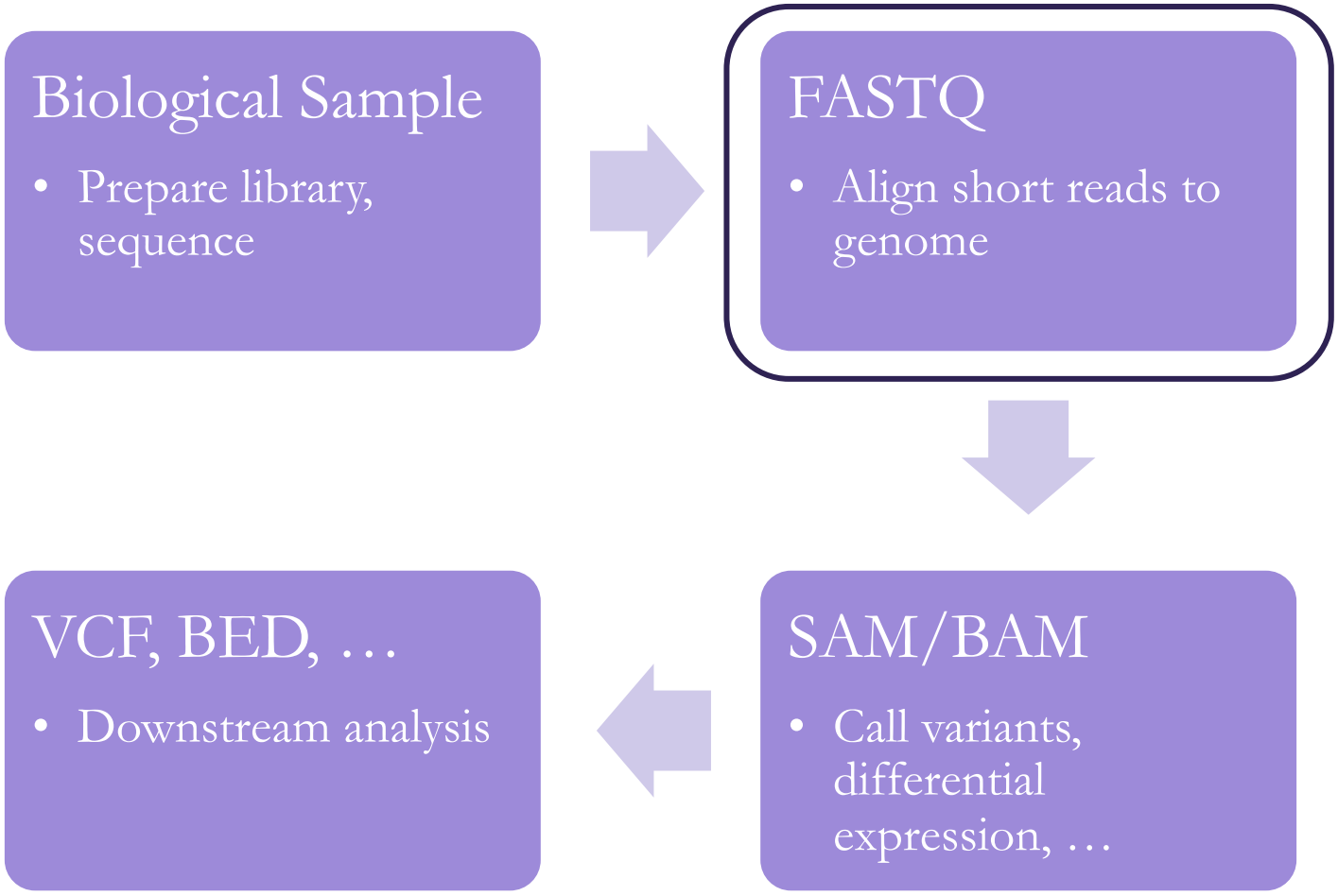
# HTS analysis workflow





# From the sequencer to you



- Sequencing is usually done by core facilities
- Each sequencing run will generate millions of short (~100 bp) reads
  - + read quality score for each base
- They often perform initial processing
  - Adaptor trimming
  - Basic quality control
  - Demultiplexing
- You will (usually) receive a FASTQ file

# FASTQ Files



- FASTQ = FASTA + Quality

- So what is FASTA?

# FASTA Format (.fa, .fasta)



```
>CHROMOSOME_1
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTC
AACTCACAGTTTGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATT
…
>CHROMOSOME_2
TCACAGTTTGGTTCAAAGCAGTATCGATCATATCGATCAAATAGTAAA
…
```

- Format for raw DNA/protein sequences
- For each sequence:
  1. > NAME
  2. Nucleotides, with line breaks every ~60 bp

# FASTQ format (.fq, .fastq)



```
@HWI-ST169:285:C0PPAACXX:1:1101:1241:1913
NTGCGGTCAAAAAGATCCTAAGCAGACAATTTCAAACCGGAACTCGTACAACTGAAACTGATACAAATAA
+
#11=BDD??CDFFGFFIFEHEEGEFIIIIICFBCGEEGIIFFEIECCCFF@FEIEFEFFFFFFDDBDBBB
@HWI-ST169:285:C0PPAACXX:1:1101:1064:1942
NTGCGGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
+
#1:DDFDDHBHHHFI><DBAGBFDDDDDDBB@B661@6BBDBDB8559BDB8BDD@B@;7<B@B>BDDB@
```

- Format for DNA sequencing reads
- For each read:
  1. @ Read ID
  2. Nucleotide sequence of the read
  3. +
  4. Quality score for each nucleotide of the read

# Illumina sequence identifiers



| | |
|---|---|
| **EAS139** | the unique instrument name |
| **136** | the run id |
| **FC706VJ** | the flowcell id |
| **2** | flowcell lane |
| **2104** | tile number within the flowcell lane |
| **15343** | 'x'-coordinate of the cluster within the tile |
| **197393** | 'y'-coordinate of the cluster within the tile |
| **1** | the member of a pair, 1 or 2 *(paired-end or mate-pair reads only)* |
| **Y** | Y if the read fails filter (read is bad), N otherwise |
| **18** | 0 when none of the control bits are on, otherwise it is an even number |

# Quality Scores



- $Q_{\text{Sanger}} = -10 \log_{10} p$

  p = probability that the base call is wrong

  - p = 0.1 → Q = 10
  - p = 0.01 → Q = 20
  - P = 0.001 → Q = 30

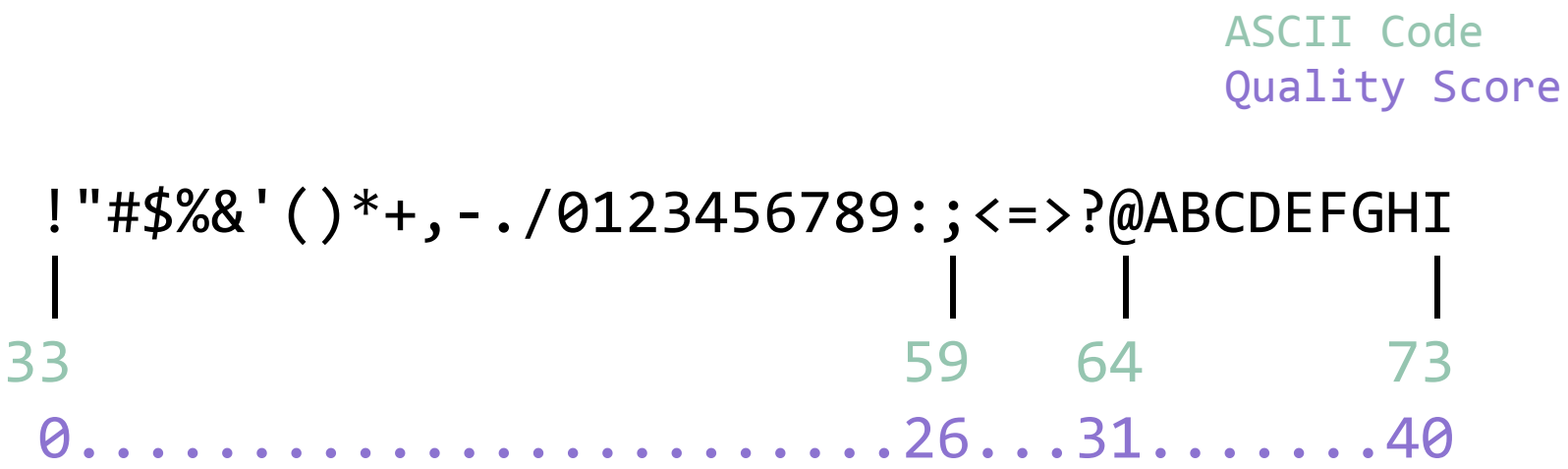
- Encoding:
  - The Sanger Phred format can encode a quality score from 0 to 93 using ASCII 33 to 126:

33 ↓ 126 ↘

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

# Quality Score Encoding






- Each character has an associated ASCII Code
- Quality Score = ASCII Code − Offset
- Normal Sanger Encoding is Phred + 33
  - Lowest: "!" = ASCII 33 = Quality 0
  - Highest: "I" = ASCII 73 = Quality 40

# Quality Encoding Example



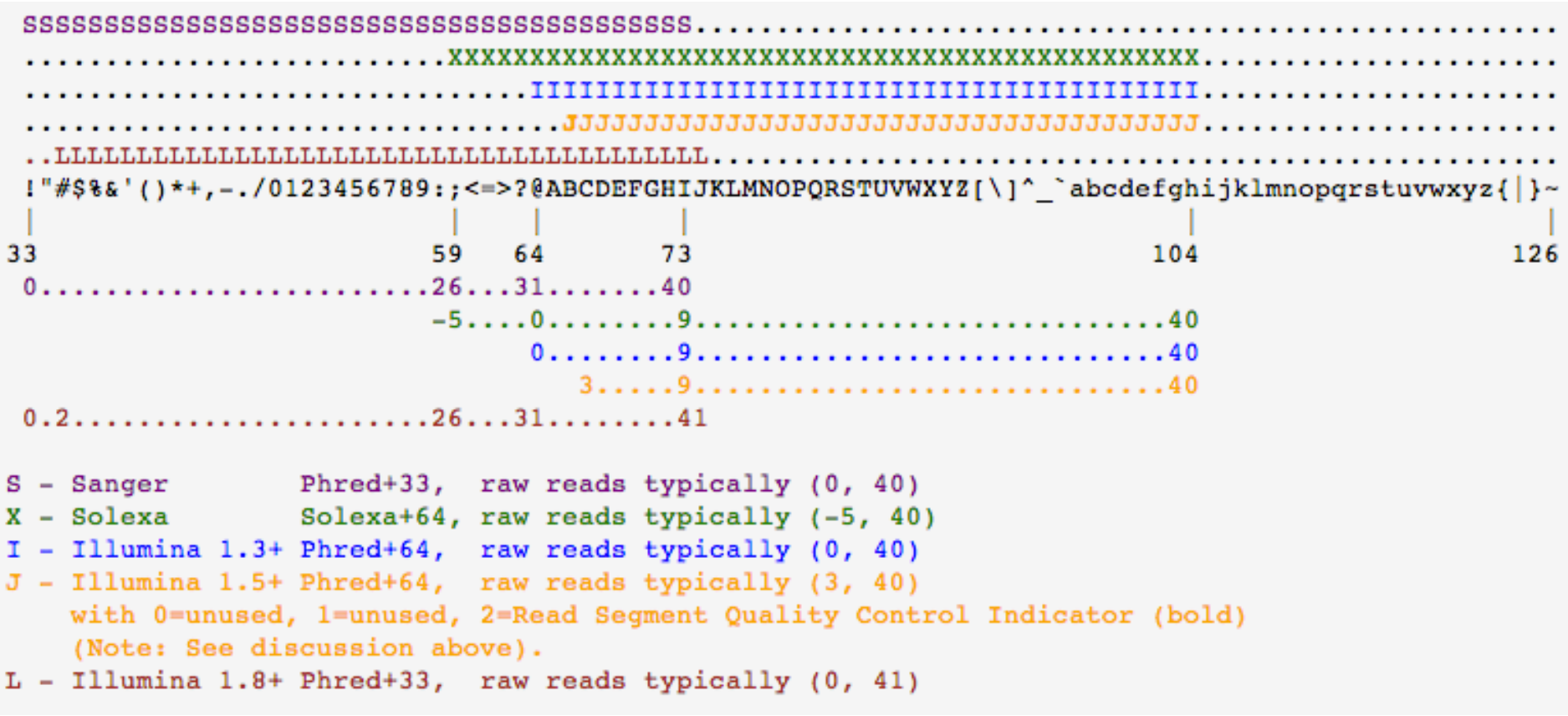
```
@HWI-ST169:285:C0PPAACXX:1:1101:1241:1913
NTGCGGTCAAAAAGATCCTAAGCAGACAATTTCAAACCGGAACTCGTACAACTGAAACTGATACAAATAA
+
#11=BDD??CDFFGFFIFEHEEGEFIIIIICFBCGEEGIIFFEIECCCFF@FEIEFEFFFFFDDBDBBB
@HWI-ST169:285:C0PPAACXX:1:1101:1064:1942
NTGCGGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
+
#1:DDFDDHBHHHFI><DBAGBFDDDDDDBB@B661@6BBDBDB8559BDB8BDD@B@;7<B@B>BDDB@
```

```
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
  |      |    |    |    |    |    |      |  |
 33      40   45   50   55   59   64     70 73
  0......7........17.......26...31.......40
```

# Different Quality Encodings



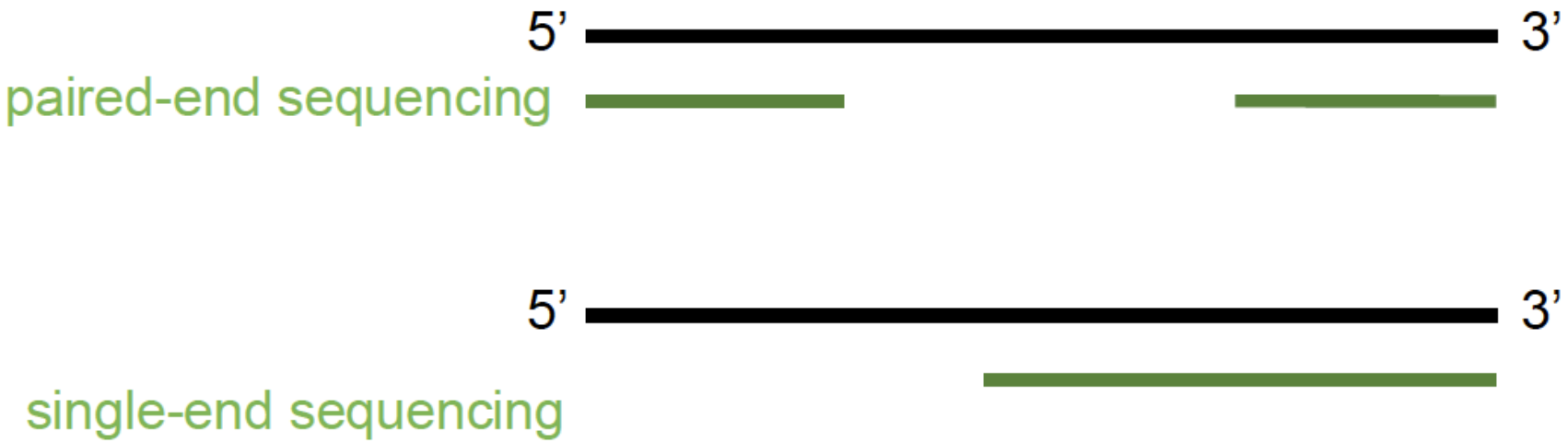
- Beware of different versions! (especially for old data)



# Paired-end reads

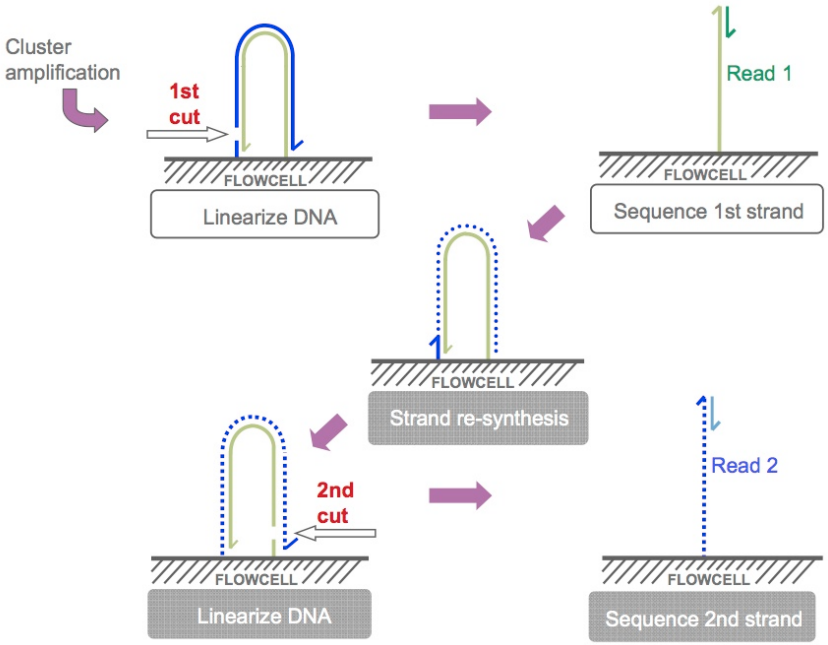


- Sequence both the 5' and 3' end of a fragment
- Will result in two FASTQ files for a single run
- $file1_n \leftrightarrow file2_n$



# single-end vs paired-end reads






(from the Illumina website)

SE

**my_sequence.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1466:1018#0/1
AAGGAAGTGCTTGTCTGGCTAACACAGCNAGNCACGTGAC
+
aVfbe`^^^_TTTSSdfffffdfffabbZbbfebafbbbbb
```

PE

**my_sequence_1.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/1
NAAATTTCGAATTTCTGTGAAGTAAGCATCTTCTTTGTCAT
+
BJJGGKIINN^^^^^QQNTUQOOTTTRTOTY^^Y^\\^^^\
```

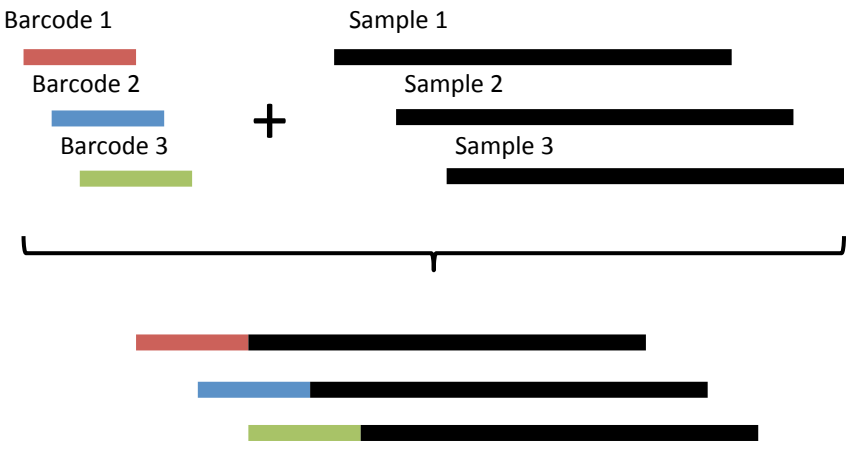
**my_sequence_2.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/2
AACCCACACAGGAGAGCAGCCTTACAGATGCAAATACTGTG
+
]K___fffffggghgegggggdgggggfgggggeggggghh
```

# Sample barcoding and de-multiplexing



## Barcoding




## De-multiplexing

- # Read 1
  - ATTAGACCTAAGCA
- # Read 2
  - GAGCAACGACTAC
- # Read 3
  - ATTAGGCCATACAT
- # Read 4
  - CCATAGGCTGACTA
- ...

# Common sequence artefacts in HTS data



- Read errors
  - Base calling errors
  - Small insertions and deletions
- Poor quality reads
- Primer / adapter contamination

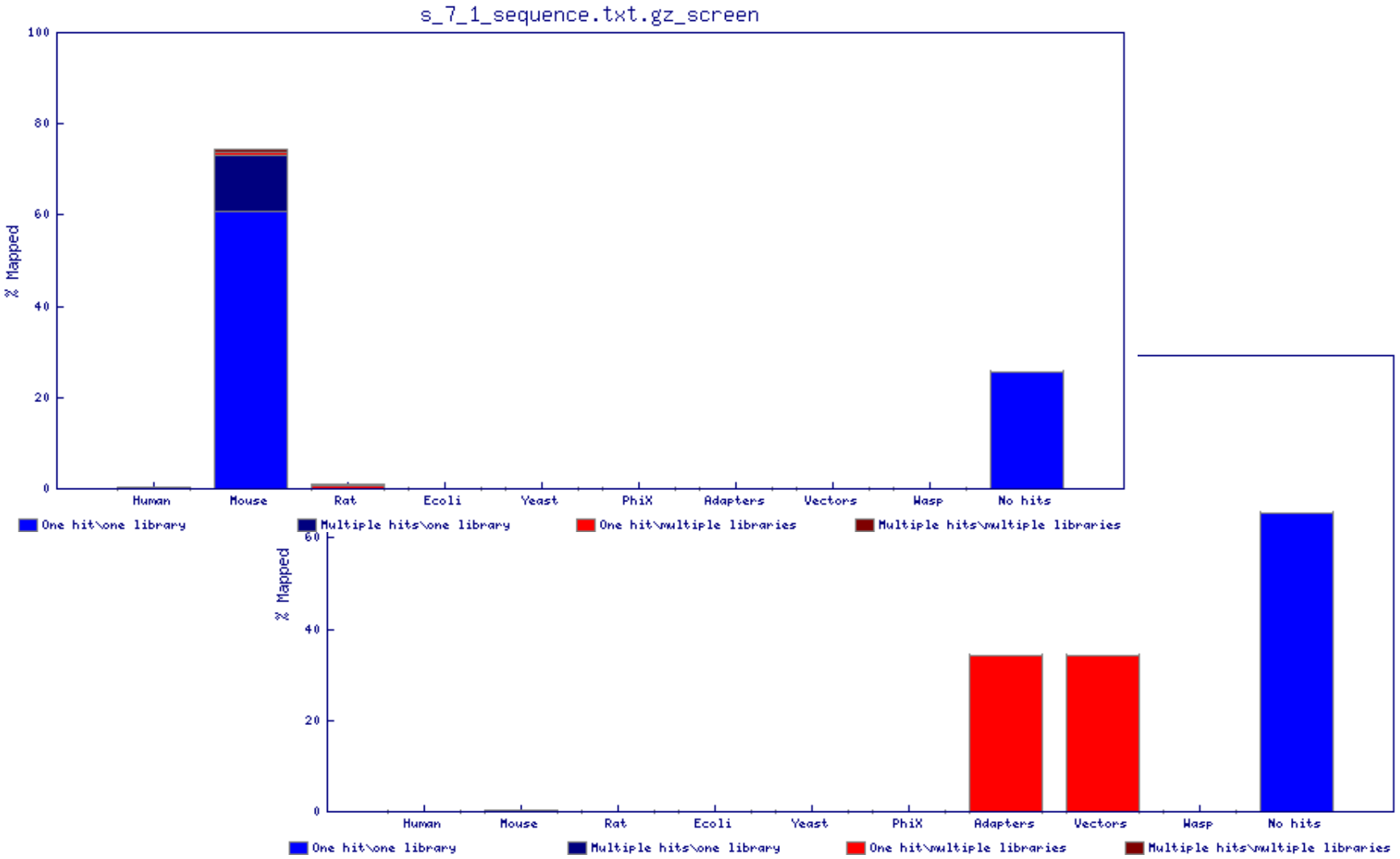
# Features across available QC tools



| Feature\Tools | NGS QC Toolkit v2.2 | FastQC v0.10.0 | PRINSEQ-lite v0.17[1] | TagDust | FASTX-Toolkit v0.0.13 | SolexaQA v1.10 | TagCleaner v0.12[1] | CANGS v1.1 |
|---|---|---|---|---|---|---|---|---|
| Supported NGS platforms | Illumina, 454 | FASTQ[2] | Illumina, 454 | Illumina, 454 | Illumina | Illumina | Illumina, 454 | 454 |
| Parallelization | Yes | Yes | No | No | No | No | No | No |
| Detection of FASTQ variants | Yes | Yes | Yes | No | No | Yes | No | No |
| Primer/Adaptor removal | Yes | No[3] | No | Yes | Yes | No | Yes[4] | Yes |
| Homopolymer trimming (Roche 454 data) | Yes | No | No | No | No | No | No | Yes |
| Paired-end data integrity | Yes | No | No | No | No | No | No | No |
| QC of 454 paired-end reads | Yes | No | No | No | No | No | No | No |
| Sequence duplication filtering | No | No[5] | Yes | No | Yes | No | No | Yes |
| Low complexity filtering | No | No | Yes | No | Yes | No | No | No |
| N/X content filtering | No | No[6] | Yes | No | Yes | No | No | Yes |
| Compatability with compressed input data file | Yes | Yes | No | No | No | No | No | No |
| GC content calculation | Yes | Yes | Yes | No | No | No | No | No |
| File format conversion | Yes | No | No | No | No | No | No | No |
| Export HQ and/or filtered reads | Yes | No | Yes | Yes | Yes | No | Yes | Yes |
| Graphical output of QC statistics | Yes | Yes | No[7] | No | Yes | Yes | No[7] | No |
| Dependencies | Perl modules: Parallel::ForkManager, String::Approx, GD::Graph (optional) | - | - | - | Perl module: GD::Graph | R, matrix2png | - | BLAST, NCBI nr database |

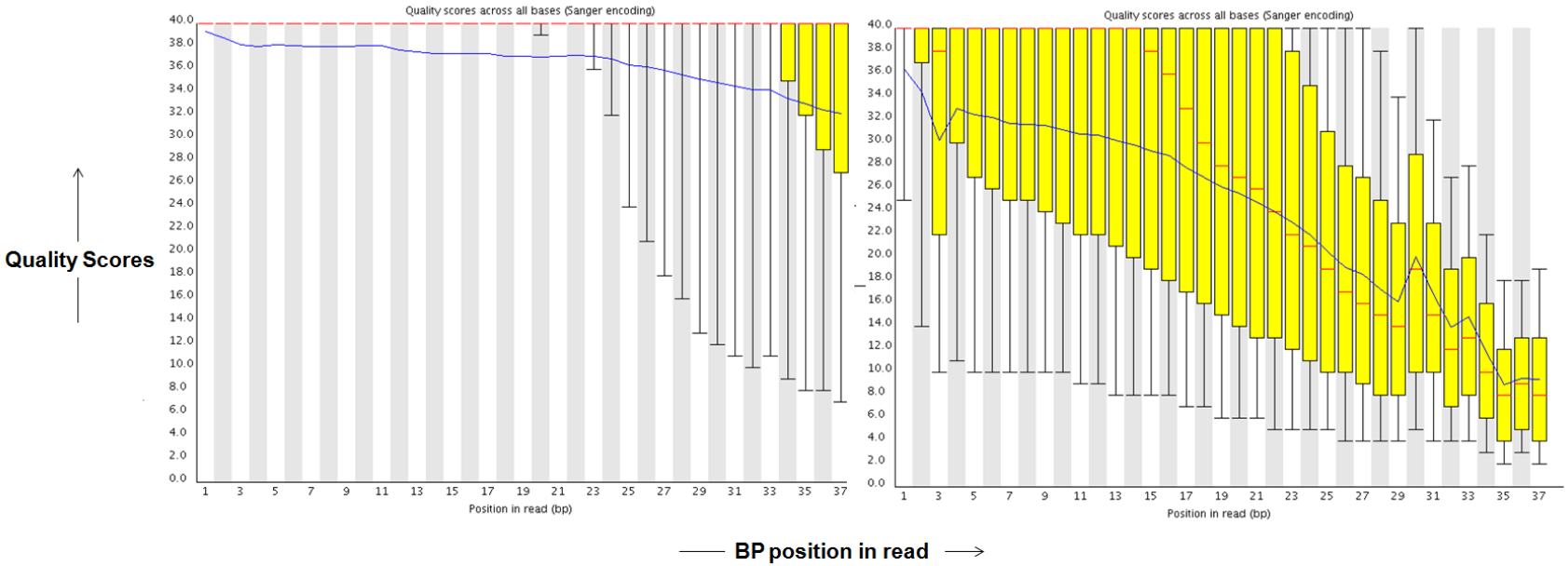
# FastqScreen: contamination





# FastQC: Per base sequence quality





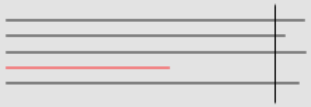

| | |
|---|---|
| **Function** | A quality control tool for high throughput sequence data. |
| **Language** | Java |
| **Requirements** | A suitable Java Runtime Environment<br>The Picard BAM/SAM Libraries (included in download) |
| **Code Maturity** | Stable. Mature code, but feedback is appreciated. |
| **Code Released** | Yes, under GPL v3 or later. |
| **Initial Contact** | Simon Andrews |
| | **Download Now** |


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

# Quality trimming



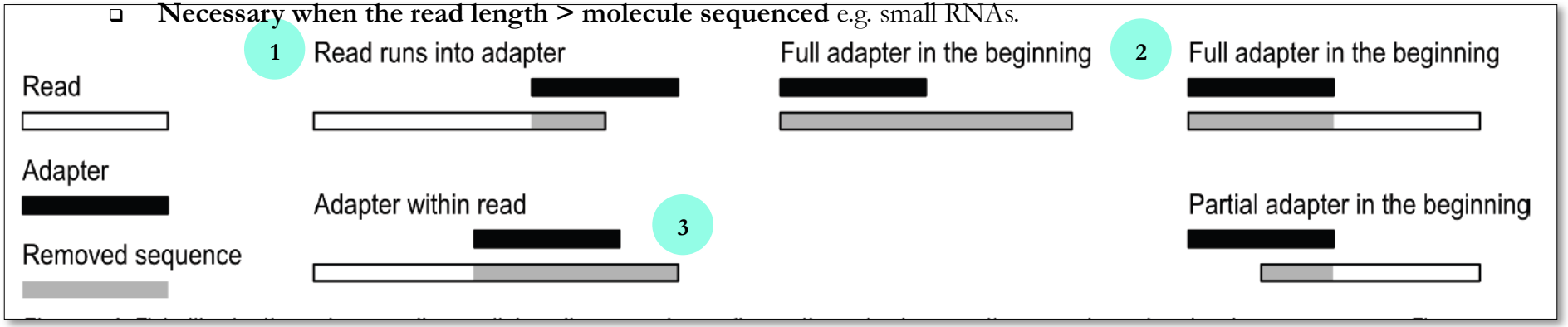
- Fixed length trimming
  - Cut-off at position x
- Adaptive trimming
  - Quality score cut-off
  - Minimum sequence length



# Removal of adapter sequences



- **Necessary when the read length > molecule sequenced** e.g. small RNAs.




- **Different scenarios requiring adapter removal**
  1. Trim the 3' end
  2. Trim/discard the reads based on the residual minimum read length.
  3. Trim the adapter region but retain reads only with a minimum read-length.
- **Tools for adapter trimming**
  - fastx_clipper (FastX-Toolkit), PRINSEQ

# How to filter?



- Fastx:
  - http://hannonlab.cshl.edu/fastx_toolkit/
- PRINSEQ:
  - http://prinseq.sourceforge.net/
- Tally and Reaper:
  - http://www.ebi.ac.uk/~stijn/reaper/tally.html
  - http://www.ebi.ac.uk/~stijn/reaper/reaper.html#recipe
  - http://www.ebi.ac.uk/~stijn/reaper/src/reaper-12-048/
- ShortRead (R):
  - http://www.bioconductor.org/packages/release/bioc/html/ShortRead.html

# FASTQ Processing – FASTX Toolkit

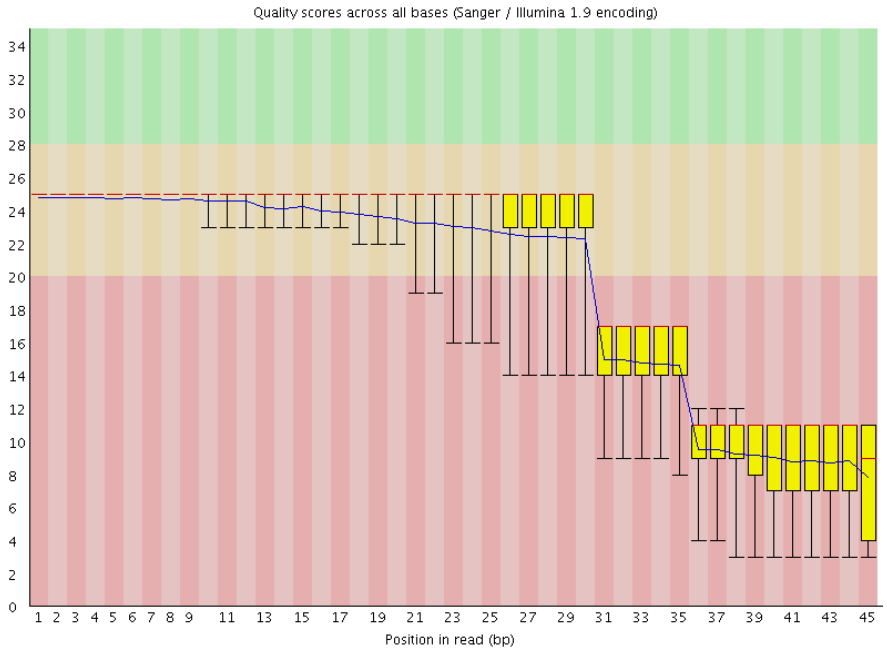


- http://hannonlab.cshl.edu/fastx_toolkit/
- Many tools for common operations on FASTQ files:
  - Conversion
  - Trimming (remove barcodes)
  - Clipping (remove adapters)
  - Quality trimmer (trim off low-quality bases)
  - Quality filter (remove low-quality reads)

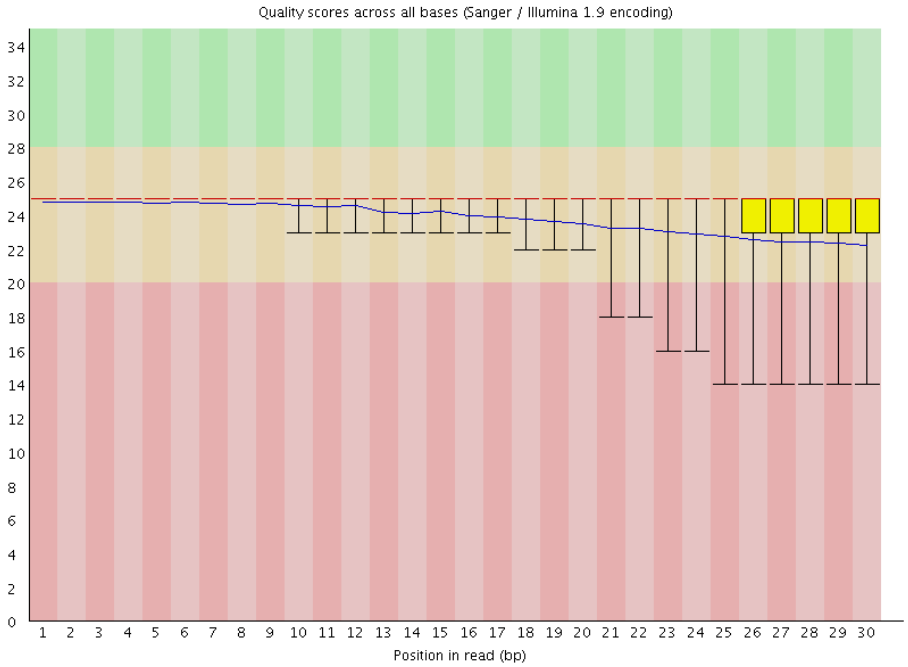
# Filtering example



## Before




## After



# Filtering comes at a price



| Measure | Value |
|---|---|
| Filename | SRR031709.fastq |
| File type | Conventional base calls |
| Encoding | Sanger / Illumina 1.9 |
| Total Sequences | 3812809 |
| Filtered Sequences | 0 |
| Sequence length | 45 |
| %GC | 49 |

| Measure | Value |
|---|---|
| Filename | SRR031709_filt1.fastq |
| File type | Conventional base calls |
| Encoding | Sanger / Illumina 1.9 |
| Total Sequences | 3668330 |
| Filtered Sequences | 0 |
| Sequence length | 30 |
| %GC | 52 |

# Important: PE files



**my_sequence_1.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/1
NAAATTTCGAATTTCTGTGAAGTAAGCATCTTCTTTGTCAT
+
BJJGGKIINN^^^^^QQNTUQOOTTTRTOTY^^Y^\\^^^\
```

**my_sequence_2.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/2
AACCCACACAGGAGAGCAGCCTTACAGATGCAAATACTGTG
+
]K___fffffggghegggggggdgggggfgggggeggggghh
```

# Important: PE files



**my_sequence_1.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/1
NAAATTTCGAATTTCTGTGAAGTAAGCATCTTCTTTGTCAT
+
BJJGGKIINN^^^^^QQNTUQOOTTTRTOTY^^Y^\\^^^\
```

**my_sequence_2.fastq**

```
@HWI-BRUNOP16X_0001:1:1:1278:989#0/2
AACCCACACAGGAGAGCAGCCTTACAGATGCAAATACTGTG
+
]K___fffffggghegggggggdgggggfgggggeggggghh
```


How?

- Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)

# Conclusions



- Quality control of sequencing data is essential for downstream analysis.
- A range of QC tools are available to remove noise.
- Decide on which data can be corrected and discard the rest.