Generating & Assessing FASTQC Outputs

Dr. Princess Rodriguez

2026-02-09

Learning Objectives:

- Describe the contents and format of a FASTQ file
- Create a quality report using FASTQC
- Evaluate the quality of your NGS data using FastQC

Recap: Advantages of Batch Job Submissions

Batch job submission on an HPC (High-Performance Computing) system offers several advantages, particularly for computationally intensive tasks like bioinformatics, genomics, and large-scale data analysis.

1. **Efficient Resource Management:**
   - Jobs are queued and scheduled based on resource availability, ensuring optimal utilization of CPUs, memory, and GPUs.
   - Users can specify resource requirements (e.g., nodes, cores, memory) to avoid wasting computational power.

Recap: Advantages of Batch Job Submissions

2. **Scalability:**
   - HPC clusters handle jobs of varying sizes, from single-threaded processes to massively parallel workloads.
   - Batch processing supports running multiple jobs concurrently, improving overall throughput.

Recap: Advantages of Batch Job Submissions

3. **Parallel Execution:**
   - Batch submission allows running thousands of jobs in parallel (e.g., processing multiple sequencing samples).
4. **Job Monitoring:**
   - Provides insights into job status, resource usage, and debugging.

Looking inside of `sra_fqdump.sh`

Purpose: Is to download FASTQ files from the SRA. FASTQ files to be downloaded from the GEO are listed in a text file as accession numbers (provided by user).

```
nano sra_fqdump.sh
```

```
#!/bin/bash
#SBATCH --partition=bluemoon
#SBATCH --nodes=1
#SBATCH --ntasks=2
#SBATCH --mem=50G
#SBATCH --time=30:00:00
#SBATCH --job-name=fastq
# %x=job-name %j=jobid
#SBATCH --output=%x_%j.out

#while there are lines in the list of SRRs file
while read p
do
#call the bash script that does the fastq dump, passing it the SRR number next $
sbatch inner_script.sh $p
done <list_of_SRRs.txt
```

To submit a script, use the command:

```
sbatch your-script.sh
```

When you submit your job, Slurm will respond with the job ID. For example, where the job ID Slurm assigns is "123456," Slurm will respond:

```
Submitted batch job 123456
```

After submitting this script you will see `.out` files:

```
[pdrodrig@vacc-user1 GSE164713_Tcf1]$ ls
fastq_6008331.out  fastq_6426350.out     SRR13422709.fastq.gz
fastq_6366635.out  fastq_6426351.out     SRR13422710.fastq.gz
fastq_6366636.out  inner_script.sh       SRR13422711.fastq.gz
fastq_6366637.out  list_of_SRRs.txt      SRR13422712.fastq.gz
fastq_6366638.out  list.txt              SRR13422713.fastq.gz
fastq_6366639.out  sra_download.sh       SRR13423162.fastq.gz
fastq_6366640.out  sra_fqdump.sh         SRR13423163.fastq.gz
fastq_6366641.out  SRR13416485.fastq.gz  SRR13423164.fastq.gz
fastq_6426340.out  SRR13416486.fastq.gz  SRR13423165.fastq.gz
fastq_6426341.out  SRR13422702.fastq.gz  SRR13423166.fastq.gz
fastq_6426342.out  SRR13422703.fastq.gz  SRR13423167.fastq.gz
fastq_6426343.out  SRR13422704.fastq.gz  SRR17379677.fastq.gz
fastq_6426344.out  SRR13422705.fastq.gz  SRR17379678.fastq.gz
fastq_6426347.out  SRR13422706.fastq.gz  SRR17379679.fastq.gz
fastq_6426348.out  SRR13422707.fastq.gz  SRR17379680.fastq.gz
fastq_6426349.out  SRR13422708.fastq.gz
```

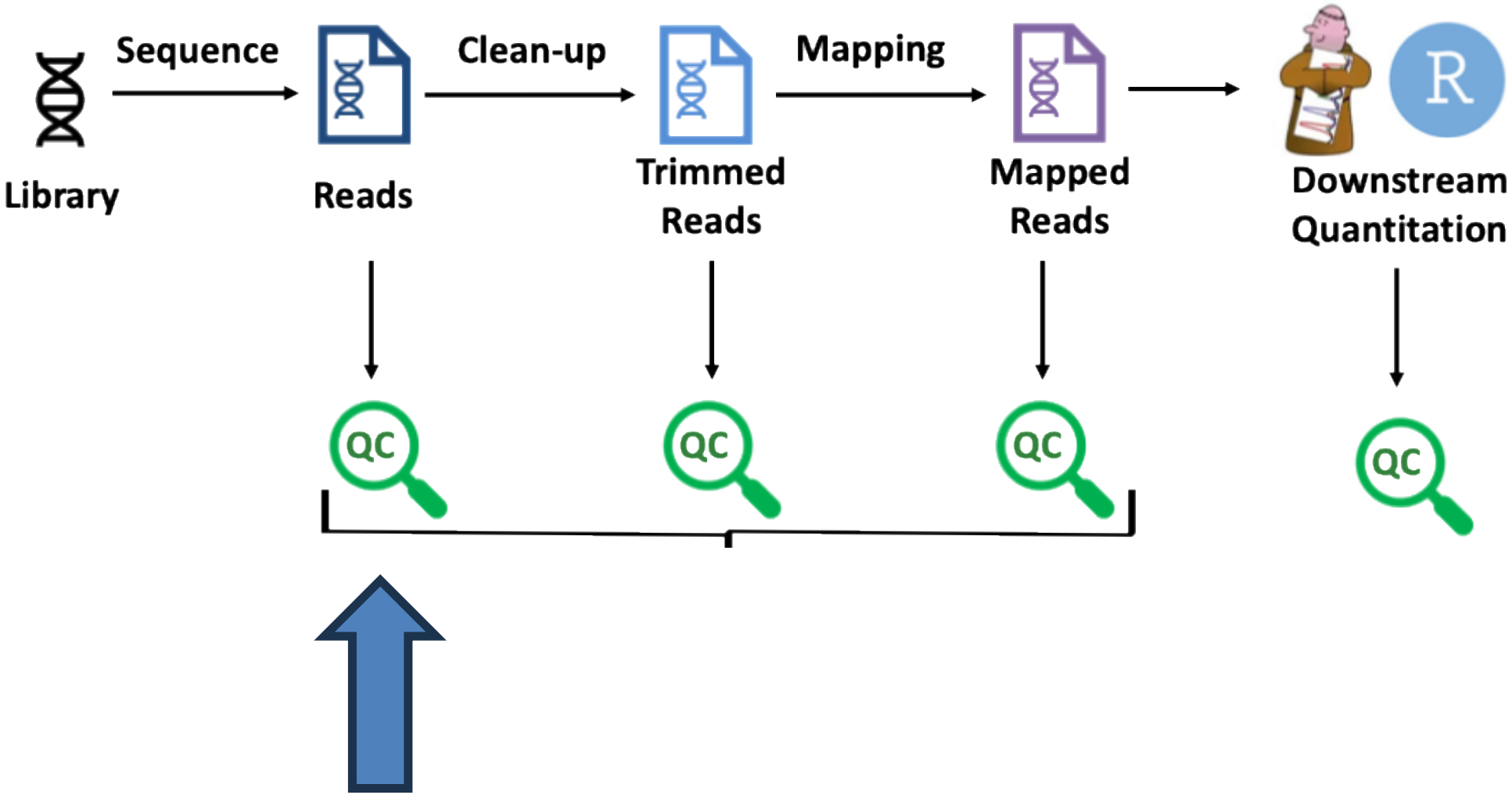
STDOUT records the output of programs

Three data streams exist for all Linux programs:

- STDIN (Standard Input - a way to send data into the program)
- STDOUT (Standard Output - a way to send expected data out of the program)
- STDERR (Standard Error - a way to send errors or warnings out of the program)

Next Step in Processing Sequencing Data

Processing Sequencing Data



What is the Point of QC?

Technical Problems …

- Don't always cause pipelines to fail
- Don't prevent hits being generated
- These hits can look biologically real

Real biology…

- Can cause unexpected, interesting behaviour of data

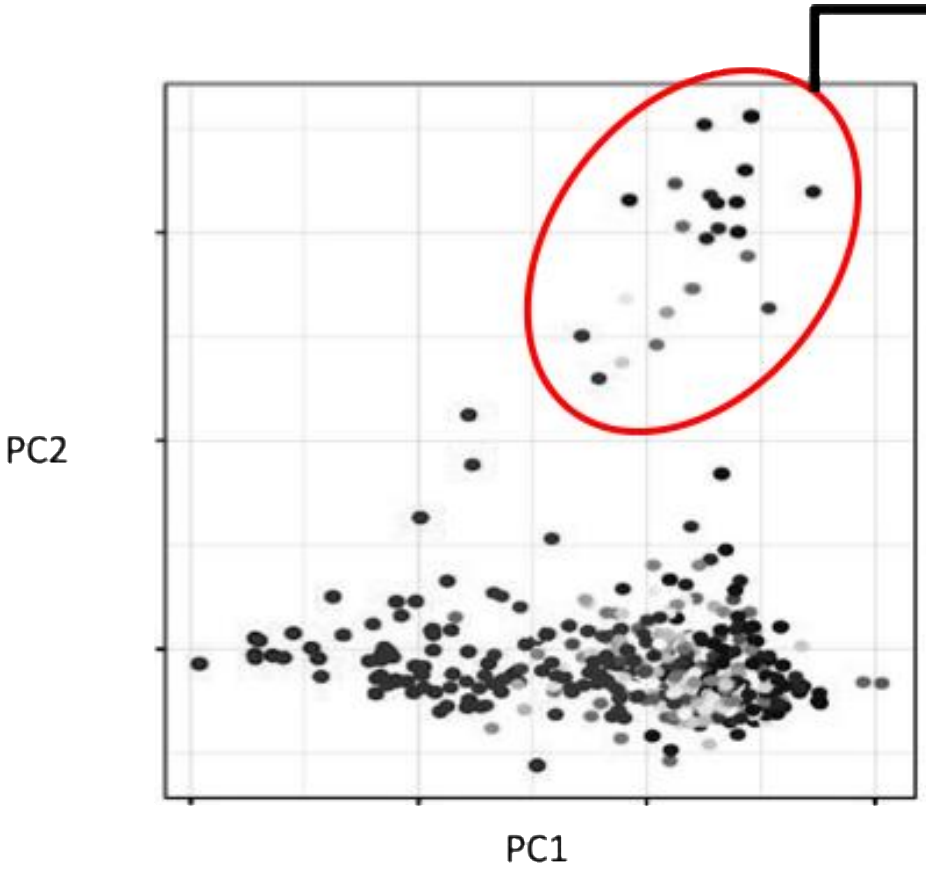
Set Pipelines can miss things….

QC Saves Time, Effort and Money!!

- Better to know asap what you're dealing with
- Want to be sure any follow-up work will be worth it

Babraham Bioinformatics

What is the Point of QC? An Example…



PC2 Genes (85 total)

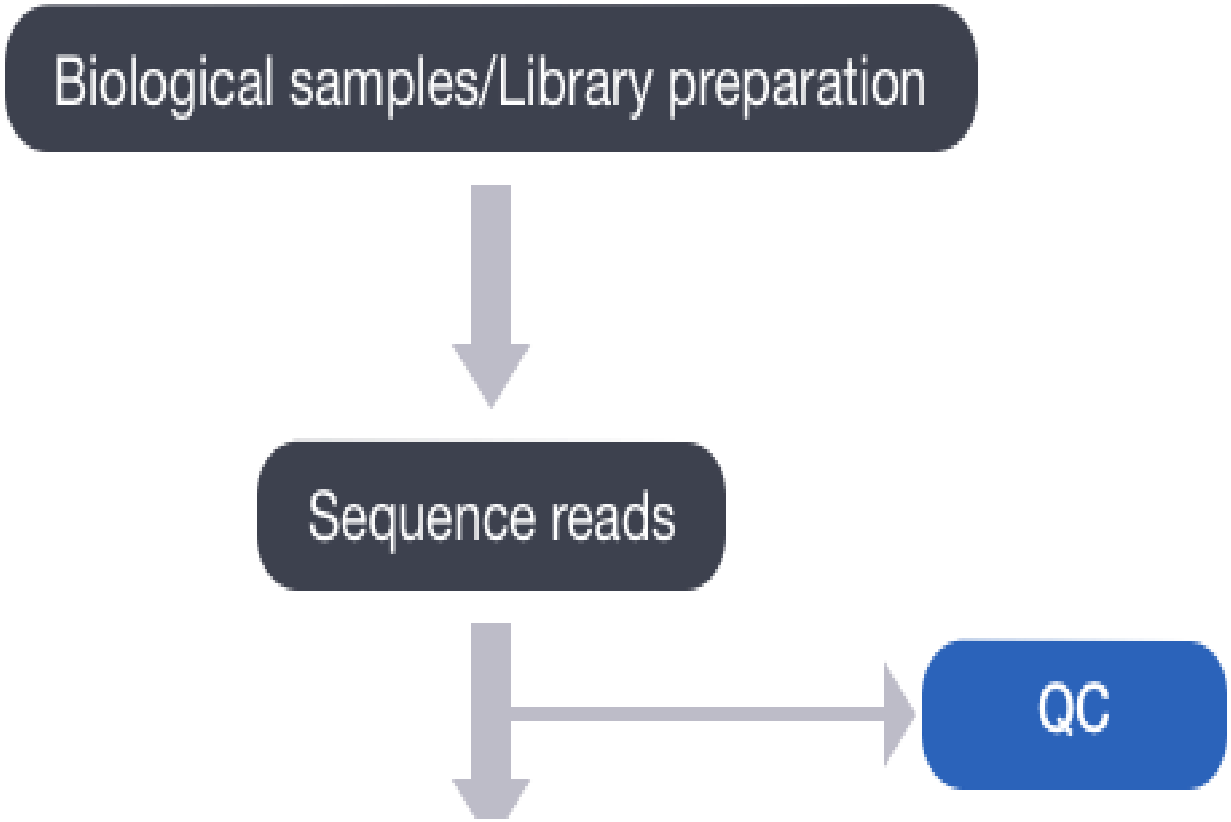
- No clear biological theme
- No clear connection to system

What is going on?

Babraham Bioinformatics

Quality Control of FASTQ files

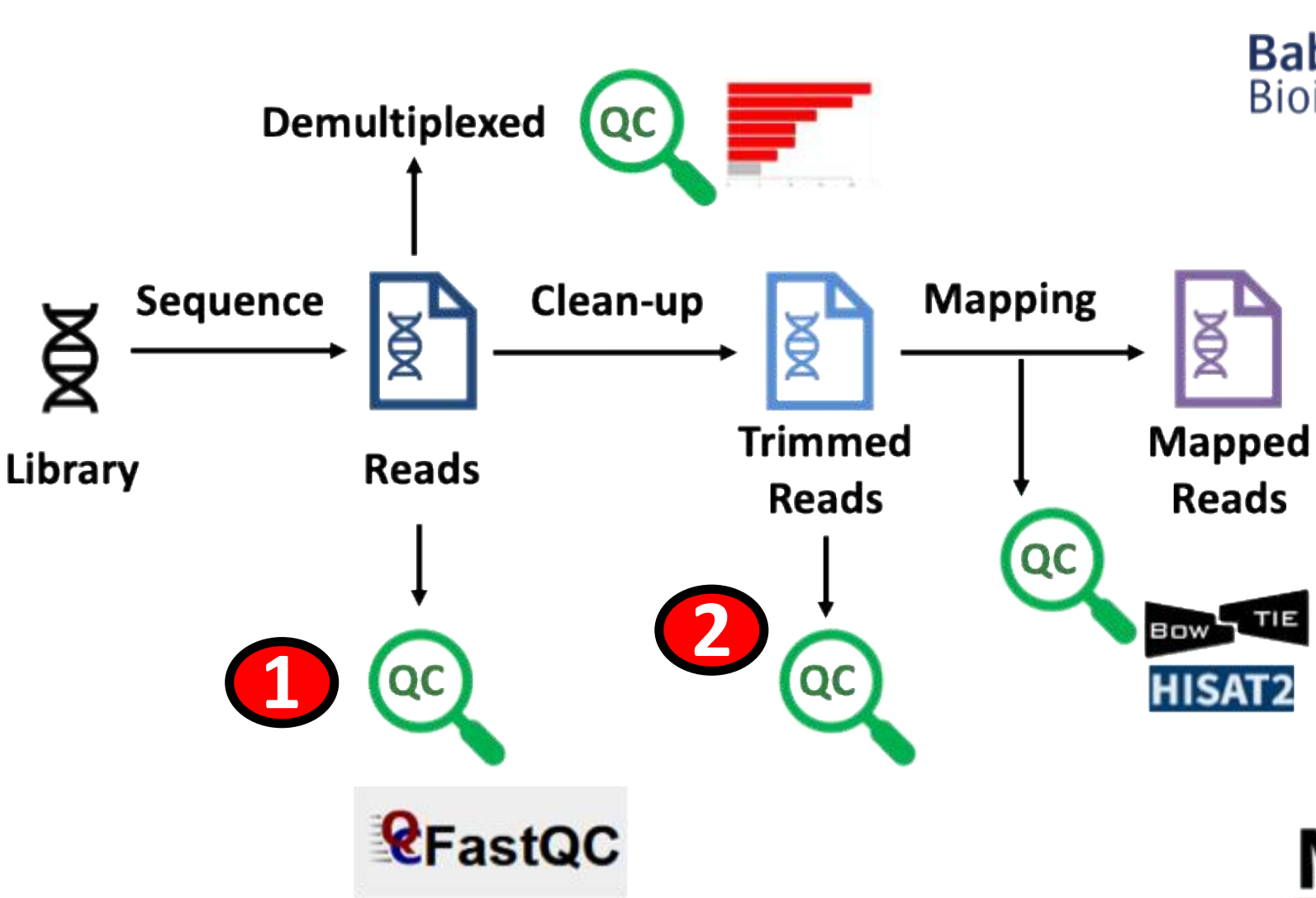
The first step in the bioinformatic pipeline is to assess the quality of the sequence reads retrieved from the sequencing facility.



In a Genomics Workflow, QC helps us answer:

1) Did sequencing work as expected?
2) Are there systematic technical artifacts?
3) Do we need trimming, filtering, or adapter removal?
4) Are problems biological or technical?

QC Programs in Data Processing



FastQC

- FastQC provides a simple way to do some quality checks on raw sequence data coming from high throughput sequencing pipelines.
- It provides a modular set of analyses, which you can use to obtain an impression of whether your data has any problems that you should be aware of before moving on to the next analysis.

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

FastQC


```
fastqc seqfile1 seqfileN
fastqc *.fastq.gz
```

- Reads raw fastq file(s)
- Performs multiple checks
  - Pass/warn/fail
  - Compares to genomic library
- Generates a HTML Report

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

Babraham Bioinformatics

Interpreting the HTML report

- Within the report, a summary of all of the modules is given on the left-hand side.
- Do not take the **yellow "WARNING"s** and **red "FAIL"s as "*this sample is not usable*"**; they should be interpreted as flags!

Context is Key for QC

QC should be about what you expect and what you see

Babraham Bioinformatics

Input: Raw FASTQ files

Similar to FASTA, the FASTQ file begins with a header line. The difference is that the FASTQ header is denoted by a @ character.

Sequence 1

```
@HWUSI-EAS611:34:6669YAAXX:1:1:5069:1159 1:N:0:
TCGATAATACCGTTTTTTTCCGTTTGATGTTGATACCATT
+
IIHIIHIIIIIIIIIIIIIIIIIIIIIIIIHIIIIHIIIII
```

Sequence 2

```
@HWUSI-EAS611:34:6669YAAXX:1:1:5243:1158 1:N:0:
TATCTGTAGATTTCACAGACTCAAATGTAAATATGCAGAG
+
DF=DBD<BBFGGGGGGGBD@GGGD4@CA3CGG>DDD:D,B
```

Sequence 3

```
@HWUSI-EAS611:34:6669YAAXX:1:1:5266:1162 1:N:0:
GGAGGAAGTATCACTTCCTTGCCTGCCTCCTCTGGGGCCT
+
:GBGGGGGGGGGDGGDEDGGDGGGGDHHDHGHHGBGG:GG
```

Babraham Bioinformatics

Illumina Header Sections (Line 1)

```
@HWUSI-EAS611:34:6669YAAXX:5:1:5069:1159 1:N:0:
```

- Starts with @ (required by fastq spec)
- Instrument ID (HWUSI-EAS611)
- Run number (34)
- Flowcell ID (6669YAAXX)
- Lane (5)
- Tile (1)
- X-position (5069)
- Y-position (1159)
- [space]
- Read number (1)
- Was filtered (Y/N) (N) - You wouldn't normally see the Ys
- Control number (0 = no control)
- Sample number (only if demultiplexed using Illumina's software)

Babraham Bioinformatics

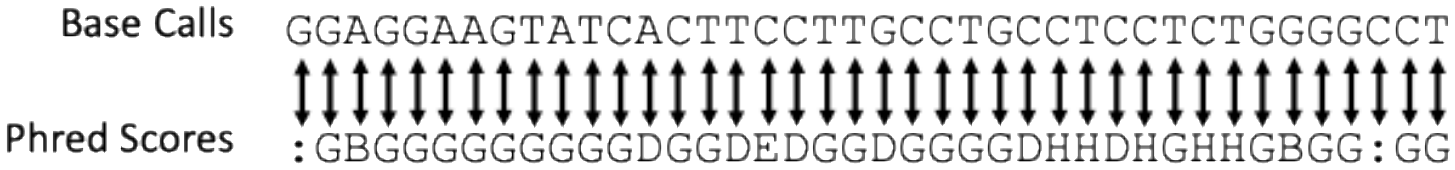
A Single FASTQ Entry

1. @HWUSI-EAS611:34:6669YAAXX:1:1:5266:1162 1:N:0:
2. GGAGGAAGTATCACTTCCTTGCCTGCCTCCTCTGGGGCCT
3. +
4. :GBGGGGGGGGGGDGGDEDGGDGGGGDHHDHGHHGBGG:GG

1. Header - starts with @
2. Base calls (can include N or IUPAC codes)
3. Mid-line - starts with + usually empty
4. Quality scores (= Phred Scores)

Babraham Bioinformatics

Phred Scores (Line 4)



- Each quality score represents the probability that the corresponding nucleotide call is incorrect.

_ This quality score is logarithmically based and is calculated as:

$Q = -10 \times \log10(P)$

- P is the probability that a base call is erroneous
  - Phred = -10 * (int)$\log_{10}(p)$
    - p=0.1 Phred = 10
    - p=0.01 Phred = 20
    - p=0.001 Phred = 30

Higher Phred Score
Higher Confidence

Phred Score Encoding

- Translation of Phred score to single ASCII letter
- Based on standard ASCII table

Different quality encoding scales exist, but the most commonly one used is fastqsanger, which is the scale output by Illumina since mid-2011.

0	NUL	17	C1	33	!	50	2	67	C
1	SOH	18	DC2	34	"	51	3	68	D
2	STX	19	DC3	35	#	52	4	69	E
3	ETX	20	DC4	36	$	53	5	70	F
4	EOT	21	NAK	37	%	54	6	71	G
5	ENQ	22	SYN	38	&	55	7	72	H
6	ACK	23	ETB	39	'	56	8	73	I
7	BEL	24	CAN	40	(	57	9	74	J
8	BS	25	EM	41	)	58	:	75	K
9	HT	26	SUB	42	*	59	;	76	L
10	LF	27	ESC	43	+	60	<	77	M
11	VT	28	FS	44	,	61	=	78	N
12	FF	29	GS	45	-	62	>	79	O
13	CR	30	RS	46	.	63	?	80	P
14	SO	31	US	47	/	64	@	81	Q
15	SI	32	(SPACE)	48	0	65	A	82	R
16	DLE			49	1	66	B	83	S

Babraham Bioinformatics

QC metrics we can work with:

Phred Scores 	**How the Sequencer Performed** • At different cycles • Across different locations • For different reads
Library Composition 	**The Nature of our Sequenced Reads** • Biases • Contaminants • Duplication
Mapping 	**Where our Sequencing Reads Come From** • Species (plural or singular!) • Region (repetitive or unique)

Babraham Bioinformatics

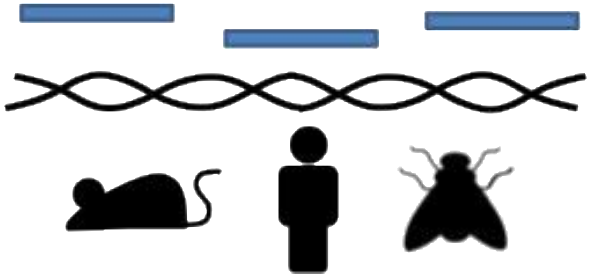
Universal QC Metrics

- Demultiplexing

- Base Call Quality

- Adapter Content

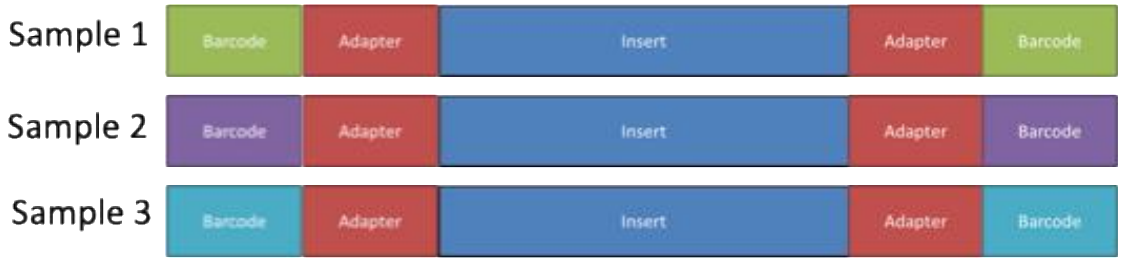
- Mapping Quality



Babraham Bioinformatics

Demultiplexing: Expectation

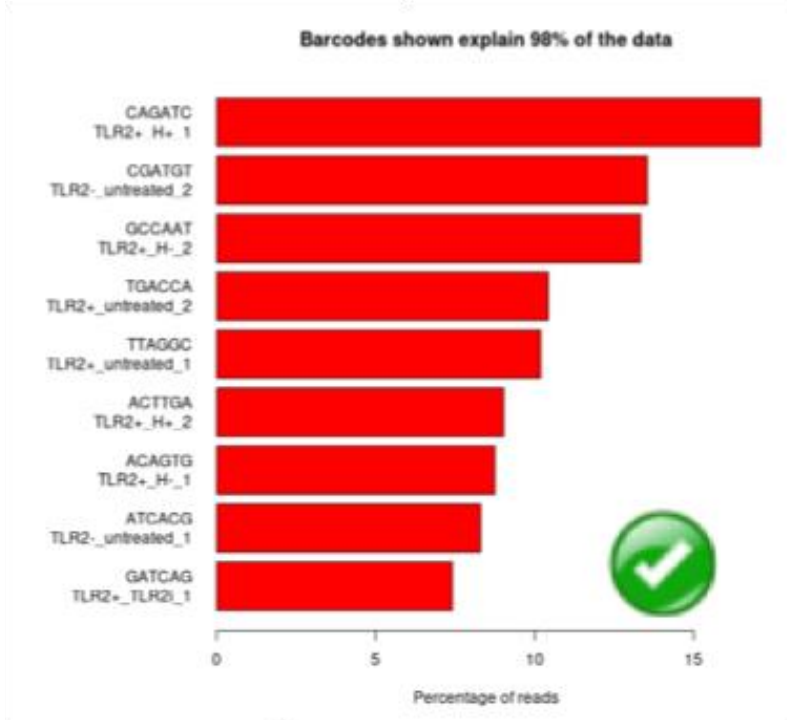
Only the barcodes we assigned to samples should be present—**no others**



ect…

What barcode sequences have we found?

Expected Barcode Unknown Sequence

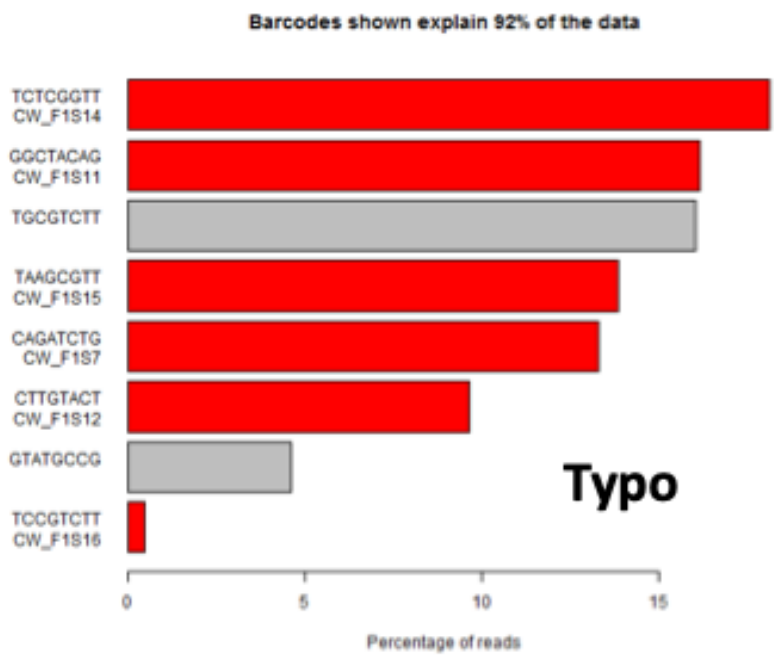


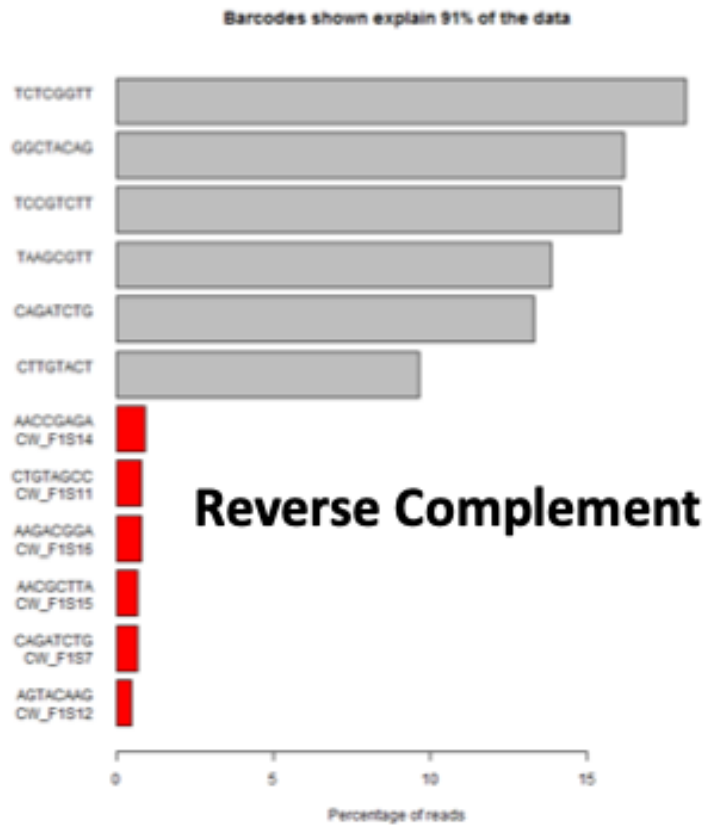
What could unknown barcode sequences mean?

Babraham Bioinformatics

Demultiplexing: Unknown

Expected Barcode Unknown Sequence

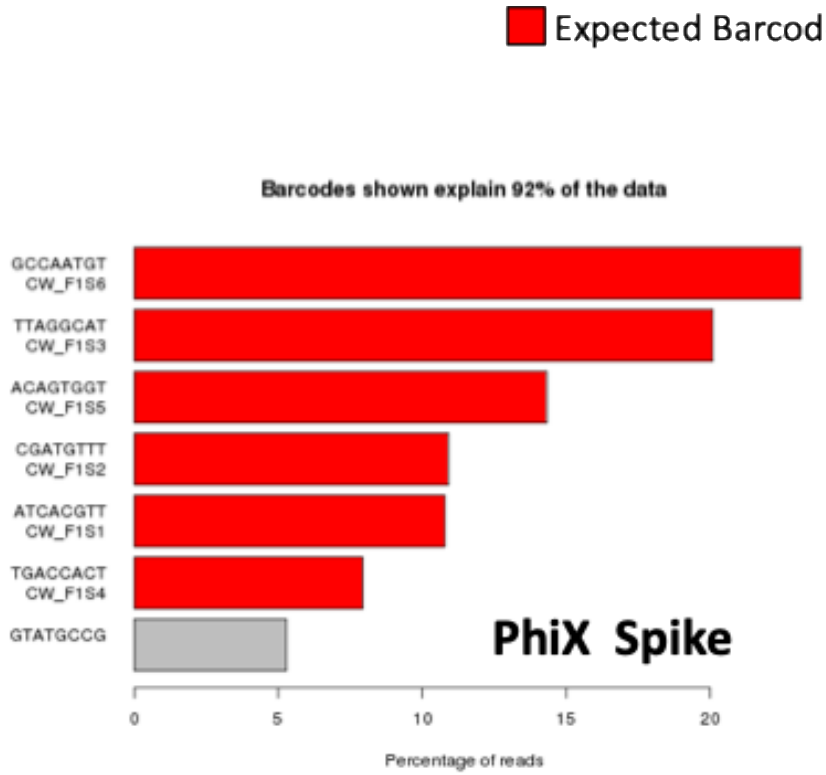




Human Error is a really common source of barcode issues

Babraham Bioinformatics

Demultiplexing: Unknown



PhiX is a well characterized bacteriophage that is used as a control in Illumina sequencing runs.

PhiX is typically added for low-complexity libraries. To add sequence diversity.

Improves flow cell clustering and reduces sequencing errors.

Sometimes more technical in nature...

Babraham Bioinformatics

Basic Statistics

Basic Statistics

Measure	Value
Filename	Mov10_oe_1.subset.fq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	305900
Sequences flagged as poor quality	0
Sequence length	100
%GC	47

Base Call Quality: Expectations

Illumina Sequencers are technically reliable, so **we expect confident calls**

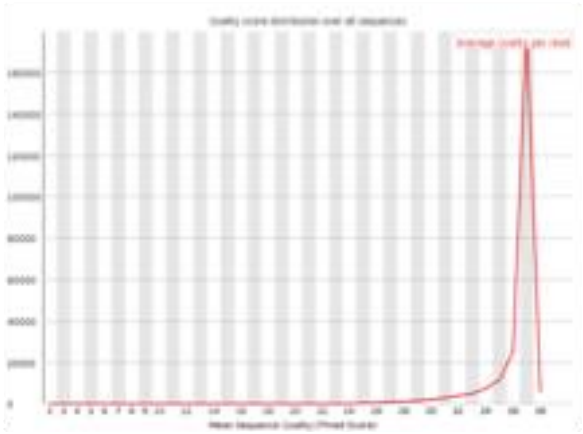
Phred scores

Per base/cycle

FastQC Report

Per base sequence quality

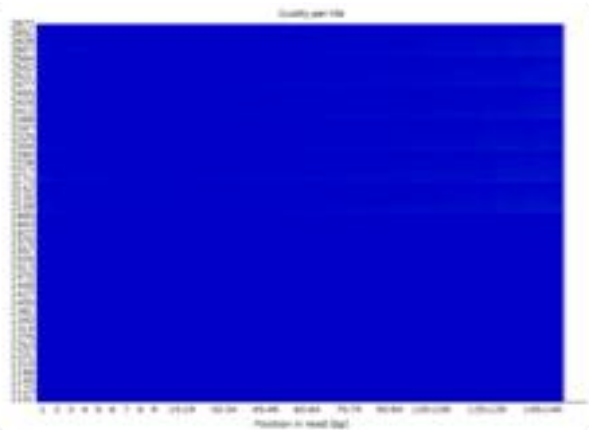
Per Read



FastQC Report

Per sequence quality scores

Location

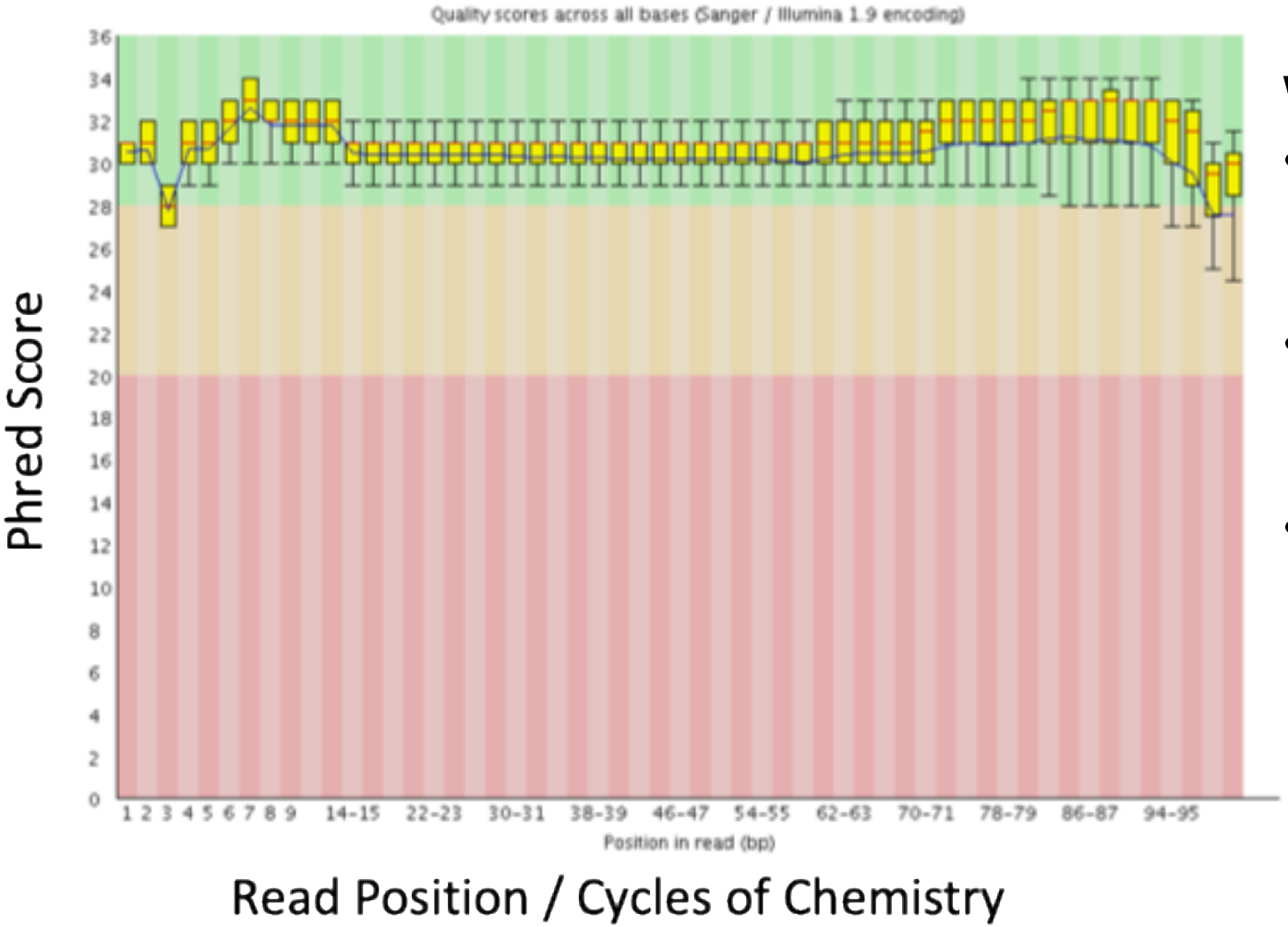


FastQC Report

Per tile sequence quality

Babraham Bioinformatics

Per base sequence quality

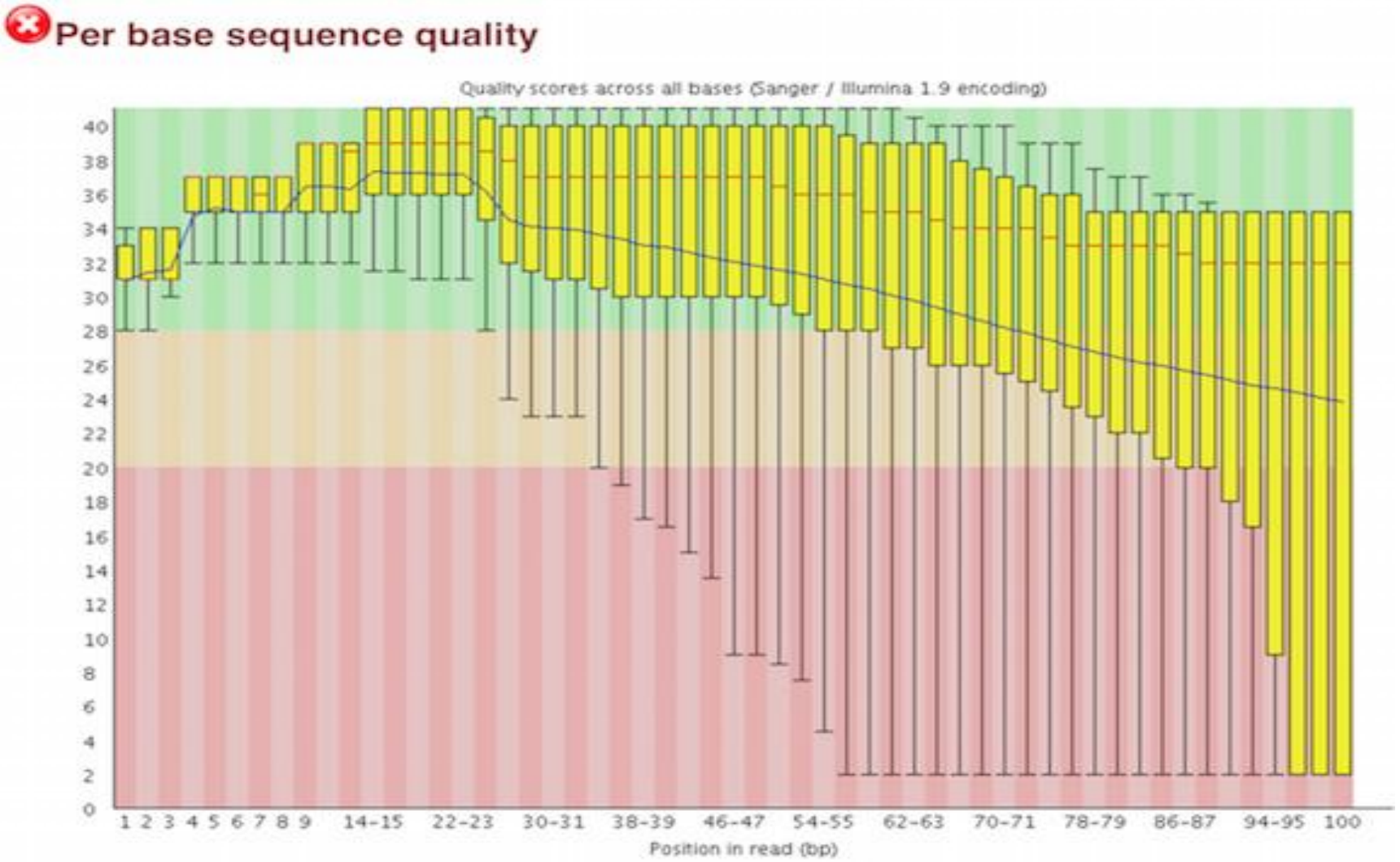


What to expect?

- High median quality at the start of reads
- Slight decline towards the 3' end
- Tight boxes (low variability

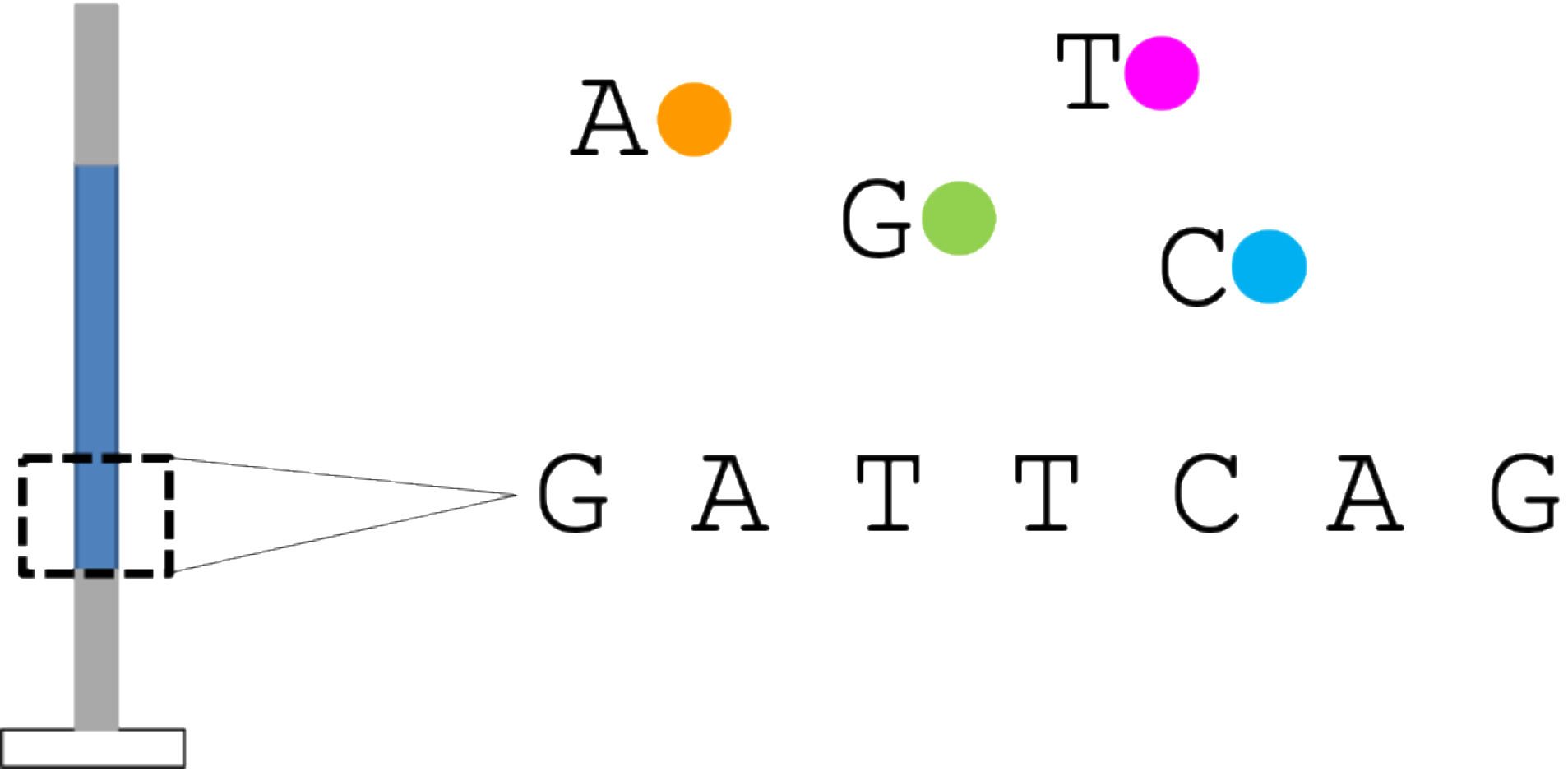
Distribution of Phred score at each position across all reads

Per base sequence quality



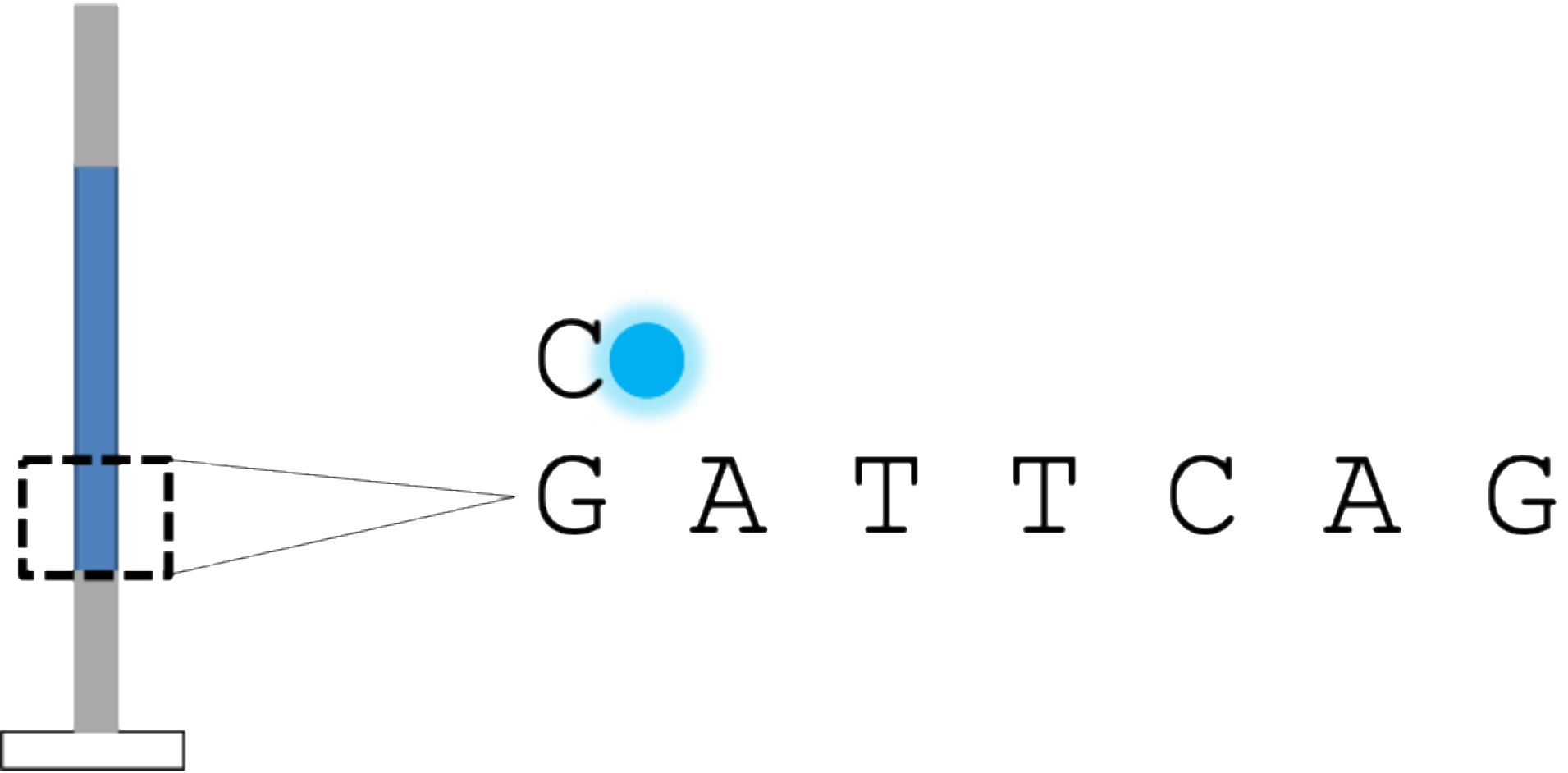
Clusters get out of sync over length of the read

Sequence by Synthesis



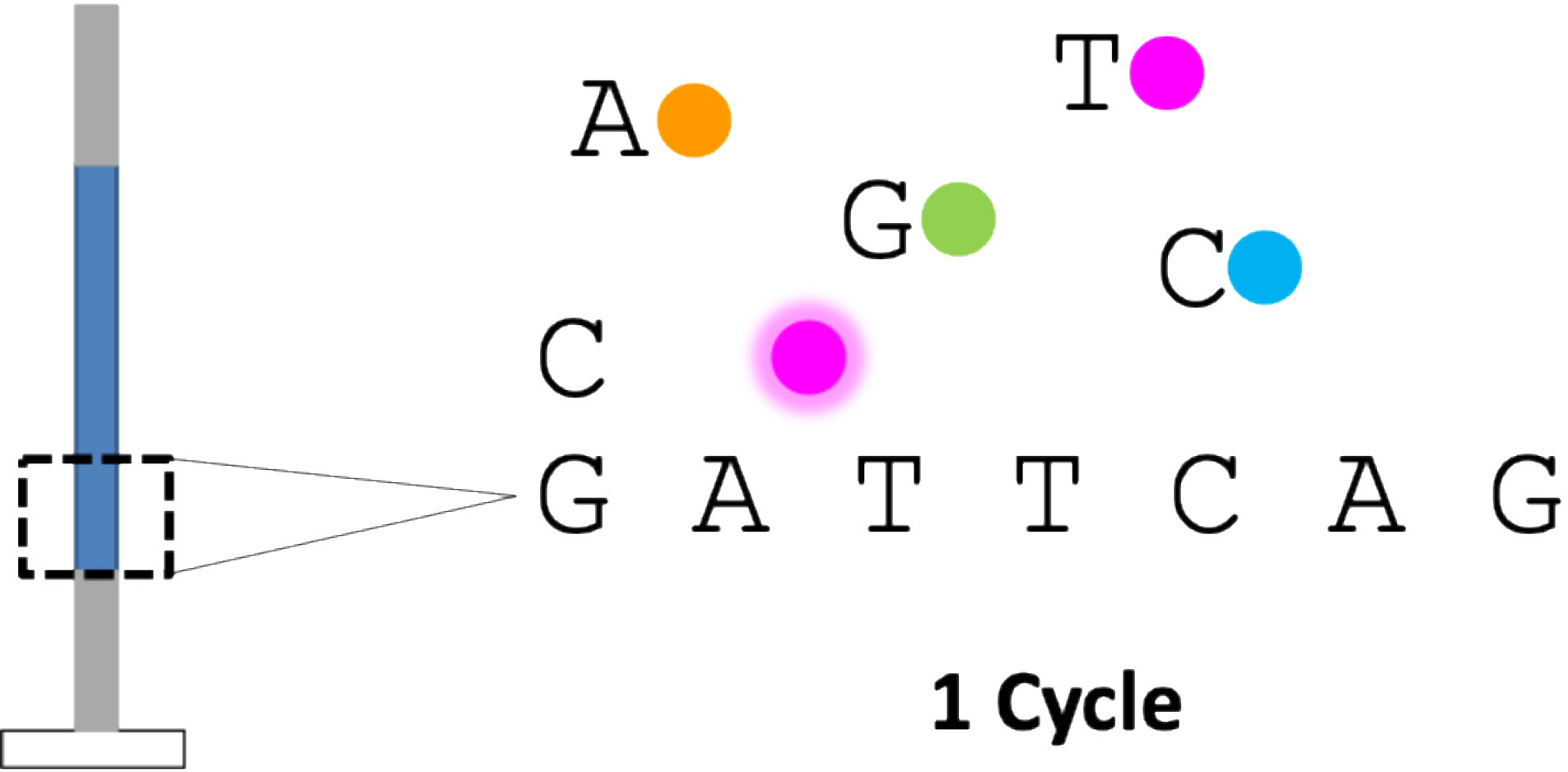
Babraham Bioinformatics

Sequence by Synthesis



Babraham Bioinformatics

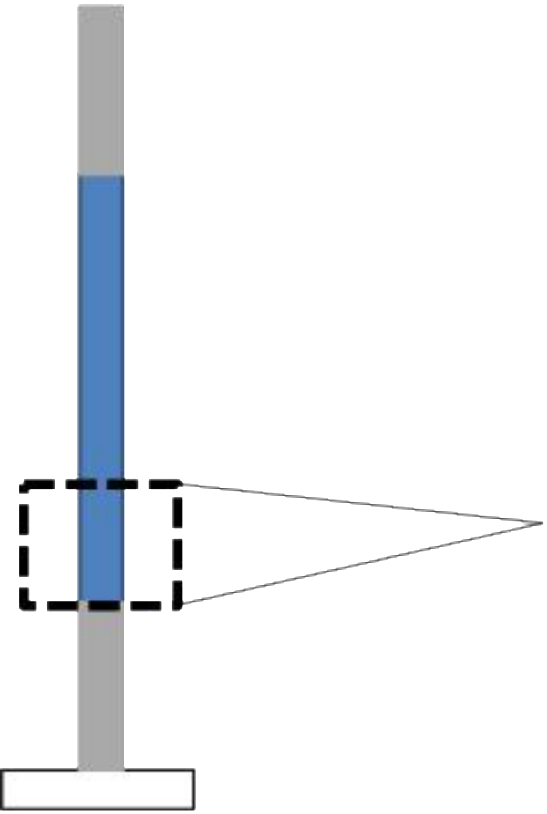
Sequence by Synthesis



Babraham Bioinformatics

A photo is taken….

Sequence by Synthesis



Problem

- As the chain grows, signal quality deteriorates
- At each cycle, accumulates noise

C T A A G T C ect...

G A T T C A G

7 Cycles ... Read length

Babraham Bioinformatics

Per sequence quality scores

What to expect?

- A single peak centered at high quality (Q30+)

Babraham Bioinformatics

Distribution of mean quality scores per read

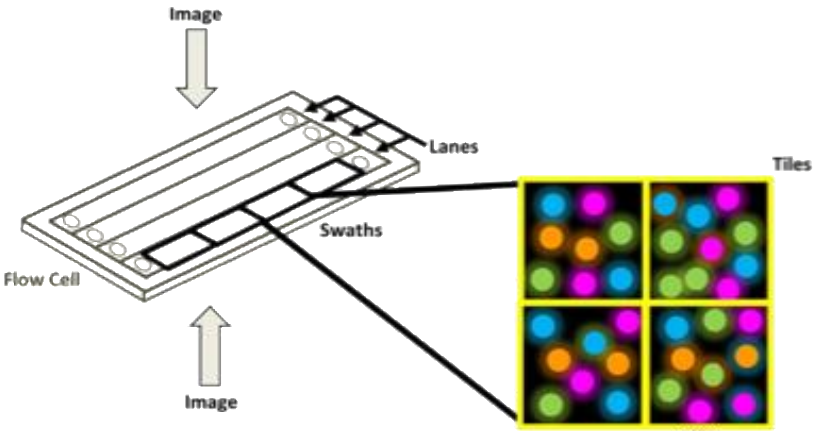
Per sequence quality scores

Mean Sequence Phred Score

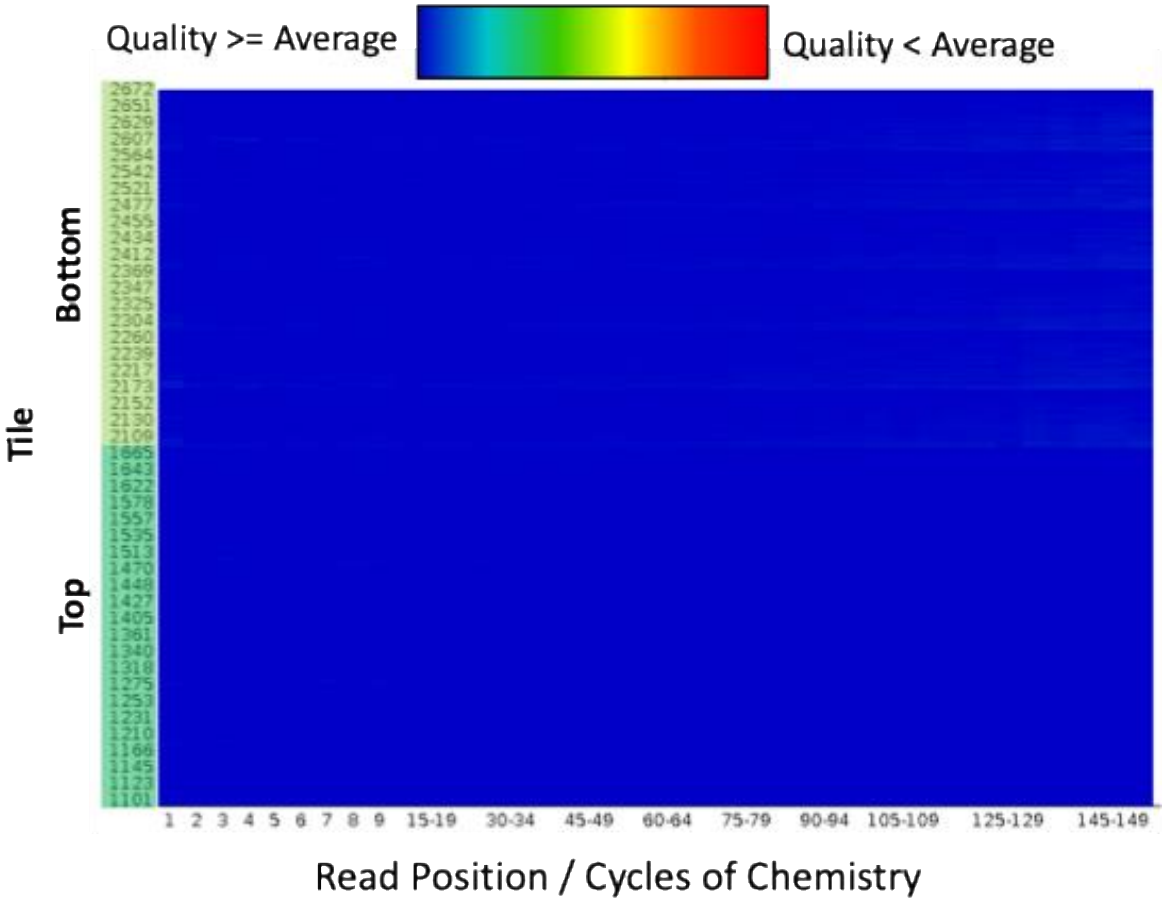
Babraham Bioinformatics

Distribution of mean quality scores per read

Positional Quality

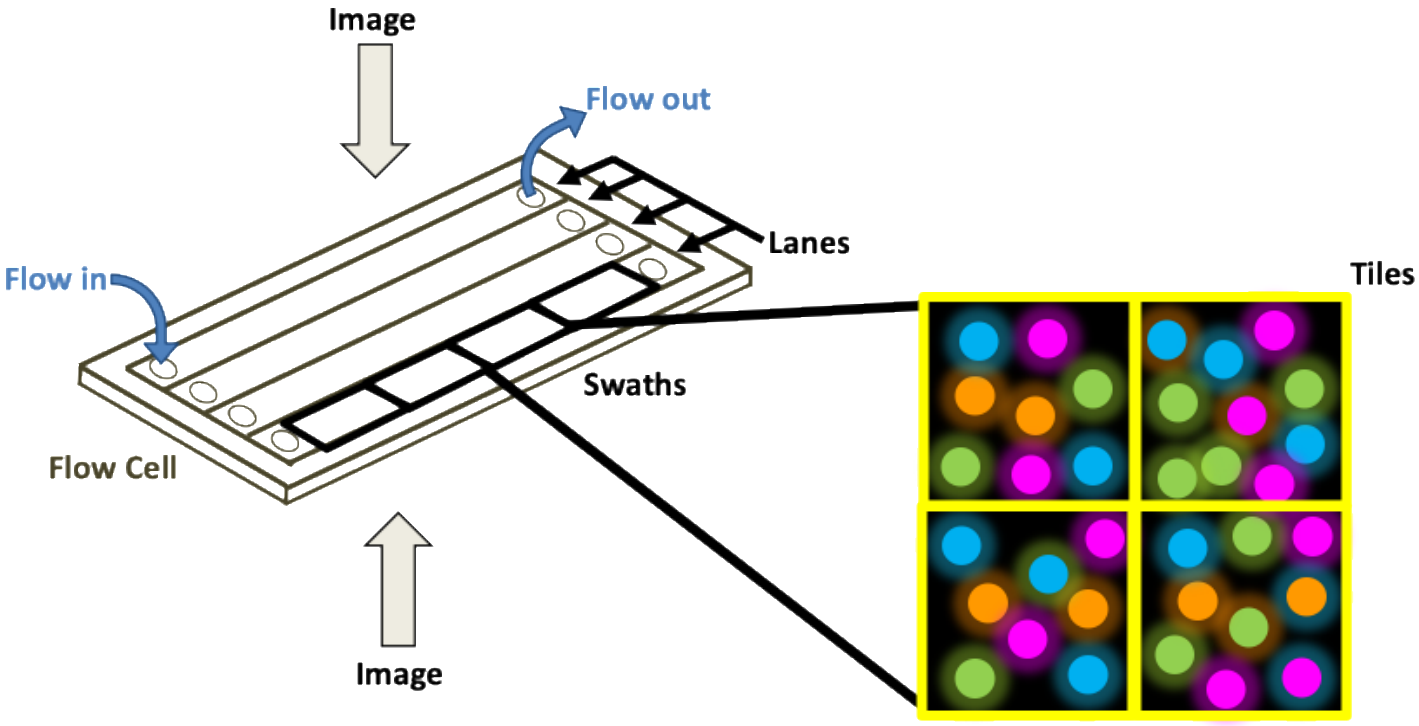


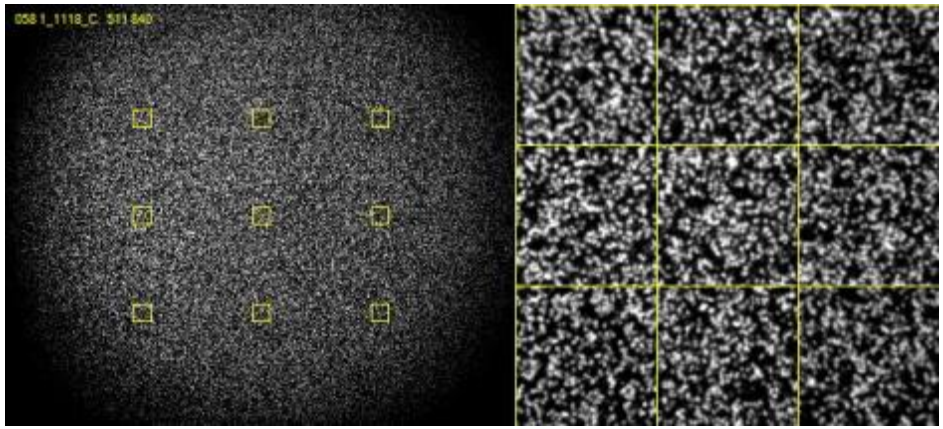
A good tile plot is a blue square



Babraham Bioinformatics

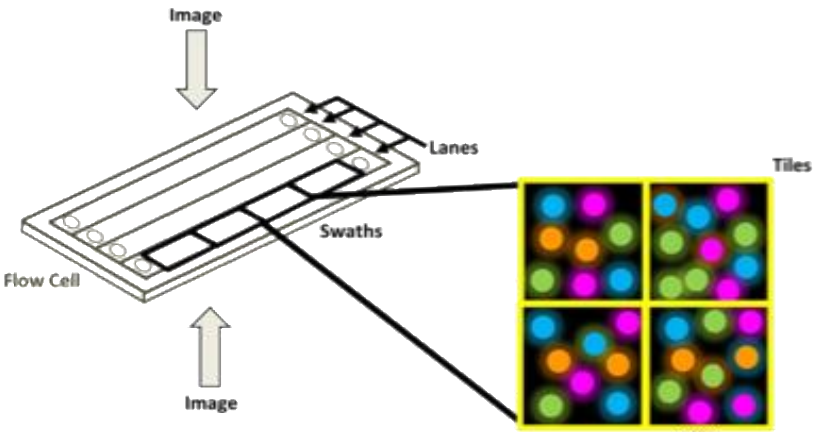
Flow Cell Imaging



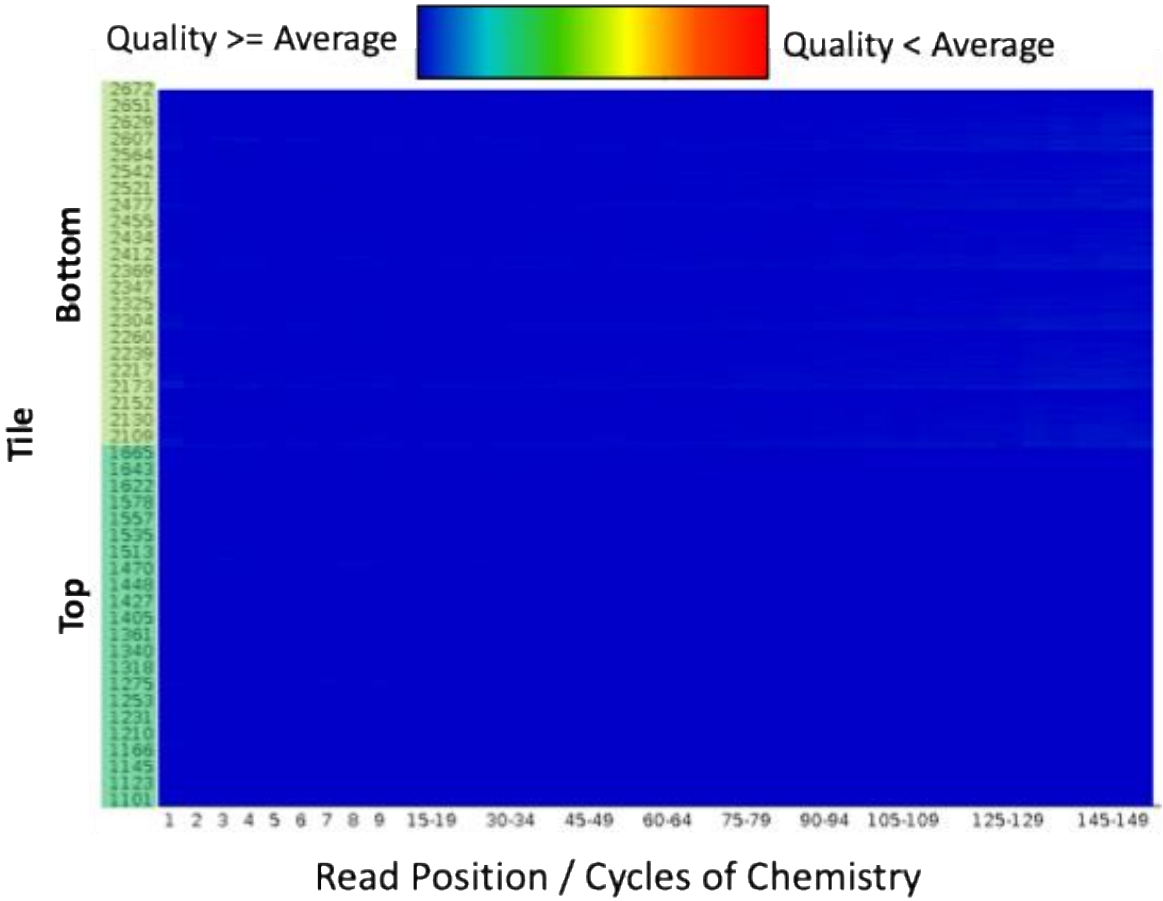


Babraham Bioinformatics

Positional Quality

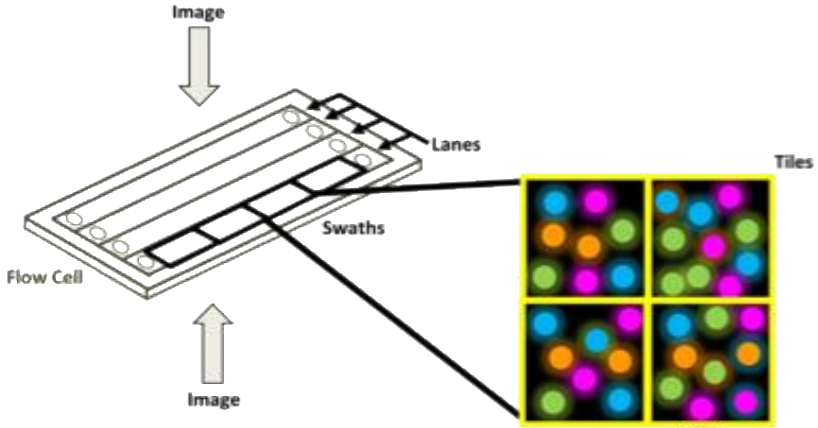


A good tile plot is a blue square

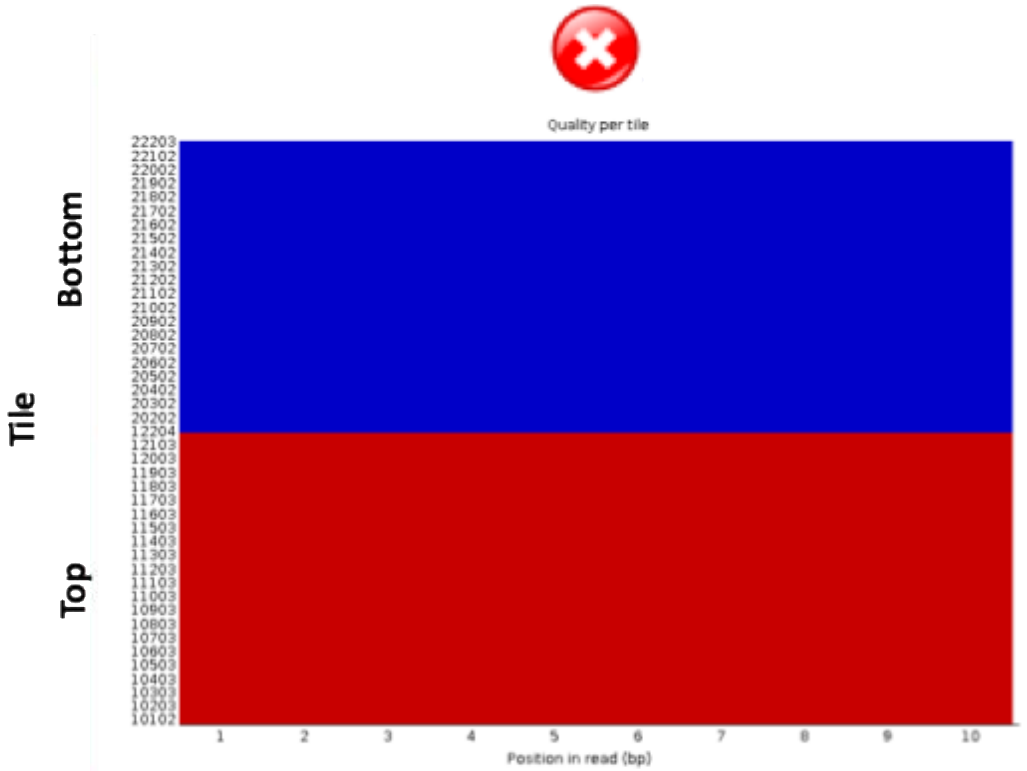


Babraham Bioinformatics

Positional Quality



A good tile plot is a blue square



Focusing Fail!

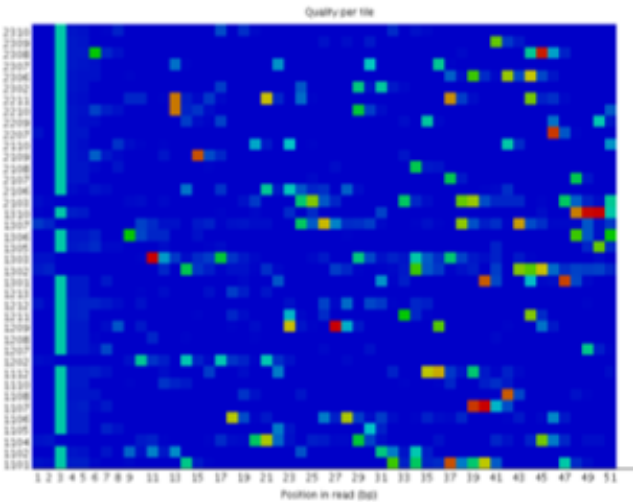
Babraham Bioinformatics

Positional Quality

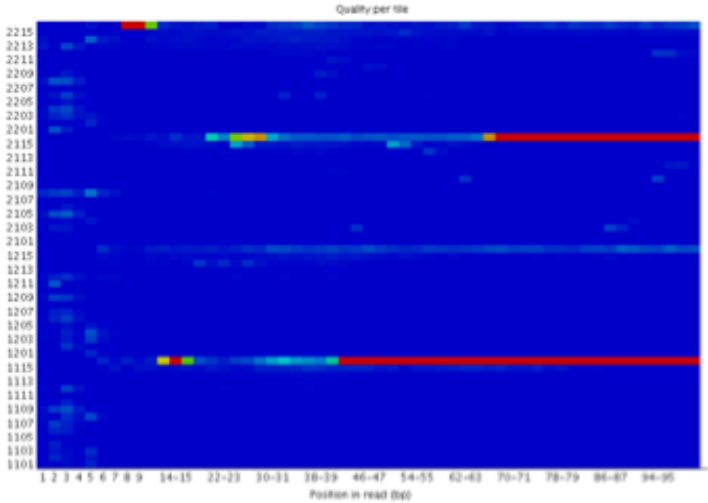
More Examples of Positional Fails

Position Specific Patterning

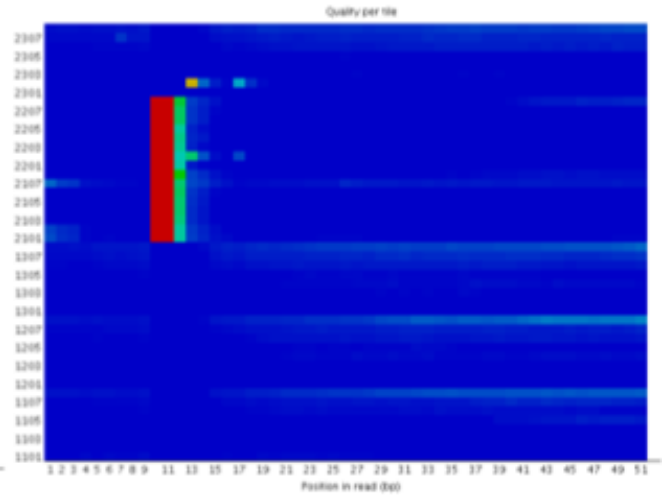
Random Pattern



Permanent



Transient



Overloading of Flow Cell

Obstruction

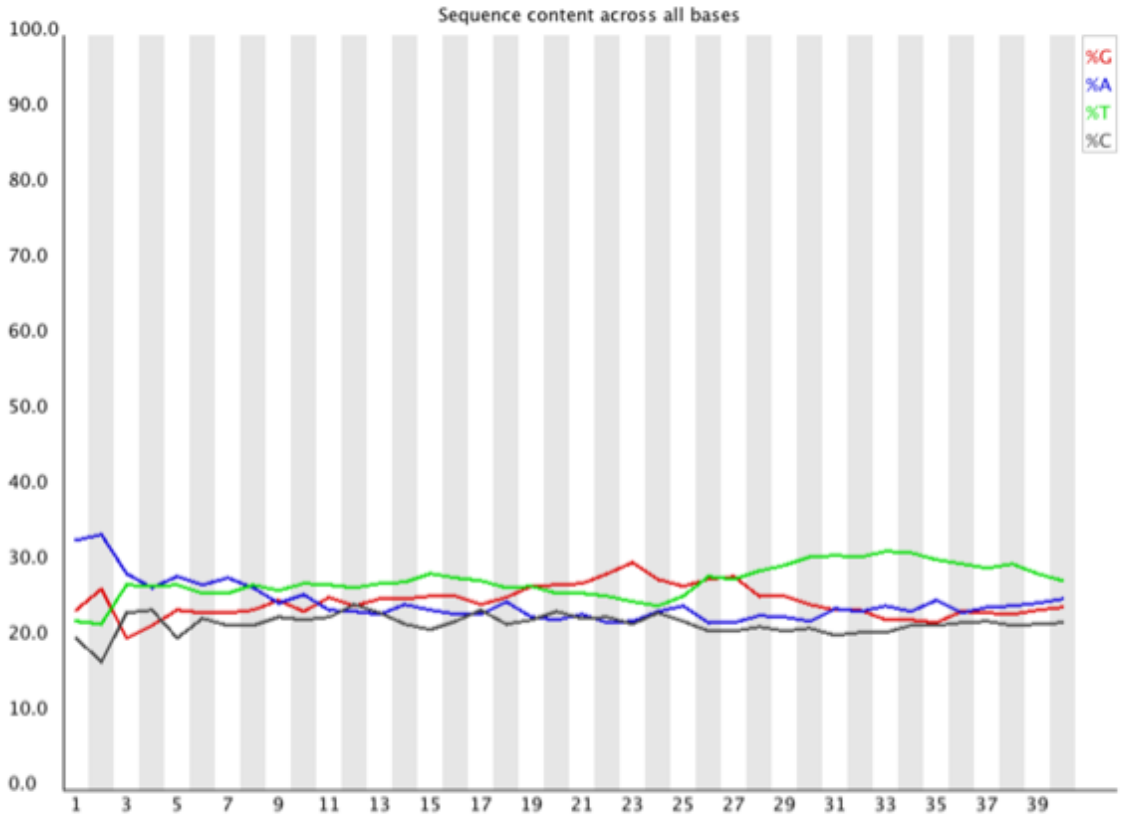
Bubble

Babraham Bioinformatics

Library Base Composition

Babraham Bioinformatics

Shows proportion of A, T, G, C's at each position

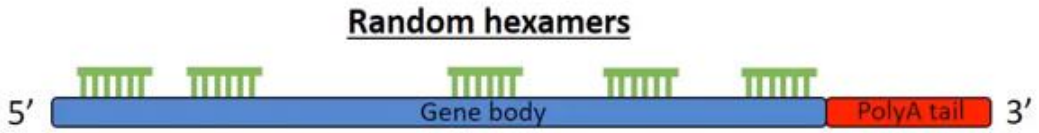


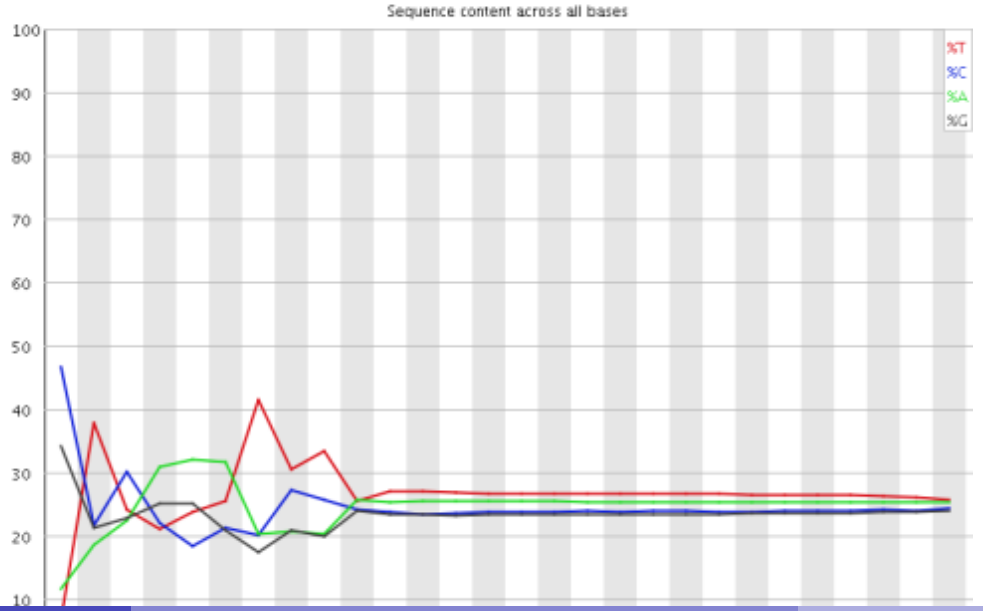
- For every chemistry cycle we can look at the number of ATGC
- The composition should be the same for all cycles

Library Base Composition

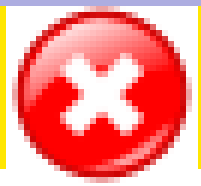
Always gives a FAIL for RNA-seq data. This is because the first 10-12 bases result from the 'random' hexamer priming that occurs during RNA-seq library preparation.

This priming is not as random as we might hope giving an enrichment in particular bases for these intial nucleotides.

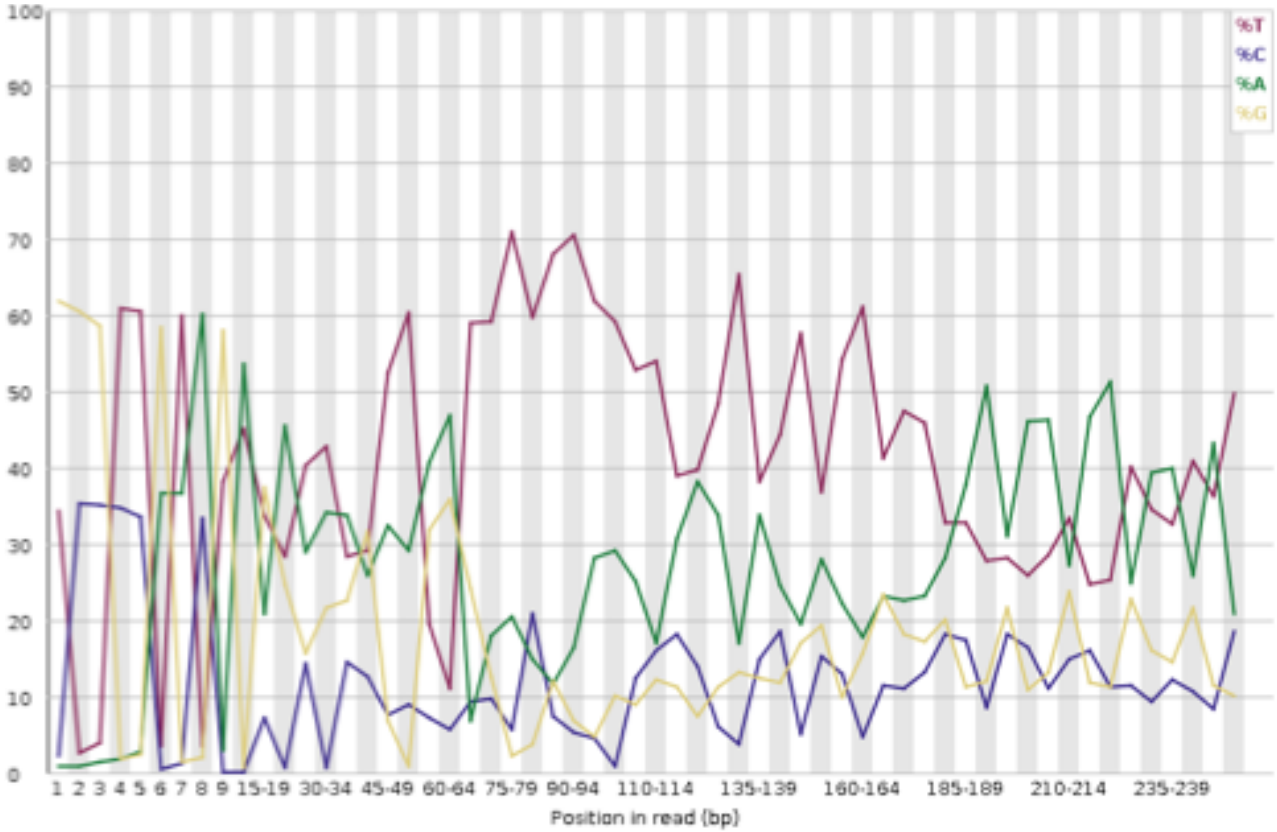




Library Base Composition: Bias



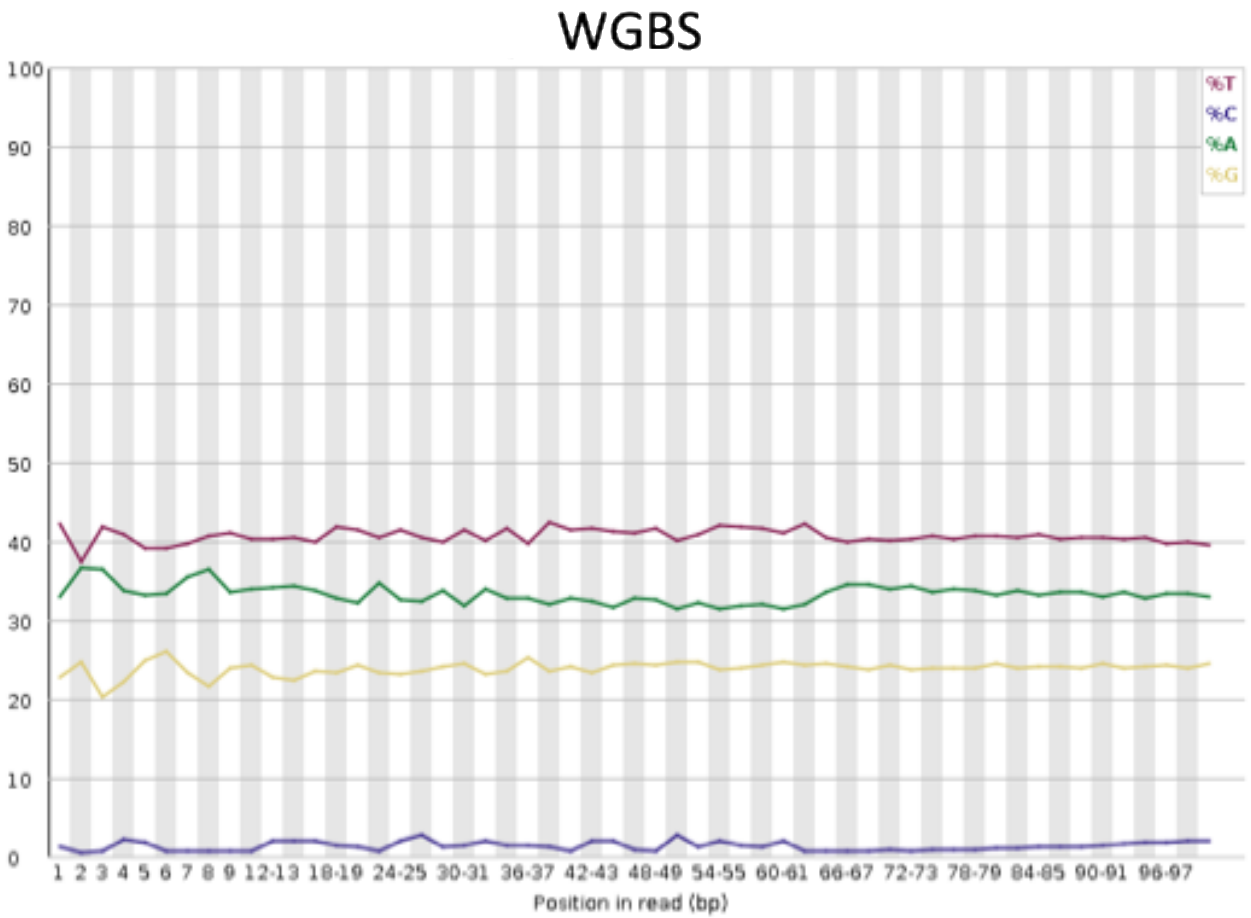
Amplicon



Very low diversity

Babraham Bioinformatics

Library Base Composition: Bias



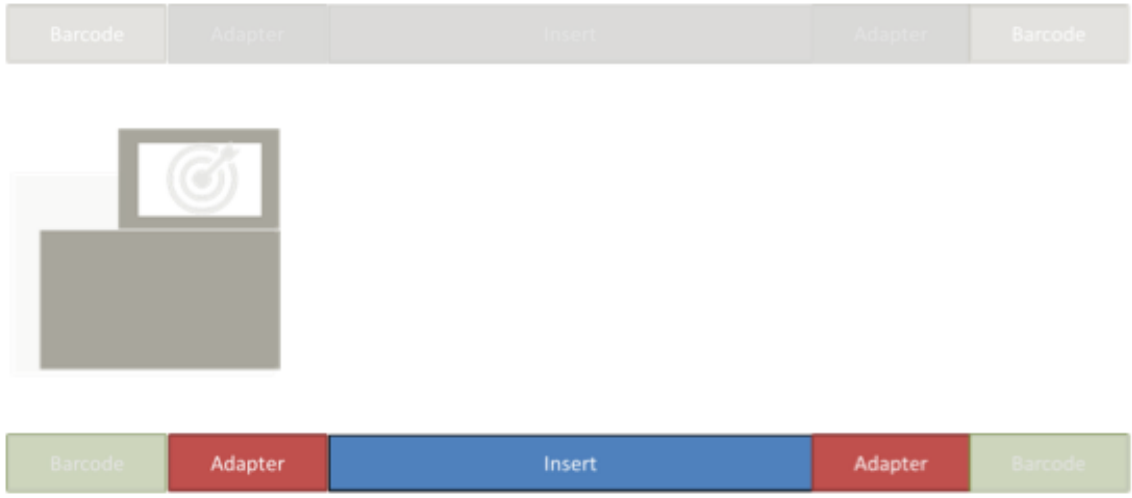
Consistent disproportional expression of bases

Bisulphite treated – C is converted to T

Babraham Bioinformatics

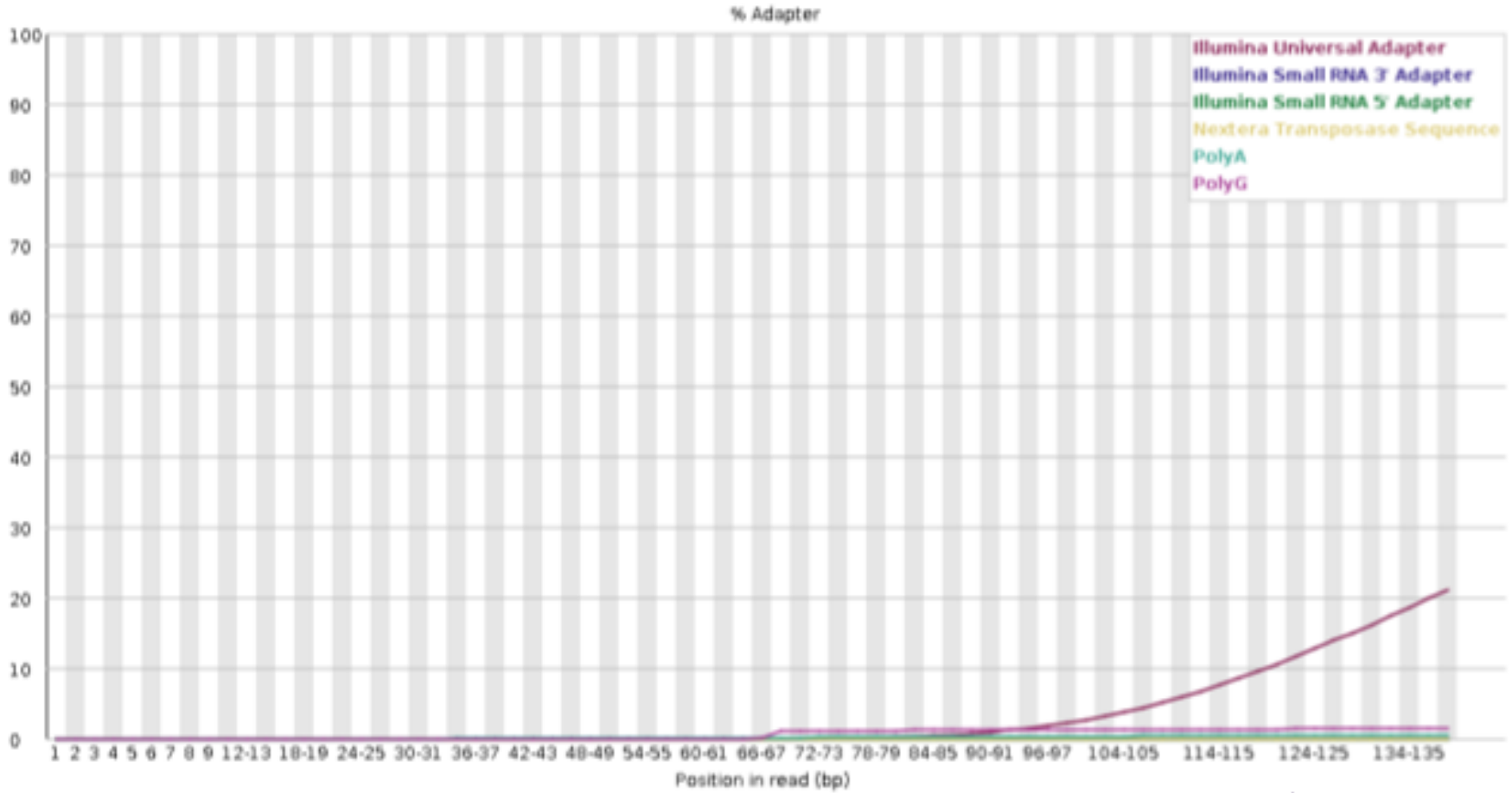
Universal QC Metrics

- Demultiplexing
- Base Call Quality
- Adapter Content
- Mapping Quality



Babraham Bioinformatics

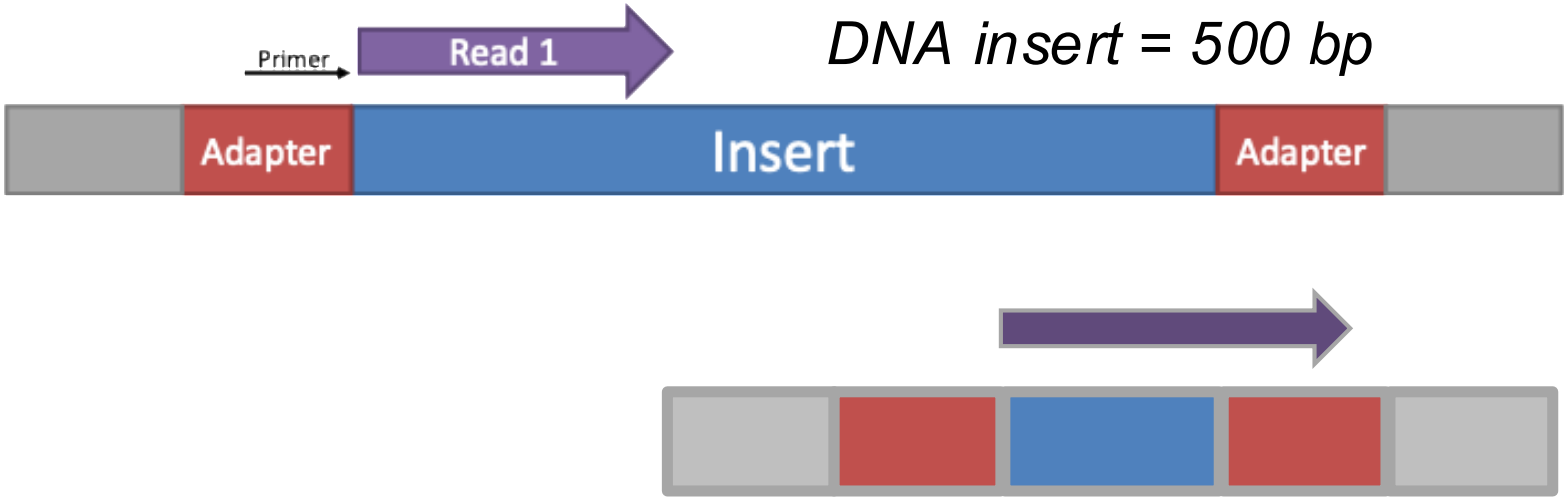
Measuring Read-through Adapters



Babraham Bioinformatics

Adapter Content

Read length = 200bp



Babraham Bioinformatics

Library Dependent QC Metrics

From the Base Sequence:

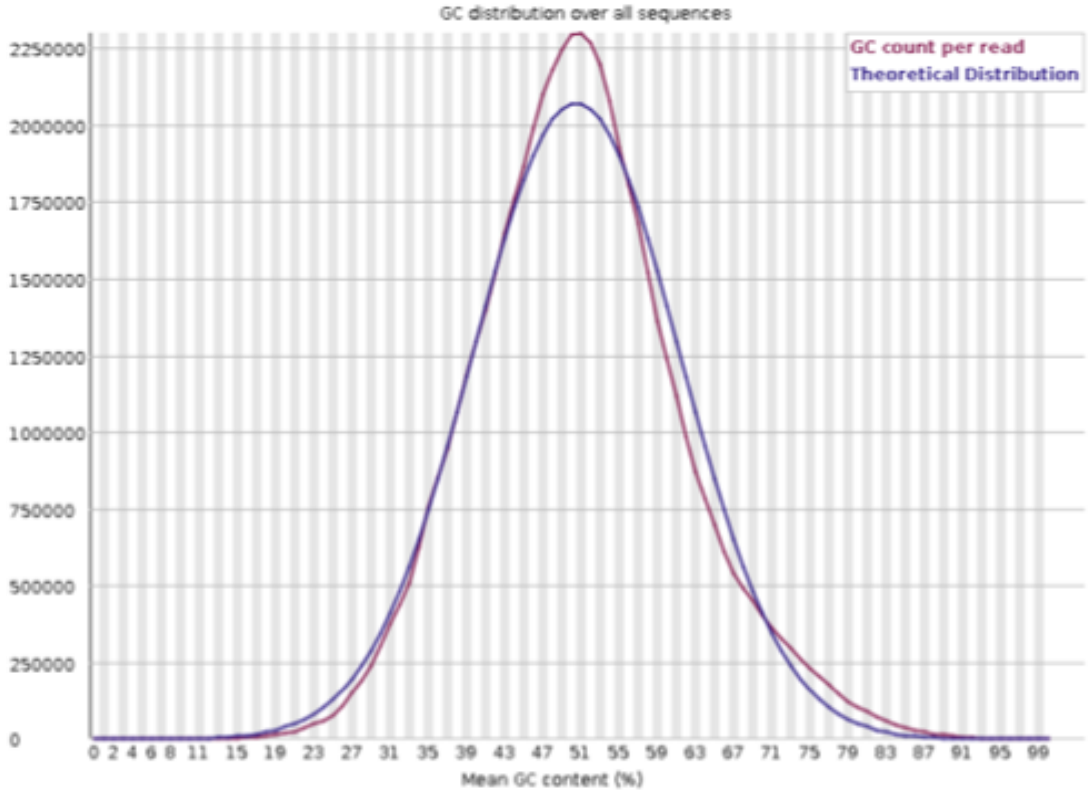
- GC Content

- Base Composition

- Duplication

Babraham Bioinformatics

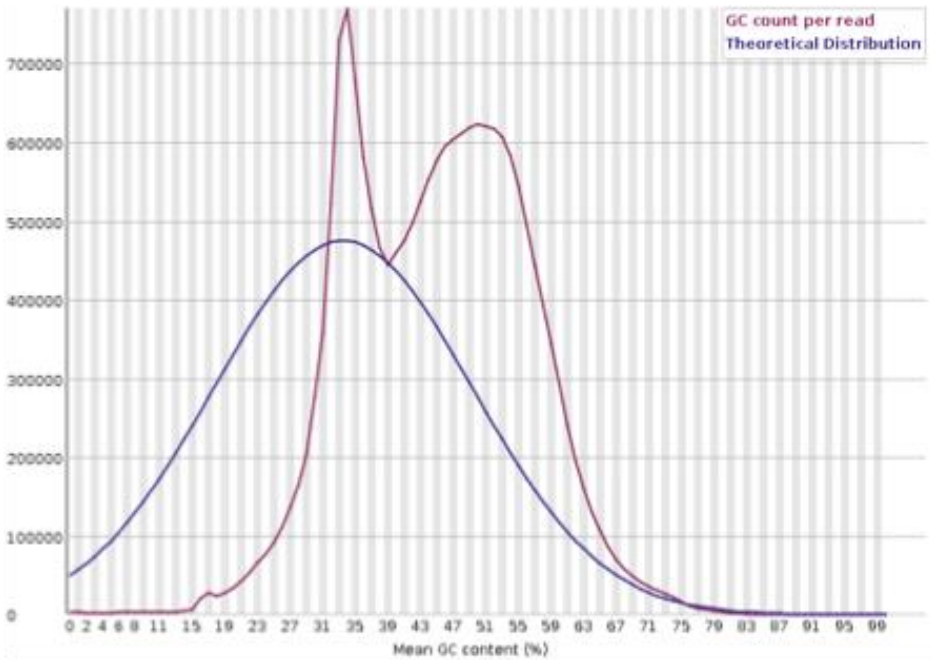
Library GC Content



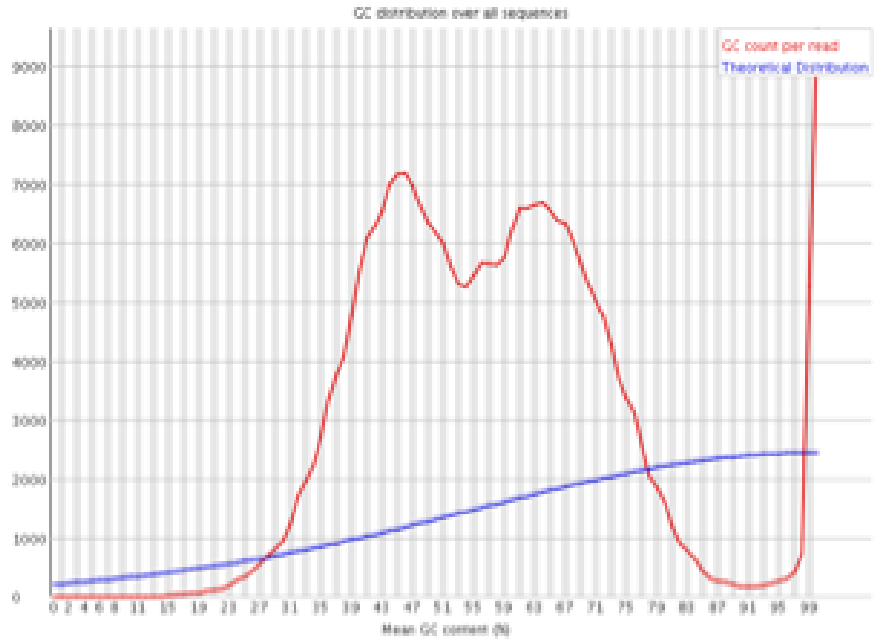
- Generic summary of library composition at a read level
- Expect a normally distributed set of values centred on the overall GC content

Per sequence GC content

This plot would indicate some type of over-represented sequence with the sharp peaks, indicating either contamination or a highly over-expressed gene.



Single contamination

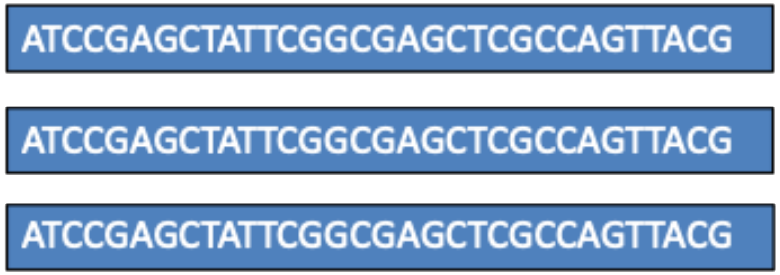


Broad contamination

Duplication

If the exact same sequence appears more than once it could be…

Technical:



- PCR duplicates

Coincidental:

- Deep sequencing
- Highly present sequences
- Restricted diversity libraries

Babraham Bioinformatics

Overrepresented Sequences

- Extreme duplication
- Displays the sequences (at least 20 bp) that occur in more than 0.1% of the total number of sequences.
- The exact same sequence is a significant proportion of the whole library (which might not be duplicated overall)

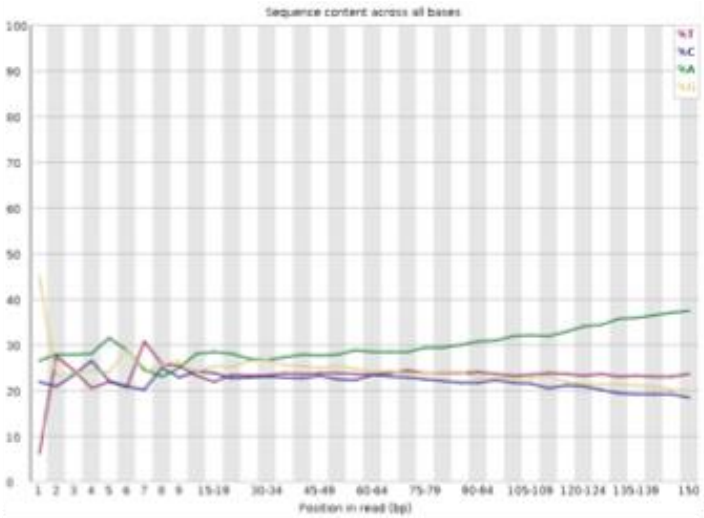
Low complexity sequences

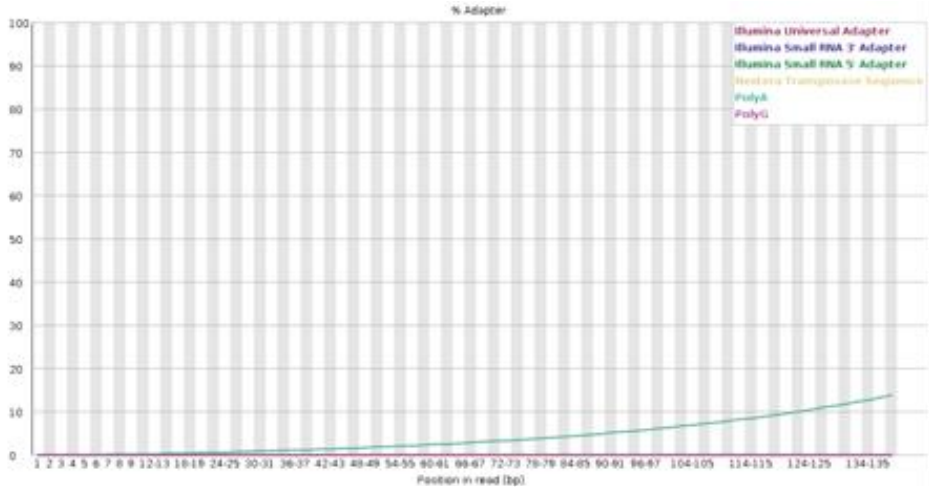
- Poly A
- Poly G
- Poly N

Overrepresented Sequences: Poly A

PolyA (or PolyT) – Common in RNA-Seq

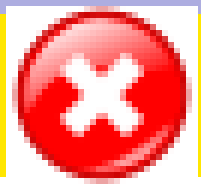
Sequence	Count	Percentage
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	68355	1.7344041279604823
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	67792	1.7201188595230343





Babraham Bioinformatics

Overrepresented Sequences: Poly N



PolyN – Quality too poor to make any calls

Sequence	Count	Percentage
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	462344	1.870897045533307
GNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	232540	0.5382147642627897
ANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	127291	0.29461553090984244
CNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	87792	0.20319493671694688
TNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	85181	0.19715176672688003
GANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	48918	0.11322090753507845

Babraham Bioinformatics

Low complexity sequences

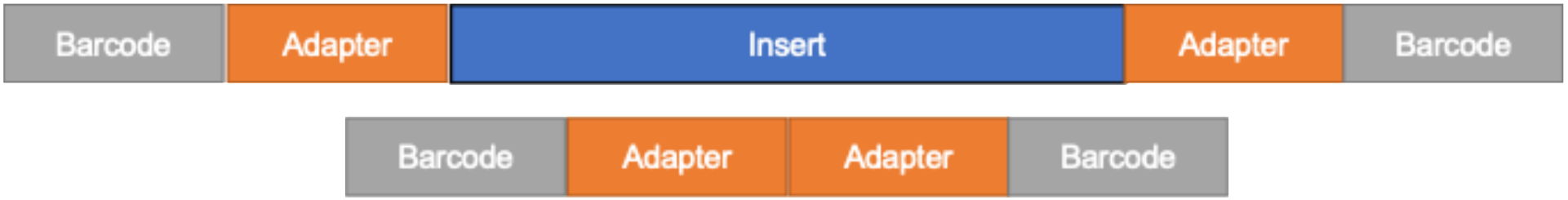
- Poly A
- Poly G
- Poly N

Why are these a problem?

- Poor alignment
- N's will break alignment step

Indication that read should be trimmed or discarded

Overrepresented Sequences: Adapter Dimers



Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGC	17957	0.14359551756800035	TruSeq Adapter, Index 12 (100% over 50bp)

Overrepresented Sequences: Specific Sequences

- Other potential sources...

Sequence	Count	Percentage	Possible Source
GGCTTCCTCGGCCCCGGGATTCGGCGAAAGCTGCGGCCGGAGGGCTGTAA	746766	1.360148419566899	No Hit

Basic Local Alignment Search Tool

Sequence	Count	Percentage	Possible Source
CTTATACACATCTCCGAGCCCACGAGACTAAGGCGAATCTCGTATGCCGT	2767629	5.149013792611521	No Hit

Ribosomal

Contaminant

Babraham Bioinformatics

FASTQC is a decision tool

FASTQC helps us decide:

- Do we trim? (quality drop, adapters present)
- How aggressively do we trim?
- Do we filter reads entirely
- Is the dataset salvageable

Real sequencing data is almost never perfect!

Run `fastqc` with the Environment Module System:

```
module load gcc/13.3.0-xp3epyt
module load fastqc/0.12.1-qxseug5
```

Class Exercise #1

- Run FASTQC on all FASTQ files in `raw_fastq`. FASTQC allows you to redirect your output into a specified location with the `-o` parameter. Be sure to use this parameter in your final code.

If successful, you will see the following outputs inside of the `fastqc` folder:

```
Irrel_kd_1.subset_fastqc.html  Irrel_kd_3.subset_fastqc.h
Irrel_kd_1.subset_fastqc.zip  Irrel_kd_3.subset_fastqc.z
Irrel_kd_2.subset_fastqc.html  Mov10_oe_1.subset_fastqc.h
Irrel_kd_2.subset_fastqc.zip  Mov10_oe_1.subset_fastqc.z
```

How to use fastqc?

```
fastqc --help
```

```
SYNOPSIS

    fastqc seqfile1 seqfile2 .. seqfileN

    fastqc [-o output dir]
    [--(no)extract]
    [-f fastq|bam|sam]
    [-c contaminant file]
    seqfile1 .. seqfileN
```

FASTQC Outputs

For each individual FASTQ file that is input to FastQC, there are **two output files that are generated**.

1. The first is **an HTML file** which is a self-contained document with various graphs embedded into it. Each of the graphs evaluate different quality aspects of our data, we will discuss in more detail in this lesson.
2. Alongside the HTML file is **a zip file**. This file contains the different plots from the report as separate image files but also contains data files which are designed to be easily parsed to allow for a more detailed and automated evaluation of the raw data on which the QC report is built.

Special Note

We are running FASTQC interactively. This is running on the login node relatively quickly. This is because this alignment for these FASTQ files was only performed for a small portion of the chromosome 1. Later on, this will take a lot longer. Therefore, you will need to generate a script.

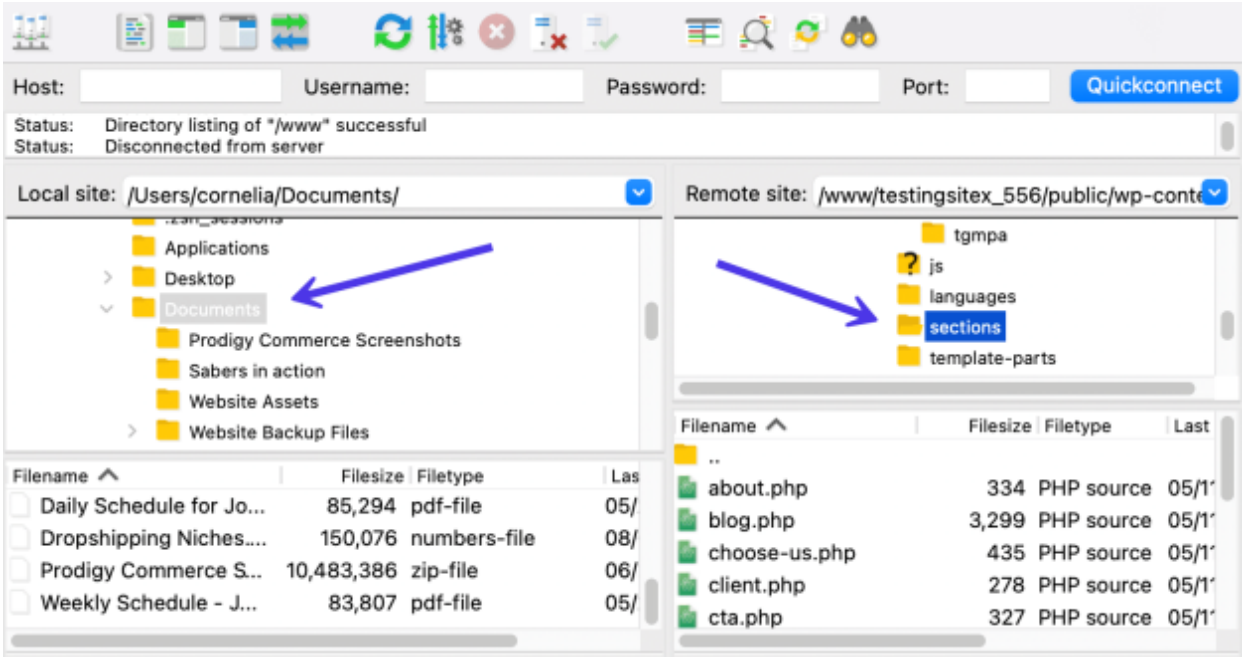
Running Parameters for FASTQC:

- 10G of memory is required
- 1 node, 2 tasks

Viewing the HTML report from FASTQC

All of the following are solutions that allow students to transfer files between remote (i.e. VACC) and local (i.e. your laptop) servers.

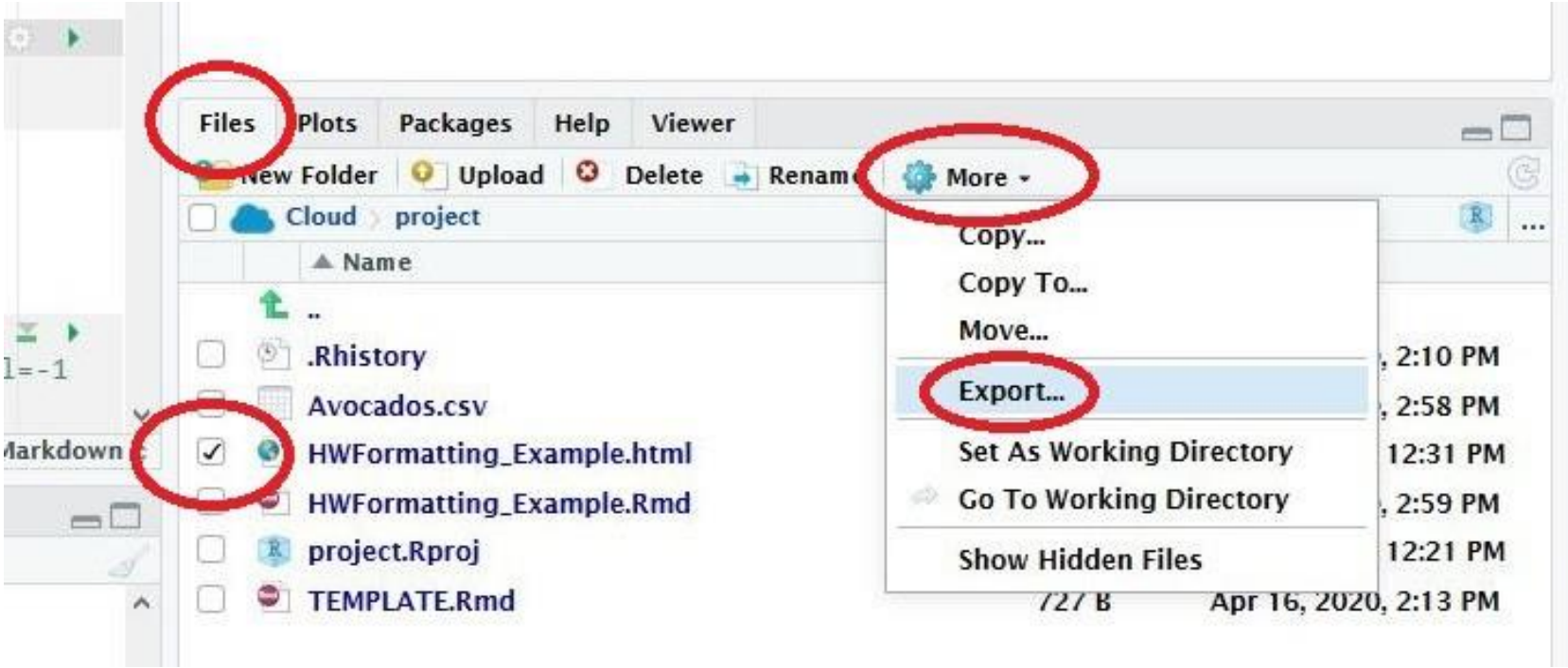
An FTP application such as Filezilla



Viewing the HTML report from FASTQC

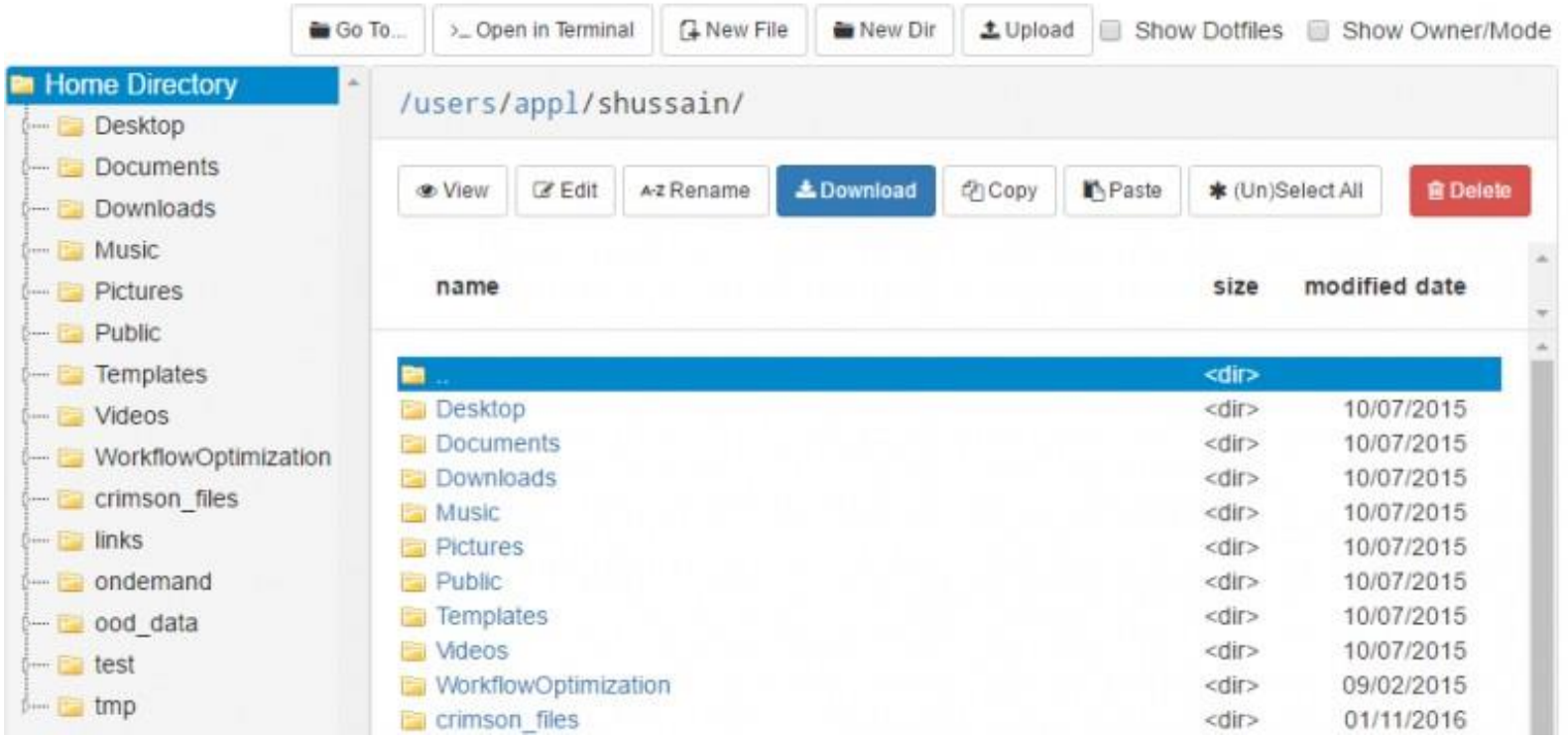
RStudio (via VACC-OOD)

You can export it or simply view it using RStudio

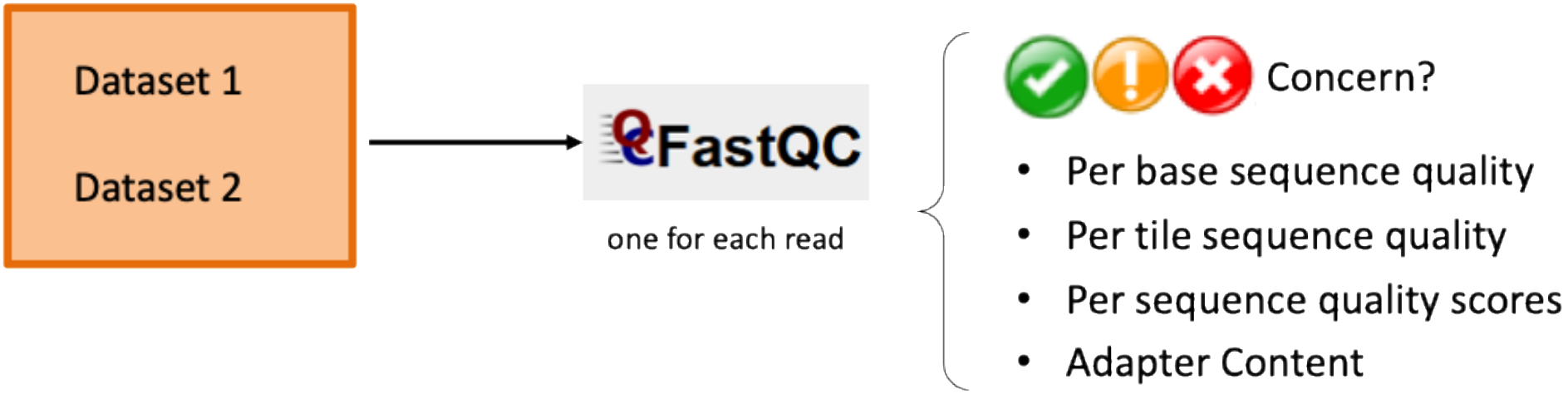


Viewing the HTML report from FASTQC

In File Explorer on OpenOnDemand, use the "Download" button



Class Exercise #2: Assessing Universal Metrics



Grab the following folder from the location below.

```
/gpfs1/cl/mmg3320/course_materials/FASTQC_example
```

Please answer questions in **ClassExercise2-FASTQC-020926.docx**

Citation

This lesson has been developed by members of the teaching team at the Harvard Chan Bioinformatics Core (HBC). These are open access materials distributed under the terms of the Creative Commons Attribution license (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.