Introduction to Bulk RNAseq data analysis

QC of Aligned Reads - exercise solutions

1. A quick look at the alignment metrics with `samtools`

Exercise 1

1. Use the `samtools flagstat` command to generate alignment metrics for the bam file `bam/SRR7657883.chr14.sorted.bam`.

```
samtools flagstat bam/SRR7657883.chr14.sorted.bam
```

```
1924847 + 0 in total (QC-passed reads + QC-failed reads)
84642 + 0 secondary
0 + 0 supplementary
0 + 0 duplicates
1863232 + 0 mapped (96.80% : N/A)
1840205 + 0 paired in sequencing
920031 + 0 read1
920174 + 0 read2
1705884 + 0 properly paired (92.70% : N/A)
1716975 + 0 with itself and mate mapped
61615 + 0 singletons (3.35% : N/A)
5261 + 0 with mate mapped to a different chr
4640 + 0 with mate mapped to a different chr (mapQ>=5)
```

Q) What percentage of the reads have aligned to the genome?

96.80% of reads have been mapped to the reference genome.

2. More detailed metrics with Picard Tools

2.1 Duplication metrics

Exercise 2.1

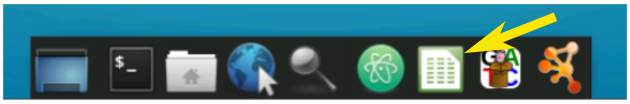
1. Run Picard's MarkDuplicates tool on the sorted bam file using the following command:

```
java -jar picard/picard.jar MarkDuplicates \
          INPUT=bam/SRR7657883.chr14.sorted.bam \
          OUTPUT=bam/SRR7657883.chr14.mkdup.bam \
          METRICS_FILE=bam/SRR7657883.chr14.mkdup_metrics.txt \
          CREATE_INDEX=true
```

⇒ *bam/SRR7657883.chr14.mkdup.bam* - The new bam file with duplicated marked
⇒ *bam/SRR7657883.chr14.mkdup.bai* - The index for the new bam file
⇒ *bam/SRR7657883.chr14.mkdup_metrics.txt* - The duplication metrics

Note: The \ at the end of each line tells the terminal that when you press `Enter`, you have not yet finished typing the command. You can if you wish, type the whole command on a single line, omitting the \ - The command is written across multiple lines here just to make it easier to read.

Q. What is the duplication rate for this bam file? You'll need to look at the metrics file. The easiest way is to open in a spreadsheet. On the course machines we have LibreOffice Calc. You can find this in the launcher bar at the bottom of the desktop.



	B	C	D	E	F	G	H	I
1	# htsjdk.samtools.metrics.StringHeader							
2	# MarkDuplicates INPUT=[bam/SRR7657883.chr14.sorted.bam] OUTPUT=temp.bam METRICS_FILE=temp.txt MAX_SEQUENCES_FOR_DISK_READ_ENDS_MAP=50000 MAX_FILE_HANDLES_FOR_READ_ENDS_MAP=8000 SORTING_COLLECTION_							
3	## htsjdk.samtools.metrics.StringHeader							
4	# Started on: Wed Mar 17 15:35:52 UTC 2021							
5								
6	picard.sam.DuplicationMetrics							
7	UNPAIRED_READS_EXAMI	READ_PAIRS_EXAMINED	SECONDARY_OR_SUPPLEMENTARY_RD	UNMAPPED_READS	UNPAIRED_READ_DUPLICATES	READ_PAIR_DUPLICATES	READ_PAIR_OPTICAL_DUPLICATES	PERCENT_DUPLICATION
8	61615	858487	84642	61615	40663	149123	0	0.190549
9								
10	java.lang.Double							
11	CoverageMult	all_sets	non_optical_sets					
12	1	591659	591659					
13	1.673975	93806	93806					
14	2.128217	15143	15143					
15	2.434366	3623	3623					

~19%. Note that although the column headers for Picard say "PERCENT" or "PCT" the number is in fact the decimal fraction and need to be multiplied by 100 for percent. Just an odd quirk of Picard.

2.2 Alignment metrics

Exercise 2.2

1. Run Picard's `CollectAlignmentSummaryMetrics` tool on the chr14 sorted bam providing the following options.
   - INPUT - The sorted chr 14 only bam file
   - OUTPUT - `bam/SRR7657883.chr14.alignment_metrics.txt`
   - REFERENCE_SEQUENCE - `references/Mus_musculus.GRCm38.dna_sm.primary_assembly.fa`

```
java -jar picard/picard.jar CollectAlignmentSummaryMetrics \
     INPUT=bam/SRR7657883.chr14.sorted.bam \
     OUTPUT=bam/SRR7657883.chr14.alignment_metrics.txt \
     REFERENCE_SEQUENCE=references/Mus_musculus.GRCm38.dna_sm.primary_assembly.fa
```

⇒ *bam/SRR7657883.chr14.alignment_metrics.txt* - The alignment metrics

2.3 Insert Size metrics

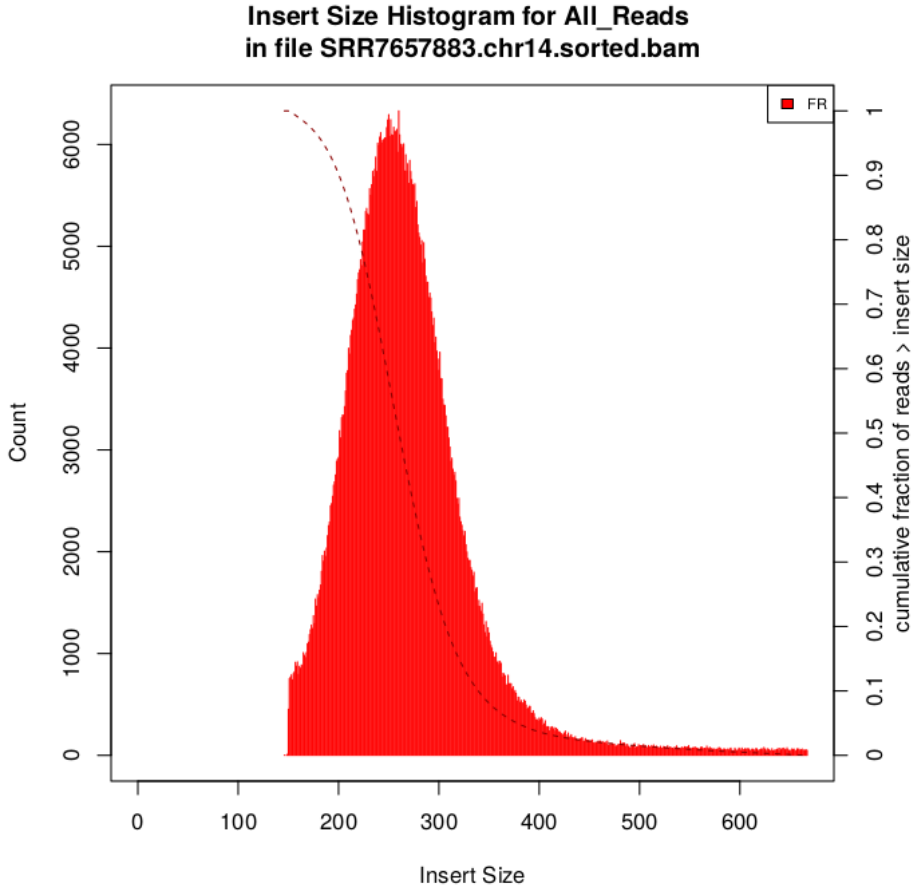
Exercise 2.3

1. Run Picard's `CollectInsertSizeMetrics` tool on the chr1 sorted bam providing the following options.
   - INPUT - The sorted chr 1 only bam file
   - OUTPUT - bam/SRR7657883.chr1.insert_size.txt
   - HISTOGRAM_FILE - bam/SRR7657883.chr1.insert_size.pdf

```
java -jar picard/picard.jar CollectInsertSizeMetrics \
     INPUT=bam/SRR7657883.chr14.sorted.bam \
     OUTPUT=bam/SRR7657883.chr14.insert_size.txt \
     HISTOGRAM_FILE=bam/SRR7657883.chr14.insert_size.pdf
```

⇒ *bam/SRR7657883.chr14.insert_size.txt* - The insert size metrics
⇒ *bam/SRR7657883.chr14.insert_size.pdf* - The PDF with a plot showing the insert size distribution

Open the PDF and look at the distribution fragment lengths (insert sizes) in the library.



Q. Considering this data is from paired 150 base reads, what are the implications of the fragment length distributions.

As we have PE 150 reads, the total amount of sequencing from each fragment is 300 bases. Looking at the distribution only ~25% of the fragments have lengths greater than 300 bases. This means that for about 80% of the fragments the reads are overlapping. From the perspective of our gene expression analysis, this doesn't matter, however, from a design perspective it means that we have unnecessarily sequenced a lot of bases twice and he more sequencing we carry out, the more expensive the study is. It would have been optimal to use a shorter read length.

2.4 RNA alignment metrics

Exercise 2.4

1. Run Picard's `CollectRnaSeqMetrics` tool on the sorted bam file providing the following options:
   - INPUT - The sorted bam file
   - OUTPUT - `bam/SRR7657883.chr14.RNA_metrics.txt`
   - REF_FLAT - the RefFlat reference file

- STRAND - `NONE`

```
java -jar picard/picard.jar CollectRnaSeqMetrics \
     INPUT=bam/SRR7657883.chr14.sorted.bam \
     REF_FLAT=references/Mus_musculus.GRCm38.102.txt \
     OUTPUT=bam/SRR7657883.chr14.RNA_metrics.txt \
     STRAND=NONE
```

⇒ *bam/SRR7657883.chr14.RNA_metrics.txt* - The RNAseq metrics

The results of this analysis are best viewed graphically, we will do this in the next exercise.

3. Visualising QC results with MultiQC

Exercise 3.1

1. Run multiqc on the bam directory:

```
multiqc -n Alignment_QC_Report.html -o bam bam
```

- `-n` - a name for the report
- `-o` - the directory in which to place the report

2. Open the html report that was generated by multiqc and inspect the QC plots The easiest way to do this is type `xdg-open multiqc_report.html`, which will open the report in a web browser.

Exercise 3.2

In the `metrics` directory you should find Picard metrics for all of the samples.

1. Run multiqc on the contents of the metrics directory.

```
multiqc -z -n Alignment_QC_Report.html -o metrics metrics
```

⇒ metrics/Alignment_QC_Report.html

2. Open the html report that was generated by multiqc and inspect the QC plots

Q. Are there any bam files that look problematic?

SRR7657893 has low alignment rate, an insert size profile that is skewed to left with a median at ~180 bp and a transcript coverage profile that shows a strong 3' bias. This suggests that the RNA in the this sample has been degraded. NOTE: This sample is not real - we have mocked up the metrics files for the purpose of illustrating a poor quality data set.