NGS Coursework (Part I)

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Question 1

The *FASTQ* file that was given in the first practice which contains reads from a simulated whole-genome sequencing experiment, the Negative split generated per barcode (using `cutadapt`), returned very poor mapping results. Here, there is an attempt to remap that *FASTQ* in order to observe an improvement in the mapping statistics.

Note: All the following commands are executed either in the departmental Crystallography Hope Server using a remote SSH connection or in a local environment using ArchLinux and the respective software installed either using `pacman`or the respective source files.

An exploratory view of the file using less shows that:

```
thoth.cryst.bbk.ac.uk> head trimmed_Negative.fq
@AFPN02.1_merge-1076320
NNNNNAAGGTCGCTAATCTCTTTACGCAGATTTTTTATTCCTTCAACTAACAGCGNNNN
+
#####BACCCGFGGGGGGGGGG1GGEGGG0GBGG1GGGGGGGG<GGGGGGGGBCG####
@AFPN02.1_merge-1076319
NNNNNCTTTATCCCGAAAGCGTTTGGTAGCTCGCTGGCATTAACGGGTTCGCCAGNNNN
+
#####BBCCB=G>GGGGGE>GGGGGGG@GGGGDGGGGGGGFGGGGGGGGG@GGGG####
@AFPN02.1_merge-1076318
NNNNNTCTTGCCGATTTCCGCGTTCGGCGCGAGGCGGGTGATCATCTCCAGCACCNNNN
```

It can be easily observed that there are poor quality bases (N) on the first 5 positions (at the 5'-end) and on the last 4 positions (at the 3'-end). Using the generated *FASTQC* Quality Report in Fig that can be seen quite clearly . Apart from this the reads seem to be of a very good quality.

To obtain a better alignment than the one in the Practical, a global alignment with the reference genome was performed using `bowtie2`while trimming the respective N-bases from the 5'-end and 3'-end.

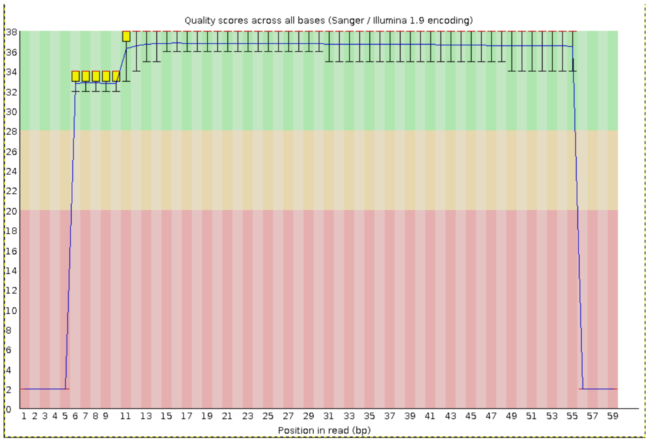


Figure 1: Quality scores for *trimmed_Negative.fq*

Note: For all the following code, the binary locations are added on the path for the session with the command:

```
PATH=${PATH}:/s/software/anaconda/python3/bin/:/s/software/samtools/v1.9/bin/
```

```
bowtie2 -p 127 -5 5 -3 4 \
--end-to-end -x ${st_path}/course_materials/genomes/AFPN02.1/\
AFPN02.1_merge -q \
${st_path}/course_materials/fastq/trimmed_Negative.fq -S \
Negative.sam 2> Negative_bowtie_stats.txt
```

This obtained a 99.97% overall alignment rate! That is the same percentage as in the trimmed Positive sample from the Practical.

```
thoth.cryst.bbk.ac.uk> more Negative_bowtie_stats.txt
1076320 reads; of these:
  1076320 (100.00%) were unpaired; of these:
    308 (0.03%) aligned 0 times
    1020259 (94.79%) aligned exactly 1 time
    55753 (5.18%) aligned >1 times
99.97% overall alignment rate
```

The respective *FASTQ* graph shows as well the alignment coverage in Fig 2

Looking for information from different sources such as (*bowtie2: Relaxed Parameters for Generous Alignments to Metagenomes*, n.d.)and (Viljetic et al., 2017) and exploring different options using the manual of the software, the parameters for `bowtie2` that gave the best result were the following:

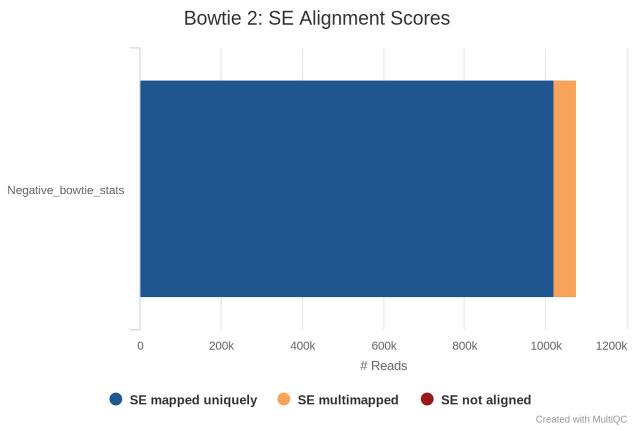


Figure 2: SE Alignment Scores

```
thoth.cryst.bbk.ac.uk> bowtie2 -a -p 8 --np 0 \
--n-ceil L,0,0.2 --non-deterministic -x \
${st_path}/course_materials/genomes/AFPN02.1/AFPN02.1_merge -q \
${st_path}/course_materials/fastq/trimmed_Negative.fq -S \
Negative_with_L.sam 2> Negative_bowtie_stats_with_L.txt

thoth.cryst.bbk.ac.uk> cat !$
1076320 reads; of these:
  1076320 (100.00%) were unpaired; of these:
    5060 (0.47%) aligned 0 times
    1018111 (94.59%) aligned exactly 1 time
    53149 (4.94%) aligned >1 times
99.53% overall alignment rate
```

The meaning of each is as following:

`non-deterministic`: Re-initialisation of pseudo-random generator for each read
`-a`: The program searches for the most distinct, valid alignments for each read
`-p 8`: Multi-threading run of the `bowtie2` alignment
`--np 0`: Penalty for having an N in either the read or the reference
`--n-ceil L,0,0.2`: It sets an upper limit on the number of positions that may contain ambiguous reference characters in a valid alignment. It sets the N function to`f(x) = 0 + 0.15 * x` as a default option. In our case a value of 0.2 multiplied by the number of reads was slightly larger than the N reads and definitely enough to ignore the ambiguous reads.

It is noticeable that the above code needs only 6 seconds to run in contrast with just a plain `-L`

parameter which takes x6 times more! Other options like local and very sensitive alignment were explored but unfortunately without any success (they only returned 0% of matches).

To conclude, two different ways of using the `bowtie2`(Langmead & Salzberg, 2012) were attempted with great success, in the first case similar with the Positive Trimmed sequence on the Practical (99.97% and 99.53 % respectively). Nevertheless, the best practice is to trim before the alignment using software like `trimmomatic` or `cutadpt`. For Whole Genome Sequencing (*WGS*) or larger number of exomes, having the full sequencing information in the *BAM*, allows to get rid of the *FASTQ* which essentially cuts storage costs by one half. Thus one can have major budget implications. Additionally, read mappers like Burrows-Wheeler Aligner (*BWA*) will just soft clip the adapter(s).

In addition to detecting and handling adapters sequence contamination it is important that the *QC* information and results are fed back into the wet lab to ensure higher quality in the future.

In the Fig 3 , Fig 4 and Fig 5 the `samtools` (Li et al., 2009) and `multiqc` (Ewels, Magnusson, Lundin, & Käller, 2016) reports can be seen. Those were generated as following, for both `Negative.sam` and `Negative_with_L.sam`

```
samtools sort Negative.sam > Negative.bam
samtools index Negative.bam
samtools stats Negative.bam > Negative_stats.txt
samtools flagstat Negative.bam > Negative_flagstat.txt
```

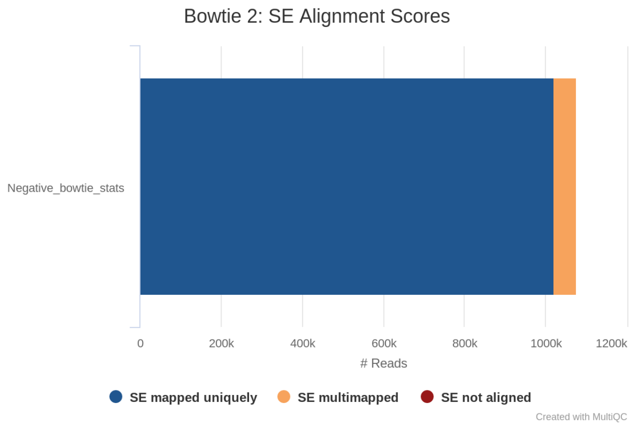


Figure 3: Bowtie2 alignement scores

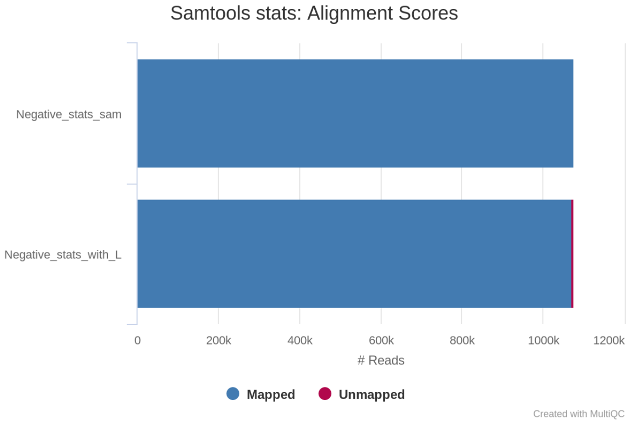
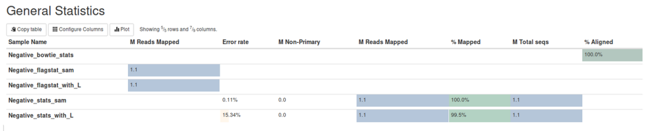


Figure 4: Samtools stats: Two different ways of aligning



General Statistics

Showing $^5/_5$ rows and $^7/_9$ columns.

Sample Name	M Reads Mapped	Error rate	M Non-Primary	M Reads Mapped	% Mapped	M Total seqs	% Aligned
Negative_bowtie_stats							100.0%
Negative_flagstat_sam	1.1						
Negative_flagstat_with_L	1.1						
Negative_stats_sam		0.11%	0.0	1.1	100.0%	1.1	
Negative_stats_with_L		15.34%	0.0	1.1	99.5%	1.1	

Figure 5: samtools error rates

As previously noted, there is an important difference between the (pre-)trimmed sequences: the error rate is only 0.11% while on the L (`--n-ceiling`) parameter there's a much higher error rate observed: 15.34%.

There is also the Integrative Genomics Viewer plot of the two different *BAM* files shown in Fig 6

Question 2

This refers to the *BQ.fq* alignment. The aim here is to achiever higher alignment rates as well as minimal alignment error rate.

In Fig 7 it's shown the Quality Report for BQ. It can be easily seen that the scores are really low quality. Similarly with the previous sequence there are also five ambiguous bases on the 5'-prime and four on the 3'-prime. That may be because of different reasons like the sample quality or the starting material, e.tc.

As in the practical the adapters were removed as well.

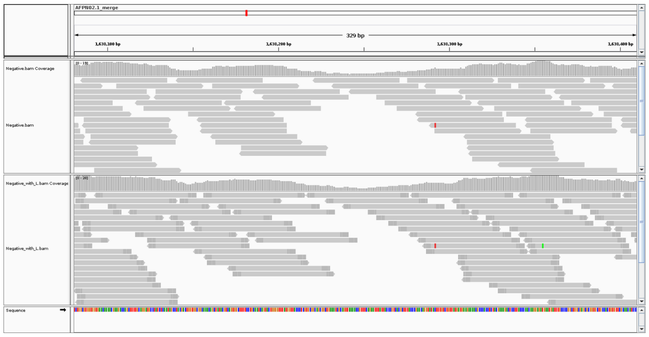


Figure 6: IGV plots

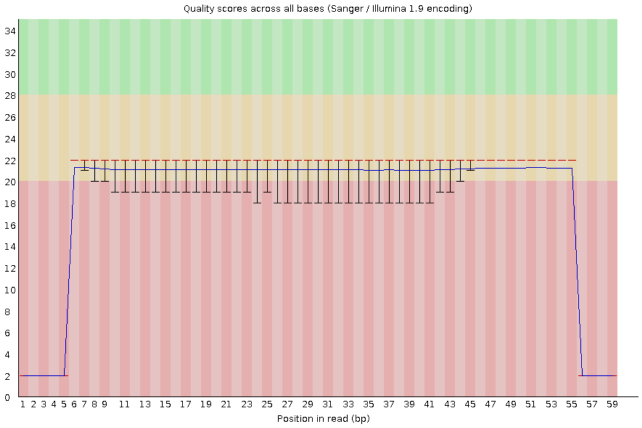


Figure 7: BQ Quality Report

```
cutadapt -g Positive=^GATACA -g \
Negative=^AGTAGT -g BQ=^CACACA -g \
Long=^AAACCC -o trimmed_'{name}'.fq \
final_merge_syntetic_reads.fq
```

`cutadapt`(Verma et al., 2018) is used to trim the ambiguous bases (N) from the 5' and 3' ends and to filter the reads according to sequence quality using the parameter `--trim-n`, which trims N's on ends of reads.

```
cutadapt -g BQ=^CACACA --trim-n -j 127 \
--quality-cutoff 15,8 -o BBQ.fq final_merge_syntetic_reads.fq
```

The parameter `-q`is used by default, only the 3' end of each read is quality-trimmed. To trim the 5' end as well, the option is used with two commaseparated cutoffs, as shown above. This parameter is used to trim low-quality ends from reads before adapter removal.

Different values were tried but this was found to have to most succesfull alignment.

The output summary of this is:

```
This is cutadapt 1.18 with Python 3.7.1
Command line parameters: -g BQ=^CACACA --trim-n -j 127 \
-q 15,8 -o BBQ.fq final_merge_syntetic_reads.fq
Processing reads on 127 cores in single-end mode ...
Finished in 2.82 s (1 us/read; 76.44 M reads/minute).

=== Summary ===

Total reads processed:               3,597,656
Reads with adapters:                 1,086,246 (30.2%)
Reads written (passing filters):     3,597,656 (100.0%)

Total basepairs processed:   256,808,830 bp
Quality-trimmed:               8,690,673 bp (3.4%)
Total written (filtered):    236,169,451 bp (92.0%)

=== Adapter BQ ===

Sequence: CACACA; Type: anchored 5’; Length: 6; Trimmed: \
1086246 times.

No. of allowed errors:
0-6 bp: 0

Overview of removed sequences
length  count   expect  max.err error counts
6   1086246 878.3   0   1086246
```

Bringing this back into `bowtie2` for alignment we get a 94.51% alignment rate!

```
thoth.cryst.bbk.ac.uk> cat BBQ_bowtie_stats.txt
3597656 reads; of these:
  3597656 (100.00%) were unpaired; of these:
    197548 (5.49%) aligned 0 times
    3252609 (90.41%) aligned exactly 1 time
    147499 (4.10%) aligned >1 times
94.51% overall alignment rate
```

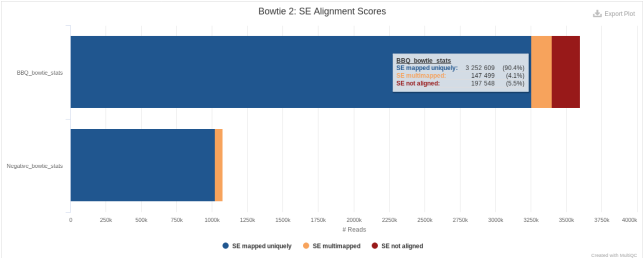
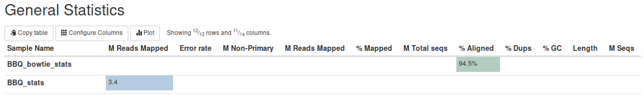


Figure 8: Alignment Scores with BQ



General Statistics

Showing 12/12 rows and 11/14 columns.

Sample Name	M Reads Mapped	Error rate	M Non-Primary	M Reads Mapped	% Mapped	M Total seqs	% Aligned	% Dups	% GC	Length	M Seqs
BBQ_bowtie_stats							94.5%				
BBQ_stats	3.4										

Figure 9: General Statistics

Finally, the `samtools` and `flagstat` analysis are shown in Fig 8 and Fig 9

As described in (Salari, Zare-Mirakabad, Sadeghi, & Rokni-Zadeh, 2018), to decrease the error rate "(we) reduce the search space for the reads which can be aligned against the genome with mismatches, insertions or deletions to decrease the probability of incorrect read mapping". This involves a quite intrinsic pipeline workflow through which isn't possible to achieve in this coursework. Despite that many different values were tested against the quality algorithm of the software in order to find the best outcome.

Question 3

Before processing the *BAM* file a report was generated in order to extract some useful information from it. For that, RSeQC (Wang, Wang, & Li, 2012) was locally installed from source and used like this:

```
thoth.cryst.bbk.ac.uk> ~/.local/bin/bam_stat.py -i BBQ.bam
Load BAM file ...  Done

#==================================================
#All numbers are READ count
#==================================================

Total records:                          3597656
```

```
QC failed:                              0
Optical/PCR duplicate:                  0
Non primary hits                        0
Unmapped reads:                         197548
mapq < mapq_cut (non-unique):           2565319

mapq >= mapq_cut (unique):              834789
Read-1:                                 0
Read-2:                                 0
Reads map to '+':                       417536
Reads map to '-':                       417253
Non-splice reads:                       834789
Splice reads:                           0
Reads mapped in proper pairs:           0
Proper-paired reads map to different chrom:0
```

In order to separate the *BBQ.bam* in two, with one part including all the mapped and the other all the unmapped reads, the following is run:

```
#samtools seperation
samtools view -h -F 4 BBQ.bam | samtools view -bS > BBQ_mapped.bam
samtools view -h -f 4 BBQ.bam | samtools view -bS > BBQ_unmapped.bam
```

To get the uniquely mapped reads the following command was run:

```
grep -v "XS:i" | grep "AS:i" BBQ.sam >| Uniquely_mapped.sam

samtools view -h BBQ.bam | grep -E -v "XS:i" \
| grep -E "@|AS:i" | samtools view -b - >| \
Uniquely_mapped.bam
```

and for multi-mapped reads respectively:

```
samtools view BBQ.bam | grep -v NH:i:1 \
| perl -pe 's/AS.+(NH:i:\d+)/\$1/' | \
cut -f1,10,12 | perl -pe 's/NH:i://' \
| sort -u -k3,3nr > sorted-multi-mapped.txt
```

The output format of this file is: `read_ID<tab>read<tab>number times mapped`

Unfortunately, I couldn't continue further with this practical as for some reason the hope server refused to accept my SSH connection.

```
[wizofe@beastie ~]$ ssh vi001@hope-ext.cryst.bbk.ac.uk
ssh: connect to host hope-ext.cryst.bbk.ac.uk port 22: Connection refused
```

Note: For all the code examples and in order to be nicely displayed for the pdf the continuity shell character has been used '\'. Sometimes, this is not parsed correctly from some PDF viewers, so the copy paste of the code may not work as it is. Thus, one can remove the character and concatenate the command.

References

bowtie2: Relaxed Parameters for Generous Alignments to Metagenomes. (n.d.). https://samnicholls.net/2016/12/24/bowtie2-metagenomes/. Retrieved from `https://samnicholls.net/2016/12/24/bowtie2-metagenomes/` (Accessed on Mon, April 01, 2019)

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016, jun). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. Retrieved from `https://doi.org/10.1093%2Fbioinformatics%2Fbtw354` doi: 10.1093/bioinformatics/btw354

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Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … and, R. D. (2009, jun). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. Retrieved from `https://doi.org/10.1093%2Fbioinformatics%2Fbtp352` doi: 10.1093/bioinformatics/btp352

Salari, F., Zare-Mirakabad, F., Sadeghi, M., & Rokni-Zadeh, H. (2018, nov). Assessing the impact of exact reads on reducing the error rate of read mapping. *BMC Bioinformatics*, *19*(1). Retrieved from `https://doi.org/10.1186%2Fs12859-018-2432-7` doi: 10.1186/s12859-018-2432-7

Verma, S., Du, P., Nakanjako, D., Hermans, S., Briggs, J., Nakiyingi, L., … Salgame, P. (2018, may). "Tuberculosis in advanced HIV infection is associated with increased expression of IFN and its downstream targets". *BMC Infectious Diseases*, *18*(1). Retrieved from `https://doi.org/10.1186%2Fs12879-018-3127-4` doi: 10.1186/s12879-018-3127-4

Viljetic, B., Diao, L., Liu, J., Krsnik, Z., Wijeratne, S. H. R., Kristopovich, R., … Rasin, M.-R. (2017, feb). Multiple roles of PIWIL1 in mouse neocorticogenesis. Retrieved from `https://doi.org/10.1101%2F106070` doi: 10.1101/106070

Wang, L., Wang, S., & Li, W. (2012, jun). RSeQC: quality control of RNA-seq experiments. *Bioinformatics*, *28*(16), 2184–2185. Retrieved from `https://doi.org/10.1093%2Fbioinformatics%2Fbts356` doi: 10.1093/bioinformatics/bts356