RNA-seq Quality Assessment

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Part 1 - Read Quality Score Distributions

Using FastQC to generate distribution plots of the quality scores of the R1 and R2 reads.

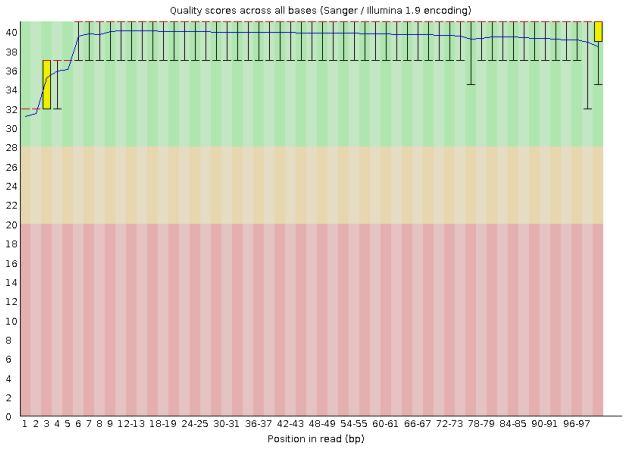
Using the following command, FastQC plots were generated to display the distribution of both mean quality scores per base, as well as distributions of the mean 'N' content per base.

```
cat FastQC.wrapper.sh
```

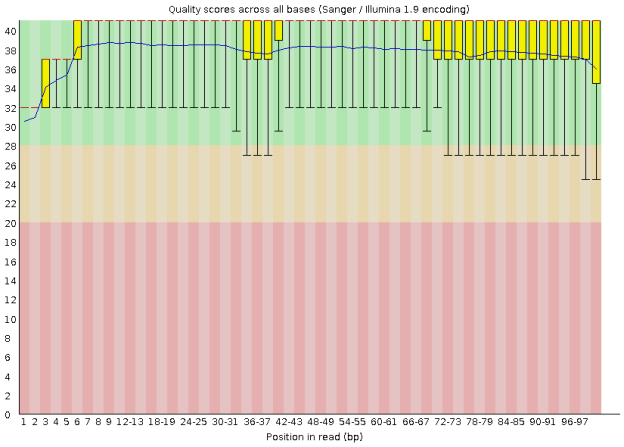
```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=1
## #SBATCH --time=0-20:00:00
## #SBATCH --output=FastQC.output.%j
## #SBATCH --error=FastQC.output.err
##
##
## #Files I am using: 23_4A_control_S17_L008 and 22_3H_both_S16_L008
## file1read1='/projects/bgmp/shared/2017_sequencing/demultiplexed/23_4A_control_S17_L008_R1_001.fastq.g
## file1read2='/projects/bgmp/shared/2017_sequencing/demultiplexed/23_4A_control_S17_L008_R2_001.fastq.g
## file2read1='/projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R1_001.fastq.gz'
## file2read2='/projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R2_001.fastq.gz'
##
## ml fastqc/0.11.5
##
## /usr/bin/time -v fastqc $file1read1 $file1read2 $file2read1 $file2read2 \
## -o '/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/fastqc_output'
```

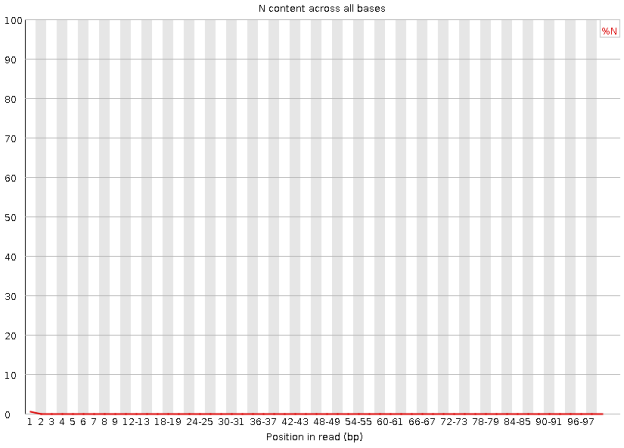
Plots for 22__3H__both__S16

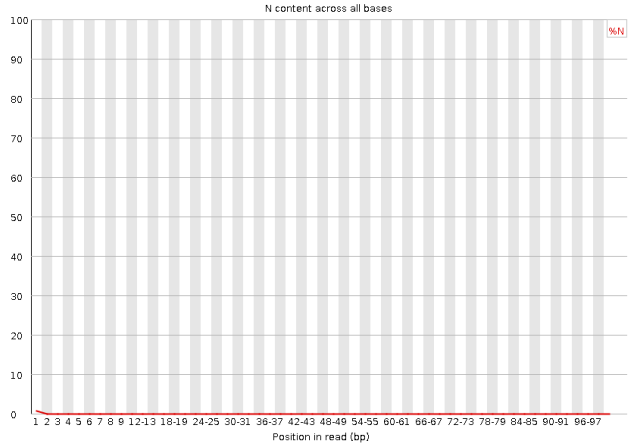
Graph order: Top two represent mean quality score distribution and the bottom two represent mean 'N' content per base. Left two represent Read 1 (forward) and



right two represent Read 2 (reverse).







Plots for 23_4A_control_S17:

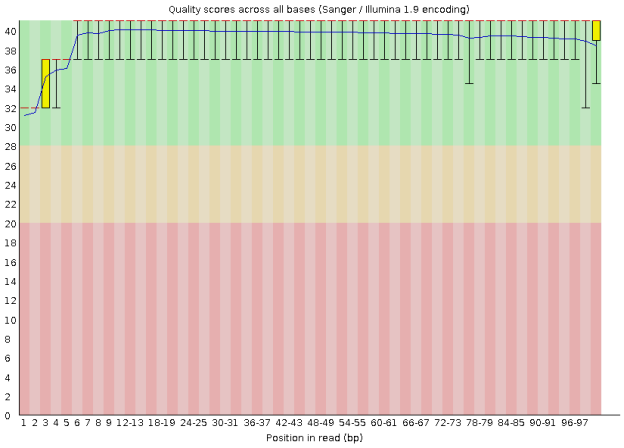
Graph order: Top two represent mean quality score distribution and the bottom two represent mean 'N' content per base. Left two represent Read 1 (forward) and right two represent Read 2 (reverse).

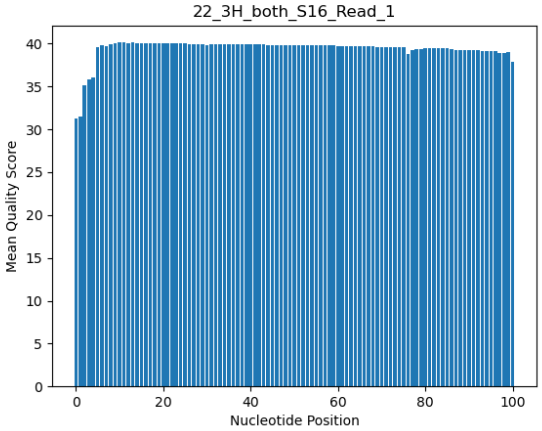
Looking at distribution plots produced by FastQC, it appears that all of the reads have an average quality score above 30, with the majority of values being between 38-40, which is consistent with the distribution plots of 'n' content per base which shows almost no 'N's' in the reads.

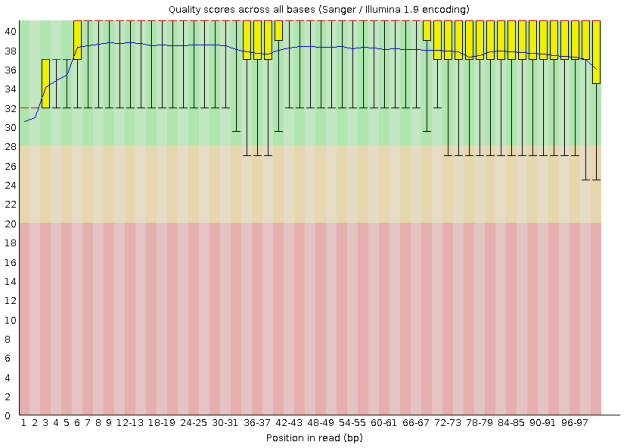
The following is a comparison of the FastQC mean quality score distribution plots with those generated by the script I created. *Note* that mean quality score plots represent the distribution of the mean quality score, based on Phred + 33 for each base position across all sequences.

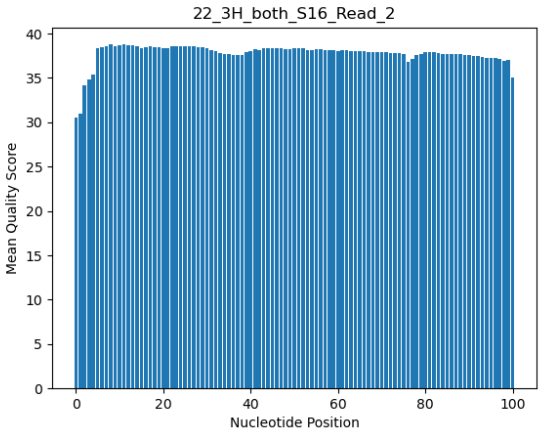
Plots for 22_3H_both:

Graph order: Top two represent Read 1 (forward) and bottom two represent Read 2 (reverse).



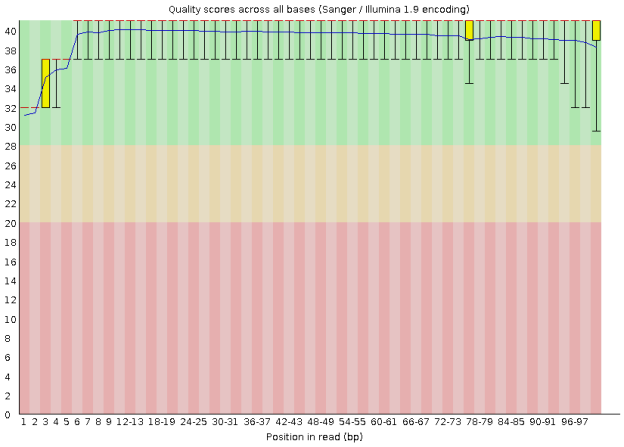


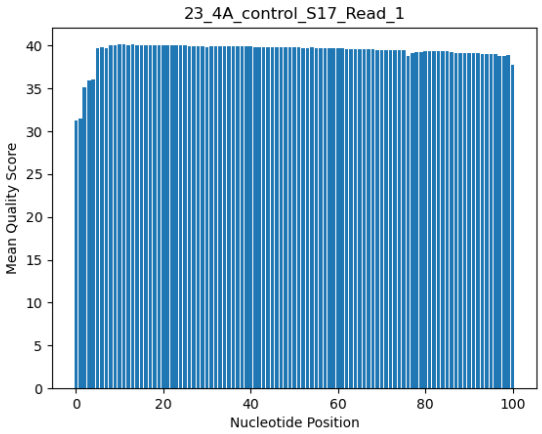


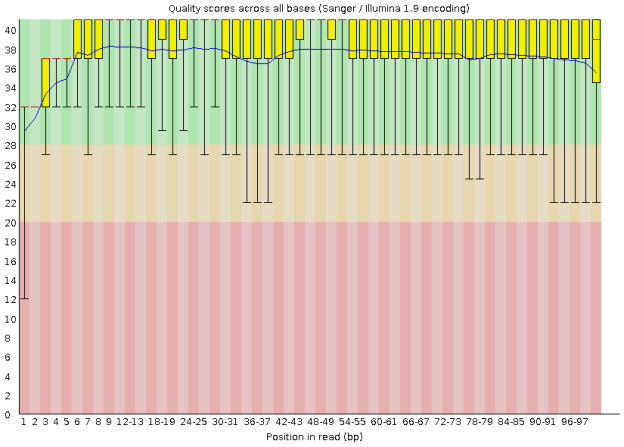


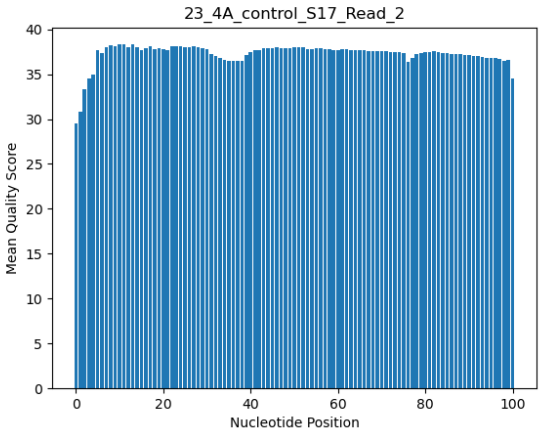
Plots for 23__4A__control:

Graph order: Top two represent Read 1 (forward) and bottom two represent Read 2 (reverse).









In comparing the distribution plots generated by fastQC compared to those I generated, it appears the distribution plots all show an identical pattern to the FastQC distribution plots, without the standard deviation bars. In comparing the run time of my script compared to FastQC, my script ran much slower, with a total completion time for all four graphs of about 40 minutes. The FastQC software generated all the plots in 10 minutes. When considering the cause of this difference, I would think that the fastQC software is optimized for speed and efficieny, whereas my script is not. Additionally, FastQC is written in java, which is a compiled language compared to python, which is an interpreted language, and so it runs slower.

In considering the overall data quality of these two libraries, I would say it is excellent. There are very few 'N's and almost all base-calls have Qscores above 38, which represents an error probability of 0.00016. The only point of concern pertains to 22__3H__both__S16 in that the per-base-quality displays a seemingly high degree of variability in base calls towards the end of the sequence read. While more variable, the variability still seems to lie within the range of high Qscores so it is not much of a concern.

Adaptor trimming comparison

Cutadapt (https://cutadapt.readthedocs.io/en/stable/) was used with the following parameters to trim adapter sequences:

```
cat cutadapt.wrapper.sh
```

```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=1
## #SBATCH --time=0-16:00:00
## #SBATCH --output=Cutadapt.out.%j
##
## file1read1='/projects/bgmp/shared/2017_sequencing/demultiplexed/23_4A_control_S17_L008_R1_001.fastq.g
## file1read2='/projects/bgmp/shared/2017_sequencing/demultiplexed/23_4A_control_S17_L008_R2_001.fastq.g
## file2read1='/projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R1_001.fastq.gz'
## file2read2='/projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R2_001.fastq.gz'
##
## conda activate QAA
##
## cutadapt -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT \
## -o '/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/23_4A_control_S17_L008_R1.fastq.gz'
## -p '/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/23_4A_control_S17_L008_R2.fastq.gz'
## $file1read1 $file1read2
##
## cutadapt -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT \
## -o '/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/22_3H_both_S16_L008_R1.fastq.gz' \
## -p '/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/22_3H_both_S16_L008_R2.fastq.gz' \
## $file2read1 $file2read2
```

The following table summarizes the proportion of reads that were trimmed:

File	Total Proportion trimmed	Proportion of forward reads trimmed	Proportion of reverse reads trimmed
22_3H_both	8.4%	3.8%	4.6%
23_4A_control	6.8%	3.1%	3.7%

To confirm the adapter sequence orientation, the following command was used to identify sequence lines that contain the adapter:

for Read 1 check (performed on both files, but only one file example shown):

```
zcat /projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R1_00fastq.gz |  grep 'AGA
```

```
output: 7563
```

for Read 2 check (performed on both files, but only one file example shown):

```
zcat /projects/bgmp/shared/2017_sequencing/demultiplexed/22_3H_both_S16_L008_R2_001.fastq.gz |  grep 'A
```

```
output: 7848
```

In short, the commands are used to read in all lines of the FASTQ file, and grab only those lines which contain the appropriate adapter-sequence string. Finally the code outputs a number representing how many lines contain that sequence. Additionally, by printing out a the grepped lines, we can see that the adapter sequence consistently occurs at the end of the read, confirming its presence. Furthermore, reading into Illumina's adapter kits, I can see that this library was built using the TruSeq single index, which reports the same forward and reverse indexes. https://support.illumina.com/bulletins/2016/12/what-sequences-do-i-use-for-adapter-trimming.html

Next, Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) was used with the following parameters to trim the sequence reads.

```
cat trimmomatic.wrapper.sh
```

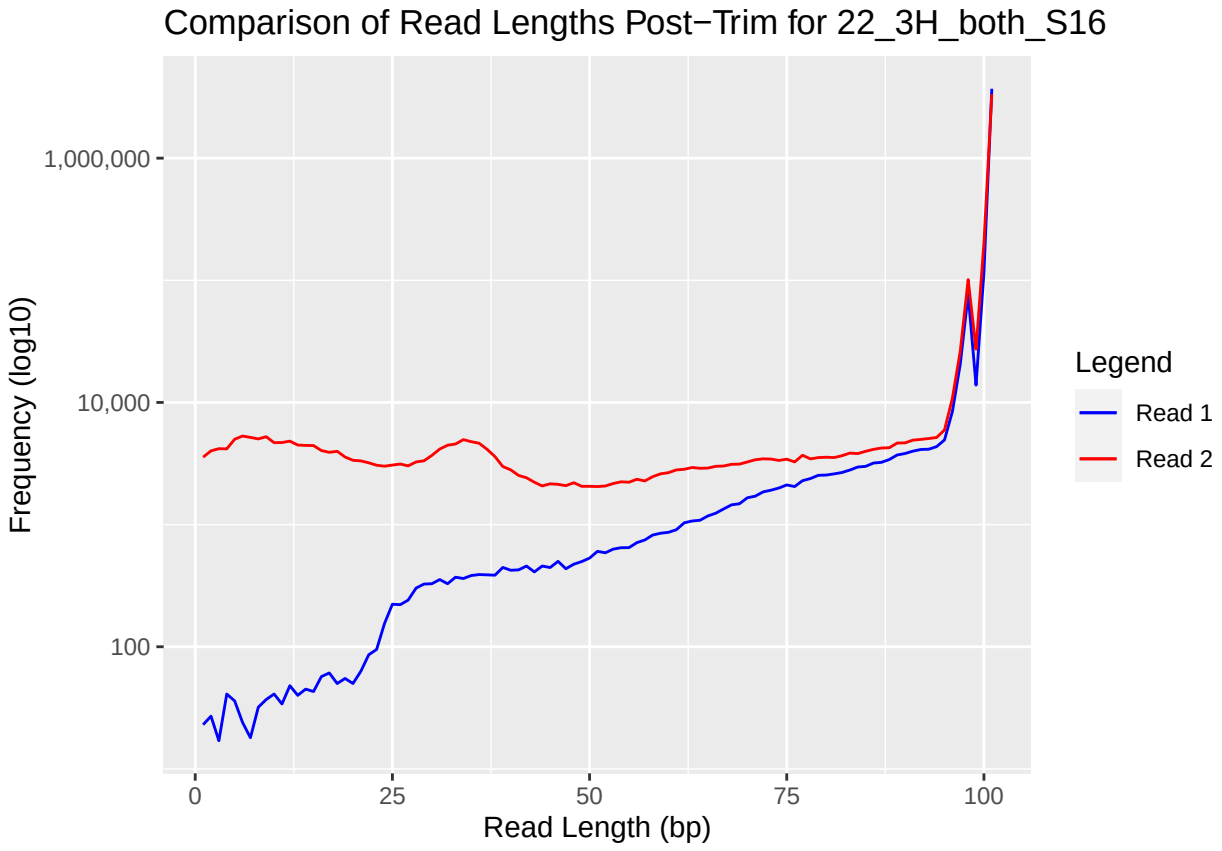
```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=8
## #SBATCH --time=0-16:00:00
## #SBATCH --output=trimmomatic.out.%j
##
## #files are taken from 'Cutadapt_output' directory within /QAA
## file1read1='/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/22_3H_both_S16_L008_R1.fast
## file1read2='/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/22_3H_both_S16_L008_R2.fast
## file2read1='/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/23_4A_control_S17_L008_R1.fa
## file2read2='/home/zsisson2/bgmp/bioinformatics/Bi623/QAA/Cutadapt_output/23_4A_control_S17_L008_R2.fa
##
```

```
## conda activate QAA
##
## /usr/bin/time -v  trimmomatic PE -threads 8 $file1read1 $file1read2 \
## 'Trimmomatic_output/22_3H_both_S16_L008_R1.trimmed.fastq.gz' \
## 'Trimmomatic_output/22_3H_both_S16_L008_R1un.trimmed.fastq.gz' \
## 'Trimmomatic_output/22_3H_both_S16_L008_R2.trimmed.fastq.gz' \
## 'Trimmomatic_output/22_3H_both_S16_L008_R2un.trimmed.fastq.gz' \
## LEADING:3 \
## TRAILING:3 \
## SLIDINGWINDOW:5:15 \
##
## /usr/bin/time -v  trimmomatic PE -threads 8 $file2read1 $file2read2 \
## 'Trimmomatic_output/23_4A_control_S17_L008_R1.trimmed.fastq.gz' \
## 'Trimmomatic_output/23_4A_control_S17_L008_R1un.trimmed.fastq.gz' \
## 'Trimmomatic_output/23_4A_control_S17_L008_R2.trimmed.fastq.gz' \
## 'Trimmomatic_output/23_4A_control_S17_L008_R2un.trimmed.fastq.gz' \
## LEADING:3 \
## TRAILING:3 \
## SLIDINGWINDOW:5:15 \
```

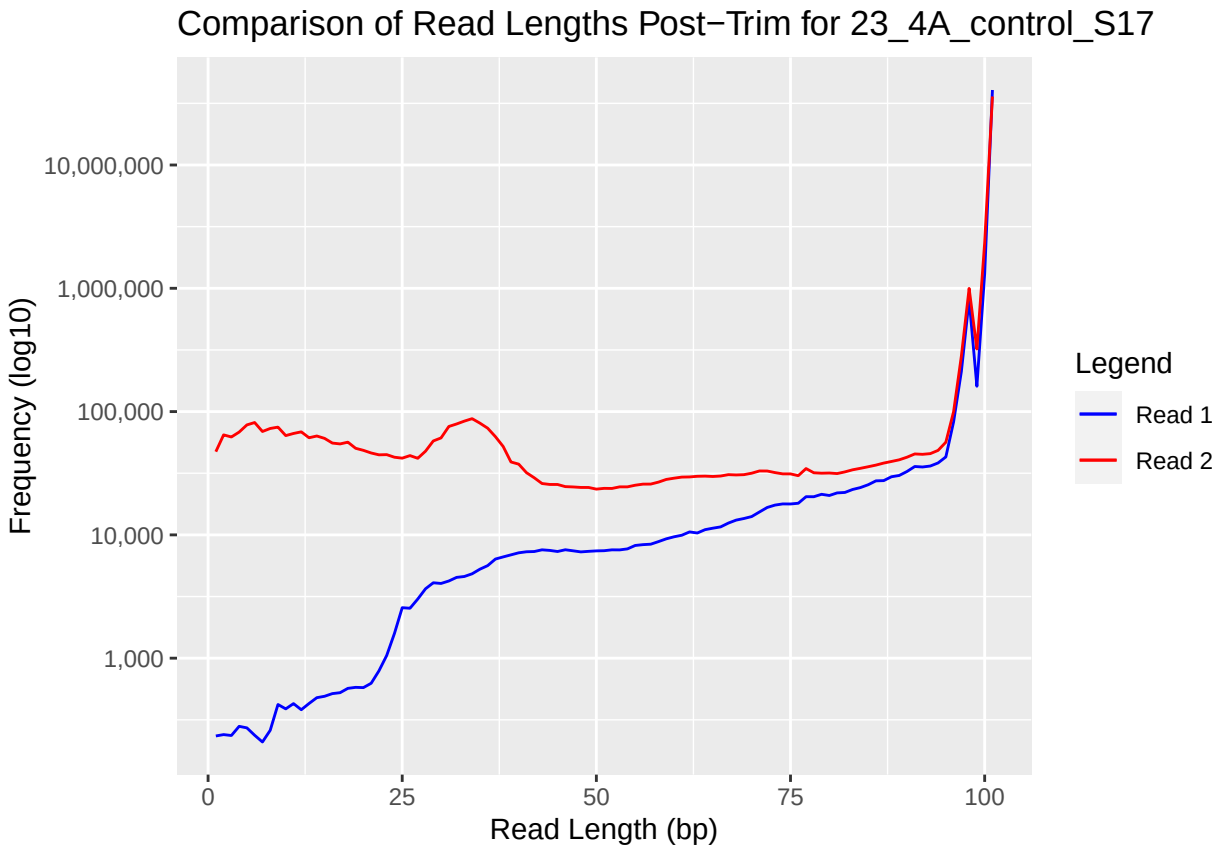
To compare the amount of trimming each read underwent, the following code was used to generate a plot to visualize the difference.

```
#Reading in the Sequence Data as Vectors
w = c(readLines('22_3H_both_S16_L008_R1.trimmed.seqonly.txt'))

x = c(readLines('22_3H_both_S16_L008_R2.trimmed.seqonly.txt'))

y = c(readLines('23_4A_control_S17_L008_R1.trimmed.seqonly.txt'))

z = c(readLines('23_4A_control_S17_L008_R2.trimmed.seqonly.txt'))

##Create Data Frames for Read1/Read2 of the sequence Data
df_22_3H <- data.frame(w,x)
df_23_4A <- data.frame(y,z)
names(df_22_3H) <- c("Read_1_(forward)","Read_2_(reverse)")
names(df_23_4A) <- c("Read_1_(forward)","Read_2_(reverse)")

##Create a new column containing the counts of the sequences
df_22_3H <- mutate(df_22_3H,Read_1_count=str_length(`Read_1_(forward)`))
df_22_3H <- mutate(df_22_3H,Read_2_count=str_length(`Read_2_(reverse)`))

df_23_4A <- mutate(df_23_4A,Read_1_count=str_length(`Read_1_(forward)`))
df_23_4A <- mutate(df_23_4A,Read_2_count=str_length(`Read_2_(reverse)`))

##Group the sequences by read length
Seq_counts_22_3H <- count(df_22_3H,`Read_1_count`)
Seq_counts_22_3H <- cbind(Seq_counts_22_3H, count(df_22_3H,`Read_2_count`))
colnames(Seq_counts_22_3H) <- c("Post_Trim_Sequence_Length", "count_R1","len","count_R2")

Seq_counts_23_4A <- count(df_23_4A,`Read_1_count`)
Seq_counts_23_4A <- cbind(Seq_counts_23_4A, count(df_23_4A,`Read_2_count`))
```

```
colnames(Seq_counts_23_4A) <- c("Post_Trim_Sequence_Length", "count_R1","len","count_R2")

#Plotting the distributions
colors <- c("Read 1" = "blue", "Read 2" = "red")

distrib_22_3H = ggplot(data = Seq_counts_22_3H, aes(x = Post_Trim_Sequence_Length)) + geom_line(aes(y =
  geom_line(aes(y = count_R2, color = "Read 2")) +
  labs(x="Read Length (bp)", y="Frequency (log10)", title = "Comparison of Read Lengths Post-Trim for 2
  scale_color_manual(values = colors) +
  scale_y_continuous(trans = 'log10', labels = scales::comma)

distrib_23_4A = ggplot(data = Seq_counts_23_4A, aes(x = Post_Trim_Sequence_Length)) + geom_line(aes(y =
  geom_line(aes(y = count_R2, color = "Read 2")) +
  labs(x="Read Length (bp)", y="Frequency (log10)", title = "Comparison of Read Lengths Post-Trim for 2
  scale_color_manual(values = colors) +
  scale_y_continuous(trans = 'log10', labels = scales::comma)

print(distrib_22_3H)
```




```
print(distrib_23_4A)
```



The Read 2 sequences appear to have undergone more trimming. This falls in line with what I would expect to see, considering that during Illumina sequencing, Read 1 begins at the primer binding and extends towards the 3' end. If the polymerase extends past the index, then the adapter will also get sequenced, resulting in more sequences in read 2 that need to be trimmed than in Read 1.

Alignment and strand-specificity

To align the reads from the RNA-seq, STAR, a splice aware aligner https://github.com/alexdobin/STAR was used. First, GTF and Fasta files for the Mouse genome were acquired from Ensembl via http://ftp.ensembl.org/pub/release-104/gtf/mus_musculus/Mus_musculus.GRCm39.104.gtf.gz and http://ftp.ensembl.org/pub/release-104/fasta/mus_musculus/dna/Mus_musculus.GRCm39.dna.primary_assembly.fa.gz, respectively. A reference database was generated using the following command with STAR installed.

```
cat STAR_generate.sh
```

```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=8
```

```
## #SBATCH --time=0-20:00:00
## #SBATCH --output=STAR_generate.out.%j
##
## GTF='Ensembl_data/Mus_musculus.GRCm39.104.gtf'
## Fasta='Ensembl_data/Mus_musculus.GRCm39.dna.primary_assembly.fa'
##
## conda activate QAA
## /usr/bin/time -v  STAR --runThreadN 8 \
## --runMode genomeGenerate \
## --genomeDir STAR_mouse_database \
## --genomeFastaFiles $Fasta \
## --sjdbGTFfile $GTF \
```

Sequences were then aligned with the following run parameters.

```
cat STAR_Align_RNAseq.sh
```

```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=8
## #SBATCH --time=0-20:00:00
## #SBATCH --output=STARalign.out.%j
##
## Read1_22_3H='Trimmomatic_output/22_3H_both_S16_L008_R1.trimmed.fastq.gz'
## Read2_22_3H='Trimmomatic_output/22_3H_both_S16_L008_R2.trimmed.fastq.gz'
##
## Read1_23_4A='Trimmomatic_output/23_4A_control_S17_L008_R1.trimmed.fastq.gz'
## Read2_23_4A='Trimmomatic_output/23_4A_control_S17_L008_R2.trimmed.fastq.gz'
##
## /usr/bin/time -v  STAR --runThreadN 8 \
## --runMode alignReads \
## --outFilterMultimapNmax 3 \
## --outSAMunmapped Within KeepPairs \
## --alignIntronMax 1000000 \
## --alignMatesGapMax 1000000 \
## --readFilesCommand zcat \
## --readFilesIn $Read1_22_3H $Read2_22_3H \
## --genomeDir STAR_mouse_database \
## --outFileNamePrefix 'RNA_seq_aligned/Mus_22_3H_both_16_L008.'
##
##
## /usr/bin/time -v  STAR --runThreadN 8 \
## --runMode alignReads \
## --outFilterMultimapNmax 3 \
## --outSAMunmapped Within KeepPairs \
## --alignIntronMax 1000000 \
## --alignMatesGapMax 1000000 \
## --readFilesCommand zcat \
## --readFilesIn $Read1_23_4A $Read2_23_4A \
```

```
## --genomeDir STAR_mouse_database \
## --outFileNamePrefix 'RNA_seq_aligned/Mus_23_4A_control_S17_L008.'
```

From the SAM file produced during this alignment, the following read mapping statistics were produced.

For 22__3H__both__S16__L008:

```
Sequences Mapped:  7869231
Sequences Unmapped:  215473
```

And for 23__4A__control__S17__L008:

```
Sequences Mapped:  82156195
Sequences Unmapped:  6154403
```

Next, a count of reads that mapped to features was produced using HTseq (https://htseq.readthedocs.io/en/master/) using the following command. The count was performed two times for each file, with the parameter '–stranded' being set to both 'yes' and 'no' in order to compare results.

```
cat HTseq_count.sh
```

```
## #!/usr/bin/bash
## #SBATCH --account=bgmp
## #SBATCH --partition=bgmp
## #SBATCH --nodes=1
## #SBATCH --ntasks-per-node=1
## #SBATCH --cpus-per-task=1
## #SBATCH --time=0-20:00:00
## #SBATCH --output=HTseq_count.out.%j
##
##
## file_22_3H_both_16='RNA_seq_aligned/Mus_22_3H_both_16_L008.Aligned.out.sam'
## file_23_4A_control_17='RNA_seq_aligned/Mus_23_4A_control_S17_L008.Aligned.out.sam'
## GTF='Ensembl_data/Mus_musculus.GRCm39.104.gtf'
##
## conda activate QAA
##
## /usr/bin/time -v htseq-count --stranded=yes $file_22_3H_both_16 $GTF > 'HTseq_output/22_3H_both_stra
## /usr/bin/time -v htseq-count --stranded=no $file_22_3H_both_16 $GTF > 'HTseq_output/22_3H_both_unstr
##
## /usr/bin/time -v htseq-count --stranded=yes $file_23_4A_control_17 $GTF > 'HTseq_output/23_4A_contro
## /usr/bin/time -v htseq-count --stranded=no $file_23_4A_control_17 $GTF > 'HTseq_output/23_4A_control
```

The results are summarized in the following tables:

For 22__3H__both:

Statistic	Stranded = yes	Stranded = no
No_feature	3,573,473	190,786
ambiguous	2,333	195,352
too_low_aQual	80,996	80,996
not_aligned	64,525	64,525
alignment_not_unique	176,655	176,655

For 23_4A_control:

Statistic	Stranded = yes	Stranded = no
No_feature	36,845,000	4,087,870
ambiguous	35,387	2,011,666
too_low_aQual	1,167,001	1,167,001
not_aligned	2,438,035	2,438,035
alignment_not_unique	2,123,419	2,123,419

The HTseq output files were further parsed using the following script to generate additional statistics on each file:

```
cat HTseq_parser.py
```

```
## #!/usr/bin/env python
##
## import argparse
## import re
##
## def get_args():
##  parser = argparse.ArgumentParser(description="A script to parse the .tsv HTseq output files that rep
##  parser.add_argument("-f", "--file_name", help = "File to run script on", type=str, required=True)
##  parser.add_argument("-p", "--outpath", help = "Path to output directory", type=str, required=True)
##  parser.add_argument("-o", "--outfile", help = "Name of output stats file", type=str, required=True)
##  return parser.parse_args()
##
## args = get_args()
##
## with open(args.file_name, "r") as fh:
##  #Initiate counter for total genes, number of total hits, and number of genes with hits
##  hit_counter = 0
##  total_hit_counter = 0
##  gene_counter = 0
##  #Grab the number of times each gene was a hit (represented by the number) and add it to the hit_coun
##  for line in fh:
##      cut_line = re.split('\t',line.strip('\n'))
##      hitval = int(cut_line[1])
##      total_hit_counter += hitval
##      gene_counter += 1
##      if hitval > 0:
##          hit_counter += 1
##      else:
```

```
##          pass
##
## out = str(str(args.outpath)+str(args.outfile))
##
## #print statistics
## with open(out, "w") as fto:
##  fto.write(str("Displaying statistics for: " +str(args.outfile) + '\n'))
##  fto.write(str("Number of sequences with gene feature hits: " + str(hit_counter) +'\n'))
##  fto.write(str("Total number of gene feature hits across all sequences: " + str(total_hit_counter)+
##  percent = round((hit_counter/gene_counter)*100,2)
##  fto.write(str("Percentage of gene features with hits: " + str(percent)+ str("%")))
```

Statistics produced by the script are summarized in the following tables:

For 22__3H__both:

Statistic	Stranded = yes	Stranded = no
Number of sequences with gene feature hits	8,147	15,631
Total number of gene feature hits across all sequences	4,042,352	4,042,352
Percentage of gene features with hits	14.7%	28.2%

For 23__4A__control:

Statistic	Stranded = yes	Stranded = no
Number of sequences with gene feature hits	15,511	23,330
Total number of gene feature hits across all sequences	44,155,299	44,155,299
Percentage of gene features with hits	27.99%	42.1%

From these data, I would conclude that the the RNA-seq libraries were made without a strand-specific kit. In unstranded kits, about 50% of the reads would match the reference genome with the correct directionality. Looking at the tables, it appears that when the stranded flag = no, the percentage of gene features with hits just about doubles. I would expect to see this if the reads were mapping to both the forward and reverse strand of the reference genome. Furthermore, comparing the output tables from HTSeq, it appears that there are many more instances of sequences being classified as 'No__feature' when the kit is assumed to be stranded. Given these results, the reads must be a mixture of antisense/sense strands, thus suggesting the kit used to generate these libraries was unstranded.