Wrangling genomics

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Preface

A lot of genomics analysis is done using command-line tools for three reasons:

1. you will often be working with a large number of files, and working through the command-line rather than through a graphical user interface (GUI) allows you to automate repetitive tasks,
2. you will often need more compute power than is available on your personal computer, and connecting to and interacting with remote computers requires a command-line interface, and
3. you will often need to customize your analyses, and command-line tools often enable more customization than the corresponding GUI tools (if in fact a GUI tool even exists).

In a previous lesson, you learned how to use the bash shell to interact with your computer through a command line interface. In this lesson, you will be applying this new knowledge to carry out a common genomics workflow - identifying variants among sequencing samples taken from multiple individuals within a population. We will be starting with a set of sequenced reads (`.fastq` files), performing some quality control steps, aligning those reads to a reference genome, and ending by identifying and visualizing variations among these samples.

As you progress through this lesson, keep in mind that, even if you aren't going to be doing this same workflow in your research, you will be learning some very important lessons about using command-line bioinformatic tools. What you learn here will enable you to use a variety of bioinformatic tools with confidence and greatly enhance your research efficiency and productivity.

Prerequisites

This lesson assumes a working understanding of the bash shell. If you haven't already completed the Shell Genomics lesson, and aren't familiar with the bash shell, please review those materials before starting this lesson.
This lesson also assumes some familiarity with biological concepts, including the structure of DNA, nucleotide abbreviations, and the concept of genomic variation within a population.

Schedule

	Setup	Download files required for the lesson
00:00	1. Background and metadata	What data are we using? Why is this experiment important?
00:15	2. Assessing read quality	How can I describe the quality of my data?
01:05	3. Trimming and filtering	How can I get rid of sequence data that does not meet my quality standards?
02:00	4. Variant calling workflow	How do I find sequence variants between my sample and a reference genome?
03:00	5. Automating a variant calling workflow	How can I make my workflow more efficient and less error-prone?
03:45	Finish	

The actual schedule may vary slightly depending on the topics and exercises chosen by the instructor.

1 Background and metadata

Time

- Teaching: 10 min
- Exercises: 5 min

Questions

- What data are we using?
- Why is this experiment important?
- Why study E. coli?
- What is hypermutability?

Objectives

- Understand the data set.

1.1 Background

We are going to use a long-term sequencing data set from a population of *Escherichia coli*.

1.1.1 What is *E. coli*?

E. coli are rod-shaped bacteria that can survive under a wide variety of conditions including variable temperatures, nutrient availability, and oxygen levels. Most strains are harmless, but some are associated with food-poisoning.

1.1.2 Why is *E. coli* important?

E. coli are one of the most well-studied model organisms in science. As a single-celled organism, *E. coli* reproduces rapidly, typically doubling its population every 20 minutes, which means it can be manipulated easily in experiments. In addition, most naturally occurring strains of *E.*

coli are harmless. Most importantly, the genetics of *E. coli* are fairly well understood and can be manipulated to study adaptation and evolution.

1.2 The data

The data we are going to use is part of a long-term evolution experiment led by Richard Lenski.

The experiment was designed to assess adaptation in *E. coli*. A population was propagated for more than 40,000 generations in a glucose-limited minimal medium (in most conditions glucose is the best carbon source for *E. coli*, providing faster growth than other sugars). This medium was supplemented with citrate, which *E. coli* cannot metabolize in the aerobic conditions of the experiment. Sequencing of the populations at regular time points revealed that spontaneous citrate-using variant (Cit+) appeared between 31,000 and 31,500 generations, causing an increase in population size and diversity. In addition, this experiment showed hypermutability in certain regions. Hypermutability is important and can help accelerate adaptation to novel environments, but also can be selected against in well-adapted populations.

To see a timeline of the experiment to date, check out this figure, and this paper by Blount, Borland, and Lenski (2008) titled "Historical contingency and the evolution of a key innovation in an experimental population of Escherichia coli".

1.2.1 View the metadata

We will be working with three sample events from the **Ara-3** strain of this experiment, one from 5,000 generations, one from 15,000 generations, and one from 50,000 generations. The population changed substantially during the course of the experiment, and we will be exploring how (the evolution of a **Cit+** mutant and **hypermutability**) with our variant calling workflow. The metadata file associated with this lesson can be downloaded directly here or viewed in Github. If you would like to know details of how the file was created, you can look at some notes and sources here.

This metadata describes information on the *Ara-3* clones and the columns represent:

Column	Description
strain	strain name
generation	generation when sample frozen
clade	based on parsimony-based tree
reference	study the samples were originally sequenced for
population	ancestral population group
mutator	hypermutability mutant status
facility	facility samples were sequenced at

Column	Description
run	Sequence read archive sample ID
read_type	library type of reads
read_length	length of reads in sample
sequencing_depth	depth of sequencing
cit	citrate-using mutant status

Exercise

Based on the metadata, can you answer the following questions?

1. How many different generations exist in the data?
2. How many rows and how many columns are in this data?
3. How many citrate+ mutants have been recorded in Ara-3?
4. How many hypermutable mutants have been recorded in Ara-3?

Solution

1. 25 different generations
2. 62 rows, 12 columns
3. 10 citrate+ mutants
4. 6 hypermutable mutants

1.3 Summary

! Key point

It is important to record and understand your experiment's metadata.

2 Assessing read quality

Time

- Teaching: 30 min
- Exercises: 20 min

Question

- How can I describe the quality of my data?

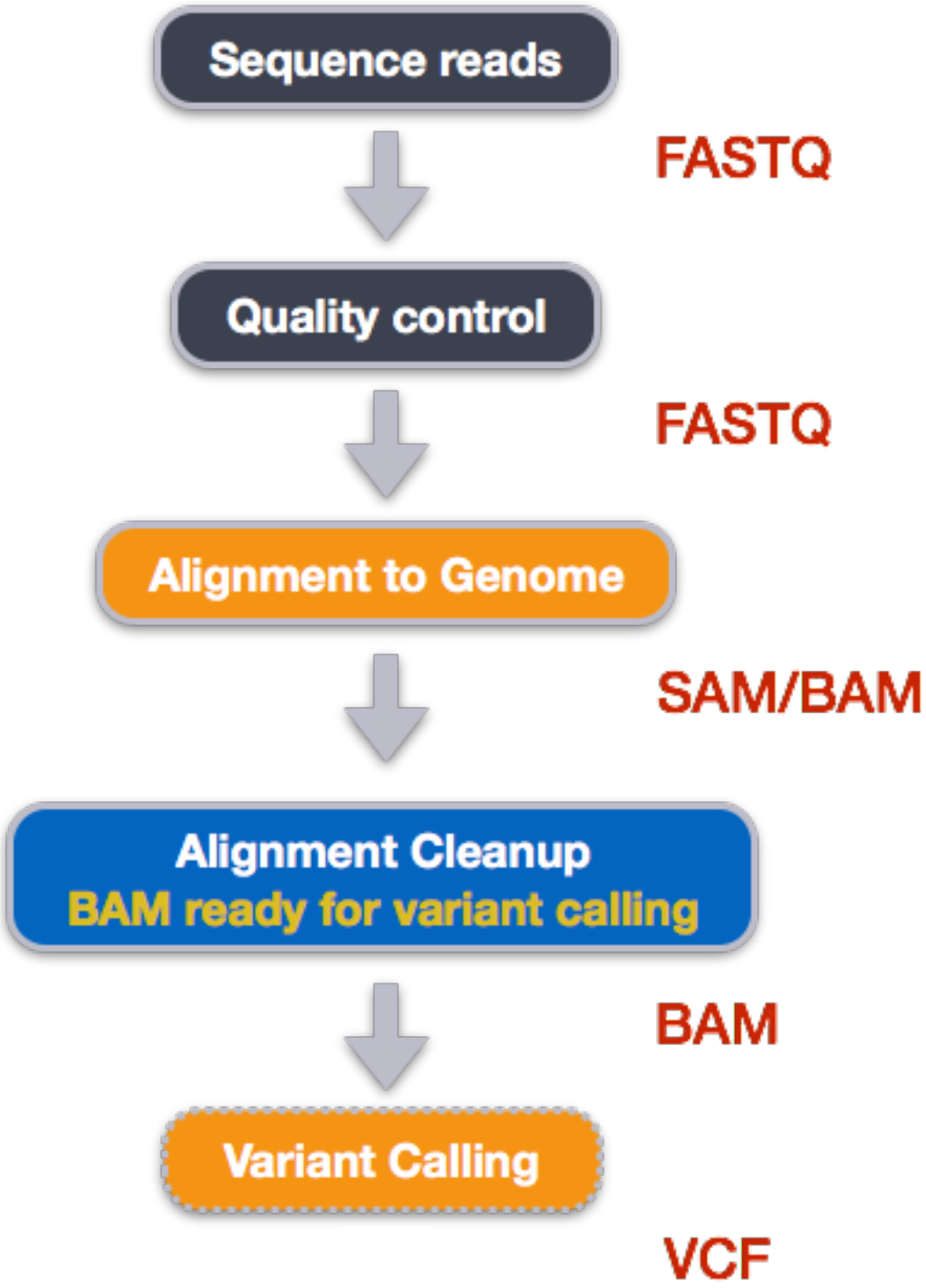
Objectives

- Explain how a FASTQ file encodes per-base quality scores.
- Interpret a FastQC plot summarizing per-base quality across all reads.
- Use for loops to automate operations on multiple files.

2.1 Bioinformatic workflows

When working with high-throughput sequencing data, the raw reads you get off of the sequencer will need to pass through a number of different tools in order to generate your final desired output. The execution of this set of tools in a specified order is commonly referred to as a workflow or a pipeline.

An example of the workflow we will be using for our variant calling analysis is provided below with a brief description of each step.



1. Quality control - Assessing quality
2. Quality control - Trimming and/or filtering reads (if necessary)

3. Align reads to reference genome
4. Perform post-alignment clean-up
5. Variant calling

These workflows in bioinformatics adopt a plug-and-play approach in that the output of one tool can be easily used as input to another tool without any extensive configuration. Having standards for data formats is what makes this feasible. Standards ensure that data is stored in a way that is generally accepted and agreed upon within the community. The tools that are used to analyze data at different stages of the workflow are therefore built under the assumption that the data will be provided in a specific format.

2.2 Get the data

Often times, the first step in a bioinformatic workflow is getting the data you want to work with onto a computer where you can work with it. If you have outsourced sequencing of your data, the sequencing center will usually provide you with a link that you can use to download your data. Today we will be working with publicly available sequencing data.

We are studying a population of *Escherichia coli* (designated Ara-3), which were propagated for more than 50,000 generations in a glucose-limited minimal medium. We will be working with three samples from this experiment, one from 5,000 generations, one from 15,000 generations, and one from 50,000 generations. The population changed substantially during the course of the experiment, and we will be exploring how with our variant calling workflow.

The data are paired-end, so we will download two files for each sample. We will use the European Nucleotide Archive to get our data. The ENA "provides a comprehensive record of the world's nucleotide sequencing information, covering raw sequencing data, sequence assembly information and functional annotation." The ENA also provides sequencing data in the fastq format, an important format for sequencing reads that we will be learning about today.

2.2.1 Copy directory containing data on the HPC

Using the terminal, you can access the remote server using the `ssh` command (use your username)

```
$ ssh [username]@hpc.tsl.ac.uk
```

Now that you are logged in on the server, we must start an `interactive` session.

```
$ interactive
```

Copy the directory containing the dataset to your home directory.

```
$ cp -r /tsl/data/dc_workshop/ ~
```

Now you can access the directory that now should contain the fastq files.

```
$ cd ~/dc_workshop/data/untrimmed_fastq
```

The data comes in a compressed format, which is why there is a `.gz` at the end of the file names. This makes it faster to transfer, and allows it to take up less space on our computer. Let's unzip one of the files so that we can look at the fastq format.

```
$ gunzip SRR2584863_1.fastq.gz
```

It takes a few seconds.

2.3 Quality control

2.3.1 Details on the FASTQ format

Although it looks complicated (and it is), we can understand the fastq format with a little decoding. Some rules about the format include…

Line	Description
1	Always begins with '@' and then information about the read
2	The actual DNA sequence
3	Always begins with a '+' and sometimes the same info in line 1
4	Has a string of characters which represent the quality scores; must have same number of characters as line 2

We can view the first complete read in one of the files our data set by using `head` to look at the first four lines.

```
$ head -n 4 SRR2584863_1.fastq
@SRR2584863.1 HWI-ST957:244:H73TDADXX:1:1101:4712:2181/1
TTCACATCCTGACCATTCAGTTGAGCAAAATAGTTCTTCAGTGCCTGTTTAACCGAGTCACGCAGGGGTTTTTGGGTTACCTGATCCTGA
+
CCCFFFFFGHHHHJIJJJJIJJJIIJJJJIIIJJGFIIIJEDDFEGGJIFHHJIJJDECCGGEGIIJFHFFFACD:BBBDDACCCCAA@@
```

Line 4 shows the quality for each nucleotide in the read. Quality is interpreted as the probability of an incorrect base call (e.g. 1 in 10) or, equivalently, the base call accuracy (e.g. 90%). To make it possible to line up each individual nucleotide with its quality score, the numerical score is converted into a code where each individual character represents the numerical quality score for an individual nucleotide. For example, in the line above, the quality score line is:

```
CCCFFFFFGHHHHJIJJJJIJJJIIJJJJIIIJJGFIIIJEDDFEGGJIFHHJIJJDECCGGEGIIJFHFFFACD:BBBDDACCCCAA@@
```

The numerical value assigned to each of these characters depends on the sequencing platform that generated the reads. The sequencing machine used to generate our data uses the standard Sanger quality PHRED score encoding, using Illumina version 1.8 onwards. Each character is assigned a quality score between 0 and 41 as shown in the chart below.

```
Quality encoding: !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJ
                   |         |         |         |         |
Quality score:    01........11........21........31........41
```

Each quality score represents the probability that the corresponding nucleotide call is incorrect. This quality score is logarithmically based, so a quality score of 10 reflects a base call accuracy of 90%, but a quality score of 20 reflects a base call accuracy of 99%. These probability values are the results from the base calling algorithm and depend on how much signal was captured for the base incorporation.

Looking back at our read:

```
@SRR2584863.1 HWI-ST957:244:H73TDADXX:1:1101:4712:2181/1
TTCACATCCTGACCATTCAGTTGAGCAAAATAGTTCTTCAGTGCCTGTTTAACCGAGTCACGCAGGGGTTTTTGGGTTACCTGATCCTGA
+
CCCFFFFFGHHHHJIJJJJIJJJIIJJJJIIIJJGFIIIJEDDFEGGJIFHHJIJJDECCGGEGIIJFHFFFACD:BBBDDACCCCAA@@
```

We can now see that there is a range of quality scores, but that the end of the sequence is very poor (`#` = a quality score of 2).

> Exercise
>
> What is the last read in the `SRR2584863_1.fastq` file? How confident are you in this read?

Solution

```
$ tail -n 4 SRR2584863_1.fastq
@SRR2584863.1553259 HWI-ST957:245:H73R4ADXX:2:2216:21048:100894/1
CTGCAATACCACGCTGATCTTTCACATGATGTAAGAAAAGTGGGATCAGCAAACCGGGTGCTGCTGTGGCTAGTTGCAGCAAACCATG
+
CCCFFFFFHHHHGJJJJJJJJJHGIJJJIJJJJIJJJJIIIIJJJJJJJJJJJJJIIJJJHHHHHFFFFFEEEEEDDDDDDDDDDDDD
```

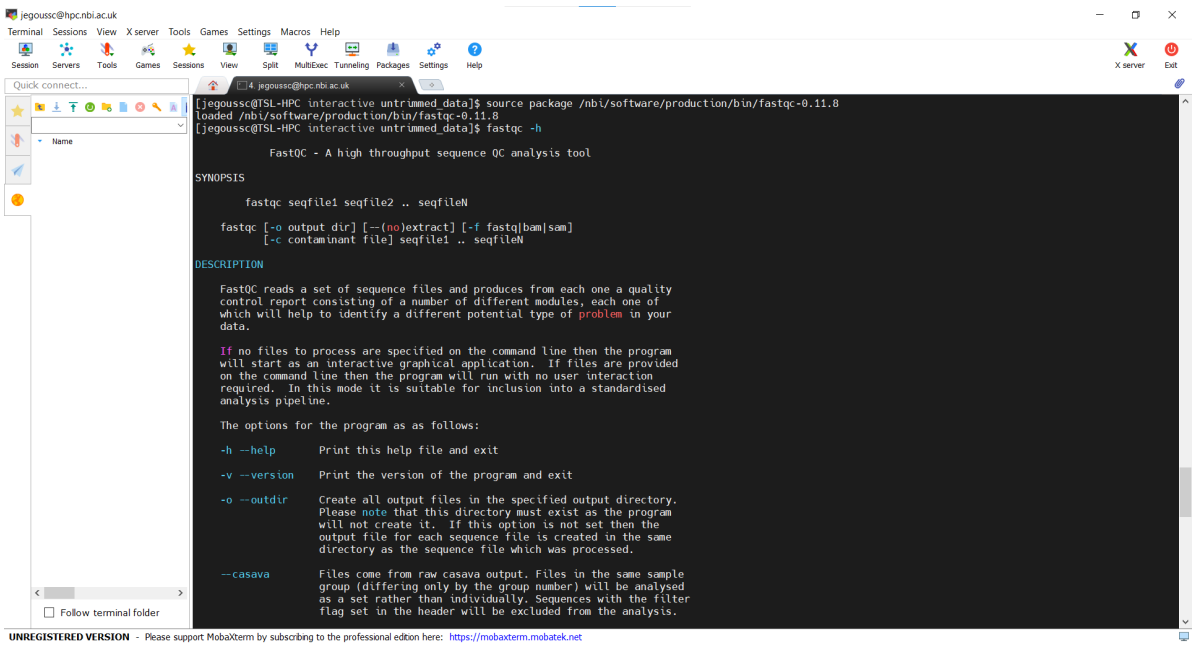
This read has more consistent quality at its end than the first read that we looked at, but still has a range of quality scores, most of them high. We will look at variations in position-based quality in just a moment.

At this point, let's load the relevant tools that are already installed on the server:

```
$ source package /nbi/software/production/bin/fastqc-0.11.8
loaded /nbi/software/production/bin/fastqc-0.11.8
```

One way to validate if the correct software is loaded and ready to work with is to check the software's manual:

```
$ fastqc -h
```



If FastQC is not installed, you will get an error message:

```
$ fastqc -h
The program 'fastqc' is currently not installed. You can install it by typing:
sudo apt-get install fastqc
```

If this happens check with your instructor before trying to install it.

2.3.2 Assessing read quality with FastQC

In real life, you will not be assessing the quality of your reads by visually inspecting your FASTQ files. Rather, you will be using a software program to assess read quality and filter out poor quality reads. We will first use a program called FastQC (**andrews2010fastqcto?**) visualize the quality of our reads. Later in our workflow, we will use another program to filter out poor quality reads.

FastQC has a number of features which can give you a quick impression of any problems your data may have, so you can take these issues into consideration before moving forward with your analyses. Rather than looking at quality scores for each individual read, FastQC looks at quality collectively across all reads within a sample. The image below shows one FastQC-generated plot that indicates a very high quality sample:

The x-axis displays the base position in the read, and the y-axis shows quality scores. In this example, the sample contains reads that are 40 bp long. This is much shorter than the reads we are working with in our workflow. For each position, there is a box-and-whisker plot showing the distribution of quality scores for all reads at that position. The horizontal red line indicates the median quality score and the yellow box shows the 1st to 3rd quartile range. This means that 50% of reads have a quality score that falls within the range of the yellow box at that position. The whiskers show the absolute range, which covers the lowest (0th quartile) to highest (4th quartile) values.

For each position in this sample, the quality values do not drop much lower than 32. This is a high quality score. The plot background is also color-coded to identify good (green), acceptable (yellow), and bad (red) quality scores.

Now let's take a look at a quality plot on the other end of the spectrum.

Here, we see positions within the read in which the boxes span a much wider range. Also, quality scores drop quite low into the "bad" range, particularly on the tail end of the reads. The FastQC tool produces several other diagnostic plots to assess sample quality, in addition to the one plotted above.

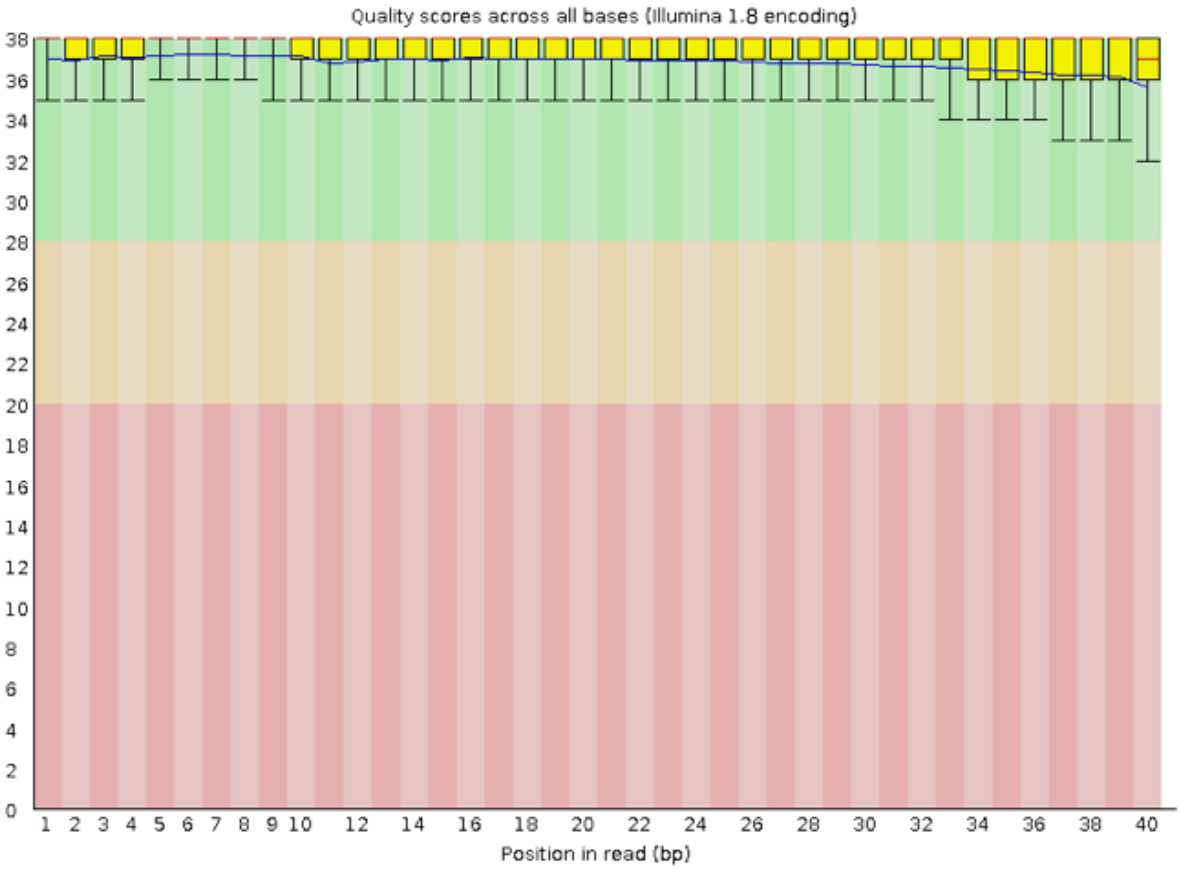


Figure 2.1: good_quality

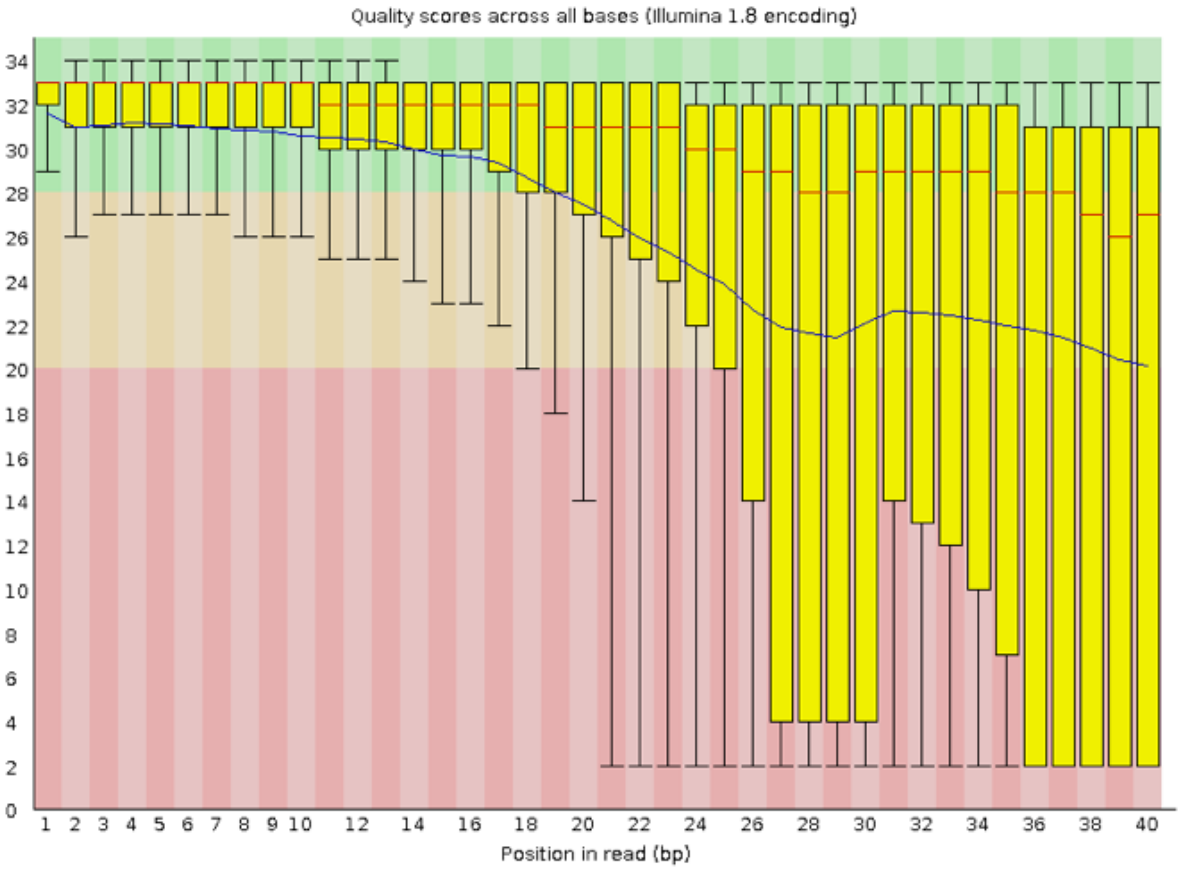


Figure 2.2: bad_quality

2.3.3 Running FastQC

We will now assess the quality of the reads that we downloaded. First, make sure you are still in the `untrimmed_fastq` directory

```
cd ~/dc_workshop/data/untrimmed_fastq/
```

Exercise

How big are the files? (Hint: Look at the options for the ls command to see how to show file sizes.)

Solution

```
$ ls -l -h
-rwx------ 1 [username] TSL_20 545M Aug 11 11:02 SRR2584863_1.fastq
-rwx------ 1 [username] TSL_20 183M Aug 11 11:03 SRR2584863_2.fastq.gz
-rwx------ 1 [username] TSL_20 309M Aug 11 11:04 SRR2584866_1.fastq.gz
-rwx------ 1 [username] TSL_20 296M Aug 11 11:07 SRR2584866_2.fastq.gz
-rwx------ 1 [username] TSL_20 124M Aug 11 11:00 SRR2589044_1.fastq.gz
-rwx------ 1 [username] TSL_20 128M Aug 11 11:01 SRR2589044_2.fastq.gz
```

There are six FASTQ files ranging from 124M (124MB) to 545M.

FastQC can accept multiple file names as input, and on both zipped and unzipped files, so we can use the *.fastq* wildcard to run FastQC on all of the FASTQ files in this directory.

```
$ fastqc *.fastq*
```

You will see an automatically updating output message telling you the progress of the analysis. It will start like this:

```
Started analysis of SRR2584863_1.fastq
Approx 5% complete for SRR2584863_1.fastq
Approx 10% complete for SRR2584863_1.fastq
Approx 15% complete for SRR2584863_1.fastq
Approx 20% complete for SRR2584863_1.fastq
Approx 25% complete for SRR2584863_1.fastq
Approx 30% complete for SRR2584863_1.fastq
Approx 35% complete for SRR2584863_1.fastq
Approx 40% complete for SRR2584863_1.fastq
Approx 45% complete for SRR2584863_1.fastq
```

In total, it should take about five minutes for FastQC to run on all six of our FASTQ files. When the analysis completes, your prompt will return. So your screen will look something like this:

```
Approx 80% complete for SRR2589044_2.fastq.gz
Approx 85% complete for SRR2589044_2.fastq.gz
Approx 90% complete for SRR2589044_2.fastq.gz
Approx 95% complete for SRR2589044_2.fastq.gz
Analysis complete for SRR2589044_2.fastq.gz
$
```

The FastQC program has created several new files within our `data/untrimmed_fastq/` directory.

```
$ ls
SRR2584863_1.fastq        SRR2584866_1_fastqc.html  SRR2589044_1_fastqc.html
SRR2584863_1_fastqc.html  SRR2584866_1_fastqc.zip   SRR2589044_1_fastqc.zip
SRR2584863_1_fastqc.zip   SRR2584866_1.fastq.gz     SRR2589044_1.fastq.gz
SRR2584863_2_fastqc.html  SRR2584866_2_fastqc.html  SRR2589044_2_fastqc.html
SRR2584863_2_fastqc.zip   SRR2584866_2_fastqc.zip   SRR2589044_2_fastqc.zip
SRR2584863_2.fastq.gz     SRR2584866_2.fastq.gz     SRR2589044_2.fastq.gz
```

For each input FASTQ file, FastQC has created a `.zip` file and a

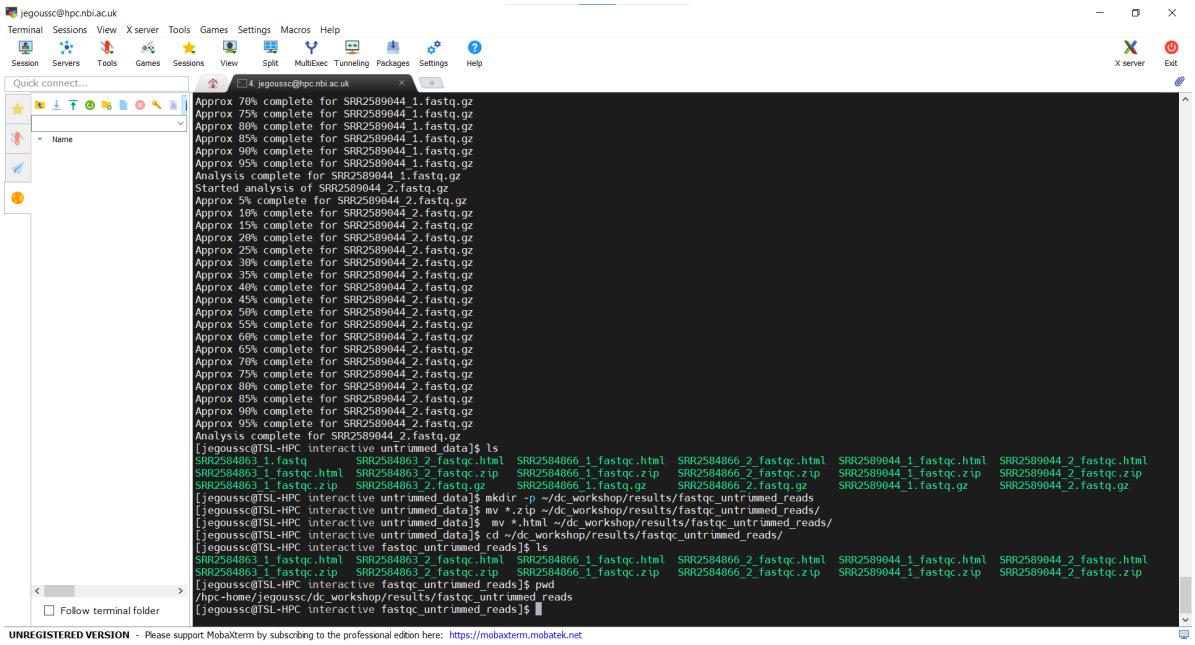
`.html` file. The `.zip` file extension indicates that this is actually a compressed set of multiple output files. We will be working with these output files soon. The `.html` file is a stable webpage displaying the summary report for each of our samples.

We want to keep our data files and our results files separate, so we will move these output files into a new directory within our `results/` directory.

```
$ mkdir -p ~/dc_workshop/results/fastqc_untrimmed_reads
$ mv *.zip ~/dc_workshop/results/fastqc_untrimmed_reads/
$ mv *.html ~/dc_workshop/results/fastqc_untrimmed_reads/
```

Now we can navigate into this results directory and do some closer inspection of our output files.

```
$ cd ~/dc_workshop/results/fastqc_untrimmed_reads/
```



2.3.4 Viewing the FastQC results

If we were working on our local computers, we would be able to look at each of these HTML files by opening them in a web browser.

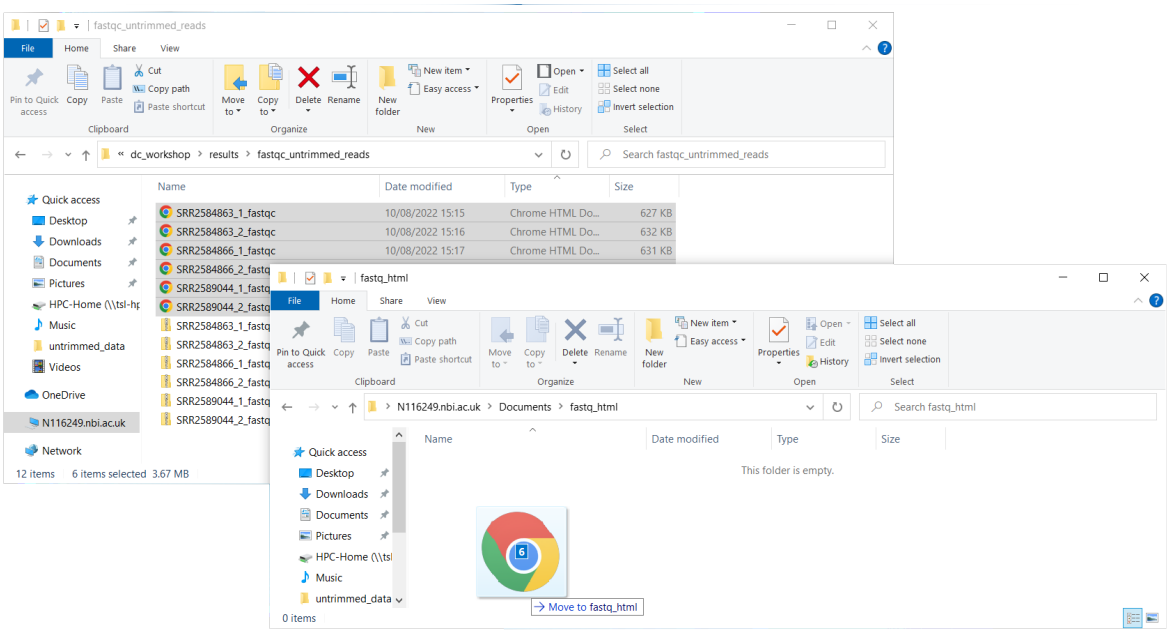
However, these files are currently sitting on our remote server, where our local computer can not see them. And, since we are only logging into the server via the command line - it does not have any web browser setup to display these files either.

So the easiest way to look at these webpage summary reports will be to transfer them to our local computers (i.e. your laptop).

To transfer a file from a remote server to our own machines, we will simply use the Windows navigation system.

1. From the Start Menu, select Run, then type `\\tsl-hpc-data\HPC-Home`
2. You may need to toggle `Use different credentials` on or off

A window with the directory showing the files in should pop up



Now we can go to our new directory and open the 6 HTML files.

Depending on your system, you should be able to select and open them all at once via a right click menu in your file browser.

Exercise

Discuss your results with a neighbor. Which sample(s) looks the best in terms of per base sequence quality? Which sample(s) look the worst?

Solution

All of the reads contain usable data, but the quality decreases toward the end of the reads.

2.3.5 Decoding the other FastQC outputs

We have now looked at quite a few "Per base sequence quality" FastQC graphs, but there are nine other graphs that we have not talked about! Below we have provided a brief overview of interpretations for each of these plots. For more information, please see the FastQC documentation here

- **Per tile sequence quality**: the machines that perform sequencing are divided into tiles. This plot displays patterns in base quality along these tiles. Consistently low scores are

often found around the edges, but hot spots can also occur in the middle if an air bubble was introduced at some point during the run.

- **Per sequence quality scores**: a density plot of quality for all reads at all positions. This plot shows what quality scores are most common.
- **Per base sequence content**: plots the proportion of each base position over all of the reads. Typically, we expect to see each base roughly 25% of the time at each position, but this often fails at the beginning or end of the read due to quality or adapter content.
- **Per sequence GC content**: a density plot of average GC content in each of the reads.
- **Per base N content**: the percent of times that 'N' occurs at a position in all reads. If there is an increase at a particular position, this might indicate that something went wrong during sequencing.
- **Sequence Length Distribution**: the distribution of sequence lengths of all reads in the file. If the data is raw, there is often on sharp peak, however if the reads have been trimmed, there may be a distribution of shorter lengths.
- **Sequence Duplication Levels**: A distribution of duplicated sequences. In sequencing, we expect most reads to only occur once. If some sequences are occurring more than once, it might indicate enrichment bias (e.g. from PCR). If the samples are high coverage (or RNA-seq or amplicon), this might not be true.
- **Overrepresented sequences**: A list of sequences that occur more frequently than would be expected by chance.
- **Adapter Content**: a graph indicating where adapater sequences occur in the reads.
- **K-mer Content**: a graph showing any sequences which may show a positional bias within the reads.

2.3.6 Working with the FastQC text output

Now that we have looked at our HTML reports to get a feel for the data, let's look more closely at the other output files. Go back to the tab in your terminal program that is connected to your interactive session on the server and make sure you are in our results subdirectory.

```
$ cd ~/dc_workshop/results/fastqc_untrimmed_reads/
$ ls
SRR2584863_1_fastqc.html  SRR2584866_1_fastqc.html  SRR2589044_1_fastqc.html
SRR2584863_1_fastqc.zip   SRR2584866_1_fastqc.zip   SRR2589044_1_fastqc.zip
SRR2584863_2_fastqc.html  SRR2584866_2_fastqc.html  SRR2589044_2_fastqc.html
SRR2584863_2_fastqc.zip   SRR2584866_2_fastqc.zip   SRR2589044_2_fastqc.zip
```

Our `.zip` files are compressed files. They each contain multiple different types of output files for a single input FASTQ file. To view the contents of a `.zip` file, we can use the program `unzip` to decompress these files. Let's try doing them all at once using a wildcard.

```
$ unzip *.zip
Archive:  SRR2584863_1_fastqc.zip
caution: filename not matched:  SRR2584863_2_fastqc.zip
caution: filename not matched:  SRR2584866_1_fastqc.zip
caution: filename not matched:  SRR2584866_2_fastqc.zip
caution: filename not matched:  SRR2589044_1_fastqc.zip
caution: filename not matched:  SRR2589044_2_fastqc.zip
```

This did not work. We unzipped the first file and then got a warning message for each of the other `.zip` files. This is because `unzip` expects to get only one zip file as input. We could go through and unzip each file one at a time, but this is very time consuming and error-prone. Someday you may have 500 files to unzip!

A more efficient way is to use a `for` loop like we learned in the Shell Genomics lesson to iterate through all of our `.zip` files. Let's see what that looks like and then we will discuss what we are doing with each line of our loop.

> **Warning**
>
> Do not copy-paste the four lines at once. You must type the `for` loop line by line!

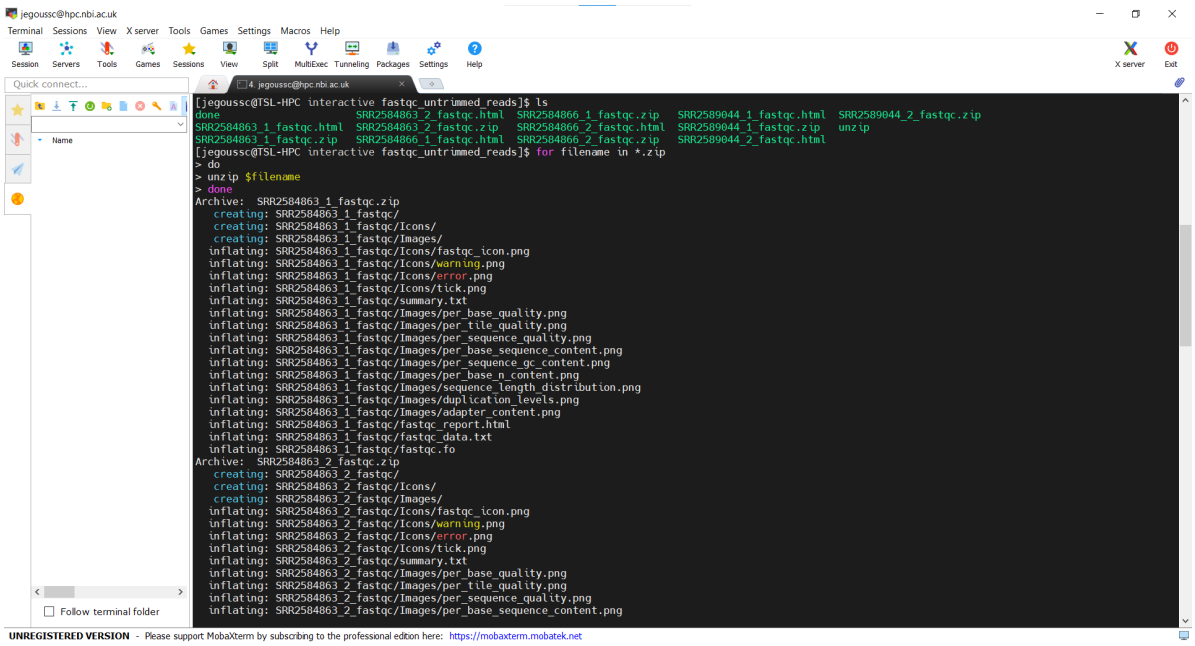
```
$ for filename in *.zip
> do
> unzip $filename
> done
```

In this example, the input is six filenames (one filename for each of our `.zip` files). Each time the loop iterates, it will assign a file name to the variable `filename` and run the `unzip` command. The first time through the loop, `$filename` is `SRR2584863_1_fastqc.zip`. The interpreter runs the command `unzip` on `SRR2584863_1_fastqc.zip`. For the second iteration, `$filename` becomes `SRR2584863_2_fastqc.zip`. This time, the shell runs `unzip` on `SRR2584863_2_fastqc.zip`. It then repeats this process for the four other `.zip` files in our directory.

When we run our `for` loop, you will see output that starts like this:

```
Archive:  SRR2589044_2_fastqc.zip
   creating: SRR2589044_2_fastqc/
   creating: SRR2589044_2_fastqc/Icons/
```

```
 creating: SRR2589044_2_fastqc/Images/
inflating: SRR2589044_2_fastqc/Icons/fastqc_icon.png
inflating: SRR2589044_2_fastqc/Icons/warning.png
inflating: SRR2589044_2_fastqc/Icons/error.png
inflating: SRR2589044_2_fastqc/Icons/tick.png
inflating: SRR2589044_2_fastqc/summary.txt
inflating: SRR2589044_2_fastqc/Images/per_base_quality.png
inflating: SRR2589044_2_fastqc/Images/per_tile_quality.png
inflating: SRR2589044_2_fastqc/Images/per_sequence_quality.png
inflating: SRR2589044_2_fastqc/Images/per_base_sequence_content.png
inflating: SRR2589044_2_fastqc/Images/per_sequence_gc_content.png
inflating: SRR2589044_2_fastqc/Images/per_base_n_content.png
inflating: SRR2589044_2_fastqc/Images/sequence_length_distribution.png
inflating: SRR2589044_2_fastqc/Images/duplication_levels.png
inflating: SRR2589044_2_fastqc/Images/adapter_content.png
inflating: SRR2589044_2_fastqc/fastqc_report.html
inflating: SRR2589044_2_fastqc/fastqc_data.txt
inflating: SRR2589044_2_fastqc/fastqc.fo
```



The `unzip` program is decompressing the `.zip` files and creating a new directory (with sub-directories) for each of our samples, to store all of the different output that is produced by FastQC. There

are a lot of files here. The one we are going to focus on is the `summary.txt` file.

If you list the files in our directory now you will see:

```
SRR2584863_1_fastqc       SRR2584866_1_fastqc       SRR2589044_1_fastqc
SRR2584863_1_fastqc.html  SRR2584866_1_fastqc.html  SRR2589044_1_fastqc.html
SRR2584863_1_fastqc.zip   SRR2584866_1_fastqc.zip   SRR2589044_1_fastqc.zip
SRR2584863_2_fastqc       SRR2584866_2_fastqc       SRR2589044_2_fastqc
SRR2584863_2_fastqc.html  SRR2584866_2_fastqc.html  SRR2589044_2_fastqc.html
SRR2584863_2_fastqc.zip   SRR2584866_2_fastqc.zip   SRR2589044_2_fastqc.zip
```

The `.html` files and the uncompressed `.zip` files are still present, but now we also have a new directory for each of our samples. We can see for sure that it is a directory if we use the `-F` flag for `ls`.

```
$ ls -F
SRR2584863_1_fastqc/      SRR2584866_1_fastqc/      SRR2589044_1_fastqc/
SRR2584863_1_fastqc.html  SRR2584866_1_fastqc.html  SRR2589044_1_fastqc.html
SRR2584863_1_fastqc.zip   SRR2584866_1_fastqc.zip   SRR2589044_1_fastqc.zip
SRR2584863_2_fastqc/      SRR2584866_2_fastqc/      SRR2589044_2_fastqc/
SRR2584863_2_fastqc.html  SRR2584866_2_fastqc.html  SRR2589044_2_fastqc.html
SRR2584863_2_fastqc.zip   SRR2584866_2_fastqc.zip   SRR2589044_2_fastqc.zip
```

Let's see what files are present within one of these output directories.

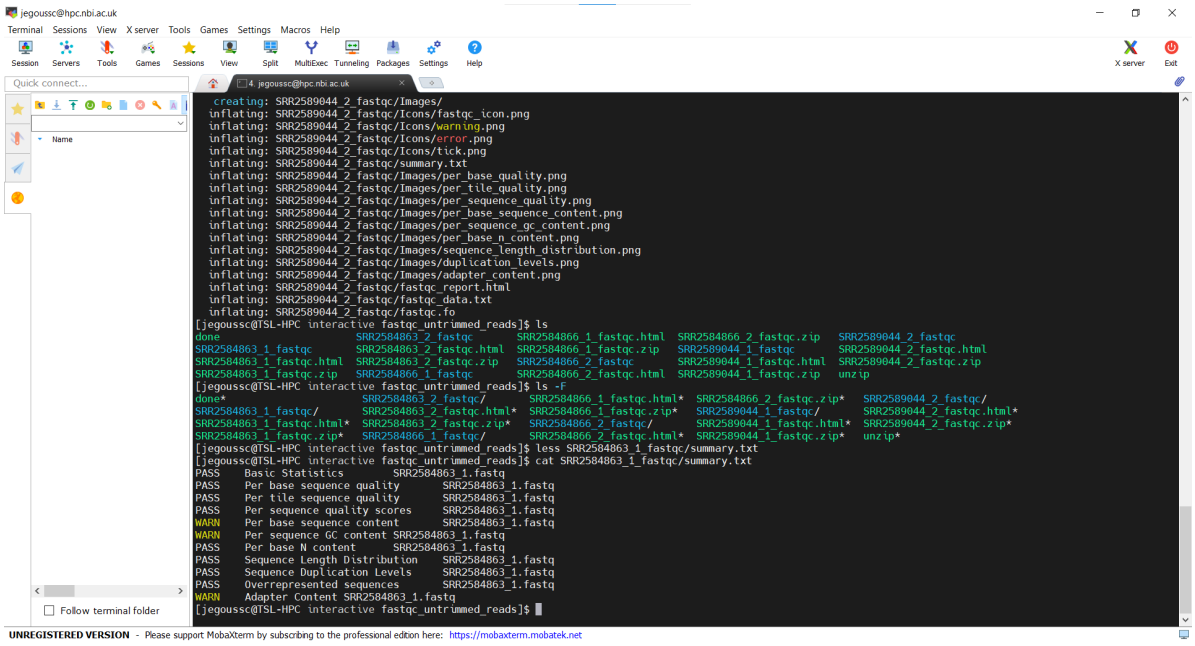
```
$ ls -F SRR2584863_1_fastqc/
fastqc_data.txt  fastqc.fo  fastqc_report.html  Icons/  Images/  summary.txt
```

Use `less` to preview the `summary.txt` file for this sample.

```
$ less SRR2584863_1_fastqc/summary.txt

PASS    Basic Statistics        SRR2584863_1.fastq
PASS    Per base sequence quality       SRR2584863_1.fastq
PASS    Per tile sequence quality       SRR2584863_1.fastq
PASS    Per sequence quality scores     SRR2584863_1.fastq
WARN    Per base sequence content       SRR2584863_1.fastq
WARN    Per sequence GC content SRR2584863_1.fastq
PASS    Per base N content      SRR2584863_1.fastq
PASS    Sequence Length Distribution    SRR2584863_1.fastq
PASS    Sequence Duplication Levels     SRR2584863_1.fastq
PASS    Overrepresented sequences       SRR2584863_1.fastq
WARN    Adapter Content SRR2584863_1.fastq
```

The summary file gives us a list of tests that FastQC ran, and tells us whether this sample passed, failed, or is borderline (`WARN`). Remember, to quit from `less` you must type `q`.



2.4 Documenting your work

We can make a record of the results we obtained for all our samples

by concatenating all of our `summary.txt` files into a single file using the `cat` command. We will call this `fastqc_summaries.txt` and move it to `~/dc_workshop/docs`.

```
$ mkdir -p ~/dc_workshop/docs/
$ cat */summary.txt > ~/dc_workshop/docs/fastqc_summaries.txt
```

Exercise

Which samples failed at least one of FastQC's quality tests? What test(s) did those samples fail?

Solution

We can get the list of all failed tests using grep.

```
$ cd ~/dc_workshop/docs
$ grep FAIL fastqc_summaries.txt
FAIL    Per base sequence quality       SRR2584863_2.fastq.gz
FAIL    Per tile sequence quality       SRR2584863_2.fastq.gz
FAIL    Per base sequence content       SRR2584863_2.fastq.gz
FAIL    Per base sequence quality       SRR2584866_1.fastq.gz
FAIL    Per base sequence content       SRR2584866_1.fastq.gz
FAIL    Adapter Content SRR2584866_1.fastq.gz
FAIL    Adapter Content SRR2584866_2.fastq.gz
FAIL    Adapter Content SRR2589044_1.fastq.gz
FAIL    Per base sequence quality       SRR2589044_2.fastq.gz
FAIL    Per tile sequence quality       SRR2589044_2.fastq.gz
FAIL    Per base sequence content       SRR2589044_2.fastq.gz
FAIL    Adapter Content SRR2589044_2.fastq.gz
```

2.5 Summary

Quality encodings vary

Although we have used a particular quality encoding system to demonstrate interpretation of read quality, different sequencing machines use different encoding systems. This means that, depending on which sequencer you use to generate your data, a `#` may not be an indicator of a poor quality base call.
This mainly relates to older Solexa/Illumina data, but it is essential that you know which sequencing platform was used to generate your data, so that you can tell your quality control program which encoding to use. If you choose the wrong encoding, you run the risk of throwing away good reads or (even worse) not throwing away bad reads!

Same symbols but different meanings

Here we see `>` being used as a shell prompt, whereas `>` is also used to redirect output. Similarly, `$` is used as a shell prompt, but, as we saw earlier, it is also used to ask the shell to get the value of a variable.
If the *shell* prints `>` or `$` then it expects you to type something, and the symbol is a prompt.
If *you* type `>` or `$` yourself, it is an instruction from you that the shell should redirect output or get the value of a variable.

! Key points

- Quality encodings vary across sequencing platforms.
- `for` loops let you perform the same set of operations on multiple files with a single command.

3 Trimming and filtering

Time

- Teaching: 30 min
- Exercises: 25 min

Question

- How can I get rid of sequence data that does not meet my quality standards?

Objectives

- Clean FASTQ reads using Trimmomatic.
- Select and set multiple options for command-line bioinformatic tools.
- Write for loops with two variables.

3.1 Cleaning reads

In the previous episode, we took a high-level look at the quality of each of our samples using FastQC. We visualized per-base quality graphs showing the distribution of read quality at each base across all reads in a sample and extracted information about which samples fail which quality checks. Some of our samples failed quite a few quality metrics used by FastQC. This does not mean, though, that our samples should be thrown out! It is very common to have some quality metrics fail, and this may or may not be a problem for your downstream application. For our variant calling workflow, we will be removing some of the low quality sequences to reduce our false positive rate due to sequencing error.

We will use a program called Trimmomatic (Bolger, Lohse, and Usadel 2014) to filter poor quality reads and trim poor quality bases from our samples.

! Important

Make sure to be connected to the server.

Let's load Trimmomatic to our interactive session on the server.

```
$ source package /nbi/software/production/bin/trimmomatic-0.39
loaded /nbi/software/production/bin/trimmomatic-0.39
```

3.1.1 Trimmomatic options

Trimmomatic has a variety of options to trim your reads. If we run the following command, we can see some of our options.

```
$ trimmomatic
```

Which will give you the following output:

```
Usage:
       PE [-version] [-threads <threads>] [-phred33|-phred64] [-trimlog <trimLogFile>] [-summ
   or:
       SE [-version] [-threads <threads>] [-phred33|-phred64] [-trimlog <trimLogFile>] [-summ
   or:
       -version
```

This output shows us that we must first specify whether we have paired end (`PE`) or single end (`SE`) reads. Next, we specify what flag we would like to run. For example, you can specify `threads` to indicate the number of processors on your computer that you want Trimmomatic to use. In most cases using multiple threads (processors) can help to run the trimming faster. These flags are not necessary, but they can give you more control over the command. The flags are followed by positional arguments, meaning the order in which you specify them is important. In paired end mode, Trimmomatic expects the two input files, and then the names of the output files. These files are described below. While, in single end mode, Trimmomatic will expect 1 file as input, after which you can enter the optional settings and lastly the name of the output file.

option	meaning
<inputFile1>	Input reads to be trimmed. Typically the file name will contain an `_1` or `_R1` in the name.
<inputFile2>	Input reads to be trimmed. Typically the file name will contain an `_2` or `_R2` in the name.
<outputFile1P>	Output file that contains surviving pairs from the `_1` file.
<outputFile1U>	Output file that contains orphaned reads from the `_1` file.
<outputFile2P>	Output file that contains surviving pairs from the `_2` file.
<outputFile2U>	Output file that contains orphaned reads from the `_2` file.

The last thing Trimmomatic expects to see is the trimming parameters:

step	meaning
`ILLUMINACLIP`	Perform adapter removal.
`SLIDINGWINDOW`	Perform sliding window trimming, cutting once the average quality within the window falls below a threshold.
`LEADING`	Cut bases off the start of a read, if below a threshold quality.
`TRAILING`	Cut bases off the end of a read, if below a threshold quality.
`CROP`	Cut the read to a specified length.
`HEADCROP`	Cut the specified number of bases from the start of the read.
`MINLEN`	Drop an entire read if it is below a specified length.
`TOPHRED33`	Convert quality scores to Phred-33.
`TOPHRED64`	Convert quality scores to Phred-64.

We will use only a few of these options and trimming steps in our analysis. It is important to understand the steps you are using to clean your data. For more information about the Trimmomatic arguments and options, see the Trimmomatic manual.

3.1.2 Running Trimmomatic

Now we will run Trimmomatic on our data. To begin, navigate to your `untrimmed_fastq` data directory:

```
$ cd ~/dc_workshop/data/untrimmed_fastq
```

We are going to run Trimmomatic on one of our paired-end samples. While using FastQC we saw that Nextera adapters were present in our samples. The adapter sequences came with the installation of Trimmomatic.

We will also use a sliding window of size 4 that will remove bases if their phred score is below 20 (like in our example above). We will also discard any reads that do not have at least 25 bases remaining after this trimming step. Three additional pieces of code are also added to the end of the ILLUMINACLIP step. These three additional numbers (2:40:15) tell Trimmomatic how to handle sequence matches to the Nextera adapters. A detailed explanation of how they work is advanced for this particular lesson. For now we will use these numbers as a default and recognize they are needed to for Trimmomatic to run properly.

```
$ trimmomatic PE SRR2589044_1.fastq.gz SRR2589044_2.fastq.gz \
                 SRR2589044_1.trim.fastq.gz SRR2589044_1un.trim.fastq.gz \
                 SRR2589044_2.trim.fastq.gz SRR2589044_2un.trim.fastq.gz \
                 SLIDINGWINDOW:4:20 \
```

```
                MINLEN:25 \
                ILLUMINACLIP:NexteraPE-PE.fa:2:40:15
```

This command will take about two minutes to run.

> **About multiline commands**
>
> Some of the commands we ran in this lesson are long! When typing a long command into your terminal, you can use the \ character to separate code chunks onto separate lines. This can make your code more readable.

code	meaning
`PE`	that it will be taking a paired end file as input
`-threads 4`	to use four computing threads to run (this will speed up our run)
`SRR2589044_1.fastq`	the first input file name
`SRR2589044_2.fastq`	the second input file name
`SRR2589044_1.trimmed.fastq`	the output file for surviving pairs from the `_1` file
`SRR2589044_1un.trimmed.fastq`	the output file for orphaned reads from the `_1` file
`SRR2589044_2.trimmed.fastq`	the output file for surviving pairs from the `_2` file
`SRR2589044_2un.trimmed.fastq`	the output file for orphaned reads from the `_2` file
`ILLUMINACLIP:SRR_adapters.fa`	clip the Illumina adapters from the input file using the adapter sequences listed in `SRR_adapters.fa`
`SLIDINGWINDOW:4:20`	to use a sliding window of size 4 that will remove bases if their phred score is below 20
`MINLEN:25`	minimum length of 25 nucleotides.

The output should look like this:

```
TrimmomaticPE: Started with arguments:
 SRR2589044_1.fastq.gz SRR2589044_2.fastq.gz SRR2589044_1.trim.fastq.gz SRR2589044_1un.trim.
Multiple cores found: Using 2 threads
Using PrefixPair: 'AGATGTGTATAAGAGACAG' and 'AGATGTGTATAAGAGACAG'
Using Long Clipping Sequence: 'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG'
Using Long Clipping Sequence: 'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG'
Using Long Clipping Sequence: 'CTGTCTCTTATACACATCTCCGAGCCCACGAGAC'
Using Long Clipping Sequence: 'CTGTCTCTTATACACATCTGACGCTGCCGACGA'
ILLUMINACLIP: Using 1 prefix pairs, 4 forward/reverse sequences, 0 forward only sequences, 0
Quality encoding detected as phred33
Input Read Pairs: 1107090 Both Surviving: 885220 (79.96%) Forward Only Surviving: 216472 (19
TrimmomaticPE: Completed successfully
```

Exercise

Use the output from your Trimmomatic command to answer the following questions.

1) What percent of reads did we discard from our sample?
2) What percent of reads did we keep both pairs?

Solution

1) 0.23%
2) 79.96%

You may have noticed that Trimmomatic automatically detected the quality encoding of our sample. It is always a good idea to double-check this or to enter the quality encoding manually.

We can confirm that we have our output files:

```
$ ls SRR2589044*
SRR2589044_1.fastq.gz       SRR2589044_1un.trim.fastq.gz  SRR2589044_2.trim.fastq.gz
SRR2589044_1.trim.fastq.gz  SRR2589044_2.fastq.gz         SRR2589044_2un.trim.fastq.gz
```

The output files are also FASTQ files. It should be smaller than our input file, because we have removed reads. We can confirm this:

```
$ ls SRR2589044* -l -h
-rwx------ 1 [username] TSL_20 124M Aug 11 11:00 SRR2589044_1.fastq.gz
-rwx------ 1 [username] TSL_20 105M Aug 11 15:56 SRR2589044_1.trim.fastq.gz
-rwx------ 1 [username] TSL_20 8.4M Aug 11 15:56 SRR2589044_1un.trim.fastq.gz
-rwx------ 1 [username] TSL_20 128M Aug 11 11:01 SRR2589044_2.fastq.gz
-rwx------ 1 [username] TSL_20 103M Aug 11 15:56 SRR2589044_2.trim.fastq.gz
-rwx------ 1 [username] TSL_20 276K Aug 11 15:56 SRR2589044_2un.trim.fastq.gz
```

We have just successfully run Trimmomatic on one of our FASTQ files! However, there is some bad news. Trimmomatic can only operate on one sample at a time and we have more than one sample. The good news is that we can use a `for` loop to iterate through our sample files quickly!

We unzipped one of our files before to work with it, let's compress it again before we run our for loop.

```
$ gzip SRR2584863_1.fastq
```

> **Warning**
>
> Do not copy-paste the four lines at once. You must type the `for` loop line by line!

```
$ for infile in *_1.fastq.gz
> do
>   base=$(basename ${infile} _1.fastq.gz)
>   trimmomatic PE ${infile} ${base}_2.fastq.gz \
>                ${base}_1.trim.fastq.gz ${base}_1un.trim.fastq.gz \
>                ${base}_2.trim.fastq.gz ${base}_2un.trim.fastq.gz \
>                SLIDINGWINDOW:4:20 \
>                MINLEN:25 \
>                ILLUMINACLIP:NexteraPE-PE.fa:2:40:15
> done
```

Go ahead and run the for loop. It should take about 12 minutes for Trimmomatic to run for each of our six input files.

Once it is done running, take a look at your directory contents.

```
$ ls
SRR2584863_1.fastq.gz          SRR2584866_2.fastq.gz
SRR2584863_1.trim.fastq.gz     SRR2584866_2.trim.fastq.gz
SRR2584863_1un.trim.fastq.gz   SRR2584866_2un.trim.fastq.gz
SRR2584863_2.fastq.gz          SRR2589044_1.fastq.gz
SRR2584863_2.trim.fastq.gz     SRR2589044_1.trim.fastq.gz
SRR2584863_2un.trim.fastq.gz   SRR2589044_1un.trim.fastq.gz
SRR2584866_1.fastq.gz          SRR2589044_2.fastq.gz
SRR2584866_1.trim.fastq.gz     SRR2589044_2.trim.fastq.gz
SRR2584866_1un.trim.fastq.gz   SRR2589044_2un.trim.fastq.gz
```

> **Note**
>
> You will notice that even though we ran Trimmomatic on file `SRR2589044` before running the for loop, there is only one set of files for it. Because we matched the ending `_1.fastq.gz`, we re-ran Trimmomatic on this file, overwriting our first results. That is ok, but it is good to be aware that it happened.

We have now completed the trimming and filtering steps of our quality control process! Before we move on, let's move our trimmed FASTQ files to a new subdirectory within our `data/` directory.

```
$ cd ~/dc_workshop/data/untrimmed_fastq
$ mkdir ../trimmed_fastq
$ mv *.trim* ../trimmed_fastq
$ cd ../trimmed_fastq
$ ls
SRR2584863_1.trim.fastq.gz    SRR2584866_1.trim.fastq.gz    SRR2589044_1.trim.fastq.gz
SRR2584863_1un.trim.fastq.gz  SRR2584866_1un.trim.fastq.gz  SRR2589044_1un.trim.fastq.gz
SRR2584863_2.trim.fastq.gz    SRR2584866_2.trim.fastq.gz    SRR2589044_2.trim.fastq.gz
SRR2584863_2un.trim.fastq.gz  SRR2584866_2un.trim.fastq.gz  SRR2589044_2un.trim.fastq.gz
```

Bonus exercise

Now that our samples have gone through quality control, they should perform better on the quality tests run by FastQC. Go ahead and re-run FastQC on your trimmed FASTQ files and visualize the HTML files to see whether your per base sequence quality is higher after trimming.

Solution

```
$ fastqc ~/dc_workshop/data/trimmed_fastq/*.fastq*
```

Then output files can be transferred to the local computer to be visualized.
After trimming and filtering, our overall quality is much higher, we have a distribution of sequence lengths, and more samples pass adapter content. However, quality trimming is not perfect, and some programs are better at removing some sequences than others. Because our sequences still contain 3' adapters, it could be important to explore other trimming tools like Cutadapt (Martin 2011) to remove these, depending on your downstream application. Trimmomatic did pretty well though, and its performance is good enough for our workflow.

3.2 Summary

! Key points

- The options you set for the command-line tools you use are important!
- Data cleaning is an essential step in a genomics workflow.

4 Variant calling workflow

Time

- Teaching: 35 min
- Exercises: 25 min

Question

- How do I find sequence variants between my sample and a reference genome?

Objectives

- Understand the steps involved in variant calling.
- Describe the types of data formats encountered during variant calling.
- Use command line tools to perform variant calling.

We mentioned before that we are working with files from a long-term evolution study of an *E. coli* population (designated Ara-3). Now that we have looked at our data to make sure that it is high quality, and removed low-quality base calls, we can perform variant calling to see how the population changed over time. We care how this population changed relative to the original population, *E. coli* strain REL606. Therefore, we will align each of our samples to the *E. coli* REL606 reference genome, and see what differences exist in our reads versus the genome.

4.1 Alignment to a reference genome

We perform read alignment or mapping to determine where in the genome our reads originated from. There are a number of tools to choose from and, while there is no gold standard, there are some tools that are better suited for particular NGS analyses. We will be using the Burrows Wheeler Aligner (BWA) by Li and Durbin (2010), which is a software package for mapping low-divergent sequences against a large reference genome.

The alignment process consists of two steps:

1. Indexing the reference genome

2. Aligning the reads to the reference genome

4.2 Setting up

4.2.1 Option 1: Copy from shared directory on the HPC

You should already have all the files from the previous section where we copied all files we need including the reference genome and small dataset to your directory. Now:

Unzip the reference genome after changing to the proper directory

```
$ cd ~/dc_workshop/data/ref_genome
$ gunzip ecoli_rel606.fasta.gz
```

Exercise

We saved this file as `data/ref_genome/ecoli_rel606.fasta.gz` and then decompressed it. What is the real name of the genome?

Solution

```
$ head data/ref_genome/ecoli_rel606.fasta
```

The name of the sequence follows the `>` character. The name is `CP000819.1 Escherichia coli B str. REL606, complete genome`. Keep this chromosome name (`CP000819.1`) in mind, as we will use it later in the lesson.

We will also have a set of trimmed FASTQ files to work with. These are small subsets of our real trimmed data, and will enable us to run our variant calling workflow quite quickly.

```
$ ls ~/dc_workshop/data/trimmed_fastq_small
```

4.3 Index the reference genome

Let's make sure we are back in the `dc_workshop` directory and that the two directories are where we hope they are:

```
$ cd ~/dc_workshop/
$ ls -F data/
ref_genome/  trimmed_fastq_small/  untrimmed_fastq/
$ ls -F data/ref_genome/
ecoli_rel606.fasta*
```

You will also need to create directories for the results that will be generated as part of this workflow. We can do this in a single line of code, because `mkdir` can accept multiple new directory names as input.

```
$ mkdir -p results/sam results/bam results/bcf results/vcf
```

Our first step is to index the reference genome for use by BWA.

```
$ source package /nbi/software/production/bin/bwa-0.7.5
loaded /nbi/software/production/bin/bwa-0.7.5
```

Indexing allows the aligner to quickly find potential alignment sites for query sequences in a genome, which saves time during alignment. Indexing the reference only has to be run once. The only reason you would want to create a new index is if you are working with a different reference genome or you are using a different tool for alignment.

```
$ bwa index data/ref_genome/ecoli_rel606.fasta
```

While the index is created, you will see output that looks something like this:

```
[bwa_index] Pack FASTA... 0.15 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 3.01 seconds elapse.
[bwa_index] Update BWT... 0.05 sec
[bwa_index] Pack forward-only FASTA... 0.05 sec
[bwa_index] Construct SA from BWT and Occ... 0.89 sec
[main] Version: 0.7.5-r404
[main] CMD: bwa index data/ref_genome/ecoli_rel606.fasta
[main] Real time: 4.348 sec; CPU: 4.160 sec
```

i BWA alignment options

BWA consists of three algorithms: BWA-backtrack, BWA-SW and BWA-MEM. The first algorithm is designed for Illumina sequence reads up to 100bp, while the other two are for sequences ranging from 70bp to 1Mbp. BWA-MEM and BWA-SW share similar features

such as long-read support and split alignment, but BWA-MEM, which is the latest, is generally recommended for high-quality queries as it is faster and more accurate.

4.4 Align reads to the reference genome

The alignment process consists of choosing an appropriate reference genome to map our reads against and then deciding on an aligner. We will use the BWA-MEM algorithm, which is the latest and is generally recommended for high-quality queries as it is faster and more accurate.

An example of what a `bwa` command looks like is below. This command will not run, as we do not have the files `ref_genome.fa`, `input_file_R1.fastq`, or `input_file_R2.fastq`.

```
$ bwa mem ref_genome.fasta input_file_R1.fastq input_file_R2.fastq > output.sam
```

Have a look at the bwa options page. While we are running `bwa` with the default parameters here, your use case might require a change of parameters.

> **Tip**
>
> *Always read the manual page for any tool before using and make sure the options you use are appropriate for your data.*

We are going to start by aligning the reads from just one of the samples in our data set (`SRR2584866`). Later, we will be iterating this whole process on all of our sample files.

```
$ bwa mem data/ref_genome/ecoli_rel606.fasta \
  data/trimmed_fastq_small/SRR2584866_1.trim.sub.fastq \
  data/trimmed_fastq_small/SRR2584866_2.trim.sub.fastq > \
  results/sam/SRR2584866.aligned.sam
```

You will see output that starts like this:

```
[M::main_mem] read 77446 sequences (10000033 bp)...
[M::mem_pestat] # candidate unique pairs for (FF, FR, RF, RR): (48, 36779, 21, 61)
[M::mem_pestat] analyzing insert size distribution for orientation FF...
[M::mem_pestat] (25, 50, 75) percentile: (420, 660, 1774)
[M::mem_pestat] low and high boundaries for computing mean and std.dev: (1, 4482)
[M::mem_pestat] mean and std.dev: (784.68, 700.87)
[M::mem_pestat] low and high boundaries for proper pairs: (1, 5836)
[M::mem_pestat] analyzing insert size distribution for orientation FR...
[M::mem_pestat] (25, 50, 75) percentile: (221, 361, 576)
```

```
[M::mem_pestat] low and high boundaries for computing mean and std.dev: (1, 1286)
```

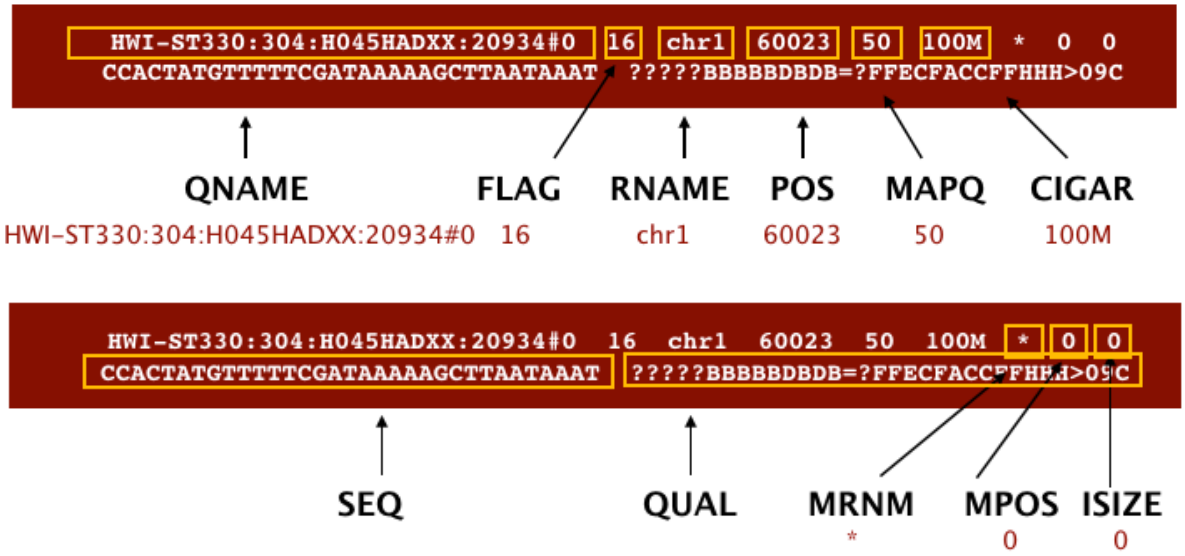
It should take less than a minute to be complete.

4.4.1 SAM/BAM format

The SAM file for "sequence alignment map", is a tab-delimited text file that contains information for each individual read and its alignment to the genome. While we do not have time to go into detail about the features of the SAM format, the paper by Li et al. (2009) provides a lot more detail on the specification.

The compressed binary version of SAM is called a BAM file for "binary sequence alignment map". We use this version to reduce size and to allow for *indexing*, which enables efficient random access of the data contained within the file.

The file begins with a **header**, which is optional. The header is used to describe the source of data, reference sequence, method of alignment, etc., this will change depending on the aligner being used. Following the header is the **alignment section**. Each line that follows corresponds to alignment information for a single read. Each alignment line has **11 mandatory fields** for essential mapping information and a variable number of other fields for aligner specific information. An example entry from a SAM file is displayed below with the different fields highlighted.



We will convert the SAM file to BAM format using the `samtools` program (Li et al. 2009) with the `view` command and tell this command that the input is in SAM format (`-S`) and to output BAM format (`-b`):

```
$ source package /tsl/software/testing/bin/samtools-1.9
$ samtools view -S -b results/sam/SRR2584866.aligned.sam > results/bam/SRR2584866.aligned.
[samopen] SAM header is present: 1 sequences.
```

4.4.2 Sort BAM file by coordinates

Next we sort the BAM file using the `sort` command from `samtools`. `-o` tells the command where to write the output. Our files are pretty small, so we will not see this output. If you run the workflow with larger files, you will see something like this:

```
$ samtools sort -o results/bam/SRR2584866.aligned.sorted.bam results/bam/SRR2584866.aligne
[bam_sort_core] merging from 2 files...
```

SAM/BAM files can be sorted in multiple ways, e.g. by location of alignment on the chromosome, by read name, etc. It is important to be aware that different alignment tools will output differently sorted SAM/BAM, and different downstream tools require differently sorted alignment files as input.

You can use `samtools` to learn more about this BAM file as well.

```
$ samtools flagstat results/bam/SRR2584866.aligned.sorted.bam
```

This will give you the following statistics about your sorted bam file:

```
351169 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 secondary
1169 + 0 supplementary
0 + 0 duplicates
351103 + 0 mapped (99.98% : N/A)
350000 + 0 paired in sequencing
175000 + 0 read1
175000 + 0 read2
346688 + 0 properly paired (99.05% : N/A)
349876 + 0 with itself and mate mapped
58 + 0 singletons (0.02% : N/A)
0 + 0 with mate mapped to a different chr
0 + 0 with mate mapped to a different chr (mapQ>=5)
```

4.5 Variant calling

A variant call is a conclusion that there is a nucleotide difference vs. some reference at a given position in an individual genome or transcriptome, often referred to as a Single Nucleotide Variant (SNV). The call is usually accompanied by an estimate of variant frequency and some measure of confidence. Similar to other steps in this workflow, there are a number of tools available for variant calling. In this workshop we will be using `bcftools`, but there are a few things we need to do before actually calling the variants.

```
$ source package /tsl/software/testing/bin/bcftools-1.9
loaded /tsl/software/testing/bin/bcftools-1.9
```

4.5.1 Step 1: Calculate the read coverage of positions in the genome

Do the first pass on variant calling by counting read coverage with bcftools. We will use the command mpileup. The flag -O b tells bcftools to generate a bcf format output file, -o specifies where to write the output file, and -f flags the path to the reference genome:

```
$ bcftools mpileup -O b -o results/bcf/SRR2584866_raw.bcf \
-f data/ref_genome/ecoli_rel606.fasta results/bam/SRR2584866.aligned.sorted.bam
[mpileup] 1 samples in 1 input files
...
```

It should take less than 1 minute to finish.

We have now generated a file with coverage information for every base.

4.5.2 Step 2: Detect the single nucleotide variants (SNVs)

Identify SNVs using `bcftools call`. We have to specify ploidy with the flag `--ploidy`, which is one for the haploid *E. coli*. `-m` allows for multi-allelic and rare-variant calling, `-v` tells the program to output variant sites only (not every site in the genome), and `-o` specifies where to write the output file:

```
$ bcftools call --ploidy 1 -m -v -o results/vcf/SRR2584866_variants.vcf results/bcf/SRR258
```

4.5.3 Step 3: Filter and report the SNV variants in variant calling format (VCF)

Filter the SNVs for the final output in VCF format, using `vcfutils.pl`:

```
$ vcfutils.pl varFilter results/vcf/SRR2584866_variants.vcf  > results/vcf/SRR2584866_fina
```

4.6 Explore the VCF format

```
$ less -S results/vcf/SRR2584866_final_variants.vcf
```

You will see the header (which describes the format), the time and date the file was created, the version of `bcftools` that was used, the command line parameters used, and some additional information:

```
##fileformat=VCFv4.2
##FILTER=<ID=PASS,Description="All filters passed">
##bcftoolsVersion=1.8+htslib-1.8
##bcftoolsCommand=mpileup -O b -o results/bcf/SRR2584866_raw.bcf -f data/ref_genome/ecoli_rel
##reference=file://data/ref_genome/ecoli_rel606.fasta
##contig=<ID=CP000819.1,length=4629812>
##ALT=<ID=*,Description="Represents allele(s) other than observed.">
##INFO=<ID=INDEL,Number=0,Type=Flag,Description="Indicates that the variant is an INDEL.">
##INFO=<ID=IDV,Number=1,Type=Integer,Description="Maximum number of reads supporting an indel
##INFO=<ID=IMF,Number=1,Type=Float,Description="Maximum fraction of reads supporting an indel
##INFO=<ID=DP,Number=1,Type=Integer,Description="Raw read depth">
##INFO=<ID=VDB,Number=1,Type=Float,Description="Variant Distance Bias for filtering splice-si
##INFO=<ID=RPB,Number=1,Type=Float,Description="Mann-Whitney U test of Read Position Bias (bi
##INFO=<ID=MQB,Number=1,Type=Float,Description="Mann-Whitney U test of Mapping Quality Bias
##INFO=<ID=BQB,Number=1,Type=Float,Description="Mann-Whitney U test of Base Quality Bias (big
##INFO=<ID=MQSB,Number=1,Type=Float,Description="Mann-Whitney U test of Mapping Quality vs St
##INFO=<ID=SGB,Number=1,Type=Float,Description="Segregation based metric.">
##INFO=<ID=MQ0F,Number=1,Type=Float,Description="Fraction of MQ0 reads (smaller is better)">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="List of Phred-scaled genotype likelihoods"
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##INFO=<ID=ICB,Number=1,Type=Float,Description="Inbreeding Coefficient Binomial test (bigger
##INFO=<ID=HOB,Number=1,Type=Float,Description="Bias in the number of HOMs number (smaller is
##INFO=<ID=AC,Number=A,Type=Integer,Description="Allele count in genotypes for each ALT allel
##INFO=<ID=AN,Number=1,Type=Integer,Description="Total number of alleles in called genotypes"
##INFO=<ID=DP4,Number=4,Type=Integer,Description="Number of high-quality ref-forward , ref-re
##INFO=<ID=MQ,Number=1,Type=Integer,Description="Average mapping quality">
##bcftools_callVersion=1.8+htslib-1.8
##bcftools_callCommand=call --ploidy 1 -m -v -o results/bcf/SRR2584866_variants.vcf results/b
```

Followed by information on each of the variations observed:

```
#CHROM  POS     ID      REF     ALT     QUAL    FILTER  INFO    FORMAT  results/bam/SRR25848
CP000819.1      1521    .       C       T       207     .       DP=9;VDB=0.993024;SGB=-0.662
CP000819.1      1612    .       A       G       225     .       DP=13;VDB=0.52194;SGB=-0.676
CP000819.1      9092    .       A       G       225     .       DP=14;VDB=0.717543;SGB=-0.67
CP000819.1      9972    .       T       G       214     .       DP=10;VDB=0.022095;SGB=-0.67
CP000819.1      10563   .       G       A       225     .       DP=11;VDB=0.958658;SGB=-0.67
CP000819.1      22257   .       C       T       127     .       DP=5;VDB=0.0765947;SGB=-0.59
CP000819.1      38971   .       A       G       225     .       DP=14;VDB=0.872139;SGB=-0.68
CP000819.1      42306   .       A       G       225     .       DP=15;VDB=0.969686;SGB=-0.68
CP000819.1      45277   .       A       G       225     .       DP=15;VDB=0.470998;SGB=-0.68
CP000819.1      56613   .       C       G       183     .       DP=12;VDB=0.879703;SGB=-0.67
CP000819.1      62118   .       A       G       225     .       DP=19;VDB=0.414981;SGB=-0.69
CP000819.1      64042   .       G       A       225     .       DP=18;VDB=0.451328;SGB=-0.68
```

This is a lot of information, so let's take some time to make sure we understand our output.

The first few columns represent the information we have about a predicted variation.

column	info
CHROM	contig location where the variation occurs
POS	position within the contig where the variation occurs
ID	a `.` until we add annotation information
REF	reference genotype (forward strand)
ALT	sample genotype (forward strand)
QUAL	Phred-scaled probability that the observed variant exists at this site (higher is better)
FILTER	a `.` if no quality filters have been applied, PASS if a filter is passed, or the name of the filters this variant failed

In an ideal world, the information in the `QUAL` column would be all we needed to filter out bad variant calls. However, in reality we need to filter on multiple other metrics.

The last two columns contain the genotypes and can be tricky to decode.

column	info
FORMAT	lists in order the metrics presented in the final column
results	lists the values associated with those metrics in order

For our file, the metrics presented are GT:PL:GQ.

metric	definition
AD, DP	the depth per allele by sample and coverage
GT	the genotype for the sample at this loci. For a diploid organism, the GT field indicates the two alleles carried by the sample, encoded by a 0 for the REF allele, 1 for the first ALT allele, 2 for the second ALT allele, etc. A 0/0 means homozygous reference, 0/1 is heterozygous, and 1/1 is homozygous for the alternate allele.
PL	the likelihoods of the given genotypes
GQ	the Phred-scaled confidence for the genotype

The Broad Institute's VCF guide is an excellent place to learn more about the VCF file format.

Exercise

Use the `grep` and `wc` commands you have learned to assess how many variants are in the vcf file.

Solution

```
$ grep -v "#" results/vcf/SRR2584866_final_variants.vcf | wc -l
765
```

There are 765 variants in this file.

4.7 Visualize and assess the alignment

It is often instructive to look at your data in a genome browser. Visualization will allow you to get a "feel" for the data, as well as detecting abnormalities and problems. Also, exploring the data in such a way may give you ideas for further analyses. As such, visualization tools are useful for exploratory analysis. In this lesson we will describe two different tools for visualization: a light-weight command-line based one and the Broad Institute's Integrative Genomics Viewer (IGV) which requires software installation and transfer of files.

In order for us to visualize the alignment files, we will need to index the BAM file using `samtools`:

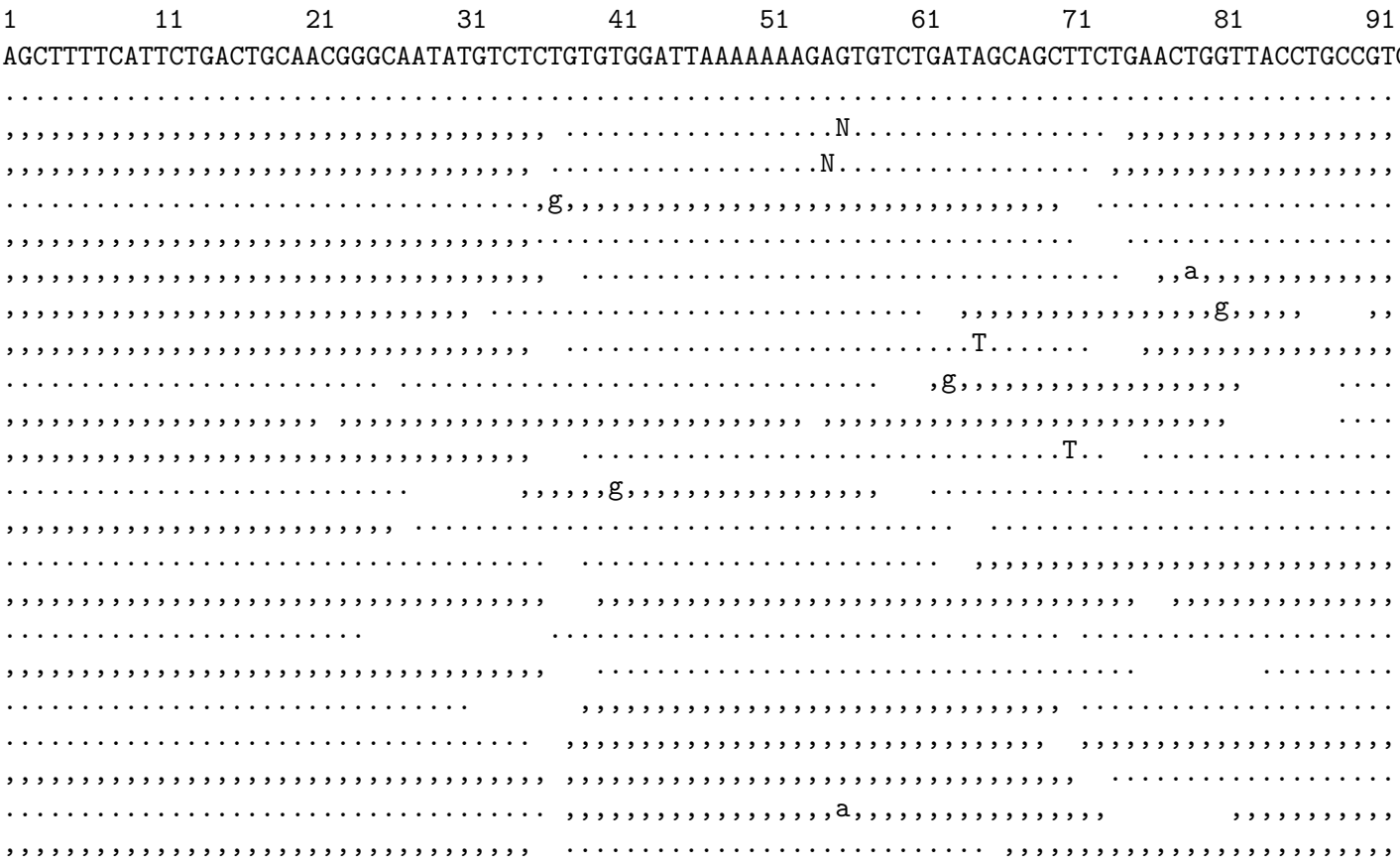
```
$ samtools index results/bam/SRR2584866.aligned.sorted.bam
```

4.7.1 Visualization with tview

Samtools implements a very simple text alignment viewer based on the GNU `ncurses` library, called `tview`. This alignment viewer works with short indels and shows MAQ consensus. It uses different colors to display mapping quality or base quality, subjected to users' choice. Samtools viewer is known to work with a 130 GB alignment swiftly. Due to its text interface, displaying alignments over network is also very fast.

In order to visualize our mapped reads, we use `tview`, giving it the sorted bam file and the reference file:

```
$ samtools tview results/bam/SRR2584866.aligned.sorted.bam data/ref_genome/ecoli_rel606.fa
```



The first line of output shows the genome coordinates in our reference genome. The second line shows the reference genome sequence. The third line shows the consensus sequence determined from the sequence reads. A `.` indicates a match to the reference sequence, so we can see that

the consensus from our sample matches the reference in most locations. That is good! If that was not the case, we should probably reconsider our choice of reference.

Below the horizontal line, we can see all of the reads in our sample aligned with the reference genome. Only positions where the called base differs from the reference are shown. You can use the arrow keys on your keyboard to scroll or type `?` for a help menu. To navigate to a specific position, type `g`. A dialogue box will appear. In this box, type the name of the "chromosome" followed by a colon and the position of the variant you would like to view (e.g. for this sample, type `CP000819.1:50` to view the 50th base. Type `Ctrl^C` or `q` to exit `tview`.

Exercise

Visualize the alignment of the reads for our SRR2584866 sample. What variant is present at position 4377265? What is the canonical nucleotide in that position?

Solution

```
$ samtools tview ~/dc_workshop/results/bam/SRR2584866.aligned.sorted.bam ~/dc_workshop/d
```

Then type `g`. In the dialogue box, type `CP000819.1:4377265`. `G` is the variant. `A` is canonical. This variant possibly changes the phenotype of this sample to hypermutable. It occurs in the gene *mutL*, which controls DNA mismatch repair.

4.7.2 Visualization with IGV

IGV is a stand-alone browser, which has the advantage of being installed locally and providing fast access. Web-based genome browsers, like Ensembl or the UCSC browser, are slower, but provide more functionality. They not only allow for more polished and flexible visualization, but also provide easy access to a wealth of annotations and external data sources. This makes it straightforward to relate your data with information about repeat regions, known genes, epigenetic features or areas of cross-species conservation, to name just a few.

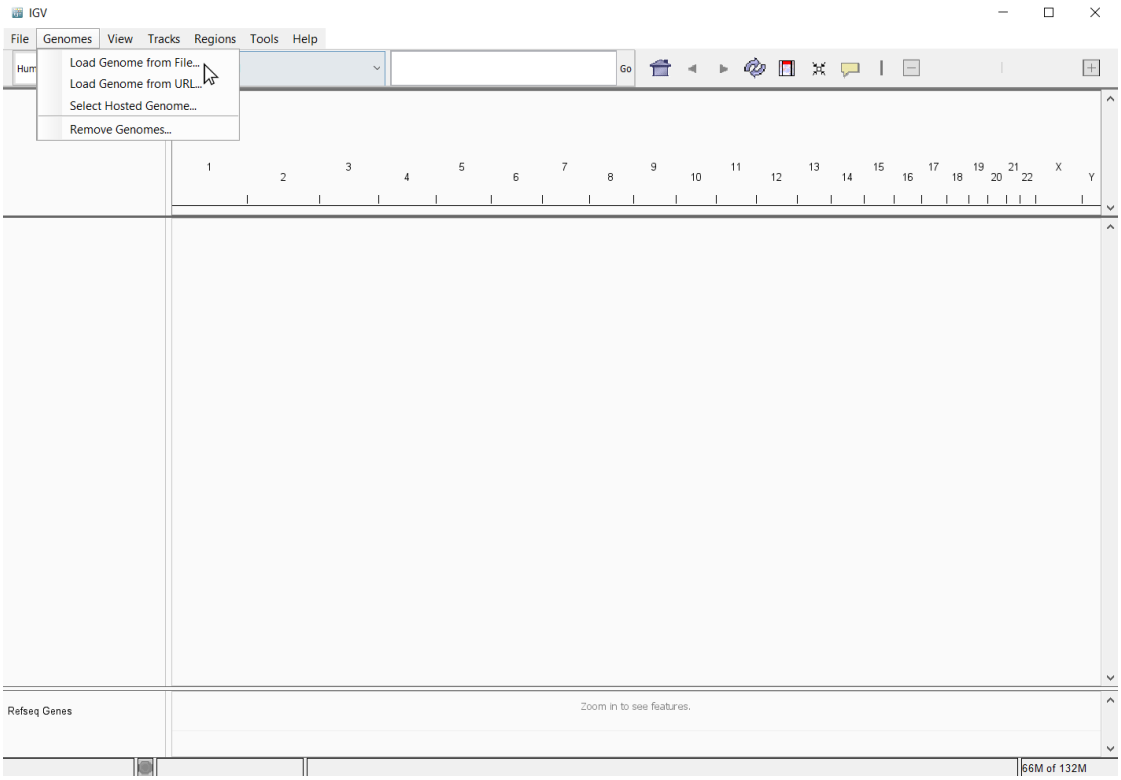
In order to use IGV, we will need to transfer some files to our local machine. We know how to do this with the Windows navigation system. Open a new tab in your terminal window and create a new folder. We will put this folder on our Desktop for demonstration purposes, but in general you should avoid proliferating folders and files on your Desktop and instead organize files within a directory structure like we have been using in our `dc_workshop` directory. Now we will transfer our files to that new local directory.

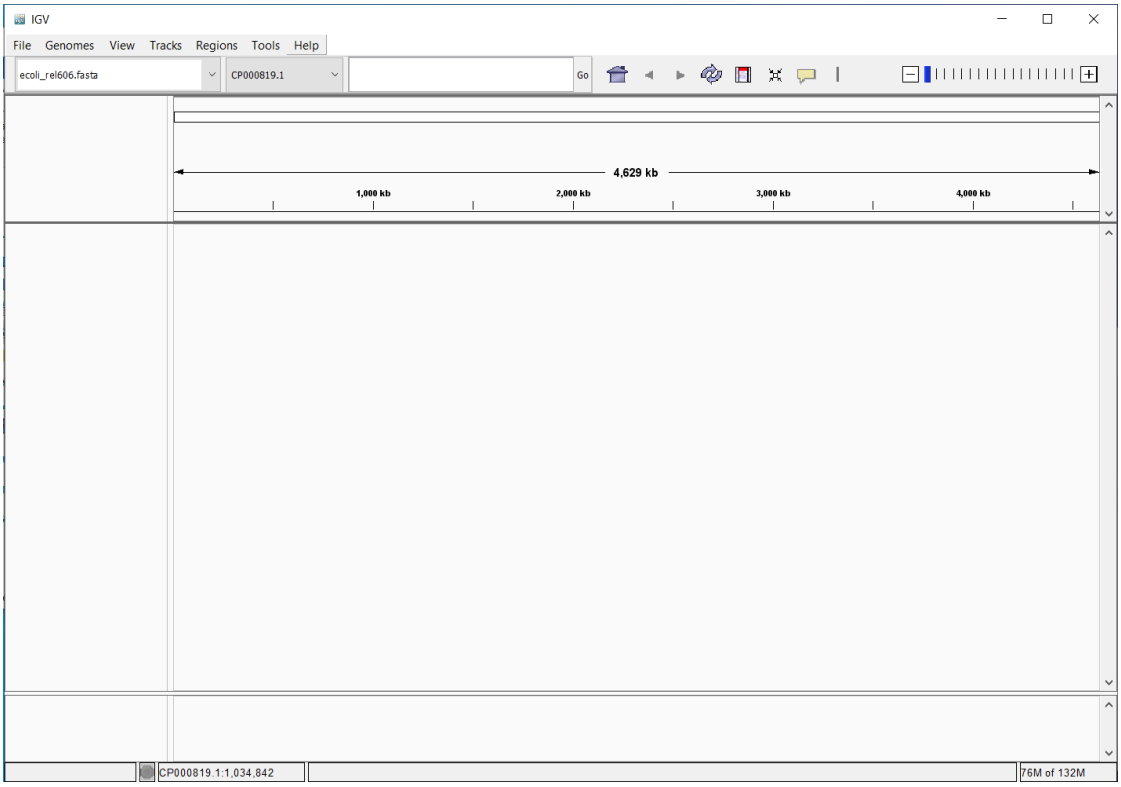
Next, we need to open the IGV software. If you have not done so already, you can download IGV from the Broad Institute's software page, double-click the `.zip` file to unzip it, and then drag the program into your Applications folder.

1. Open IGV.

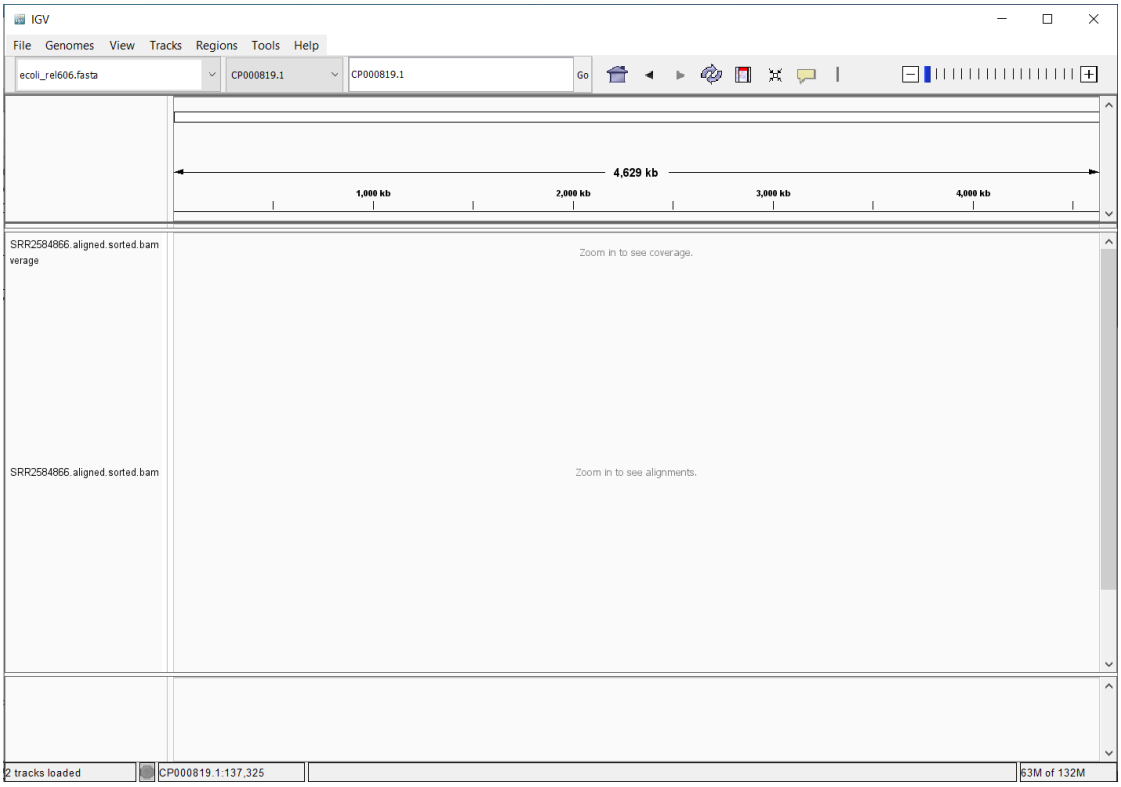
> **Tip**
>
> IGV is already installed on your computer and you can find the icon shortcut on your Desktop.

2. Load our reference genome file (`ecoli_rel606.fasta`) into IGV using the **"Load Genomes from File..."** option under the **"Genomes"** pull-down menu.

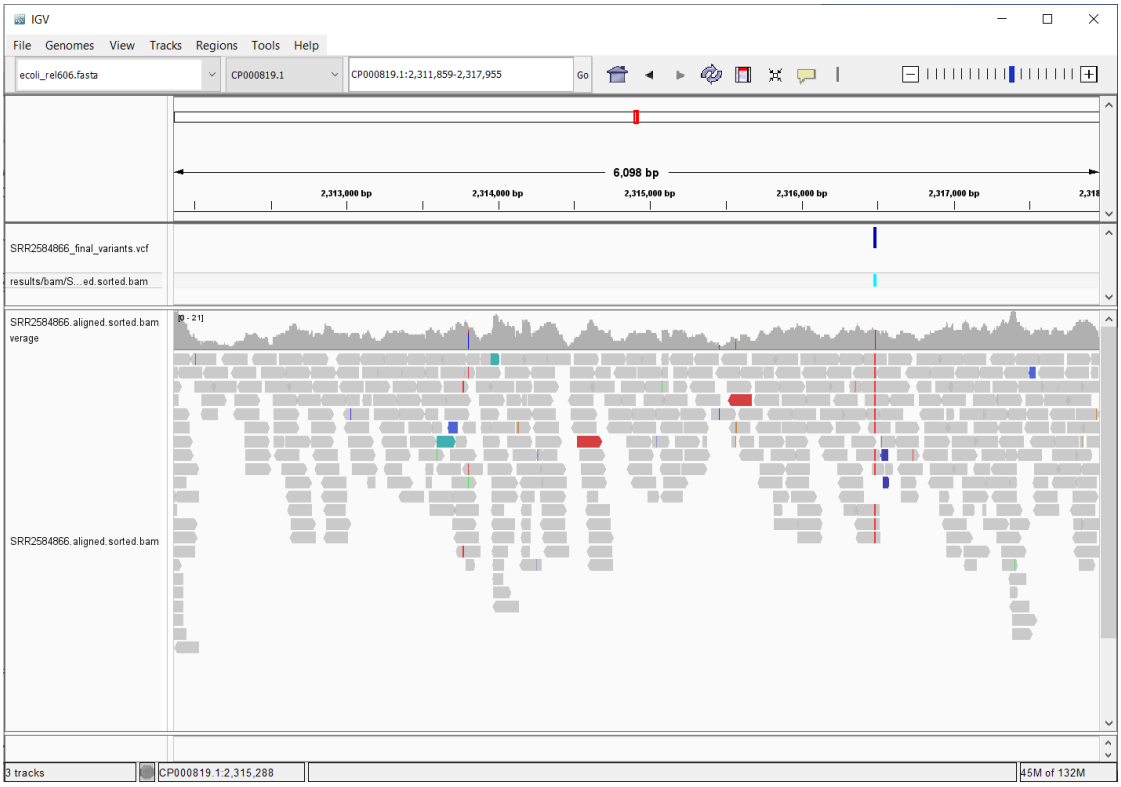




3. Load our BAM file (`SRR2584866.aligned.sorted.bam`) using the **"Load from File..."** option under the **"File"** pull-down menu.



4. Do the same with our VCF file (`SRR2584866_final_variants.vcf`).



There should be two tracks: one corresponding to our BAM file and the other for our VCF file.

In the **VCF track**, each bar across the top of the plot shows the allele fraction for a single locus. The second bar shows the genotypes for each locus in each *sample*. We only have one sample called here, so we only see a single line.

- Dark blue = heterozygous,
- Cyan = homozygous variant,
- Grey = reference.
- Filtered entries are transparent.

Zoom in to inspect variants you see in your filtered VCF file to become more familiar with IGV. See how quality information corresponds to alignment information at those loci. Use this website and the links therein to understand how IGV colors the alignments.

Now that we have run through our workflow for a single sample, we want to repeat this workflow for our other five samples. However, we do not want to type each of these individual steps again five more times. That would be very time consuming and error-prone, and would become impossible as we gathered more and more samples. Luckily, we already know the tools

we need to use to automate this workflow and run it on as many files as we want using a single line of code. Those tools are: wildcards, for loops, and bash scripts. We will use all three in the next lesson.

4.8 Summary

! Key points

- Bioinformatic command line tools are collections of commands that can be used to carry out bioinformatic analyses.
- To use most powerful bioinformatic tools, you will need to use the command line.
- There are many different file formats for storing genomics data. It is important to understand what type of information is contained in each file, and how it was derived.

5 Automating a variant calling workflow

Time

- Teaching: 30 min
- Exercises: 15 min

Question

- How can I make my workflow more efficient and less error-prone?

Objectives

- Write a shell script with multiple variables.
- Incorporate a for loop into a shell script.

5.1 What is a shell script?

You wrote a simple shell script in a previous lesson that we used to extract bad reads from our FASTQ files and put them into a new file.

Here is the script you wrote:

```
grep -B1 -A2 NNNNNNNNNN *.fastq > scripted_bad_reads.txt

echo "Script finished!"
```

That script was only two lines long, but shell scripts can be much more complicated than that and can be used to perform a large number of operations on one or many files. This saves you the effort of having to type each of those commands over for each of your data files and makes your work less error-prone and more reproducible. For example, the variant calling workflow we just carried out had about eight steps where we had to type a command into our terminal. Most of these commands were pretty long. If we wanted to do this for all six of our data files, that would be forty-eight steps. If we had 50 samples (a more realistic number), it would be 400 steps! You can see why we want to automate this.

We have also used `for` loops in previous lessons to iterate one or two commands over multiple input files. In these `for` loops, the filename was defined as a variable in the `for` statement, which enabled you to run the loop on multiple files. We will be using variable assignments like this in our new shell scripts.

Here is the `for` loop you wrote for unzipping `.zip` files:

```
$ for filename in *.zip
> do
> unzip $filename
> done
```

And here is the one you wrote for running Trimmomatic on all of our `.fastq` sample files:

```
$ for infile in *_1.fastq.gz
> do
>   base=$(basename ${infile} _1.fastq.gz)
>   trimmomatic PE ${infile} ${base}_2.fastq.gz \
>                ${base}_1.trim.fastq.gz ${base}_1un.trim.fastq.gz \
>                ${base}_2.trim.fastq.gz ${base}_2un.trim.fastq.gz \
>                SLIDINGWINDOW:4:20 MINLEN:25 ILLUMINACLIP:NexteraPE-PE.fa:2:40:15
> done
```

Notice that in this `for` loop, we used two variables, `infile`, which was defined in the `for` statement, and `base`, which was created from the filename during each iteration of the loop.

> **Creating variables**
>
> Within the Bash shell you can create variables at any time (as we did above, and during the 'for' loop lesson). Assign any name and the value using the assignment operator: '='. You can check the current definition of your variable by typing into your script: echo $variable_name.

In this lesson, we will use two shell scripts to automate the variant calling analysis: one for FastQC analysis (including creating our summary file), and a second for the remaining variant calling. To write a script to run our FastQC analysis, we will take each of the commands we entered to run FastQC and process the output files and put them into a single file with a `.sh` extension. The `.sh` is not essential, but serves as a reminder to ourselves and to the computer that this is a shell script.

5.2 Analysing quality with FastQC

We will use the command `touch` to create a new file where we will write our shell script. We will create this script in a new directory called `scripts/`. Previously, we used `nano` to create and open a new file. The command `touch` allows us to create a new file without opening that file.

```
$ mkdir -p ~/dc_workshop/scripts
$ cd ~/dc_workshop/scripts
$ touch read_qc.sh
$ ls
read_qc.sh
```

We now have an empty file called `read_qc.sh` in our `scripts/` directory. We will now open this file in `nano` and start building our script.

```
$ nano read_qc.sh
```

> **Warning**
>
> Enter the following pieces of code into your shell script using `nano`, not into your terminal prompt (with `$`).

Our first line will ensure that our script will exit if an error occurs, and is a good idea to include at the beginning of your scripts. The second line will move us into the `untrimmed_fastq/` directory when we run our script. Then we load the software needed.

```
set -e
cd ~/dc_workshop/data/untrimmed_fastq/

source package /nbi/software/production/bin/fastqc-0.11.8
source package /nbi/software/production/bin/trimmomatic-0.39
```

These next two lines will give us a status message to tell us that we are currently running FastQC, then will run FastQC on all of the files in our current directory with a `.fastq` extension.

```
echo "Running FastQC ..."
fastqc *.fastq*
```

Our next line will create a new directory to hold our FastQC output files. Here we are using the `-p` option for `mkdir` again. It is a good idea to use this option in your shell scripts to avoid running into errors if you do not have the directory structure you think you do.

```
mkdir -p ~/dc_workshop/results/fastqc_untrimmed_reads
```

Our next three lines first give us a status message to tell us we are saving the results from FastQC, then moves all of the files with a `.zip` or a `.html` extension to the directory we just created for storing our FastQC results.

```
echo "Saving FastQC results…"
mv .zip ~/dc_workshop/results/fastqc_untrimmed_reads/
mv .html ~/dc_workshop/results/fastqc_untrimmed_reads/
```

The next line moves us to the results directory where we have stored our output.

```
cd ~/dc_workshop/results/fastqc_untrimmed_reads/
```

The next five lines should look very familiar. First we give ourselves a status message to tell us that we are unzipping our ZIP files. Then we run our for loop to unzip all of the `.zip` files in this directory.

```
echo "Unzipping..."
for filename in *.zip
    do
    unzip $filename
    done
```

Next we concatenate all of our summary files into a single output file, with a status message to remind ourselves that this is what we are doing.

```
echo "Saving summary..."
cat */summary.txt > ~/dc_workshop/docs/fastqc_summaries.txt
```

5.3 Using `echo` statements

We have used `echo` statements to add progress statements to our script. Our script will print these statements as it is running and therefore we will be able to see how far our script has progressed.

Your full shell script should now look like this:

```
set -e
cd ~/dc_workshop/data/untrimmed_fastq/

source package /nbi/software/production/bin/fastqc-0.11.8
source package /nbi/software/production/bin/trimmomatic-0.39

echo "Running FastQC ..."
source package /nbi/software/production/bin/fastqc-0.11.8
fastqc *.fastq*

mkdir -p ~/dc_workshop/results/fastqc_untrimmed_reads

echo "Saving FastQC results..."
mv *.zip ~/dc_workshop/results/fastqc_untrimmed_reads/
mv *.html ~/dc_workshop/results/fastqc_untrimmed_reads/

cd ~/dc_workshop/results/fastqc_untrimmed_reads/

echo "Unzipping..."
for filename in *.zip
    do
    unzip $filename
    done

echo "Saving summary..."
cat */summary.txt > ~/dc_workshop/docs/fastqc_summaries.txt
```

Save your file and exit `nano`. We can now run our script:

```
$ bash read_qc.sh

Running FastQC ...
Started analysis of SRR2584866.fastq
Approx 5% complete for SRR2584866.fastq
```

```
Approx 10% complete for SRR2584866.fastq
Approx 15% complete for SRR2584866.fastq
Approx 20% complete for SRR2584866.fastq
Approx 25% complete for SRR2584866.fastq
.
.
.
```

For each of your sample files, FastQC will ask if you want to replace the existing version with a new version. This is because we have already run FastQC on this samples files and generated all of the outputs. We are now doing this again using our scripts. Go ahead and select `A` each time this message appears. It will appear once per sample file (six times total).

```
replace SRR2584866_fastqc/Icons/fastqc_icon.png? [y]es, [n]o, [A]ll, [N]one, [r]ename:
```

5.4 Automating the rest of our variant calling workflow

We can extend these principles to the entire variant calling workflow. To do this, we will take all of the individual commands that we wrote before, put them into a single file, add variables so that the script knows to iterate through our input files and write to the appropriate output files. This is very similar to what we did with our `read_qc.sh` script, but will be a bit more complex.

The complete script can be found here.

```
$ cd ~/dc_workshop/scripts
$ nano run_variant_calling.sh
```

Then copy and paste the script and close the text editor.

> **Possibility to download the script**
>
> Download the script from here to `~/dc_workshop/scripts`.
>
> ```
> curl -O https://raw.githubusercontent.com/datacarpentry/wrangling-genomics/gh-pages/files/r
> ```

Our variant calling workflow has the following steps:

1. Index the reference genome for use by `bwa` and `samtools`.
2. Align reads to reference genome.
3. Convert the format of the alignment to sorted BAM, with some intermediate steps.
4. Calculate the read coverage of positions in the genome.

5. Detect the single nucleotide variants (SNVs).
6. Filter and report the SNVs in VCF (variant calling format).

Let's go through this script together:

```
$ cd ~/dc_workshop/scripts
$ less run_variant_calling.sh
```

The script should look like this:

```
set -e
cd ~/dc_workshop/results

source package /nbi/software/production/bin/bwa-0.7.5
source package /tsl/software/testing/bin/samtools-1.9
source package /tsl/software/testing/bin/bcftools-1.9

genome=~/dc_workshop/data/ref_genome/ecoli_rel606.fasta

bwa index $genome

mkdir -p sam bam bcf vcf

for fq1 in ~/dc_workshop/data/trimmed_fastq_small/*_1.trim.sub.fastq
    do
    echo "working with file $fq1"

    base=$(basename $fq1 _1.trim.sub.fastq)
    echo "base name is $base"

    fq1=~/dc_workshop/data/trimmed_fastq_small/${base}_1.trim.sub.fastq
    fq2=~/dc_workshop/data/trimmed_fastq_small/${base}_2.trim.sub.fastq
    sam=~/dc_workshop/results/sam/${base}.aligned.sam
    bam=~/dc_workshop/results/bam/${base}.aligned.bam
    sorted_bam=~/dc_workshop/results/bam/${base}.aligned.sorted.bam
    raw_bcf=~/dc_workshop/results/bcf/${base}_raw.bcf
    variants=~/dc_workshop/results/vcf/${base}_variants.vcf
    final_variants=~/dc_workshop/results/vcf/${base}_final_variants.vcf

    bwa mem $genome $fq1 $fq2 > $sam
    samtools view -S -b $sam > $bam
    samtools sort -o $sorted_bam $bam
    samtools index $sorted_bam
```

```
    bcftools mpileup -O b -o $raw_bcf -f $genome $sorted_bam
    bcftools call --ploidy 1 -m -v -o $variants $raw_bcf
    vcfutils.pl varFilter $variants > $final_variants

    done
```

Now, we will go through each line in the script before running it.

First, notice that we change our working directory so that we can create new results subdirectories in the right location.

```
cd ~/dc_workshop/results
```

Next we tell our script where to find the reference genome by assigning the `genome` variable to the path to our reference genome:

```
genome=~/dc_workshop/data/ref_genome/ecoli_rel606.fasta
```

Next we index our reference genome for BWA:

```
bwa index $genome
```

And create the directory structure to store our results in:

```
mkdir -p sam bam bcf vcf
```

Then, we use a loop to run the variant calling workflow on each of our FASTQ files. The full list of commands within the loop will be executed once for each of the FASTQ files in the `data/trimmed_fastq_small/` directory. We will include a few `echo` statements to give us status updates on our progress.

The first thing we do is assign the name of the FASTQ file we are currently working with to a variable called `fq1` and tell the script to `echo` the filename back to us so we can check which file we are on.

```
for fq1 in ~/dc_workshop/data/trimmed_fastq_small/*_1.trim.sub.fastq
    do
    echo "working with file $fq1"
```

We then extract the base name of the file (excluding the path and `.fastq` extension) and assign it to a new variable called `base`.

```
base=$(basename $fq1 _1.trim.sub.fastq)
echo "base name is $base"
```

We can use the `base` variable to access both the `base_1.fastq` and `base_2.fastq` input files, and create variables to store the names of our output files. This makes the script easier to read because we do not need to type out the full name of each of the files: instead, we use the `base` variable, but add a different extension (e.g. `.sam`, `.bam`) for each file produced by our workflow.

```
#input fastq files
fq1=~/dc_workshop/data/trimmed_fastq_small/${base}_1.trim.sub.fastq
fq2=~/dc_workshop/data/trimmed_fastq_small/${base}_2.trim.sub.fastq

# output files
sam=~/dc_workshop/results/sam/${base}.aligned.sam
bam=~/dc_workshop/results/bam/${base}.aligned.bam
sorted_bam=~/dc_workshop/results/bam/${base}.aligned.sorted.bam
raw_bcf=~/dc_workshop/results/bcf/${base}_raw.bcf
variants=~/dc_workshop/results/bcf/${base}_variants.vcf
final_variants=~/dc_workshop/results/vcf/${base}_final_variants.vcf
```

And finally, the actual workflow steps:

1) align the reads to the reference genome and output a `.sam` file:

```
bwa mem $genome $fq1 $fq2 > $sam
```

2) convert the SAM file to BAM format:

```
samtools view -S -b $sam > $bam
```

3) sort the BAM file:

```
samtools sort -o $sorted_bam $bam
```

4) index the BAM file for display purposes:

```
samtools index $sorted_bam
```

5) calculate the read coverage of positions in the genome:

```
bcftools mpileup -O b -o $raw_bcf -f $genome $sorted_bam
```

6) call SNVs with bcftools:

```
bcftools call --ploidy 1 -m -v -o $variants $raw_bcf
```

7) filter and report the SNVs in variant calling format (VCF):

```
vcfutils.pl varFilter $variants  > $final_variants
```

> Exercise
>
> It is a good idea to add comments to your code so that you (or a collaborator) can make sense of what you did later. Look through your existing script. Discuss with a neighbor where you should add comments. Add comments (anything following a # character will be interpreted as a comment, bash will not try to run these comments as code).

Now we can run our script:

```
$ bash run_variant_calling.sh
```

> Exercise
>
> The samples we just performed variant calling on are part of the long-term evolution experiment introduced at the beginning of our variant calling workflow. From the metadata table, we know that SRR2589044 was from generation 5000, SRR2584863 was from generation 15000, and SRR2584866 was from generation 50000. How did the number of mutations per sample change over time? Examine the metadata table. What is one reason the number of mutations may have changed the way they did?
> Hint: You can find a copy of the output files for the subsampled trimmed FASTQ file variant calling in the `~/.solutions/wrangling-solutions/variant_calling_auto/` directory.

Solution

```
$ for infile in ~/dc_workshop/results/vcf/*_final_variants.vcf
> do
>     echo ${infile}
>     grep -v "#" ${infile} | wc -l
> done
```

For SRR2589044 from generation 5000 there were 10 mutations, for SRR2584863 from generation 15000 there were 25 mutations, and SRR2584866 from generation 766 mutations. In the last generation, a hypermutable phenotype had evolved, causing this strain to have more mutations.

Bonus exercise

If you have time after completing the previous exercise, use run_variant_calling.sh to run the variant calling pipeline on the full-sized trimmed FASTQ files. You should have a copy of these already in ~/dc_workshop/data/trimmed_fastq, but if you do not, there is a copy in ~/.solutions/wrangling-solutions/trimmed_fastq. Does the number of variants change per sample?

5.5 Summary

! Key points

- We can combine multiple commands into a shell script to automate a workflow.
- Use `echo` statements within your scripts to get an automated progress update.

References

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