2017 Workshop on Genomics

Objectives – Part 1: Short Read Genomics Introduction

- Understand how short reads are generated.
- Understand paired-end reads
- See possible sources of errors
- Learn about adaptors

Objectives - Part 2: QC, alignment and variant calling

- Interpret FASTQ quality metrics
- Remove poor quality data
- Trim adaptor/contaminant sequences from FASTQ data
- Count the number of reads before and after trimming and quality control
- Align reads to a reference sequence to form a SAM file (Sequence AlignMent file) using BWA
- Convert the SAM file to BAM format (Binary AlignMent format)
- Identify and select high quality SNPs and Indels using SAMtools
- Identify missing or truncated genes with respect to the reference genome
- Identify SNPs which overlap with known coding regions

Objectives – Part 3: Examining unmapped reads

- Extract reads which do not map to the reference sequence
- Assemble these reads de novo using SPAdes
- Generate summary statistics for the assembly
- Identify potential genes within the assembly
- Search for matches within the NCBI database via BLAST and against the Pfam database
- Visualize the taxonomic distribution of BLAST hits
- Perform gene prediction and annotation using RAST

Objectives – Part 4: De-novo assembly using short reads

- Perform QC and adaptor-trim Illumina reads.
- Assemble these reads de novo using SPAdes
- Generate summary statistics for the assembly
- Understand how to incorporate long PacBio reads into the assembly.
- Identify open reading frames within the assembly
- Search for matches within the NCBI database via BLAST and against the Pfam database
- Visualize species distribution of potential matches

Objectives - Part 5 (optional):

- Run parts 2-4 of the workshop on up to new 6 datasets
- Use pre-prepared scripts to compare SNPs and Indels between strains
- Generate pseudo-sequences based on synonymous SNPs
- Draw simple trees to illustrate the likely evolutionary relationship between strains
- Compare Pfam matches between strains

Objectives - Part 6: Long read Introduction and de-novo assembly using long reads

- Understand how to perform basic QC on PacBio and Oxford Nanopore MinION data
- Generate and polish long read assemblies using PacBio and Oxford Nanopore MinION data
- Generate and polish long read assemblies using PacBio data and Illumina
- Compare your assemblies to the reference genome using Quast

Part 1:
Short read genomics: Introduction

Introduction

Welcome to the Genomics workshop. Generating reams of data in Biology is easy these days. In little more than a fortnight we can generate more data than the entire human genome project generated in over a decade of work. Making biological sense out of that data, understanding its limitations and how the analysis algorithms work is now the major challenge for researchers. The aim of this workshop is to take you through an example project. On the way you will learn how to evaluate the quality of data as provided by a sequencing facility, how to align the data against a known and annotated reference genome and how to perform a de-novo assembly. In addition you will also learn how to compare results between different samples.

This workshop is broken into 5 parts. You should feel free to take as long as you like on each part. It is much more important that you have a thorough understanding of each part, rather than try to race through the entire workshop.

The five parts are:

1. Introduction
2. Remapping a strain of *E.coli* to a reference sequence
3. Assembly of unmapped reads
4. Complete *de-novo* assembly of all reads
5. Repeating parts 3-5 on strains of *Vibrio parahaemolyticus* and comparing them

For this first workshop we will assume little background knowledge, save a basic familiarity with the Linux operating system and the Amazon cloud. We will cover the basics of how genomic DNA libraries are generated and sequenced, and the principles behind short read paired-end sequencing. We will look at why data can vary in quality, why adaptor sequences need to be filtered out and how to quality control data.

In the second part we will take the plunge and align the filtered reads to a reference genome, call variants and compare them against the published genome to identify missing, truncated or altered genes. This will involve the use of a publicly available set of bacterial *E.coli* Illumina reads and reference genome.

In parts 3 and 4 we will look at how one can identify novel sequences which are not present in the reference genome. In part 5, you will be asked to repeat the steps 2, 3 and 4 on other data sets and to compare the results.

A word on notation. If you see something like this:

```
cd ~/genomics_tutorial/reference_sequence
```

It means, type the highlighted text into your terminal.

Principles of Illumina-based sequencing:

There are several second generation sequencers currently on the market. These include the Ion Torrent, and the Illumina HiSeq, NextSeq and MiSeq systems. Other (now obsolescent) platforms included Life Tech SoLID and Roche 454. All of these systems rely on making hundreds of thousands of clonal copies of a fragment of DNA and sequencing the ensemble of fragments using DNA polymerase or in the case of the SOLiD via ligation. This is simply because the detectors (basically souped-up digital cameras), cannot detect fluorescence (Illumina, SolID, 454) or pH changes (Ion Torrent) from a single molecule. The 'third-generation' Pacific Biosciences SMRT (Single Molecule Real Time) RSII and Sequel sequencers are able to detect fluorescence from a single molecule of DNA. However, the machines are very large (the RSII is almost 2 tons) and produces less than a tenth of the data of an Illumina MiSeq run and for long reads >10kb error rates are generally around 10-12%. The Oxford Nanopore MinION is another 'third-generation' single-molecule system which measures changes in electrical current through a Nanopore as a single molecule is ratcheted through it. Although error rates are higher (5-10%) and per-base costs are higher the technology has improved rapidly and will probably replace second generation systems over the next few years. Currently however, the second generation sequencers are dominating the sequencing world.

We will mainly look at the Illumina sequencing pipeline here, but the basic principles apply to other second-generation sequencers. If you would like further details on other platforms then I recommend reading *Mardis ER. Next-generation DNA sequencing methods. Annual Reviews Genomics Hum Genet 2008; 9 :387–402.*
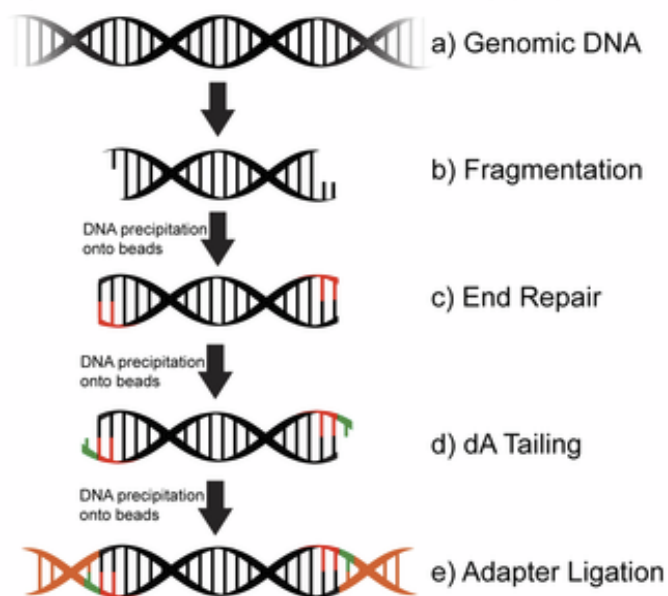
A typical sequencing run would begin with the user supplying 1ng-1 ug of genomic DNA to a facility along with quality control information in the form of an automated electrophoresis output (e.g. Agilent Bioanalyser/Tapestation trace) or gel image and quantification information.
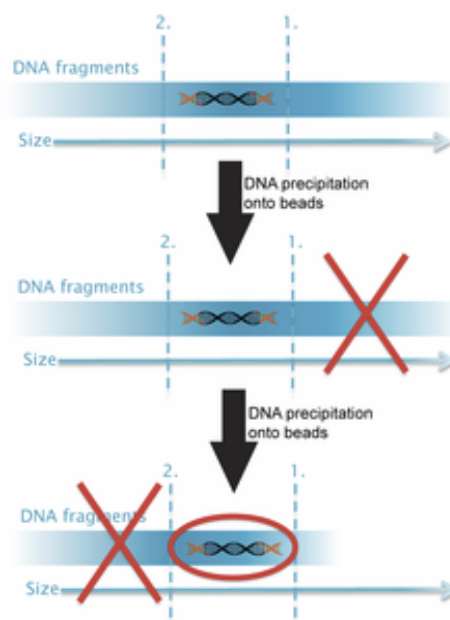
DNA Library preparation

For most sequencing applications, paired-end libraries are generated. Genomic DNA is sheared into 300-500bp fragments (usually via sonication) and size-selected accordingly. Ends are repaired and an overhanging adenine base is added, after which oligonucleotide adaptors are ligated. In many cases the adaptors contain unique DNA sequences of 6-12bp which can be used to identify the sample if they are 'multiplexed' together for sequencing. This type of sequencing is used extensively when sequencing small genomes such as those of bacteria because it lowers the overall per-genome cost.

Part 1:   Short read genomics: Introduction

A) Workflow of the automated library preparation

a) Genomic DNA

b) Fragmentation

DNA precipitation onto beads

c) End Repair

DNA precipitation onto beads

d) dA Tailing

DNA precipitation onto beads

e) Adapter Ligation

B) Automated size selection

A) Steps a through e explain the main steps in Illumina sample preparation: a) the initial genomic DNA, b) fragmentation of genomic DNA into 500bp fragments, c) end repair, d) addition of A bases to the fragment ends and e) ligation of the adaptors to the fragments.

B) Overview of the automated the size selection protocol: The first precipitation discards fragments larger than the desired interval. The second precipitation selects all fragments larger than the lower boundary of the desired interval.

Borgström E, Lundin S, Lundeberg J, 2011 Large Scale Library Generation for High Throughput Sequencing. PLoS ONE 6: e19119. doi:10.1371/journal.pone.0019119
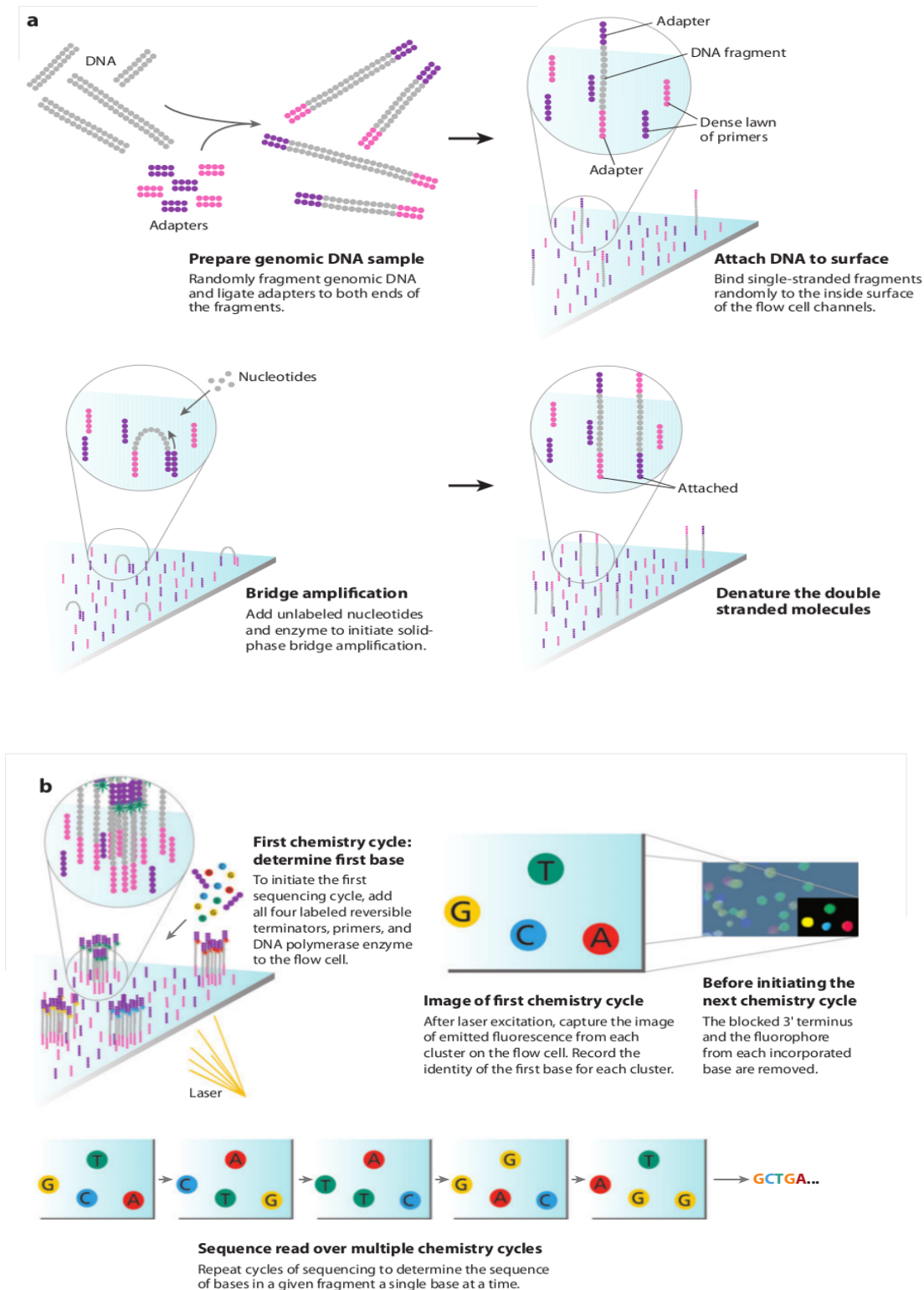
Sequencing

(adapted from Margulis, E.R., reference below)

Once sufficient libraries have been prepared, the task is to amplify single strands of DNA to form monoclonal clusters. The single molecule amplification step for the Illumina HiSeq 2500 starts with an Illumina-specific adapter library and takes place on the oligo-derivatized surface of a flow cell, and is performed by an automated device called a cBot Cluster Station. The flow cell is either a 2 or 8-channel sealed glass microfabricated device that allows bridge amplification of fragments on its surface, and uses DNA polymerase to produce multiple DNA copies, or clusters, that each represent the single molecule that initiated the cluster amplification.

Separate or multiple libraries can be added to each of the eight channels, or the same library can be used in all eight, or combinations thereof. Each cluster contains approximately one million copies of the original fragment, which is sufficient for reporting incorporated bases at the required signal intensity for detection during sequencing. The Illumina system utilizes a sequencing- by-synthesis approach in which all four nucleotides are added simultaneously to the flow cell channels, along with DNA polymerase, for incorporation into the oligo-primed cluster fragments (see figure below for details). Specifically, the nucleotides carry a base-unique fluorescent label and the 3 -OH group is chemically blocked such that each incorporation is a unique event. An imaging step follows each base incorporation step, during which each flow cell lane is imaged in three 100-tile segments by the instrument optics at a cluster density of 600,000-800,000 per mm^2. After each imaging step, the 3' blocking group is chemically removed to prepare each strand for the next incorporation by DNA polymerase. This series of steps continues for a specific number of cycles, as determined by user-defined instrument settings, which permits discrete read lengths of 40–300 bases. A base-calling algorithm assigns sequences and associated quality values to each read and a quality checking pipeline evaluates the Illumina data from each run.

The figure on the following page summarises the process:

Part 1: Short read genomics: Introduction

a

Prepare genomic DNA sample
Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

Attach DNA to surface
Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

Bridge amplification
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

Denature the double stranded molecules

b

First chemistry cycle: determine first base
To initiate the first sequencing cycle, add all four labeled reversible terminators, primers, and DNA polymerase enzyme to the flow cell.

Image of first chemistry cycle
After laser excitation, capture the image of emitted fluorescence from each cluster on the flow cell. Record the identity of the first base for each cluster.

Before initiating the next chemistry cycle
The blocked 3' terminus and the fluorophore from each incorporated base are removed.

Sequence read over multiple chemistry cycles
Repeat cycles of sequencing to determine the sequence of bases in a given fragment a single base at a time.

The Illumina sequencing-by-synthesis approach: Cluster strands created by bridge amplification are primed and all four fluorescently labelled, 3 -OH blocked nucleotides are added to the flow cell with DNA polymerase. The cluster strands are extended by one nucleotide. Following the incorporation step, the unused nucleotides and DNA polymerase molecules are washed away, a scan buffer is added to the flow cell, and the optics system scans each lane of the flow cell by imaging units called tiles. Once imaging is completed, chemicals that effect cleavage of the fluorescent labels and the 3 -OH blocking

Base-calling:

Base-calling involves evaluating the raw intensity values for each fluorophore and comparing them to determine which base is actually present at a given position during a cycle. To call bases on the Illumina or SOLiD platform, the positions of clusters need to be identified during the first few cycles. This is because they are formed in random positions on the flowcell as the annealing process is stochastic. This is in contrast to the 454 system where the position of each cluster is defined by steel plate with pico-litre sized holes in which the reaction takes place.

If there are too many clusters, the edges of the clusters will begin to merge and the image analysis algorithms will not be able to distinguish one cluster from another (remember, the software is dealing with upwards of half a million clusters per square millimeter – that's a lot of dots!).
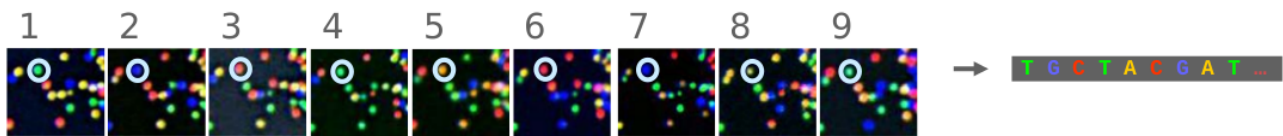
The above figure illustrates the principles of base-calling from cycles 1 to 9. If we focus on the highlighted cluster, one can observe that the colour (wavelength) of light observed at each cycle changes along with the brightness (intensity). This is due to the incorporation of complementary ddNTPs containing fluorophores. So at cycle 1 we have a T base, at 2 a G base and so on. If the colour or intensity is ambiguous the sequencer will mark it as an N. Other clusters are also visible in the images; these will represent different monoclonal clusters with different sequences.

The base calling algorithms turn the raw intensity values into T,G,C,A or N base calls. There are a variety of methods to do this and the one mentioned here is by no means the only one available, but it is often used as the default method on the Illumina systems. Known as the 'Chastity filter' it will only call a base if the intensity divided by the sum of the highest and second highest intensity is less than a given threshold (usually 0.6). Otherwise the base is marked with an N. In addition the standard Illumina pipeline will reject an entire read if two or more of these failures occur in the first 4 bases of a read (it uses these cycles to determine the boundary of a cluster).

Note that these processes are carried out at the sequencing facility and you will not need to perform any of these tasks under normal circumstances. They are explained here as useful background information.

CHASTITY formula:

$$C = \frac{I_A}{I_A + I_B}$$
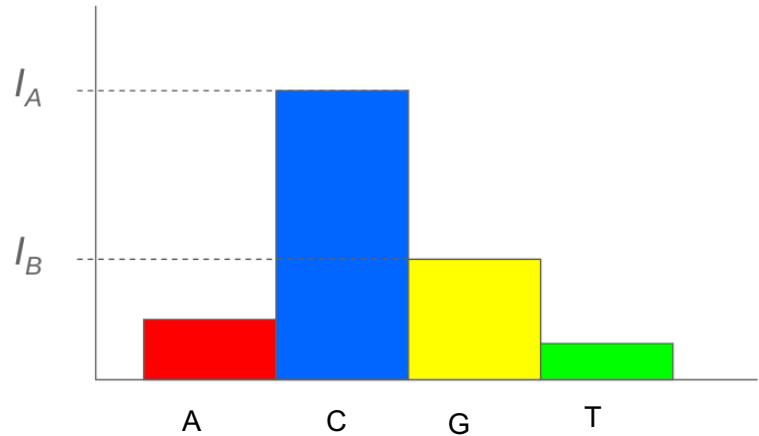
What are paired-end reads and why are they necessary?

Paired-end sequencing is a remarkably simple and powerful modification to the standard sequencing protocol. It is nearly always worth obtaining paired-end reads if performing genomic sequencing. Typically sequencers of any type are only able to sequence a portion of DNA (e.g. 100bp in the case of Illumina) before the fidelity of the enzyme and de-phasing of clusters (see later) increase the error rate beyond tolerable levels. As a result, on the Illumina system, a fragment which is 500bp long will only have the first 100bp sequenced.

If the size selection is tight enough and you know that nearly all the fragments are close to 500bp long, you can repeat the sequencing reaction from the other end of the fragment. This will yield two reads for each DNA fragment separated by a known distance. In the figure below the dashed regions represent the complete DNA fragment and the solid lines the regions we are able to sequence:

Read 1
100bp

Single-end read

Known distance (~300bp)

Read 1
100bp

Read 2
100bp

Paired-end read
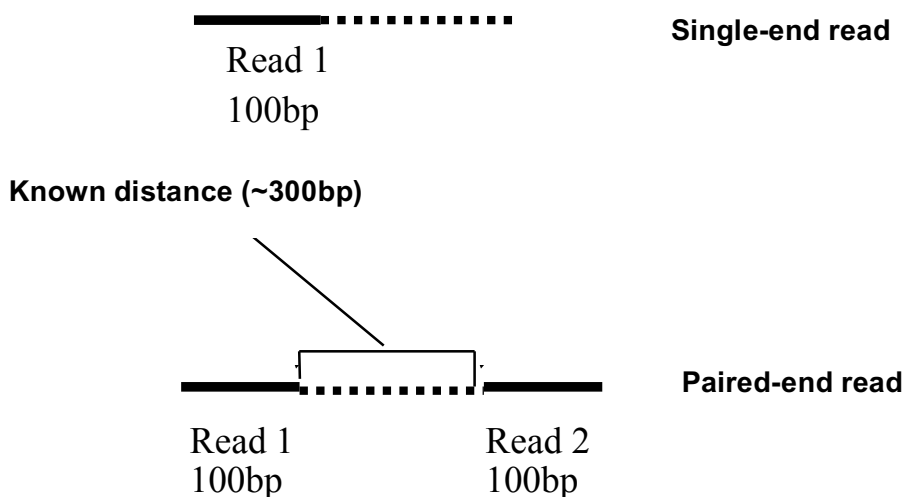
The added information gained by knowing the distance between the two reads can be invaluable for spanning repetitive regions. In the figure below, the light coloured regions indicate repetitive sections of DNA. If a read contains only repetitive DNA, an alignment algorithm will be able to map the read to many locations in a reference genome. However, with paired-end reads, there is a greater chance that

at least one of the two reads will map to a unique region of DNA. In this way one of the reads can be used to anchor the other read in the pair and help resolve the repetitive region. Paired-end reads are often used when performing de-novo genome sequencing (i.e. when a reference is not available to align against) because they enable contiguous regions of DNA to be ordered, or when characterizing variants such as large insertions or deletions.

Other forms of paired-end sequencing with much larger distances (e.g. 10kb) are possible with so called 'mate-pair' libraries. These are usually used in specific projects to help order contigs in de-novo sequencing projects. We will not cover them here, but the principles behind them are similar.
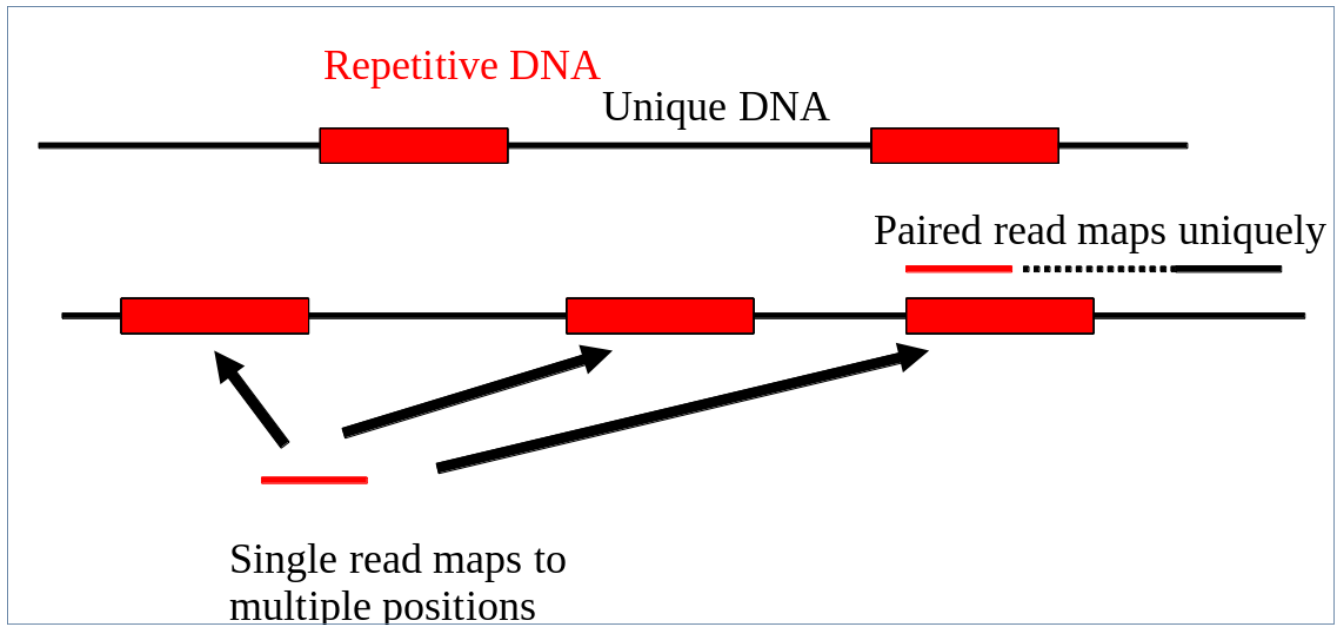
Inherent sources of error

No measurement is without a certain degree of error. This is true in sequencing. As such there is a finite probability that a base will not be called correctly. There are several possible sources:

Frequency cross-talk and normalisation errors:

When reading an A base, a small amount of C will also be measured due to frequency overlap and vice-versa. Similarly with G and T bases. Additionally, from the figure below, it should be clear that the extent to which the dyes fluoresce differs. As such it is necessary to normalize the intensities. This normalisation process can also introduce errors.
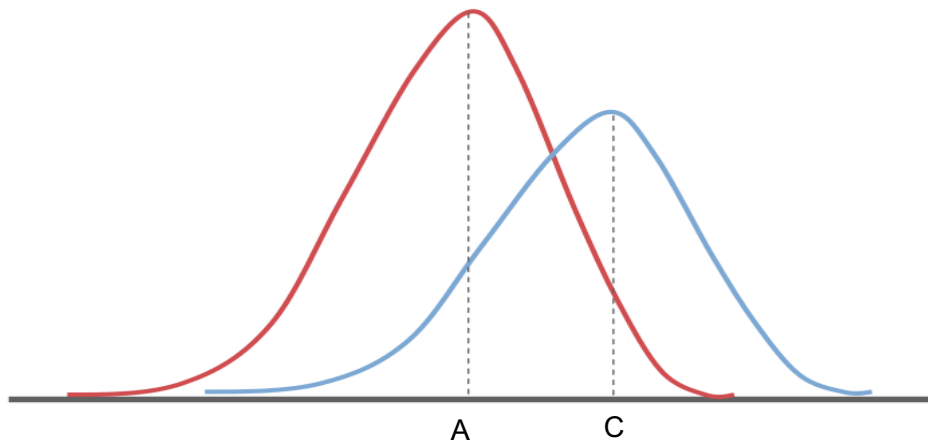
Frequency response curve for A and C dyes
(Intensity y-axis and frequency on the x-axis)

Phasing/Pre-phasing:

This occurs when a strand of DNA lags or leads the other DNA strands within a cluster. This introduces additional background noise into the signal and reduces the intensity of the true base. In the example below we have a cluster with 7 strands of DNA (very small, but this is just an example). Five strands are on a C-base, whilst 1 is lagging behind (called phasing) on a G base and the remaining strand is running ahead of the pack (confusingly called pre-phasing) on an A base. As such the C signal will be reduced and A and G boosted for the rest of the sequencing run. Too much phasing or pre-phasing (i.e. > 15-20%) usually causes problems for the base calling algorithm and result in clusters being filtered out.
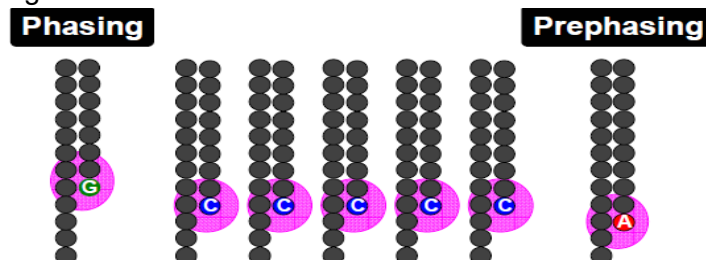
Other issues:
- **Biases introduced by sample preparation** – your sequencing is only as good as your experimental design and DNA extraction. Also, remember that your sample will be put through several cycles of PCR before sequencing. This also introduces a potential source of bias.
- **High AT or GC content sequences** – this reduces the complexity of the sequence and can result in higher error rates.
- **Homopolymeric sequences** – long stretches of a single base can make it difficult to determine phasing and pre-phasing rates. This can introduce errors in determining the precise length of a hompolymeric stretch of sequence. This much more of a problem on the 454 and Ion Torrent than Illumina platforms but still worth bearing in mind. Especially if you encounter indels which have been called in homopolymeric tracts.
- Some motifs can cause loops and other steric clashes

See Nakamura et al, Sequence-specific error profile of Illumina sequencers Nuc. Acid Res. first published online May 16, 2011 doi:10.1093/nar/gkr344

Quality scores

To account for the possible errors and provide an estimate of confidence in a given base-call, the Illumina sequencing pipeline assigns a quality score to each base called. Most quality scores are calculated using the Phred scale. Each base call has an associated base call quality which estimates the chance that the base call is incorrect.

Q10 = 1 in 10 chance of incorrect base call
Q20 = 1 in 100 chance of incorrect base call
Q30 = 1 in 1000 chance of incorrect base call
Q40 = 1 in 10,000 chance of incorrect base call

For most 454, SOLiD and Illumina runs you should see quality scores between Q20 and Q40. Note that these as only estimates of base-quality based on calibration runs performed by the manufacturer against a sample of known sequence with (typically) a GC content of 50%. Extreme GC bias and/or particular motifs or homopolymers can cause the quality scores to become unreliable.

Accurate base qualities are an essential part in ensuring variant calls are correct. As a rough and ready rule we generally assume that with Illumina data anything less than Q20 is not useful data and should be excluded from the analysis.

Reads containing adaptors

Some reads will contain adaptor sequences after sequencing, usually at the end of the read. This is usually because of short sample DNA fragments, which result in the polymerase reading into the adaptor region. Occasionally this can also happen because of mis-priming. It is important to remove or trim sequences containing these reads as the adaptor sequences can prevent reads mapping to a reference sequence and will adversely affect de-novo assembly

Part 2:
Short read genomics: remapping

Introduction

In this section of the workshop we will be analysing a strain of *E.coli* which was sequenced at Exeter. It is closely related to the K-12 substrain MG1655 (http://www.ncbi.nlm.nih.gov/nuccore/U00096). We want to obtain a list of single nucleotide polymorphisms (SNPs), insertions/deletions (Indels) and any genes which have been deleted.

Quality control

In this section of the workshop we will be learning about evaluating the quality of an Illumina MiSeq sequencing run. The process described here can be used with any FASTQ formatted file from any platform (e.g 454, Illumina, Ion Torrent, PacBio etc).

2nd (and 3rd) generation sequencers produce vast quantities of data. A single Illumina MiSeq lane will produce over 10Gbases of data. However, the error rates of these platforms are 10-100x higher than Sanger sequencing. They also have very different error profiles. Unlike Sanger sequencing, where the most reliable sequences tend to be in the middle, NGS platforms tend to be most reliable near the beginning of each read.

Quality control usually involves:
- Calculating the number of reads before quality control
- Calculating GC content, identifying over-represented sequences
- Remove or trim reads containing adaptor sequences
- Remove or trim reads containing low quality bases
- Calculating the number of reads after quality control
- Rechecking GC content, identifying over-represented sequences

Quality control is necessary because:
- CPU time required for alignment and assembly is reduced
- Data storage requirements are reduced
- Reduce potential for bias in variant calling and/or de-novo assembly

Quality scores:

Most quality scores are calculated using the Phred scale (*Ewing B, Green P: Basecalling of automated sequencer traces using phred. II. Error probabilities. Genome Research 8:186-194 (1998)*). Each base call has an associated base call quality which estimates chance that the base call is incorrect.

Q10 = 1 in 10 chance of incorrect base call
Q20 = 1 in 100 chance of incorrect base call

Part 2:   Short read genomics: remapping

Q30 = 1 in 1000 chance of incorrect base call
Q40 = 1 in 10,000 chance of incorrect base call

For most 454, SoLID and Illumina runs you should see quality scores between Q20 and Q40.
Note that these as only estimates of base-quality based on calibration runs performed by the
manufacturer against a sample of known sequence with (typically) a GC content of 50%. Extreme
GC biases and/or particular motifs or homopolymers can cause the quality scores to become
unreliable. Accurate base qualities are an essential part in ensuring variant calls are correct. As a
rough and ready rule we generally assume that with Illumina data anything less than Q20 is not useful

```
@D3P26HQ1:110:d0eh1acxx:8:1101:1116:2122 1:N:0:
AGGTGTCTCCTACAACCAAAGCTACAACAGAGCAATGGGCTATCTGGTGGGATTTAAAGGGGTGAAAATGCATCCCCCTTAAAATNAAAGTGGTTTT
+
ADDADCFHHHDHGHIII<GIICH4FGCIHIEGFHGHGIIIGDHFDFG?DEHH>FGIG=E@GGADDDCCCCC@A>ABB>BBC:A>A#,228(4>:??B
```
data and should be excluded.

Once you understand the FASTQ format try to work out what is happing to the quality scores here and
why:

FASTQ format:

A FASTQ entry consists of 4 lines

1.   A header line beginning with '@' containing information about the name of the sequencer, and
the position at which the originating cluster was located and whether it passed purity filters.
2.   The DNA sequence of the read
3.   A header line or line beginning with just '+'
4.   Quality scores for each base encoded in ASCII format

Typical FASTQ formatted file:

To reduce storage requirements, the FASTQ quality scores are stored as single characters and
converted to numbers by obtaining the ASCII quality score and subtracting either 33 or 64. For
example, the above FASTQ file is Sanger formatted and the character '!' has an ASCII value of 33.
Therefore the corresponding base would have a Phred quality score of 33-33=Q0 (i.e. totally
unreliable). On the other hand a base with a quality score denoted by '@' which has an ASCII value
of 64 would have a Phred quality score of 64-33=Q31 (i.e. less than 1/1000 chance of being incorrect).

Part 2:   *Short read genomics: remapping*

Just to confuse matters, there are several different methods of encoding quality scores in the ASCII format.

```
SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS....................................
...........

........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX............
...........
  ...............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII..................
  ............................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
  LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL................................................
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                    |    |         |                                    |                    |
 33                   59   64        73                                  104                  126
  0.......................26...31.......40
                   -5....0........9............................40
                        0.......9............................40
                         3.....9............................40
  0.2.....................26...31.........41


 S - Sanger        Phred+33,  raw reads typically (0, 40)
 X - Solexa        Solexa+64, raw reads typically (-5, 40)
 I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
 J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
     with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
     (Note: See discussion above).
 L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

Note that the latest Illumina CASAVA 1.8 pipeline (released June 2011), outputs in fastq-sanger rather than Illumina 1.3+. Thus Illumina 1.3+ and other Illumina scoring metrics are unlikely to be encountered if you are using Illumina sequencing data generated after July 2011.
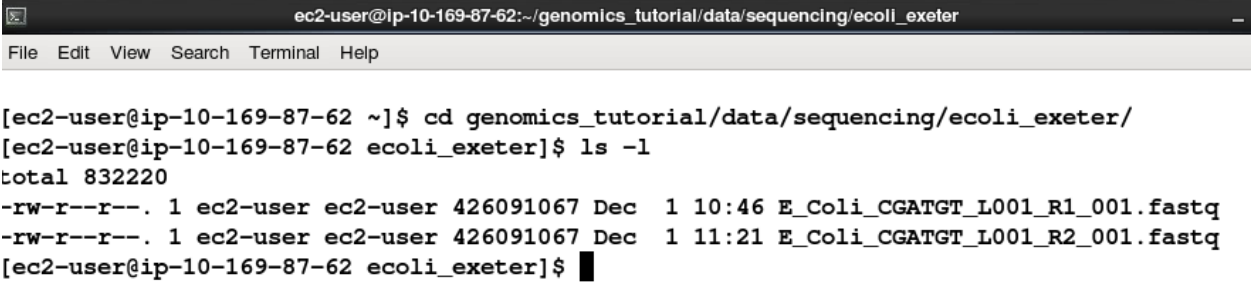
Quality control – evaluating the quality of Illumina data

The first task when one receives sequencing data is to evaluate its quality and determine whether all the cash you have handed over was well-spent! To do this we will use the FastQC toolkit (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). FastQC offers a graphical visualisation of QC metrics, but *does not* have the ability to filter data.

Task 1:

From your home directory change into the
workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/ directory and list the directory
contents. E.g.:

> cd ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/
> ls -l

```
ec2-user@ip-10-169-87-62:~/genomics_tutorial/data/sequencing/ecoli_exeter
File  Edit  View  Search  Terminal  Help

[ec2-user@ip-10-169-87-62 ~]$ cd genomics_tutorial/data/sequencing/ecoli_exeter/
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ ls -l
total 832220
-rw-r--r--. 1 ec2-user ec2-user 426091067 Dec  1 10:46 E_Coli_CGATGT_L001_R1_001.fastq
-rw-r--r--. 1 ec2-user ec2-user 426091067 Dec  1 11:21 E_Coli_CGATGT_L001_R2_001.fastq
[ec2-user@ip-10-169-87-62 ecoli_exeter]$
```
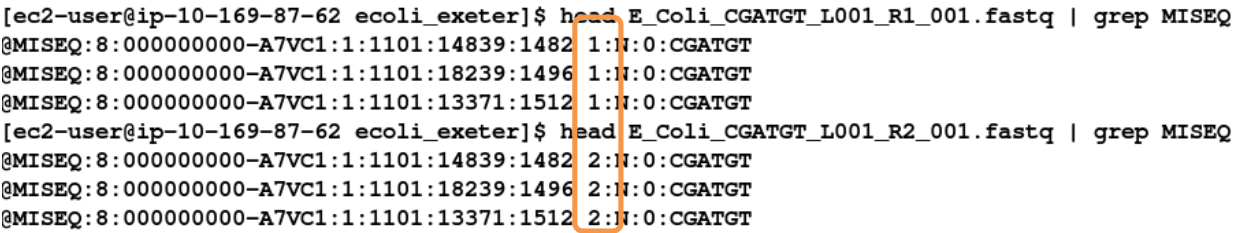
Note that this is a paired-end run. As such there are two files

- one for read 1 (E_Coli_CGATGT_L001_R1_001.fastq)

- and the other for the reverse read 2 (E_Coli_CGATGT_L001_R2_001.fastq)

Reads from the same pair can be identified because they have the same header. Many programs
require that the read 1 and read 2 files have the reads in the same order. To view the first few headers
we can use the head and grep commands:

> head E_Coli_CGATGT_L001_R1_001.fastq | grep MISEQ
> head E_Coli_CGATGT_L001_R2_001.fastq | grep MISEQ

```
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ head E_Coli_CGATGT_L001_R1_001.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:1101:14839:1482 1:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:18239:1496 1:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:13371:1512 1:N:0:CGATGT
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ head E_Coli_CGATGT_L001_R2_001.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:1101:14839:1482 2:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:18239:1496 2:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:13371:1512 2:N:0:CGATGT
```

The only difference in the headers for the two reads is the read number. Of course this is no
guarantee that all the headers in the file are consistent. To get some more confidence repeat the
above commands using 'tail' instead of 'head' to compare reads at the end of the files.

You can also check that there is an identical number of reads in each file using cat, grep and wc –l:

> cat E_Coli_CGATGT_L001_R1_001.fastq | grep MISEQ | wc –l
> cat E_Coli_CGATGT_L001_R2_001.fastq | grep MISEQ | wc –l

Part 2:   Task 1:

Now, let's start the fastqc program.

```
fastqc
```

Load the E_Coli_CGATGT_L001_R1_001.fastq file from the
workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter directory.
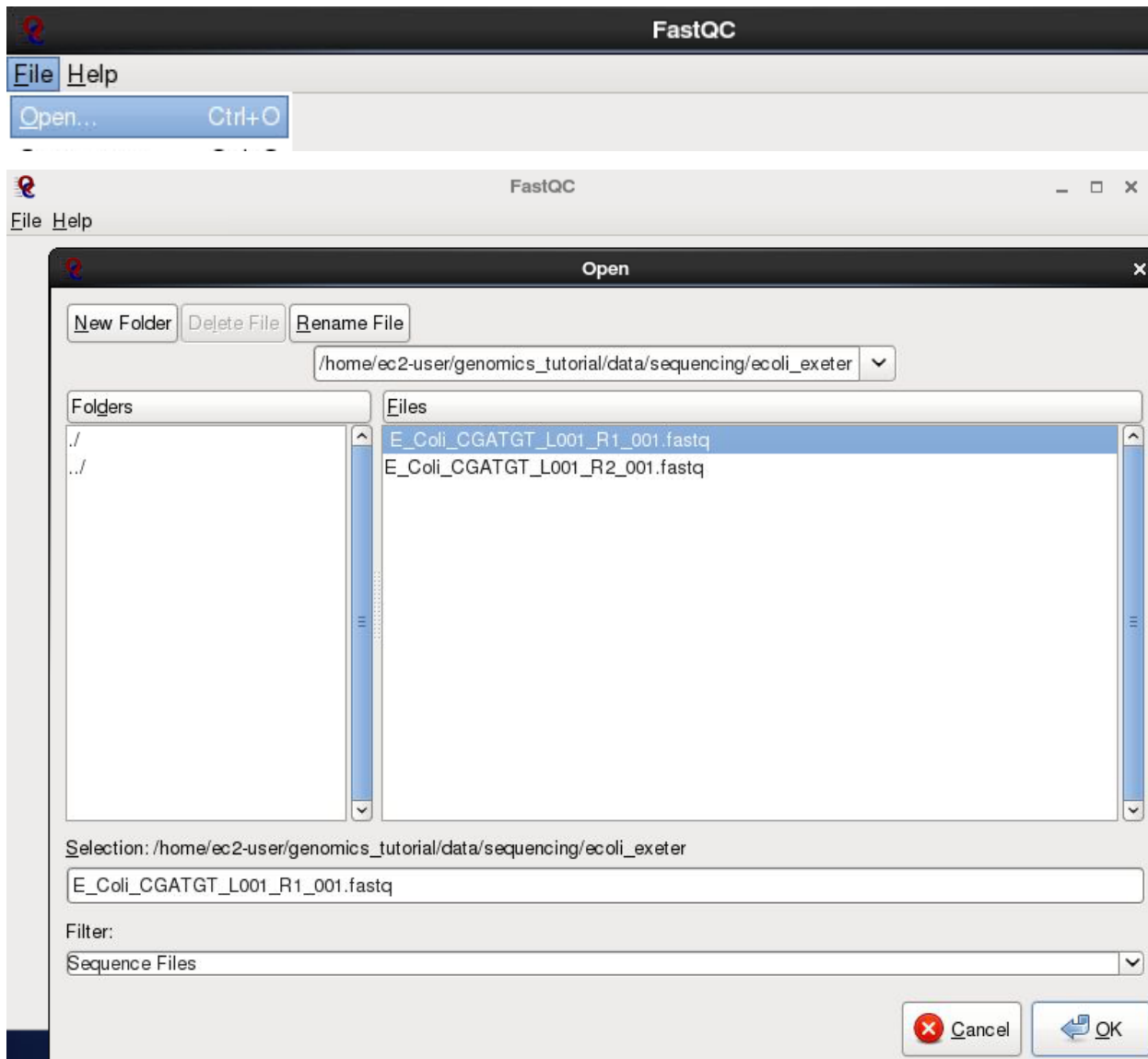
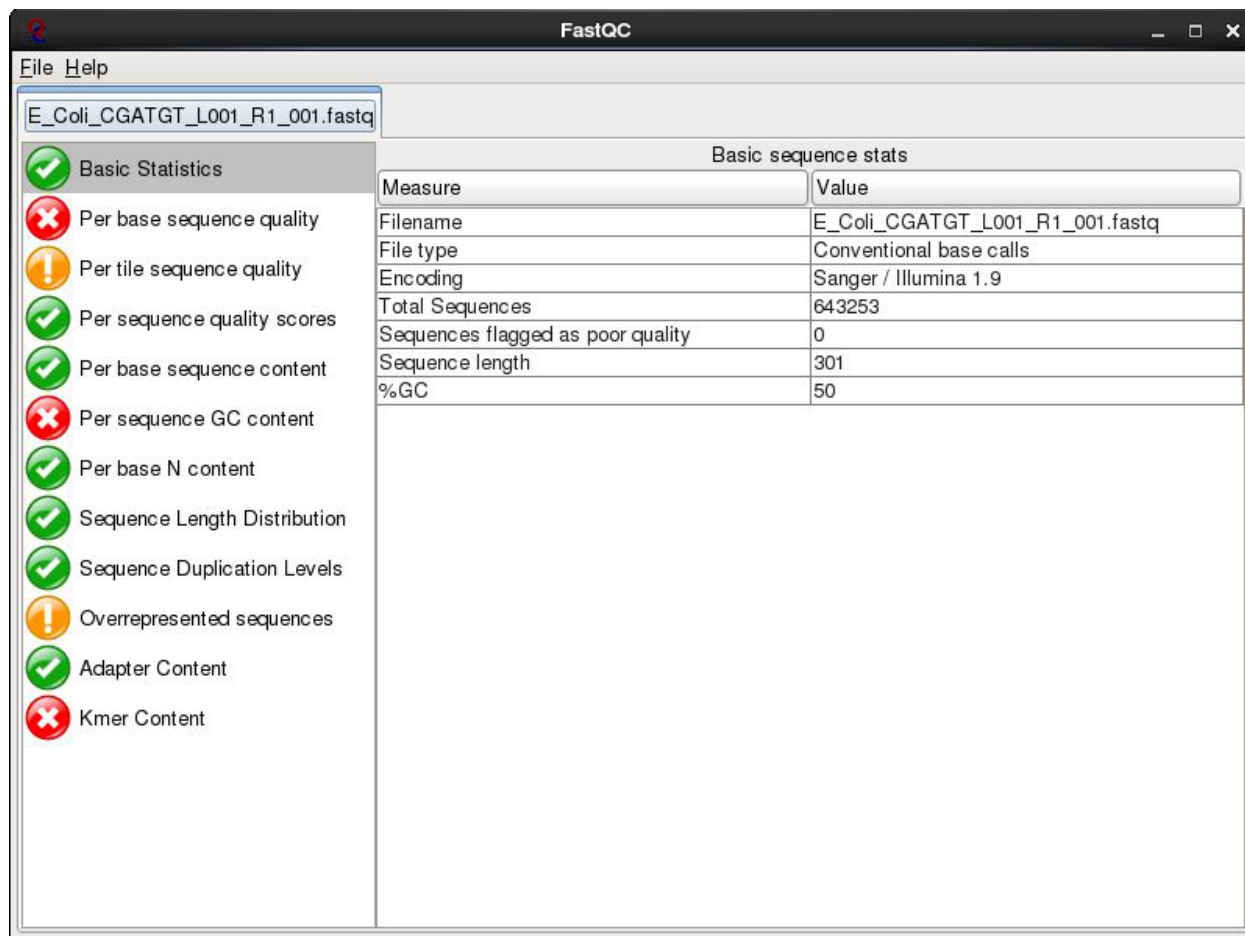
After a few minutes the program should finish analysing the FASTQ file.

While this is running, please start the filtering step described in <u>*Task 3*</u> *as this takes about 20 minutes - when it is running return here and complete these steps*

The fastqc program performs a number of tests which determines whether a green tick (pass), exclamation mark (warning) or red cross (fail) is displayed. However it is important to realise that fastqc has no knowledge of what your library is or should look like. All of its tests are based on a completely random library with 50% GC content. Therefore if you have a sample which does not match these assumptions, it may 'fail' the library. For example, if you have a high AT or high GC organism it may fail the per sequence GC content. If you have any barcodes or low complexity libraries (e.g. small RNA libraries) they may also fail some of the sequence complexity tests.

The bottom line is that you need to be aware of what your library is and whether what fastqc is reporting makes sense for that type of library.

FastQC

File Help

E_Coli_CGATGT_L001_R1_001.fastq

Basic Statistics

❌ Per base sequence quality

⚠️ Per tile sequence quality

✅ Per sequence quality scores

✅ Per base sequence content

❌ Per sequence GC content

✅ Per base N content

✅ Sequence Length Distribution

✅ Sequence Duplication Levels

⚠️ Overrepresented sequences

✅ Adapter Content

❌ Kmer Content

Basic sequence stats

Measure	Value
Filename	E_Coli_CGATGT_L001_R1_001.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	643253
Sequences flagged as poor quality	0
Sequence length	301
%GC	50

In this case we have a number of errors and warnings which at first sight suggest there has been a problem - but don't worry too much yet. Let's go through them in turn.

Part 2:   Task 1:

Quality scores:

This is one of the most important metrics. If the quality scores are poor, either the wrong FASTQ encoding has been guessed by fastqc (see the title of the chart), or the data itself is poor quality. This view shows an overview of the range of quality values across all bases at each position in the FASTQ file. Generally anything with a median quality score greater than Q20 is regarded as acceptable; anything above Q30 is regarded as 'good'. For more details, see the help documentation in fastqc.
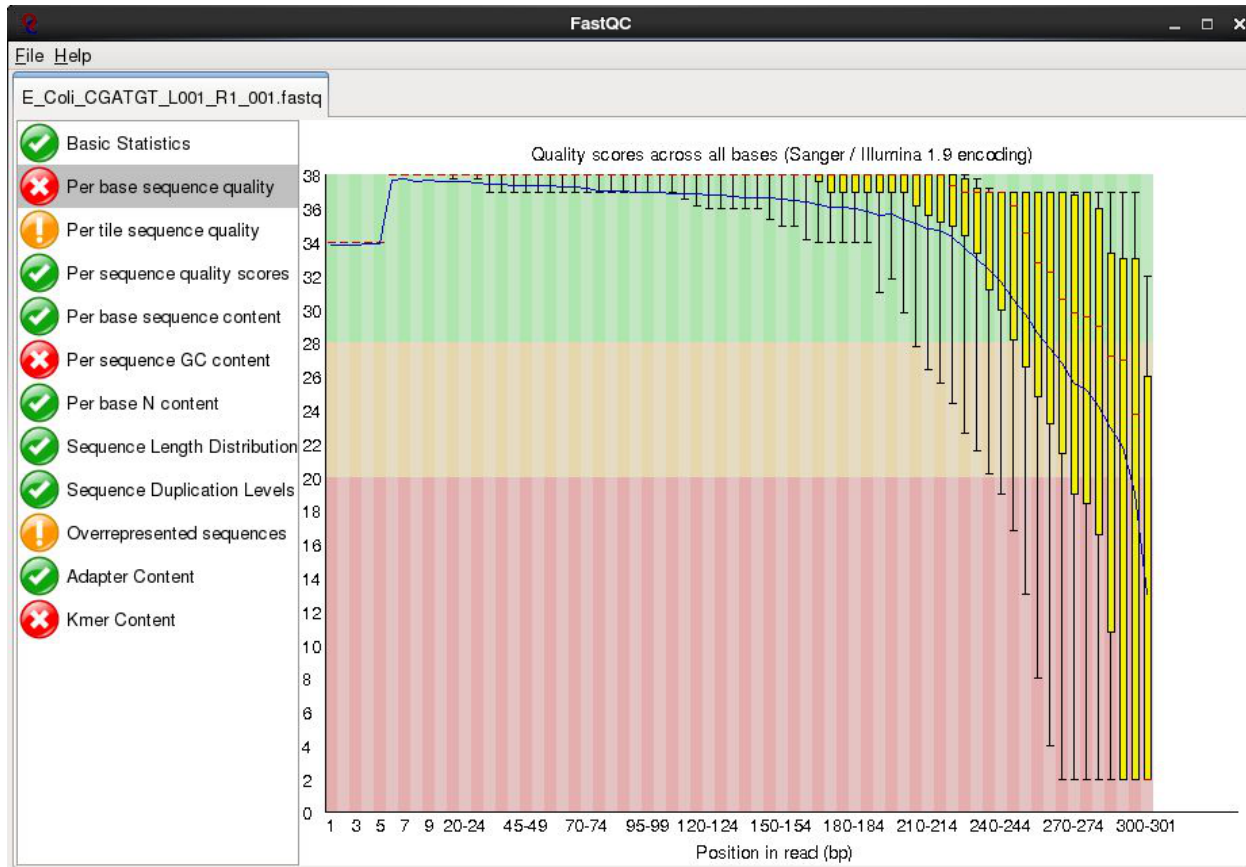
In this case this check is red - and it is true that the quality drops off at the end of the reads. It is normal for read quality to get worse towards the end of the read. You can see that at 250 bases the quality is still very good, we will later trim off the low quality bases so reserve judgment for now..

Part 2:   Task 1:

Per tile sequence quality

This is a purely technical view on the sequencing run, it is more important for the team running the sequencer. The sequencing flowcell is divided up into areas called cells. You can see that the read quality drops off in some cells faster than others. This maybe because of the way the sample flowed over the flowcell or a mark or smear on the lens of the optics.
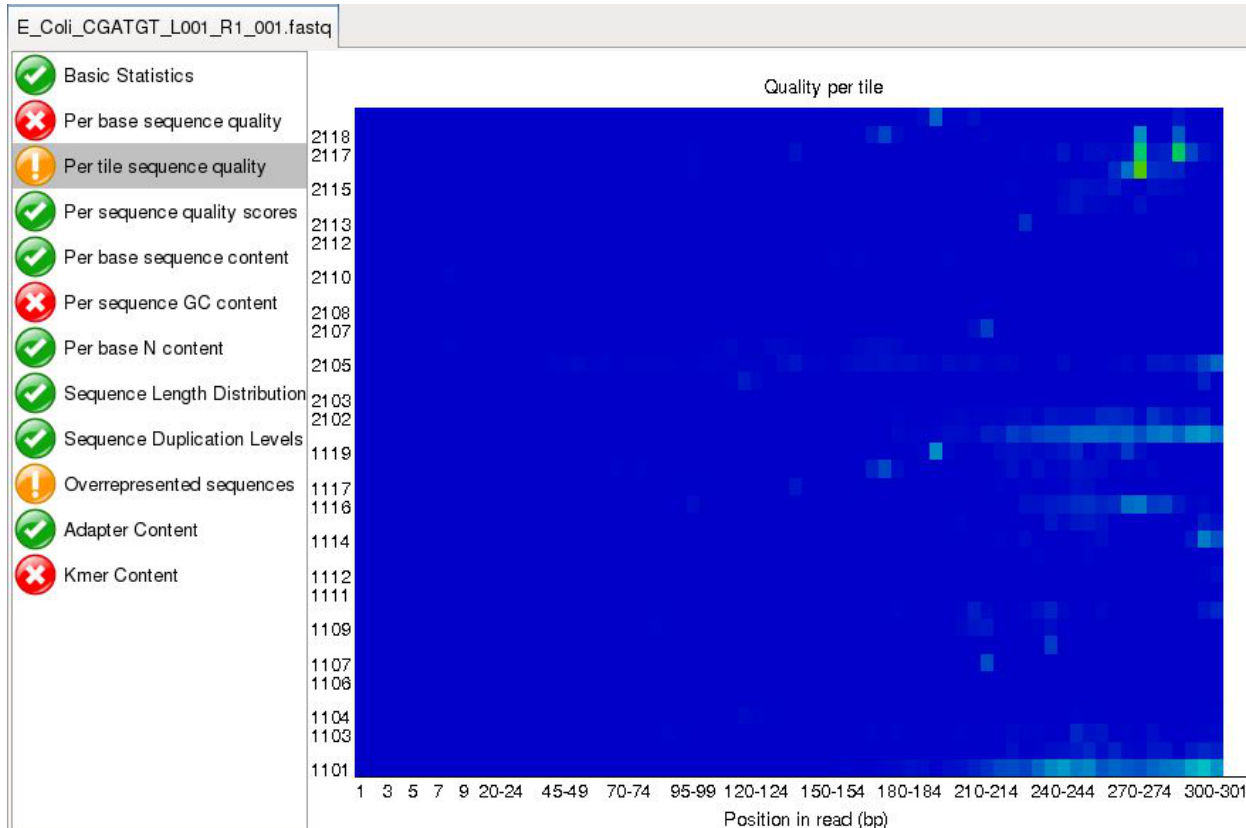
Per base sequence content:

For a completely randomly generated library with a GC content of 50% one expects that at any given position within a read there will be a 25% chance of finding an A,C,T or G base. Here we can see that our library satisfies these criteria, although there appears to be some minor bias at the beginning of the read. This may be due to PCR duplicates during amplification or during library preparation. It is unlikely that one will ever see a perfectly uniform distribution. See *http://biosciences.exeter.ac.uk/facilities/sequencing/dataguide/qualitycontrol/* for examples of good vs bad runs as well as the fastqc help for more details.
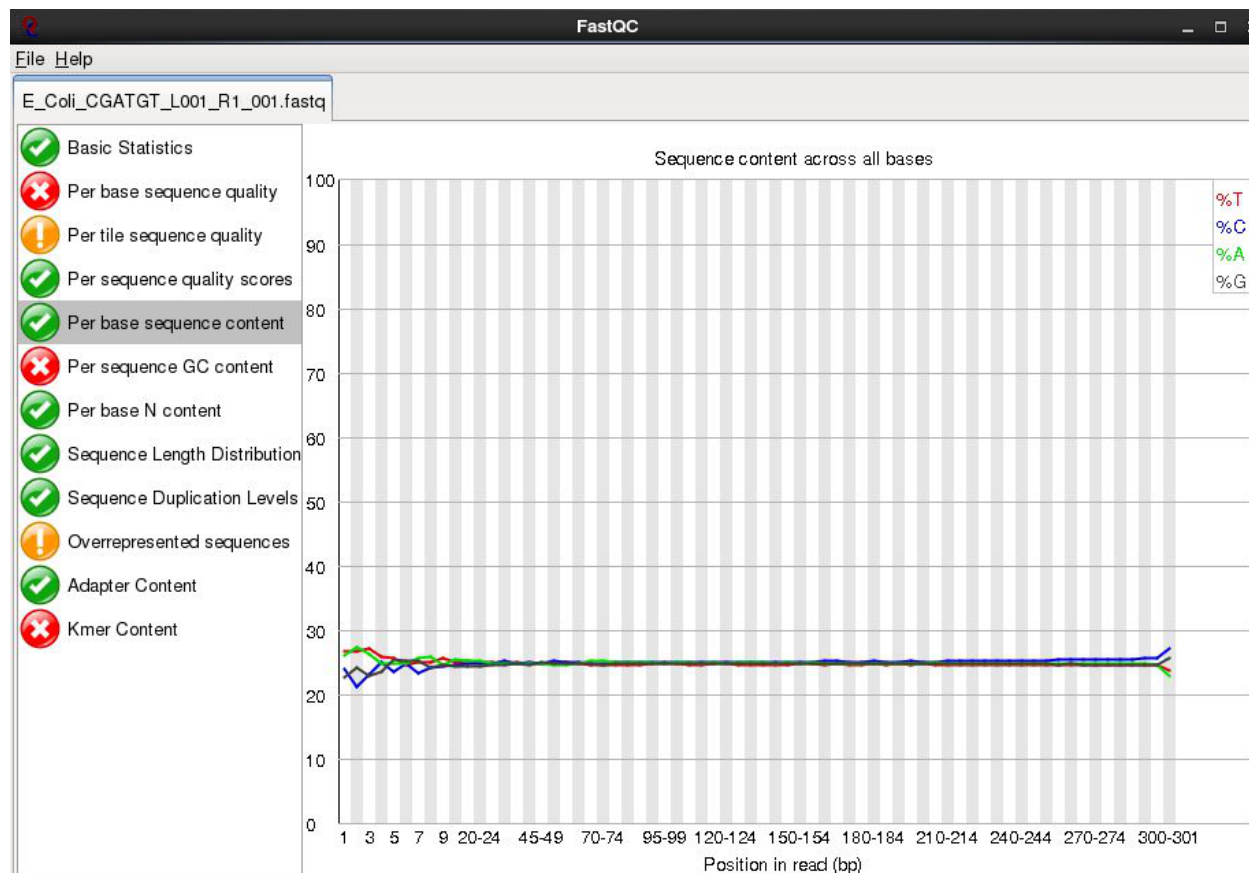
Sequence duplication levels:

In a library that covers a whole genome uniformly most sequences will occur only once in the final set. A low level of duplication may indicate a very high level of coverage of the target sequence, but a high level of duplication is more likely to indicate some kind of enrichment bias (e.g. PCR over-amplification).

This module counts the degree of duplication for every sequence in the set and creates a plot showing the relative number of sequences with different degrees of duplication.
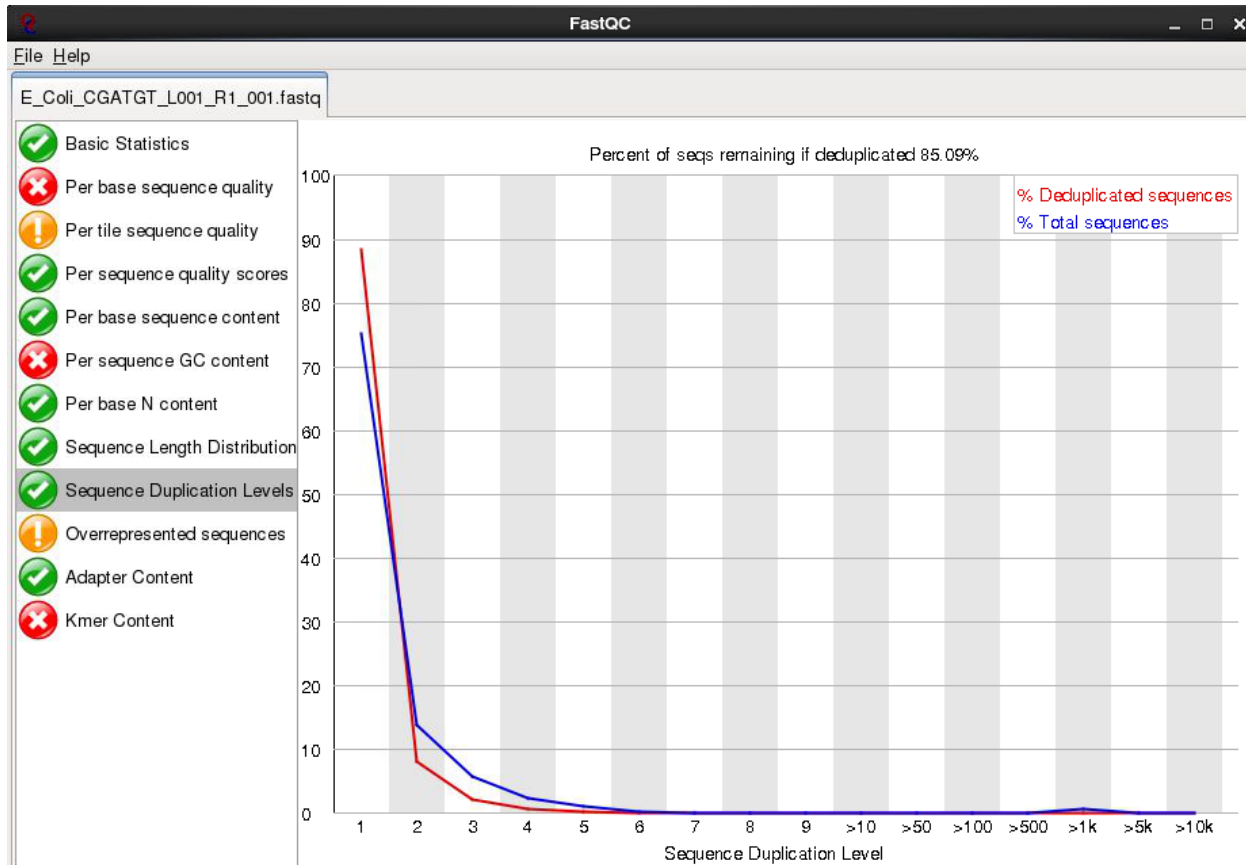
Overrepresented Sequences

This check for sequences that occur more frequently than expected in your data. It also checks any sequences it finds against a small database of known sequences. In this case it has found that a small number of reads 4000 out of 600000 appear to contain a sequence used in the preparation for the library. A typical cause is that the original DNA was shorter than the length of the read - so the sequencing overruns the actual DNA and runs in to the adaptors used to bind it to the flowcell.

At this level there is nothing to worry about - they will be trimmed in later stages.
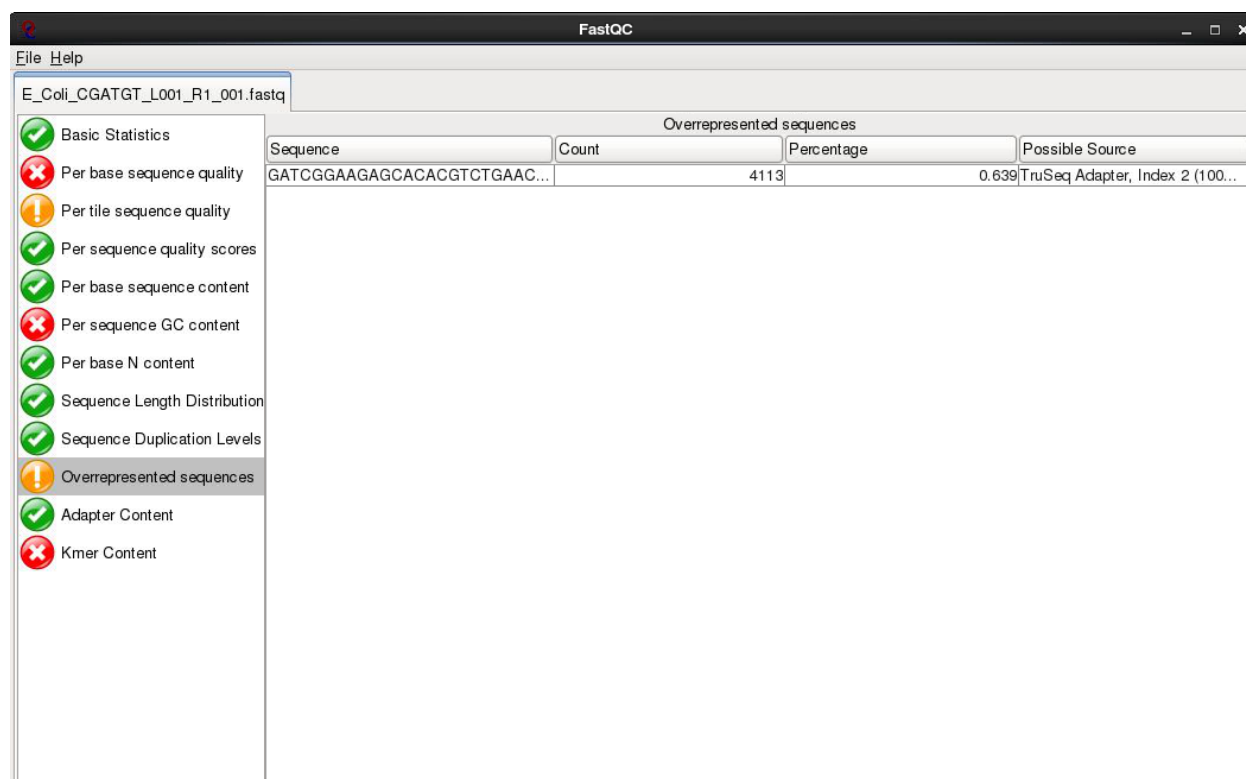
There are other reports available:

Have a look at them and at what the author of FastQC has to say.

http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/

Remember the error and warning flags are his (albeit experienced) judgement of what typical data should look like. It is up to you to use some initiative and understand whether what you are seeing is typical for your dataset and how that might affect any analysis you are performing.

Task 2

Do the same for read 2 as we have for read 1. Open fastqc and analyse the read 2 file. Look at the various plots and metrics which are generated. How similar are they?

Note that the number of reads reported in both files is identical. This is because if one read fails to pass the Illumina chastity filter, its partner is automatically excluded too.

Overall, both read 1 and read 2 can be regarded as 'good' data-sets.

Quality control – filtering of Illumina data

In this section we will be filtering the data to ensure any low quality reads are removed and that any sequences containing adaptor sequences are either trimmed or removed altogether. To do this we will use the fastq-mcf program from the ea-utils package (available at http://code.google.com/p/ea-utils/). This package is remarkably fast and ensures that after filtering both read 1 and read 2 files are in the correct order.

Note: Typically when submitting raw Illumina data to NCBI or EBI you would submit unfiltered data, so don't delete your original fastq files!

A note on checking for contaminants:

A number of tools are available now which also enable to you to quickly search reads and assign them to particular species or taxonomic groups. These can serve as a quick check to make sure your samples or libraries are not contaminated with DNA from other sources. If you are performing a de-novo assembly for example and have unwittingly have DNA sequence present from multiple organisms, you will risk poor results and chimeric contigs.

Some 'contaminants' can turn out to be inevitable by-products of sampling and DNA extraction. This is often the case with algae or other symbionts. In addition some groups have made some amazing discoveries such as the discovery of a third symbiont (which turned out to be a yeast) in lichen. http://science.sciencemag.org/content/353/6298/488.full

Some tools you can use to check the taxonomic classification of reads include:

- Kraken
- Centrifuge
- Blobology
- Blast (in conjunction with sub-sampling your reads) and Krona to plot results

We won't do this now but will do an example in the final section of the workshop on long reads.

Task 3

Make sure you are in the ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/ directory. We will execute the fastq-mcf program which performs both adaptor sequence trimming and low quality bases. To remove adaptor sequences, we need to supply the adaptor sequences to the program. A list of the most common adaptors used is given in the file

~/workshop_materials/genomics_tutorial/data/reference/adaptors/adaptors.fasta :

View it by typing:

> more ~/workshop_materials/genomics_tutorial/data/reference/adaptors/adaptors.fasta

```
genomics@genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter] more ~/workshop_data/genomics_tutorial/data/reference/adaptors/adaptors.fasta
>Nextera_enrichment
CTGTCTCTTATACACATCT
>TruSeq_Read1
AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
>TruSeq_Read2
AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
>Nextera_mate_pair_Read1
CTGTCTCTTATACACATCT
>Nextera_mate_pair_Read2
AGATGTGTATAAGAGACAG
>PolyA
AAAAAAAAAAAAAAAAAAAAAAAAAA
```

To run the fastq-mcf program, type the following (all on one line):

fastq-mcf ../../reference/adaptors/adaptors.fasta E_Coli_CGATGT_L001_R1_001.fastq E_Coli_CGATGT_L001_R2_001.fastq -o E_Coli_CGATGT_L001_R1_001.filtered.fastq -o E_Coli_CGATGT_L001_R2_001.filtered.fastq -C 1000000 -q 20 -p 10 -u -x 0.01

While this is running enter the command fastq-mcf in another terminal and try to understand what all the options do. We have found that these parameters generally work well for Illumina data.

If you would like to learn more about these options, you can look at the manual here https://code.google.com/p/ea-utils/wiki/FastqMcf . In short we are using 1 million reads to form a model of the sequence quality and then applying the filters which remove bases with q-score less than 20, trims adaptors allowing up to 10% mismatch in the adaptor sequence, allowing only pass-filter reads (virtually all sequencing data is pass-filter these days, so this is just included to be safe) and trims back reads which contain more than 1% Ns until they contain 1% or less Ns.

In case you jumped here from the Task 1, you can go back now. FastQC should be done by now.

Part 2:   *Task 3*

After a few minutes the filtering should be complete and you should see something similar to:

```
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ fastq-mcf ../../reference/adaptors/adaptors.fasta E_Coli_CGATGT_L001_R1_0
01.fastq E_Coli_CGATGT_L001_R2_001.fastq -o E_Coli_CGATGT_L001_R1_001.filtered.fastq -o E_Coli_CGATGT_L001_R2_001.
filtered.fastq -C 1000000 -q 20 -p 10 -u -x 0.01
Command Line: ../../reference/adaptors/adaptors.fasta E_Coli_CGATGT_L001_R1_001.fastq E_Coli_CGATGT_L001_R2_001.fa
stq -o E_Coli_CGATGT_L001_R1_001.filtered.fastq -o E_Coli_CGATGT_L001_R2_001.filtered.fastq -C 1000000 -q 20 -p 10
 -u -x 0.01
Scale used: 2.2
Filtering Illumina reads on purity field
Phred: 33
Threshold used: 1609 out of 643253
Adapter TruSeq_Read1 (AGATCGGAAGAGCACACGTCTGAACTCCAGTCA): counted 8548 at the 'end' of 'E_Coli_CGATGT_L001_R1_001.
fastq', clip set to 5
Adapter TruSeq_Read2 (AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT): counted 6204 at the 'end' of 'E_Coli_CGATGT_L001_R2_001.
fastq', clip set to 5
Adapter Short Nextera fragment of adaptor (TCGGAAGAGCACACGT): counted 12634 at the 'end' of 'E_Coli_CGATGT_L001_R1
_001.fastq', clip set to 4
Adapter Nextera_read_1_external_adapter (ATCGGAAGAGCACACGTCTGAACTCCAGTCAC): counted 12786 at the 'end' of 'E_Coli_
CGATGT_L001_R1_001.fastq', clip set to 4
Adapter Nextera_read_2_external_adapter (GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT): counted 6254 at the 'end' of 'E_Coli_C
GATGT_L001_R2_001.fastq', clip set to 5

Too short after clip: 8895
Clipped 'end' reads (E_Coli_CGATGT_L001_R1_001.fastq): Count 18042, Mean: 34.11, Sd: 40.08
Trimmed 505652 reads (E_Coli_CGATGT_L001_R1_001.fastq) by an average of 16.89 bases on quality < 20
Clipped 'end' reads (E_Coli_CGATGT_L001_R2_001.fastq): Count 9426, Mean: 52.99, Sd: 40.18
Trimmed 621151 reads (E_Coli_CGATGT_L001_R2_001.fastq) by an average of 60.69 bases on quality < 20
```

You can see that the trimming has been harsher on the R2 reads than on the R1 - this is generally to be expected in Illumina paired end runs.

If we look at the sizes of the files produced:

> `ls -l`

```
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ ls -l
total 1568644
-rw-r--r--. 1 ec2-user ec2-user 426091067 Dec  1 10:46 E_Coli_CGATGT_L001_R1_001.fastq
-rw-rw-r--. 1 ec2-user ec2-user 405632367 Dec  1 13:33 E_Coli_CGATGT_L001_R1_001.filtered.fastq
-rw-r--r--. 1 ec2-user ec2-user 426091067 Dec  1 11:21 E_Coli_CGATGT_L001_R2_001.fastq
-rw-rw-r--. 1 ec2-user ec2-user 348453609 Dec  1 13:33 E_Coli_CGATGT_L001_R2_001.filtered.fastq
```

You can see that the original files are exactly the same size, but the R2 filtered file is smaller than R1. Now count the lines in all the files

> `wc -l *.filtered.fastq`

```
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ wc -l *.filtered.fastq
  2537432 E_Coli_CGATGT_L001_R1_001.filtered.fastq
  2537432 E_Coli_CGATGT_L001_R2_001.filtered.fastq
```

Although the reads have been trimmed differently - the number of reads in the R1 and R2 files are identical. This is required for all the tools we will use to analyse paired end data.

Task 4

Check the quality scores and sequence distribution in the fastqc program for the two filtered fastq files. You should notice very little change (since comparatively few reads were filtered).

However, you should notice a significant improvement in quality and the absence of adaptor sequences.

Task 5

We can perform a quick check (although this by no means guarantees) that the sequences in read 1 and read 2 are in the same order by checking the ends of the two files and making sure that the headers are the same.

head E_Coli_CGATGT_L001_R1_001.filtered.fastq | grep MISEQ
head E_Coli_CGATGT_L001_R2_001.filtered.fastq | grep MISEQ
tail E_Coli_CGATGT_L001_R1_001.filtered.fastq | grep MISEQ
tail E_Coli_CGATGT_L001_R2_001.filtered.fastq | grep MISEQ

```
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ head E_Coli_CGATGT_L001_R1_001.filtered.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:1101:17200:1633 1:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:10456:1673 1:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:16582:1688 1:N:0:CGATGT
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ head E_Coli_CGATGT_L001_R2_001.filtered.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:1101:17200:1633 2:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:10456:1673 2:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:1101:16582:1688 2:N:0:CGATGT
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ tail E_Coli_CGATGT_L001_R1_001.filtered.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:2119:19669:25236 1:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:2119:10145:25237 1:N:0:CGATGT
[ec2-user@ip-10-169-87-62 ecoli_exeter]$ tail E_Coli_CGATGT_L001_R2_001.filtered.fastq | grep MISEQ
@MISEQ:8:000000000-A7VC1:1:2119:19669:25236 2:N:0:CGATGT
@MISEQ:8:000000000-A7VC1:1:2119:10145:25237 2:N:0:CGATGT
```

Task 6

Check the number of reads in each filtered file. They should be the same. To do this use the grep command to search for the number of times the header appears. E.g:

grep -c MISEQ E_Coli_CGATGT_L001_R1_001.filtered.fastq

Do the same for the E_Coli_CGATGT_L001_R2_001.filtered.fastq file.

Aligning Illumina data to a reference sequence

Now that we have checked the quality of our raw data, we can begin to align the reads against a reference sequence. In this way we can compare how the reference sequence and the strain we have sequenced compare.

To do this we will be using a program called BWA (Burrows Wheeler Aligner *Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 25:1754-60.*). This uses an algorithm called (unsurprisingly) Burrows Wheeler to rapidly map reads to the reference genome. BWA also allows for a certain number of mismatches to account for variants which may be present in strain 1 vs the reference genome. Unlike other alignment packages such as Bowtie (version 1) BWA allows for insertions or deletions as well.

By mapping reads against a reference, what we mean is that we want to go from a FASTQ file listing lots of reads, to another type of file (which we'll describe later) which lists the reads AND where/if it maps against the reference genome. The figure below illustrates what we are trying to achieve here. Along the top in grey is the reference sequence. The coloured sequences below indicate individual sequences and how they map to the reference. If there is a real variant in a bacterial genome we would expect that (nearly) all the reads would contain the variant at the relevant position rather than the same base as the reference genome. Remember that error rates for any single read on second generation platforms tend to be around 0.5-1%. Therefore a 300bp read is on average likely to contain at 2-3 errors.

Let's look at 2 potential sources of artefacts.

1.   Sequencing error:

The region highlighted in green on the right shows that most reads agree with the reference sequence (i.e. C-base). However, 2 reads near the bottom show an A-base. In this situation we can safely assume that the A-bases are due to a sequencing error rather than a genuine variant since the 'variant' has only one read supporting it. If this occurred at a higher frequency however, we would struggle to determine whether it was a genuine variant or an error.

2.   PCR duplication:

The highlighted region red on the left shows where there appears to be a variant. A C-base is present in the reference and half the reads, whilst an A-base is present in a set of reads which all start at the same position.

Is this a genuine difference or a sequencing or sample prep error? What do you think? If this was a real sample, would you expect all the reads containing an A to start at the same location?

The answer is probably not. This 'SNP' is in fact probably an artefact of PCR duplication. I.e. the same fragment of DNA has been replicated many times more than the average and happens to contain an error at the first position. We can filter out such reads during after alignment to the reference (see later).

Note that the entire region above seems to contain lots of PCR duplicates with reads starting at the same location. In the case of the region highlighted in red, this will likely cause a false SNP call. The area in green also contains PCR duplicates – the As at these positions are probably either sequencing errors or errors introduced during PCR.

It's always important to think critically about any finding - don't assume that whatever bioinformatic tools you are using are perfect. Or that you have used them perfectly.

Indexing a reference genome:

Before we can start aligning reads to a reference genome, the genome sequence needs to be indexed. This means sorting the genome into easily searched chunks.

Task 7: Generating an index file from the reference sequence

Change directory to the reference directory:

cd ~/workshop_materals/genomics_tutorial/data/reference/U00096/

List the files:

ls -l

```
[ec2-user@ip-10-169-87-62 U00096]$ ls -l
total 6760
-rw-r--r--. 1 ec2-user ec2-user 4708048 Dec  1 10:43 U00096.fna
-rw-r--r--. 1 ec2-user ec2-user 2208485 Dec  1 10:43 U00096.gff
```

In this directory we have 2 files. U00096.fna is a FASTA file which contains the reference genome sequence. The U00096.gff file contains the annotation for this genome. We will use this later.

First, let's looks at the bwa command itself. Type:

bwa

This should yield something like:

```
Program: bwa (alignment via Burrows-Wheeler transformation)
Version: 0.7.15-r1142-dirty
Contact: Heng Li <lh3@sanger.ac.uk>

Usage:   bwa <command> [options]

Command: index         index sequences in the FASTA format
         mem           BWA-MEM algorithm
         fastmap       identify super-maximal exact matches
         pemerge       merge overlapping paired ends (EXPERIMENTAL)
         aln           gapped/ungapped alignment
         samse         generate alignment (single ended)
         sampe         generate alignment (paired ended)
         bwasw         BWA-SW for long queries

         shm           manage indices in shared memory
         fa2pac        convert FASTA to PAC format
         pac2bwt       generate BWT from PAC
         pac2bwtgen    alternative algorithm for generating BWT
         bwtupdate     update .bwt to the new format
         bwt2sa        generate SA from BWT and Occ

Note: To use BWA, you need to first index the genome with `bwa index'.
      There are three alignment algorithms in BWA: `mem', `bwasw', and
      `aln/samse/sampe'. If you are not sure which to use, try `bwa mem'
      first. Please `man ./bwa.1' for the manual.
```

BWA is actually a suite of programs which all perform different functions. We are only going to use two during this workshop, bwa index, bwa mem

If we type:

bwa index

Part 2:   Task 7: Generating an index file from the reference sequence

We can see more options for the bwa index command:

```
Usage:    bwa index [options] <in.fasta>

Options: -a STR    BWT construction algorithm: bwtsw, is or rb2 [auto]
         -p STR    prefix of the index [same as fasta name]
         -b INT    block size for the bwtsw algorithm (effective with -a bwtsw) [10000000]
         -6        index files named as <in.fasta>.64.* instead of <in.fasta>.*

Warning: `-a bwtsw' does not work for short genomes, while `-a is' and
         `-a div' do not work not for long genomes.
```

By default bwa index will use the IS algorithm to produce the index. This works well for most genomes, but for very large ones (e.g. vertebrate) you may need to use bwtsw. For bacterial genomes the default algorithm will work fine.

Now we will create a reference index for the genome using BWA:

```
bwa index U00096.fna
```

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/master_data/reference/U00096$ bwa index U00096.fna
[bwa_index] Pack FASTA... 0.04 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 0.83 seconds elapse.
[bwa_index] Update BWT... 0.02 sec
[bwa_index] Pack forward-only FASTA... 0.02 sec
[bwa_index] Construct SA from BWT and Occ... 0.44 sec
[main] Version: 0.7.15-r1142-dirty
[main] CMD: bwa index U00096.fna
[main] Real time: 1.720 sec; CPU: 1.352 sec
```

If you now list the directory contents using the 'ls' command, you will notice that the BWA index program has created a set of new files. These are the index files BWA needs.

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/master_data/reference/U00096$ ls
U00096.fna  U00096.fna.amb  U00096.fna.ann  U00096.fna.bwt  U00096.fna.pac  U00096.fna.sa  U00096.gff
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/master_data/reference/U00096$ 
```

Task 8: Aligning reads to the indexed reference sequence:

Now we can begin to align read 1 and read 2 to the reference genome. First of all change back into the ~/workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter/ directory and create a subdirectory to contain our remapping results.

> cd ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/
> mkdir remapping_to_reference
> cd remapping_to_reference

```
genomics genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter] cd ~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/
genomics genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter] mkdir remapping_to_reference
genomics genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter] cd remapping_to_reference
genomics genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_reference] pwd
/home/genomics/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_reference
genomics genomics_2016 [~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_reference] █
```

Let's explore the alignment options BWA MEM has to offer. Type:

> bwa mem

Part 2:   *Task 8: Aligning reads to the indexed reference sequence:*

```
Usage: bwa mem [options] <idxbase> <in1.fq> [in2.fq]

Algorithm options:

       -t INT        number of threads [1]
       -k INT        minimum seed length [19]
       -w INT        band width for banded alignment [100]
       -d INT        off-diagonal X-dropoff [100]
       -r FLOAT      look for internal seeds inside a seed longer than {-k} * FLOAT [1.5]
       -y INT        seed occurrence for the 3rd round seeding [20]
       -c INT        skip seeds with more than INT occurrences [500]
       -D FLOAT      drop chains shorter than FLOAT fraction of the longest overlapping chain [0.50]
       -W INT        discard a chain if seeded bases shorter than INT [0]
       -m INT        perform at most INT rounds of mate rescues for each read [50]
       -S            skip mate rescue
       -P            skip pairing; mate rescue performed unless -S also in use

Scoring options:

       -A INT        score for a sequence match, which scales options -TdBOELU unless overridden [1]
       -B INT        penalty for a mismatch [4]
       -O INT[,INT]  gap open penalties for deletions and insertions [6,6]
       -E INT[,INT]  gap extension penalty; a gap of size k cost '{-O} + {-E}*k' [1,1]
       -L INT[,INT]  penalty for 5'- and 3'-end clipping [5,5]
       -U INT        penalty for an unpaired read pair [17]

       -x STR        read type. Setting -x changes multiple parameters unless overriden [null]
                     pacbio: -k17 -W40 -r10 -A1 -B1 -O1 -E1 -L0  (PacBio reads to ref)
                     ont2d: -k14 -W20 -r10 -A1 -B1 -O1 -E1 -L0  (Oxford Nanopore 2D-reads to ref)
                     intractg: -B9 -O16 -L5  (intra-species contigs to ref)

Input/output options:

       -p            smart pairing (ignoring in2.fq)
       -R STR        read group header line such as '@RG\tID:foo\tSM:bar' [null]
       -H STR/FILE   insert STR to header if it starts with @; or insert lines in FILE [null]
       -j            treat ALT contigs as part of the primary assembly (i.e. ignore <idxbase>.alt file)
       -5            always take the leftmost alignment on a read as primary

       -v INT        verbose level: 1=error, 2=warning, 3=message, 4+=debugging [3]
       -T INT        minimum score to output [30]
       -h INT[,INT]  if there are <INT hits with score >80% of the max score, output all in XA [5,200]
       -a            output all alignments for SE or unpaired PE
       -C            append FASTA/FASTQ comment to SAM output
       -V            output the reference FASTA header in the XR tag
       -Y            use soft clipping for supplementary alignments
       -M            mark shorter split hits as secondary

       -I FLOAT[,FLOAT[,INT[,INT]]]
                     specify the mean, standard deviation (10% of the mean if absent), max
                     (4 sigma from the mean if absent) and min of the insert size distribution.
                     FR orientation only. [inferred]
```

The basis format of the command is:

```
Usage: bwa mem [options] <idxbase> <in1.fq> [in2.fq]
```

We can see that we need to provide BWA with a FASTQ files containing the raw reads (denoted by <in.fq> and <in2.fq>) to align to a reference file (unhelpfully this is listed as <idxbase>). There are also a number of options. The most important are the maximum number of differences in the seed (-k i.e. the first 32 bp of the sequence vs the reference), the number of processors the program should use (-t – our machine has 2 processors).

Our reference sequence is in
~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna

Our filtered reads in
~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R1_0
01.filtered.fastq

Part 2:   *Task 8: Aligning reads to the indexed reference sequence:*

~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R2_0
01.filtered.fastq

So to align our paired reads using processors and output to file E_Coli_CGATGT_L001_filtered.sam:

type, all on one line:

bwa mem -t 2 ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna
~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R1_0
01.filtered.fastq
~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R2_0
01.filtered.fastq > E_Coli_CGATGT_L001_filtered.sam

This will take about 5 minutes to complete.
There will be quite a lot of output but the end should look like:

```
M::process] read 70094 sequences (18721937 bp)...
M::mem_pestat] # candidate unique pairs for (FF, FR, RF, RR): (1, 26431, 0, 3)
M::mem_pestat] skip orientation FF as there are not enough pairs
M::mem_pestat] analyzing insert size distribution for orientation FR...
M::mem_pestat] (25, 50, 75) percentile: (518, 580, 642)
M::mem_pestat] low and high boundaries for computing mean and std.dev: (270, 890)
M::mem_pestat] mean and std.dev: (577.01, 102.93)
M::mem_pestat] low and high boundaries for proper pairs: (146, 1014)
M::mem_pestat] skip orientation RF as there are not enough pairs
M::mem_pestat] skip orientation RR as there are not enough pairs
M::mem_process_seqs] Processed 75290 reads in 7.280 CPU sec, 3.628 real sec
M::mem_pestat] # candidate unique pairs for (FF, FR, RF, RR): (1, 24724, 0, 0)
M::mem_pestat] skip orientation FF as there are not enough pairs
M::mem_pestat] analyzing insert size distribution for orientation FR...
M::mem_pestat] (25, 50, 75) percentile: (519, 581, 641)
M::mem_pestat] low and high boundaries for computing mean and std.dev: (275, 885)
M::mem_pestat] mean and std.dev: (577.10, 100.85)
M::mem_pestat] low and high boundaries for proper pairs: (153, 1007)
M::mem_pestat] skip orientation RF as there are not enough pairs
M::mem_pestat] skip orientation RR as there are not enough pairs
M::mem_process_seqs] Processed 70094 reads in 6.572 CPU sec, 3.306 real sec
main] Version: 0.7.15-r1142-dirty
main] CMD: bwa mem -t 2 ../../reference/U00096/U00096.fna E_Coli_CGATGT_L001_R1_001.filtered.fastq E_Coli_CGATGT_L001_R2_001.filtered.fastq
main] Real time: 63.015 sec; CPU: 124.824 sec
```

Viewing the alignment

Once the alignment is complete, list the directory contents and check that the alignment file is present.

    ls -lh

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_r
total 780M
-rw-r--r-- 1 genomics workshop 780M Jan  2 15:37 E_Coli_CGATGT_L001_filtered.sam
```

Note: ls -lh outputs the size of the file in human readable format (780Mb in this case – yours may be slightly different depending on the storage options you selected when you started the AMI)

The raw alignment is stored in what is called SAM format (Simple AlignMent format). It is in plain text format and you can view it if you wish using the 'less' command. Do not try to open the whole file in a text editor as you will likely run out of memory!

    less E_Coli_CGATGT_L001_filtered.sam

```
MISEQ:8:000000000-A7VC1:1:1101:17200:1633        83        gi|545778205|gb|U00096.3|        881006  60        137M     =
        880711  -432        GGTAAAGATGCCGGGGCGACGGGAAAGCCGGAACGGCGTGGTTCATCGGTAATGTTCCGCAAACCGGGCGATCAGGTTTCGGTGGCAGACT
TGAACAAAGGTGTGATTATCCAGTCCGGTAATGACGCCTGTATTGC        @9,@D>@8+8+++>@>+?A+AE?A86+++B:+8++>+B,B:,,,8,,EA,AC,8++++,,B
@8++C@@,C:,,CC8++++EC,CC,C@<,,,CCC,CCC@C,C,CC,9E8CCFEEDGGGGGGGGGGGGGGGGGGGCCCC9        NM:i:5  MD:Z:12T13T4C2T23T78
    AS:i:112        XS:i:0
MISEQ:8:000000000-A7VC1:1:1101:17200:1633        163        gi|545778205|gb|U00096.3|        880711  60        84M     =
        881006  432        TACTCGGGTGGCCTTTCTCCCGCACTACTCCTCTCTCCTTCGTGCTCTTCCAGCGGGTTCTGCATTTTTCTTCCTTTTTTCCCC      8,A
6C,,+;++;,;CC,<,,;+8++7,;6CC<C@C<CECCC,;,,9CCC,<,,;+++88BC,<,<99@B,9:BEB@@@=+:??A        NM:i:9  MD:Z:12A3A1G0A4A19G4G
19A9G4        AS:i:39 XS:i:0
MISEQ:8:000000000-A7VC1:1:1101:10456:1673        83        gi|545778205|gb|U00096.3|        1864278 60        42M     =
        1863862 -458        GGGTAAAACTTGTGAAATCGATCTTGAATCACATGGCGAATT        CC;,@C<<,,9EAFFFC7GGGGFGGGGGGGGGGGGGGGCCCC9
    NM:i:0  MD:Z:42 AS:i:42 XS:i:0
```

Each alignment line has 11 mandatory fields for essential alignment information such as mapping position, and a variable number of optional fields for flexible or aligner specific information. For further details as to what each field means see http://samtools.sourceforge.net/SAM1.pdf

Task 9: Convert SAM to BAM file

Before we can visualise the alignment however, we need to convert the SAM file to a BAM (Binary AlignMent format) which can be read by most software analysis packages. To do this we will use another suite of programs called samtools. Type:

samtools view

```
Usage: samtools view [options] <in.bam>|<in.sam>|<in.cram> [region ...]

Options:
  -b       output BAM
  -C       output CRAM (requires -T)
  -1       use fast BAM compression (implies -b)
  -u       uncompressed BAM output (implies -b)
  -h       include header in SAM output
  -H       print SAM header only (no alignments)
  -c       print only the count of matching records
  -o FILE  output file name [stdout]
  -U FILE  output reads not selected by filters to FILE [null]
  -t FILE  FILE listing reference names and lengths (see long help) [null]
  -L FILE  only include reads overlapping this BED FILE [null]
  -r STR   only include reads in read group STR [null]
  -R FILE  only include reads with read group listed in FILE [null]
  -q INT   only include reads with mapping quality >= INT [0]
  -l STR   only include reads in library STR [null]
  -m INT   only include reads with number of CIGAR operations consuming
           query sequence >= INT [0]
  -f INT   only include reads with all bits set in INT set in FLAG [0]
  -F INT   only include reads with none of the bits set in INT set in FLAG [0]
  -x STR   read tag to strip (repeatable) [null]
  -B       collapse the backward CIGAR operation
  -s FLOAT integer part sets seed of random number generator [0];
           rest sets fraction of templates to subsample [no subsampling]
  -@, --threads INT
           number of BAM/CRAM compression threads [0]
  -?       print long help, including note about region specification
  -S       ignored (input format is auto-detected)
      --input-fmt-option OPT[=VAL]
               Specify a single input file format option in the form
               of OPTION or OPTION=VALUE
  -O, --output-fmt FORMAT[,OPT[=VAL]]...
               Specify output format (SAM, BAM, CRAM)
      --output-fmt-option OPT[=VAL]
               Specify a single output file format option in the form
               of OPTION or OPTION=VALUE
  -T, --reference FILE
               Reference sequence FASTA FILE [null]
```

We can see that we need to provide samtools view with a reference genome in FASTA format file (-T), the -b and -S flags to say that the output should be in BAM format and the input in SAM, plus the alignment file.

Remember our reference sequence is in
~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna

Type (all on one line):

> samtools view -bS -T
> ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna
> E_Coli_CGATGT_L001_filtered.sam > E_Coli_CGATGT_L001_filtered.bam

This should take around 2 minutes. Note that for larger datasets you may wish to set multiple threads as well with the --threads option.

> ls -lh

It's always good to check that your files have processed correctly if something goes wrong it's better to catch it immediately.

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/d
total 1.1G
-rw-r--r-- 1 genomics workshop 254M Jan  2 15:42 E_Coli_CGATGT_L001_filtered.bam
-rw-r--r-- 1 genomics workshop 780M Jan  2 15:37 E_Coli_CGATGT_L001_filtered.sam
```

Note that the bam file is smaller than the sam file - this is to be expected as the binary format is more efficient.

Task 10: Sort BAM file

Once this is complete we then need to sort the BAM file so that the reads are stored in the order they appear along the chromosomes (don't ask me why this isn't done automatically....). We can do this using the samtools sort command.

> samtools sort E_Coli_CGATGT_L001_filtered.bam -o E_Coli_CGATGT_L001_filtered.sorted.bam

This will take another minute or so.

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/data/seq
total 1.2G
-rw-r--r-- 1 genomics workshop 254M Jan  2 15:42 E_Coli_CGATGT_L001_filtered.bam
-rw-r--r-- 1 genomics workshop 780M Jan  2 15:37 E_Coli_CGATGT_L001_filtered.sam
-rw-r--r-- 1 genomics workshop 185M Jan  2 15:50 E_Coli_CGATGT_L001_filtered.sorted.bam
```

A note on piping BWA and samtools commands:

In tasks 8-10 we aligned reads to the reference genome, converted SAM to BAM and then sorted the resulting BAM file. For clarity we have shown these as individual steps. However, in real-life, it is faster and easier to do these simultaneously using Unix pipes!

E.g. (there is no need to do this)
 bwa mem -t 2 ~/workshop_meterials/genomics_tutorial/data/reference/U00096/U00096.fna
~/workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R1_0
01.filtered.fastq
~/workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R2_0
01.filtered.fastq > E_Coli_CGATGT_L001_filtered.sam \
| samtools sort -O bam -o E_Coli_CGATGT_L001_filtered.sorted.bam

Note that the latest versions of samtools sort can output BAM files.

Task 11: Remove suspected PCR duplicates

Especially when using paired-end reads, samtools can do a reasonably good job of removing potential PCR duplicates (see the first part of this workshop if you are unsure what this means).

Again, samtools has a great little command to do this called rmdup.

On the command-line type:

> samtools rmdup E_Coli_CGATGT_L001_filtered.sorted.bam
> E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam

```
[bam_rmdup_core] processing reference U00096.3...
[bam_rmdup_core] inconsistent BAM file for pair 'MISEQ:8:000000000-A7VC1:1:2117:24462:9451'. Continue anyway.
[bam_rmdup_core] inconsistent BAM file for pair 'MISEQ:8:000000000-A7VC1:1:2117:25993:22308'. Continue anyway.
[bam_rmdup_core] inconsistent BAM file for pair 'MISEQ:8:000000000-A7VC1:1:1113:11936:9295'. Continue anyway.
[bam_rmdup_core] 31 unmatched pairs
[bam_rmdup_core] 9680 / 458450 = 0.0211 in library '    '
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/re
total 1.4G
-rw-r--r-- 1 genomics workshop 254M Jan  2 15:42 E_Coli_CGATGT_L001_filtered.bam
-rw-r--r-- 1 genomics workshop 780M Jan  2 15:37 E_Coli_CGATGT_L001_filtered.sam
-rw-r--r-- 1 genomics workshop 185M Jan  2 15:50 E_Coli_CGATGT_L001_filtered.sorted.bam
-rw-r--r-- 1 genomics workshop 183M Jan  2 15:57 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam
```

You will notice some warnings about inconsistent BAM file for pair - this is just a warning that a pair of reads does not align together on the genome within the expected tolerance - it is normal to expect some of these, and you can ignore.

Task 12: Index the BAM file

Most programs used to view BAM formatted data require an index file to locate the reads mapping to a particular location quickly. You can think of this as an index in a book, telling you where to go to find particular phrases or words. We'll use the samtools index command to do this.

Type:
> samtools index E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam

```
total 1.4G
-rw-r--r-- 1 genomics workshop 254M Jan  2 15:42 E_Coli_CGATGT_L001_filtered.bam
-rw-r--r-- 1 genomics workshop 780M Jan  2 15:37 E_Coli_CGATGT_L001_filtered.sam
-rw-r--r-- 1 genomics workshop 185M Jan  2 15:50 E_Coli_CGATGT_L001_filtered.sorted.bam
-rw-r--r-- 1 genomics workshop 183M Jan  2 15:57 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam
-rw-r--r-- 1 genomics workshop  15K Jan  2 15:59 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam.bai
```

We should obtain a .bai file (known as a BAM-index file).

Task 13: Obtain mapping statistics
Finally we can obtain some summary statistics.

samtools flagstat E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam > mappingstats.txt

This should only take a few seconds. Once complete view the mappingstats.txt file using a text-editor (e.g. gedit or nano) or the 'more' command.

```
1250574 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 secondary
1151 + 0 supplementary
0 + 0 duplicates
898936 + 0 mapped (71.88% : N/A)
1249423 + 0 paired in sequencing
624727 + 0 read1
624696 + 0 read2
893922 + 0 properly paired (71.55% : N/A)
896371 + 0 with itself and mate mapped
1414 + 0 singletons (0.11% : N/A)
0 + 0 with mate mapped to a different chr
0 + 0 with mate mapped to a different chr (mapQ>=5)
```

So here we can see we have 1250574 reads in total, none of which failed QC.
71.88% of reads mapped to the reference genome and 71.55% mapped with the expected 500-600bp distance between them. 1414 reads could not have their read-pair mapped.

0 reads have mapped to a different chromosome than their pair (0 has a mapping quality > 5 – this is a Phred scaled quality score much as we say in the FASTQ files). If there were any such reads they would likely due to repetitive sequences (e.g phage insertion sites) or an insertion of plasmid or phage DNA into the main chromosome.

Task 15: Cleanup

We have a number of leftover intermediate files which we can now remove to save space.

Type (all on one line):

> rm E_Coli_CGATGT_L001_filtered.sam E_Coli_CGATGT_L001_filtered.bam
> E_Coli_CGATGT_L001_filtered.sorted.bam

In case you get asked if you are sure to remove 3 arguments type in "yes" and hit enter.
You should now be left with the processed alignment file, the index file and the mapping stats.

```
total 183M
-rw-r--r-- 1 genomics workshop 183M Jan  2 15:57 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam
-rw-r--r-- 1 genomics workshop  15K Jan  2 15:59 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam.bai
-rw-r--r-- 1 genomics workshop  422 Jan  2 16:00 mappingstats.txt
```

Well done! You have now mapped, filtered and sorted your first whole genome data-set!
Let's take a look at it!

Task 16: QualiMap

Qualimap (http://qualimap.bioinfo.cipf.es/) is a program that summarises the alignment in much more detail than the mapping stats file we produced. It's a technical tool which allows you to assess the sequencing for any problems and biases in the sequencing and the alignment rather than a tool to deduce biological features.

There are a few options to the program, We want to run bamqc. Type:
> qualimap bamqc
to get some help on this command.

To get the report, first make sure you are in the directory:
~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_reference
then run the command:
> qualimap bamqc -outdir bamqc -bam E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam -gff
> ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.gff

this creates a subfolder called bamqc

```
total 183M
drwxr-xr-x 5 genomics workshop 4.0K Jan  2 16:04 bamqc
-rw-r--r-- 1 genomics workshop 183M Jan  2 15:57 E_coli_CGATGT_L001_filtered.sorted.rmdup.bam
-rw-r--r-- 1 genomics workshop  15K Jan  2 15:59 E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam.bai
-rw-r--r-- 1 genomics workshop  422 Jan  2 16:00 mappingstats.txt
```

cd to this directory and run
> firefox qualimapReport.html

There is a lot in the report so just a few highlights:

Coverage across reference

This shows the number of reads that 'cover' each section of the genome. The red line shows a rolling average around 50x - this means that on average every part of the genome was sequenced 50X. It is important to have sufficient depth of coverage in order to be confident that any features you find in your data are real and not a result of sequencing errors.

What do you think the regions of low/zero coverage correspond to?

Insert Size Histogram
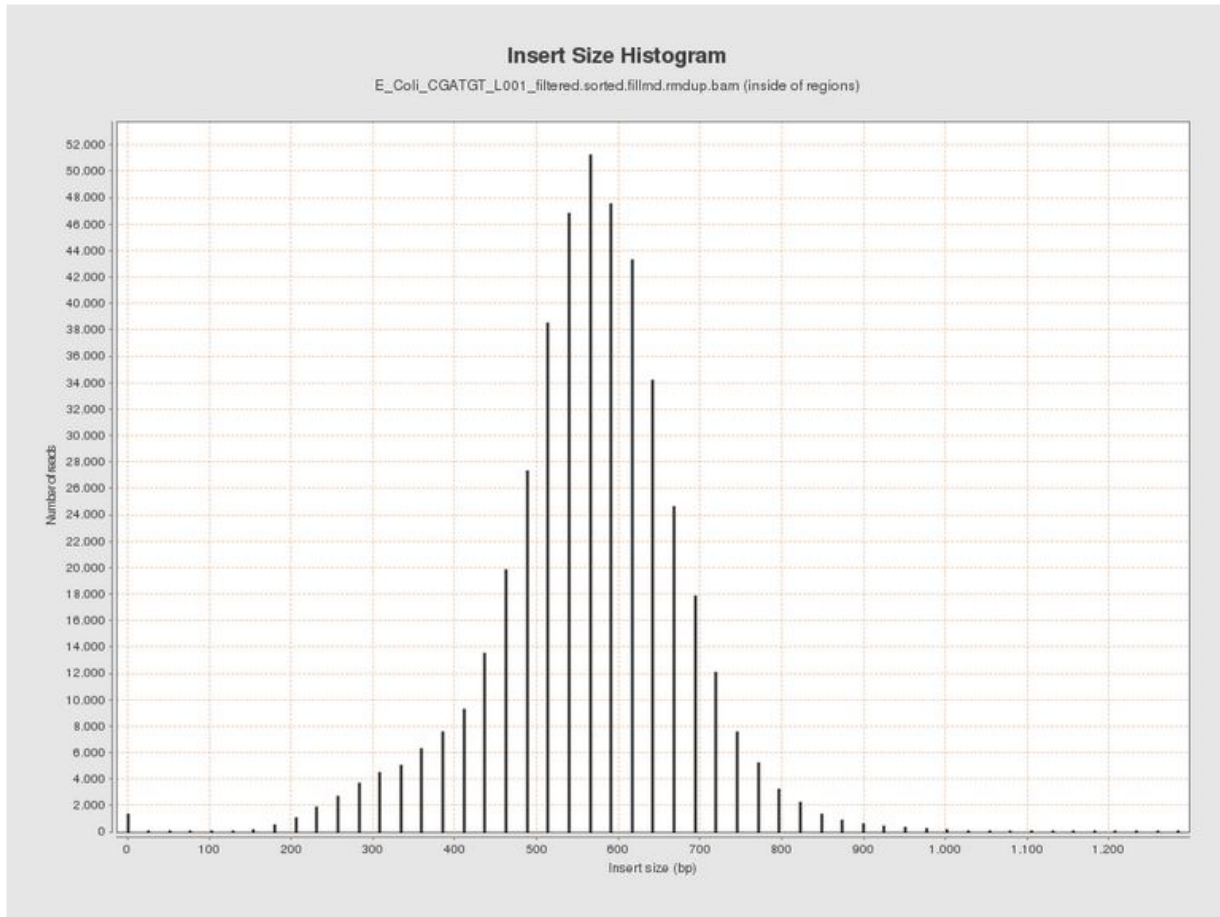
The Insert Size Histogram displays the range of sizes of the DNA fragments. It shows how well your DNA was size selected before sequencing. Note that the 'insert' refers to the DNA that was inserted between the sequencing adaptors, so equates to the size range of the DNA that was used.
In this case we have 300 paired end reads and our insert size varies around 600 bases - so there should only be a small gap between the reads that was not sequenced.

Have a look at some of the other graphs produced.

Task 17: Load the Integrative Genomics Viewer

The Integrative Genome Viewer (IGV) is a tool developed by the Broad Institute for browsing interactively the alignment data you produced. It has a wealth of features and we can only cover some basics to get you started. Go to http://www.broadinstitute.org/igv/ to get more information.

In your terminal, type
     igv.sh
Or you can click the icon on the desktop.
IGV viewer should appear:
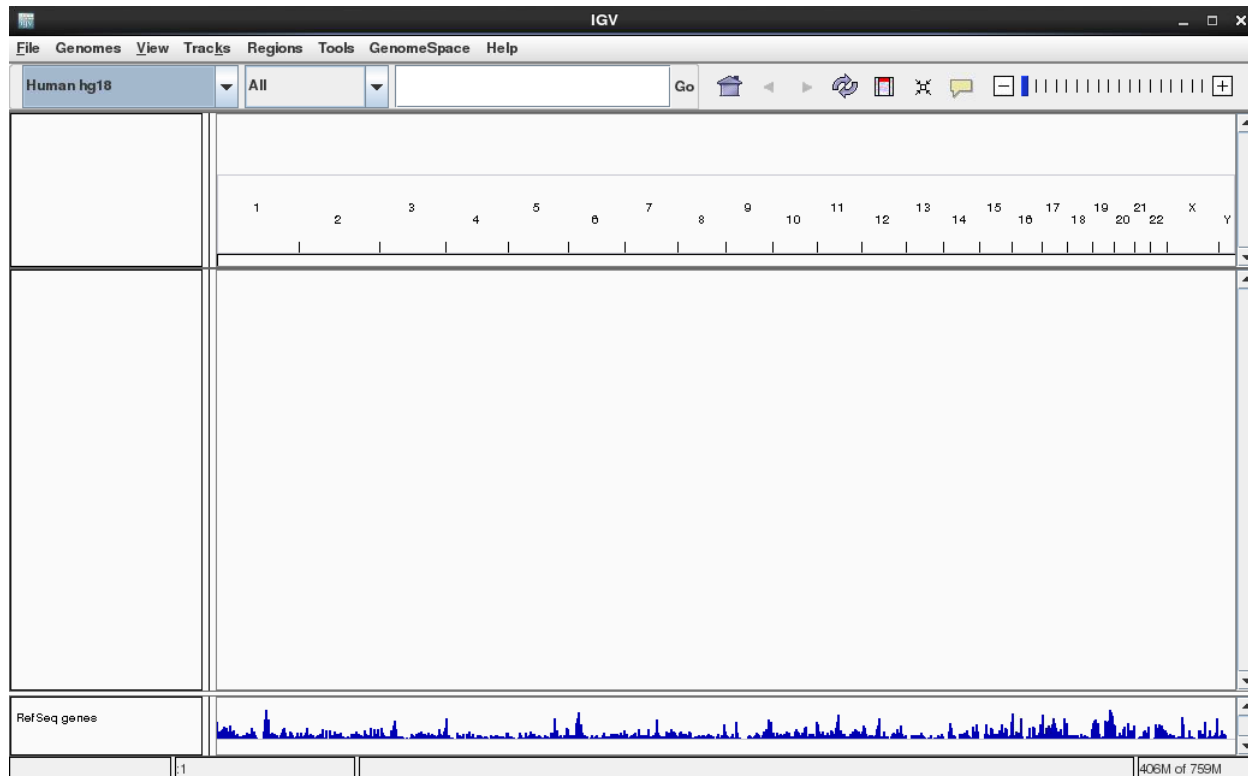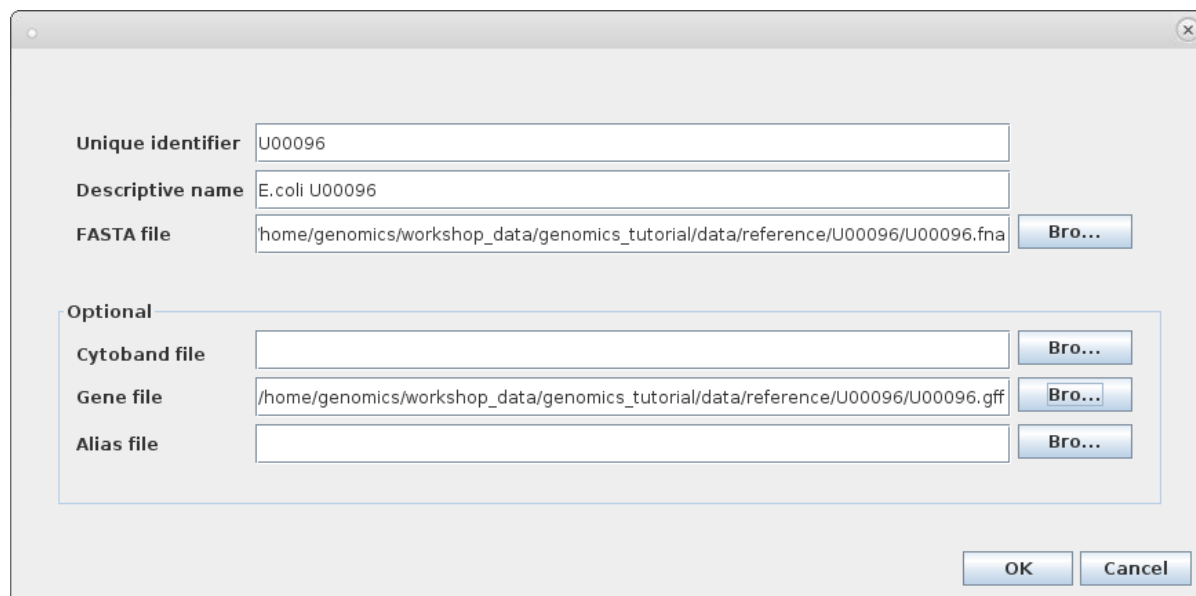
Notice that by default a human genome has been loaded.

Task 18a: Import the E.coli U0009 reference genome to IGV

By default IGV does not contain our reference genome. We'll need to import it.

Click on 'Genomes ->Create .genome file...'
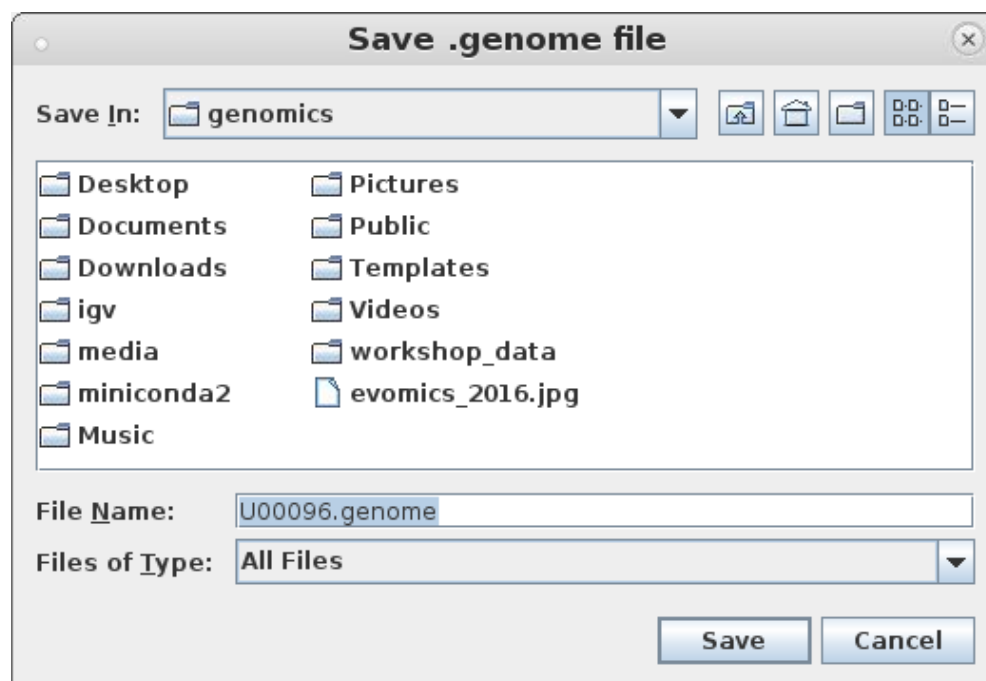
Enter the information above and click on 'OK' .

IGV will ask where it can save the genome file. Your home directory will be fine.

Click 'Save' again.

Note that the genome and the annotation have now been imported.
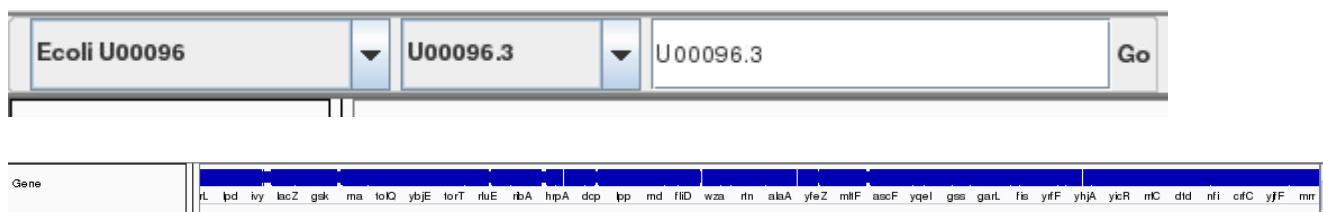
Task 18b: Load the BAM file

Load the alignment file. Note that IGV requires the .bai index file to also be in the same directory.

  Select File... and Load From File
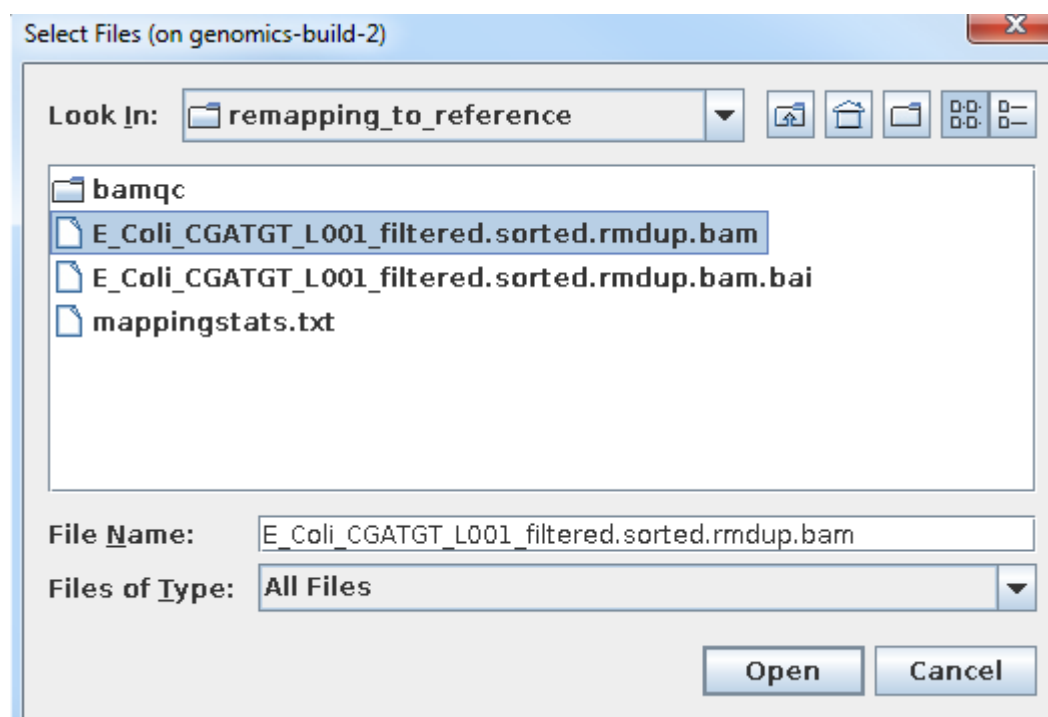
  Select the bam file and click open

Once loaded your screen should look similar to the following. Note that you can load more BAM files if you wish to compare different samples or the results of different mapping programs.
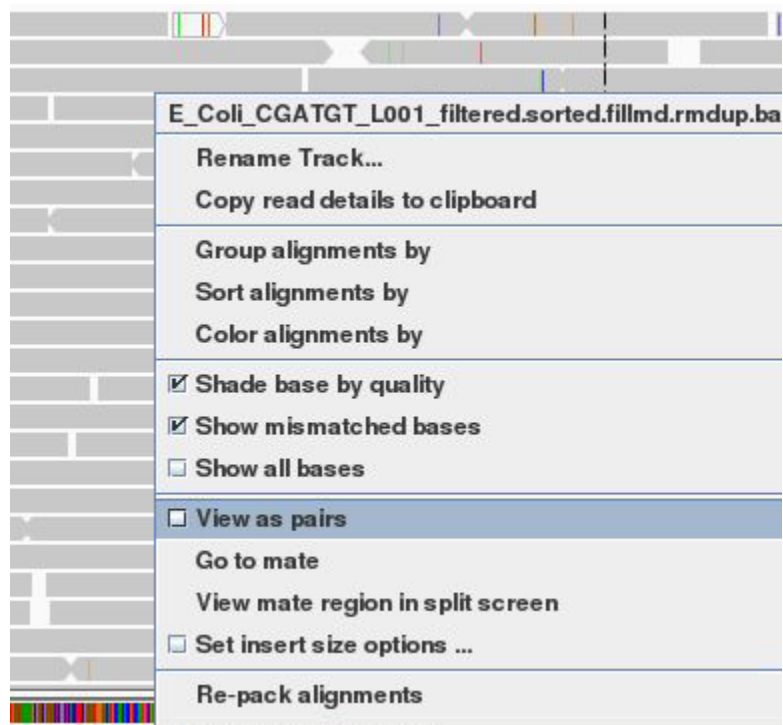
Part 2:   Task 18b: Load the BAM file
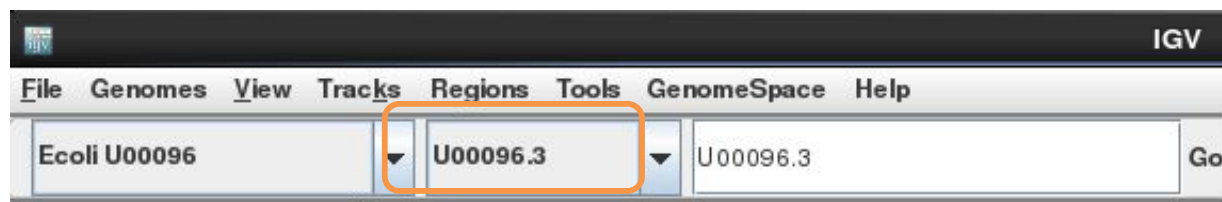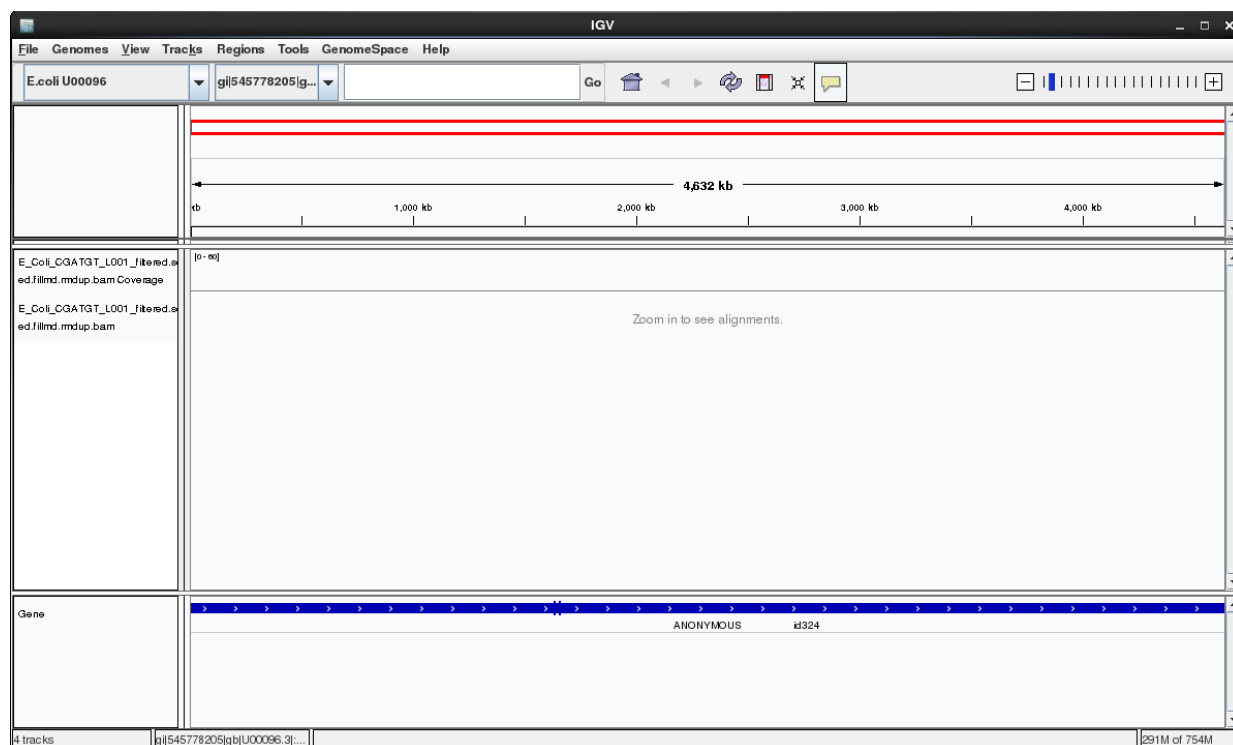
Select the chromosome U00096.3 if it is not already selected
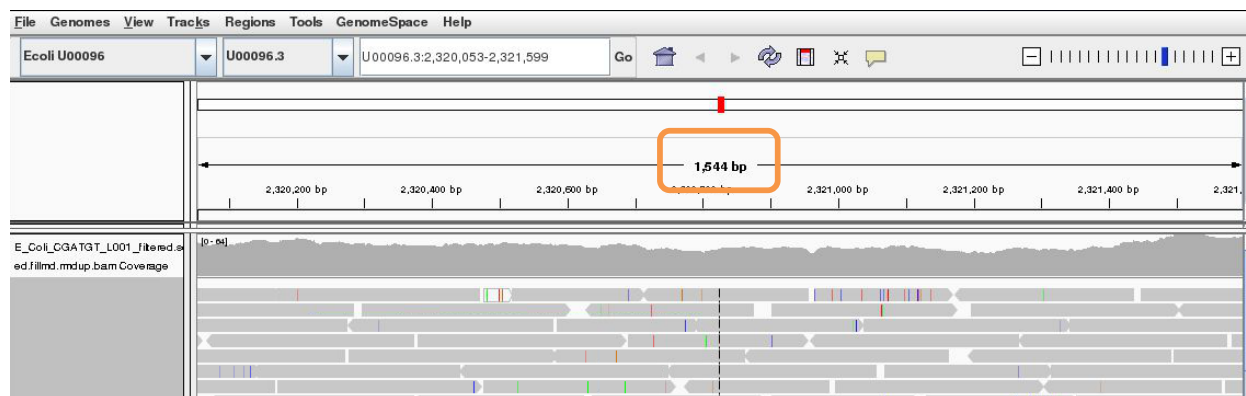
Use the +/- keys to zoom in or use the zoom bar at the top right of the screen to zoom into about 1-2kbases as above

Right click on the main area and select view as pairs

The gray graph at the top of the figure indicates the coverage of the genome:

The more reads mapping to a certain location, the higher the peak on the graph. You'll see a coloured line of blue, green or red in this coverage plot if there are any SNPs (single-nucleotide polymorphisms) present (there are none in the plot). If there are any regions in the genome which are not covered by the reads, you will see these as gaps in the coverage graph. Sometimes these gaps are caused by repetitive regions; others are caused by genuine insertions/deletions in your new strain with respect to the reference.

Below the coverage graph is a representation of each read pair as it is mapped to the genome. One pair is highlighted.

This pair consists of 2 reads with a gap (there may be no gap if the reads overlap) Any areas of mismatch either due to inconsistent distances between paired-end reads or due to differences between the reference and the read and are highlighted by a colour. The brighter the colour, the higher the base-calling quality is estimated to be. Differences in a single read are likely to be

sequencing errors. Differences consistent in all reads are likely to be mutations.

Hover over a read to get detailed information about the reads' alignment:

```
Left alignment                                              Right alignment
Read name = MISEQ:8:000000000-A7VC1:1:2112:3986:8017       Read name = MISEQ:8:000000000-A7VC1:1:2112:3986:8017
----------------------                                      ----------------------
Location = U00096.3:2,319,925                              Location = U00096.3:2,319,925
Alignment start = 2,319,293 (+)                           Alignment start = 2,319,859 (-)
Cigar = 270M                                              Cigar = 187M
Mapped = yes                                              Mapped = yes
Mapping quality = 60                                      Mapping quality = 60
Secondary = no                                            Secondary = no
Supplementary = no                                        Supplementary = no
Duplicate = no                                            Duplicate = no
Failed QC = no                                            Failed QC = no
----------------------                                    ----------------------
----------------------                                    Base = C
Mate is mapped = yes                                     Base phred quality = 25
Mate start = U00096.3:2319858 (-)                        ----------------------
Insert size = 753                                        Mate is mapped = yes
First in pair                                            Mate start = U00096.3:2319292 (+)
Pair orientation = F1R2                                  Insert size = -753
----------------------                                    Second in pair
MD = 221A10A37                                           Pair orientation = F1R2
NM = 2                                                   ----------------------
AS = 260                                                 MD = 12T27T146
XS = 0                                                   NM = 2
----------------------                                    AS = 177
```

You don't need to understand every value, but compare this to the SAM format to get an idea of what is there.

SNPs and Indels

The following 3 tasks are open-ended. Please take your time with these. Read the examples on the following page if you get stuck.

Task 19: Read about the alignment display format

Visit http://www.broadinstitute.org/software/igv/AlignmentData

Task 20a: Manually identify a region without any reads mapping.

It can be quite difficult to find even with a very small genome. Zoom out as far as you can and still see the reads. Use the coverage plot from QualiMap to try to find it. Are there genes associated?

Because of the way IGV handles BAM files, it will not display coverage information if you zoom out too far. To get coverage information across the entire genome, regardless of how far you are zoomed out,
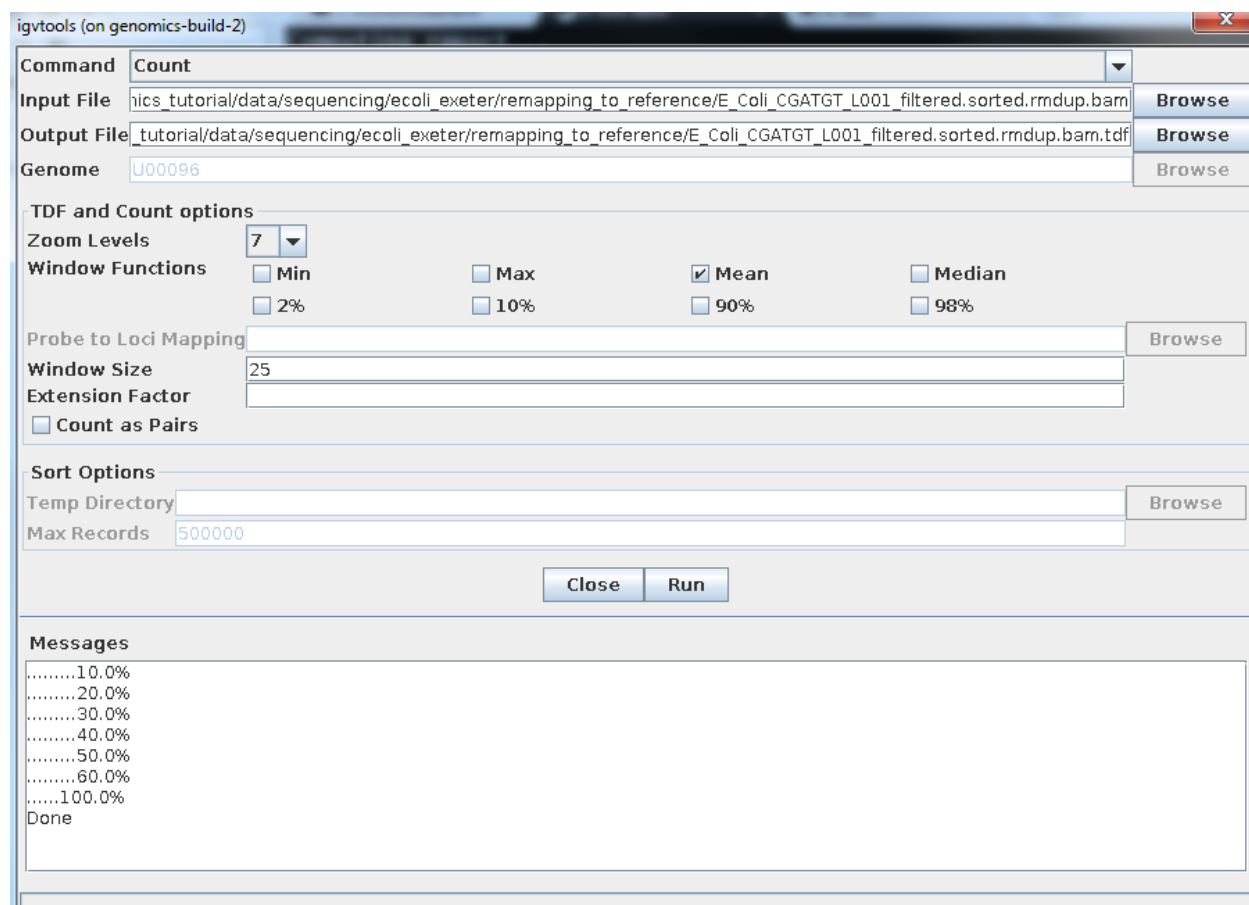
Part 2:   *Task 20a: Manually identify a region without any reads mapping.*

you'll need to create a TDF file which contains a coverage information across windows of X number of bases across the genome. You can do this within IGV:
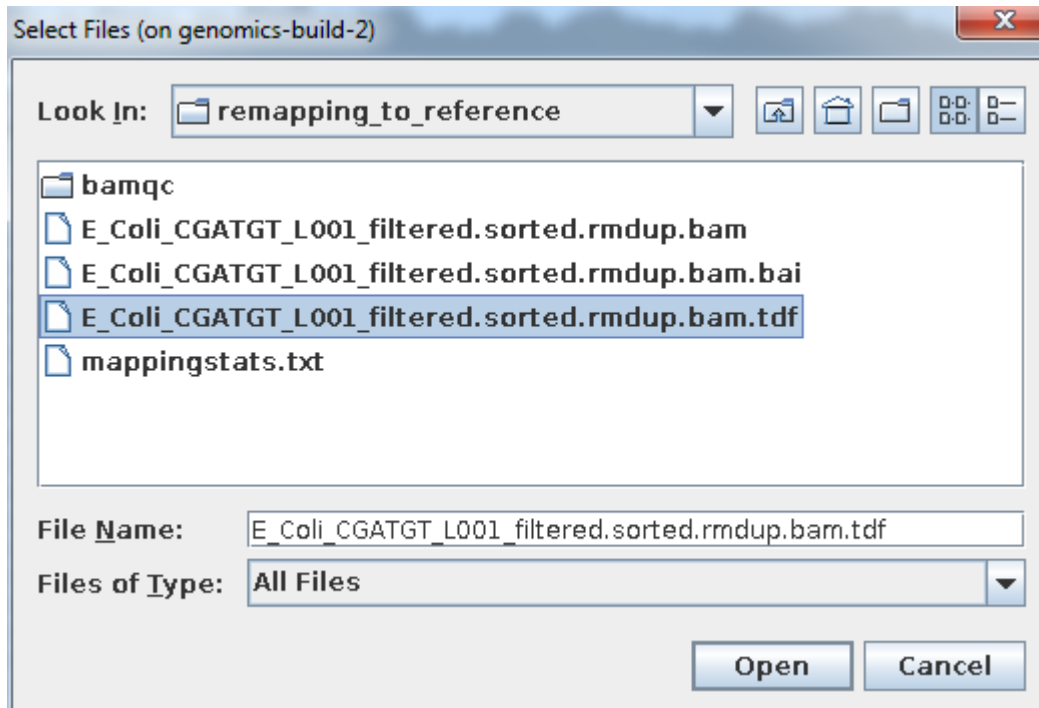
Select Tools->Run igvtools:
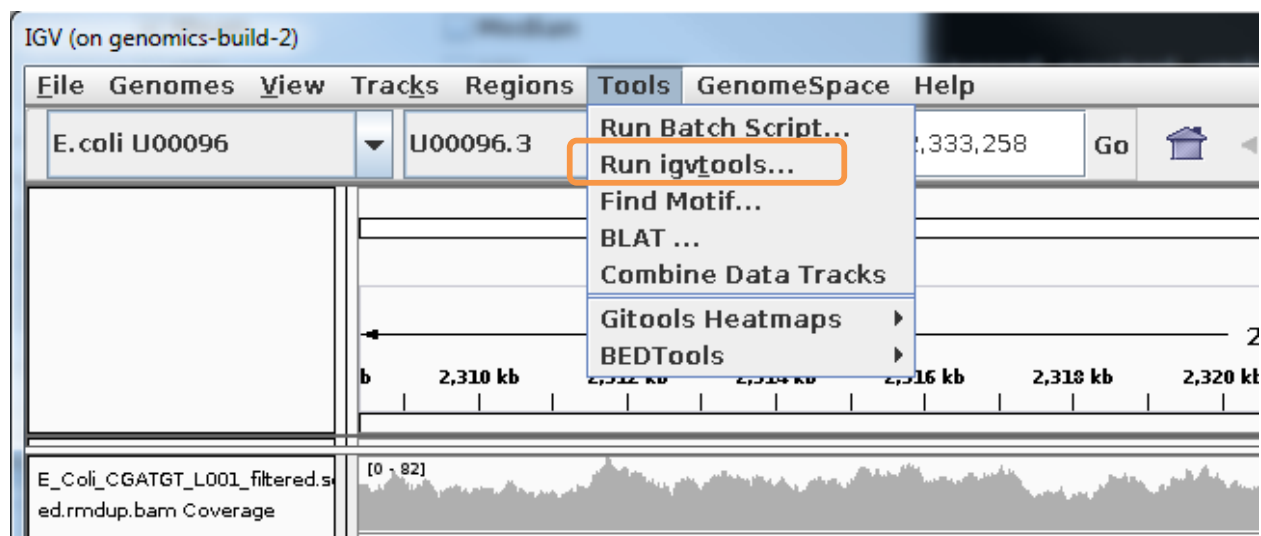
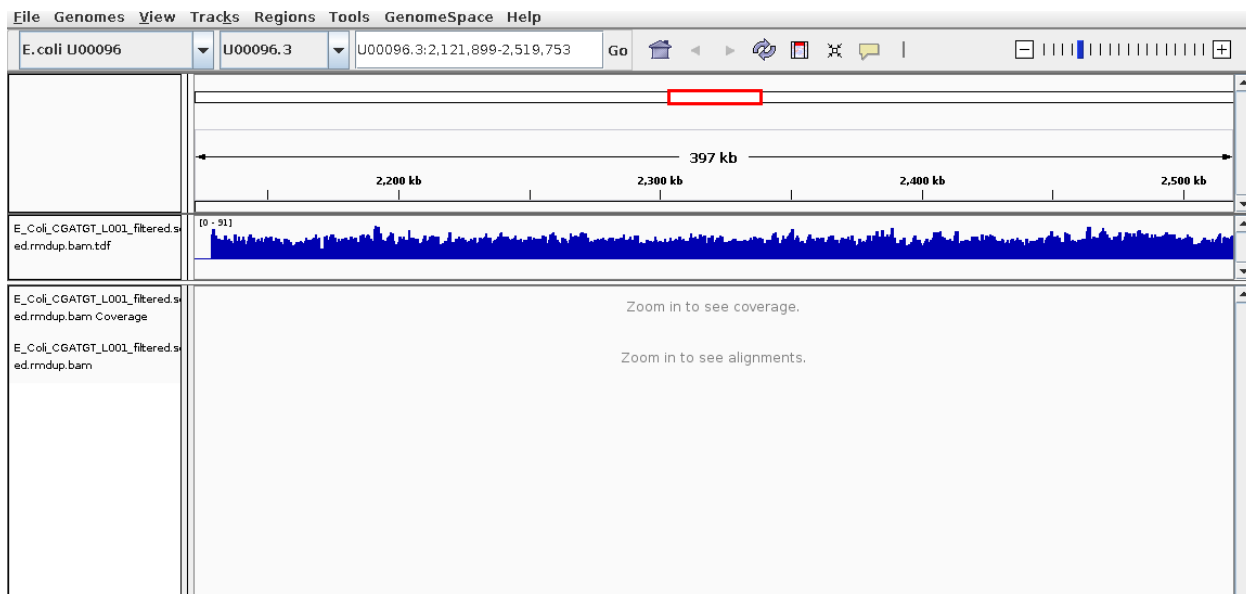Now load the BAM alignment file in the Input field and click Run:

Part 2:

Once completed, you can load this TDF file as by:

Select File -> Load from file…

You should then see the extra coverage track which remains visible even after you zoom out.

Task 20b: Manually identify a region containing repetitive sequences.

Again try to use the QualiMap report to give you an idea. What is this region? Is there a gene close-by? What do you think this is? (Think about repetitive sequences, what does BWA do if a region in the genome has been duplicated)

Task 21: Identify SNPs and Indels manually

Can you find any SNPs? Which genes (if any) are they in? How reliable do they look? (Hint – look at the number of reads mapping, their orientation - which strand they are on and how bright the base-calls are).

Zoom in to maximum magnification at the site of the SNP. Can you determine whether a SNP results in a synonymous (i.e. silent) or non-synonymous change in the amino acid? Can you use PDB (http://www.rcsb.org/pdb/home/home.do) or other resources to determine whether or not this occurs in a catalytic site or other functionally crucial region? (Note this may not always be possible).

What effect do you think this would have on the cell?

Example: Identifying Variants manually

Here are some regions where there are differences in the organism sequenced and the reference: Can you interpret what has happened to the genome of our strain? Try to work out what is going on yourself before looking at the comment

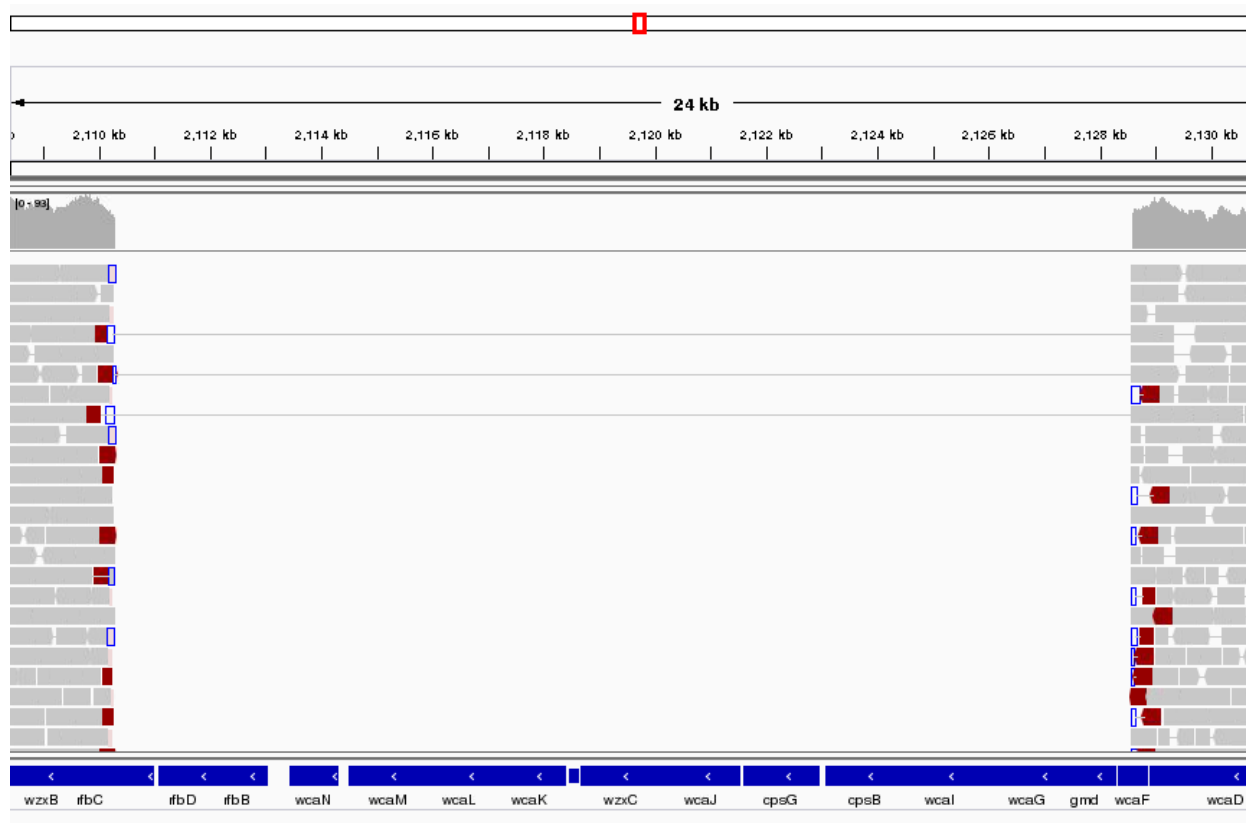Paste each of the genomic locations in this box and click go

U00096.3:2,108,392-2,133,153
U00096.3:3,662,049-3,663,291
U00096.3:4,296,249-4,296,510
U00096.3:565,965-566,489

Region U00096.3:2,108,392-2,133,153

This area corresponds to the drop in coverage identified by Qualimap. It looks like a fairly large region of about 17 kbases which was present in the reference and is missing from our sequenced genome. It looks like about 12 genes from the reference strain are not present in our strain - it this real or an artefact?

Part 2:   Task 21: Identify SNPs and Indels manually

Well it is pretty conclusive we have coverage of about 60X either side of the deletion and nothing at all within. There are nice clean edges to the start and end of the deletion. We also have paired reads which span the deletion. This is exactly what you would expect if the two regions of coverage were actually joined together.
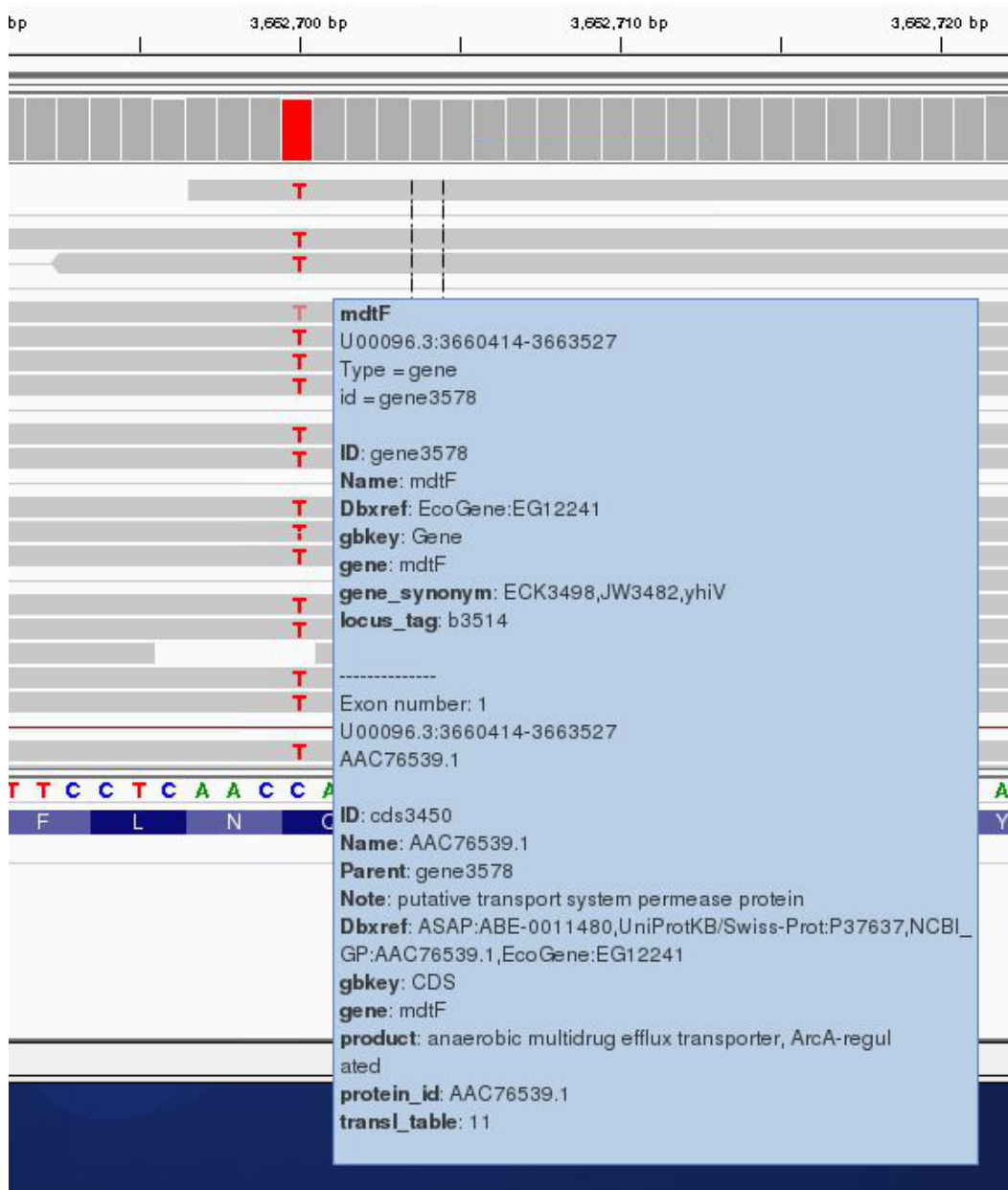
Region U00096.3:3,662,049-3,663,291
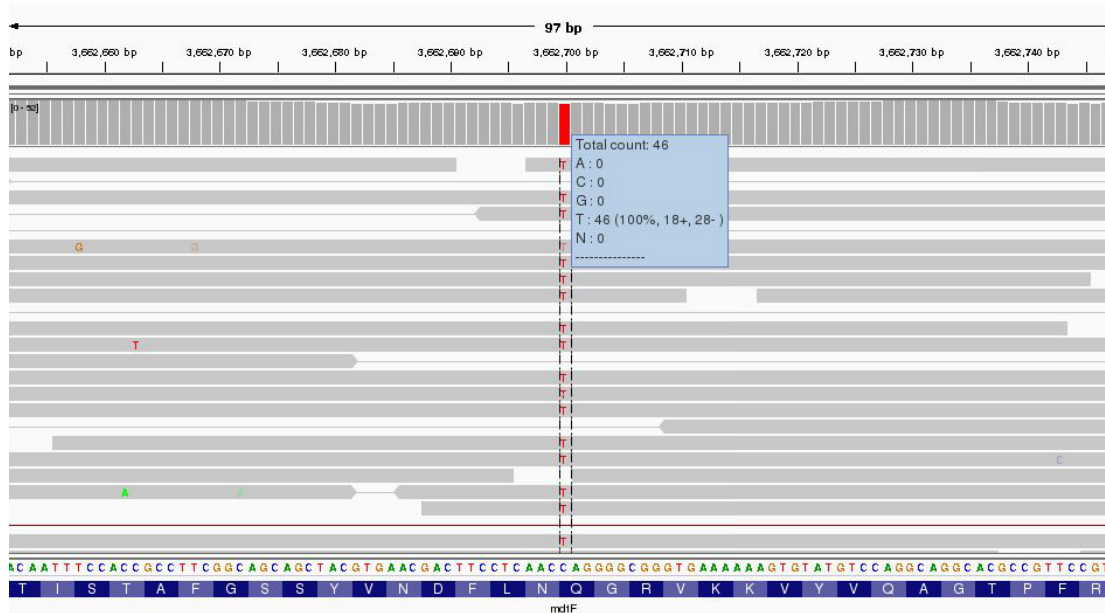
Zoom right in until you can see the reference sequence and protein sequence at the bottom of the display.

The first thing to note is that only discrepancies with respect to the reference are shown. If a read is entirely the same as its reference, it will appear entirely grey. Blue and red blocks indicate the presence of an 'abnormal' distance between paired-end reads. Note that unless this is consistent across most of the reads at a given position, it is not significant.

Here we have a C->T SNP. This changes the codon from CAG->TAG (remember to check what strand the gene is on this one is on the forward strand, if it was on the reverse strand you would have to take the reverse complement of the codon to interpret the amino acid it codes for.) and results in a Gln->Stop mutation in the final protein product which is very likely to change the effect of the protein product.

Hover over the gene to get some more information from the annotation... Since it is a drug resistance protein it could be very significant.

One additional check is that the SNPs occur when reading the forward strand. We can check this by looking at the direction of the grey reads,or by hovering over the coverage graph - see previous diagram. We can see that approximately half of the bases reporting the C->T mutation occur in read 1 (forward arrow), and half in read 2 (reverse arrow). This adds confidence to the base-call as it reduces the likelihood of this SNP being the result of a PCR duplication error.

Note that sequencing errors in Illumina data are quite common (look at the odd bases showing up in the screen above. We rely on depth of sequencing to average out these errors. This is why people often mention that a minimum median coverage of 20-30x across the genome is required for accurate SNP-calling with Illumina data. This is not necessarily true for simple organisms such as prokaryotes, but for diploid and polyploid organisms it becomes important because each position may have one, two or many alleles changed.

Regions U00096.3:2,106,835-2,140,480
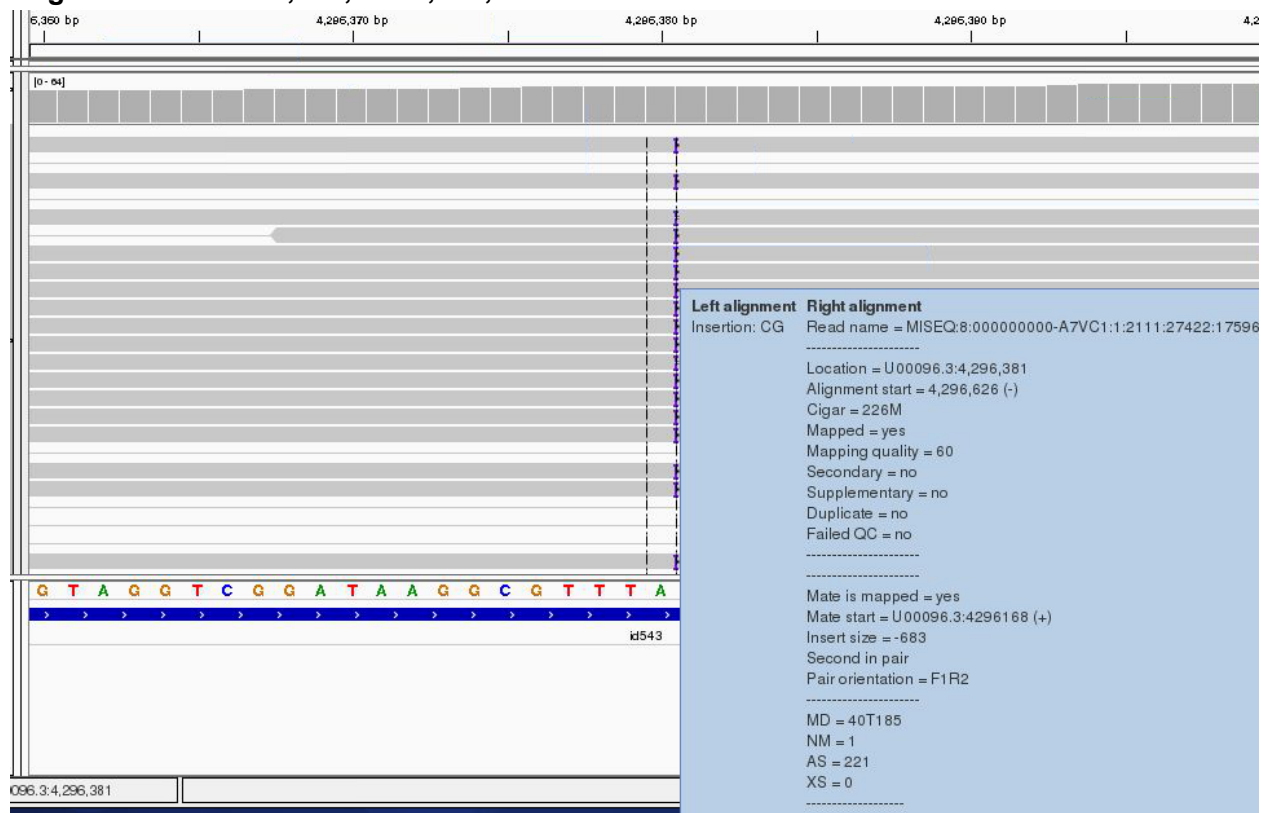
Much the same guidelines apply for indels as they do for SNPs. Here we have an insertion of two bases CG in our sample compared to the reference. Again, we can see how much confidence we have that the insertion is real by checking that the indel is found on both read 1 and read 2 and on both strands.

The insertion is signified by the presence of a purple bar. Hover your mouse over it to get more details as above

We can hover our mouse over the reference sequence to get details of the gene. We can see that it occurs in a repeat region and is unlikely to have very significant effects.

One can research the effect that a SNP or Indel may have by finding the relevant gene at http://www.uniprot.org (or google 'mdtF uniprot' in the previous case).

It should be clear from this quick exercise that trying to work out where SNPs and Indels are manually is a fairly tedious process if there are many mutations. As such, the next section will look at how to obtain spread-sheet friendly summary details of these.

Region U00096.3:565,965-566,489

This last region is more complex try to understand what genomic mutation could account for this pattern - discuss with a colleague or an instructor.

Recap: SNP/Indel identification

1.   **Only changes from the reference sequence are displayed in IGV**
2.   **Genuine SNPs/Indels should be present on both read 1 and read 2**
3.   **Genuine SNPs/Indels should be present on both strands**
4.   **Genuine SNPs/Indels should be supported by a good (i.e. 20-30x) depth of coverage**
4.   **Very important mutations (i.e. ones relied upon in a paper) should be confirmed via PCR/Sanger sequencing.**

Automated analyses

Viewing alignments is useful when convincing yourself or others that a particular mutation is real rather than an artefact and for getting a feel for short read sequencing datasets. However, if we want to quickly and easily find variants we need to be able to generate lists of variants, in which gene they occur (if any) and what effect they have. We also need to know which (if any) genes are missing (i.e. have zero coverage).

Automated variant calling

To call variants we can use a number of packages (e.g. VarScan, GTK). However here, we will show you how to use the bcftools package which comes with samtools. First we need to generate a pileup file which contains only locations with the variants and pass this to bcftools.

Task 22: Identify SNPs and Indels using automated variant callers

Make sure you are in the directory.
~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/remapping_to_reference
 Type the following:

        samtools mpileup

You should see a screen similar to the following

```
Usage: samtools mpileup [options] in1.bam [in2.bam [...]]

Input options:
  -6, --illumina1.3+      quality is in the Illumina-1.3+ encoding
  -A, --count-orphans     do not discard anomalous read pairs
  -b, --bam-list FILE     list of input BAM filenames, one per line
  -B, --no-BAQ            disable BAQ (per-Base Alignment Quality)
  -C, --adjust-MQ INT     adjust mapping quality; recommended:50, disable:0 [0]
  -d, --max-depth INT     max per-file depth; avoids excessive memory usage [250]
  -E, --redo-BAQ          recalculate BAQ on the fly, ignore existing BQs
  -f, --fasta-ref FILE    faidx indexed reference sequence file
  -G, --exclude-RG FILE   exclude read groups listed in FILE
  -l, --positions FILE    skip unlisted positions (chr pos) or regions (BED)
  -q, --min-MQ INT        skip alignments with mapQ smaller than INT [0]
  -Q, --min-BQ INT        skip bases with baseQ/BAQ smaller than INT [13]
  -r, --region REG        region in which pileup is generated
  -R, --ignore-RG         ignore RG tags (one BAM = one sample)
  --rf, --incl-flags STR|INT  required flags: skip reads with mask bits unset []
  --ff, --excl-flags STR|INT  filter flags: skip reads with mask bits set
                                       [UNMAP,SECONDARY,QCFAIL,DUP]
  -x, --ignore-overlaps   disable read-pair overlap detection

Output options:
  -o, --output FILE       write output to FILE [standard output]
  -g, --BCF               generate genotype likelihoods in BCF format
  -v, --VCF               generate genotype likelihoods in VCF format

Output options for mpileup format (without -g/-v):
  -O, --output-BP         output base positions on reads
  -s, --output-MQ         output mapping quality

Output options for genotype likelihoods (when -g/-v is used):
  -t, --output-tags LIST  optional tags to output:
             DP,AD,ADF,ADR,SP,INFO/AD,INFO/ADF,INFO/ADR []
  -u, --uncompressed      generate uncompressed VCF/BCF output

SNP/INDEL genotype likelihoods options (effective with -g/-v):
  -e, --ext-prob INT       Phred-scaled gap extension seq error probability [20]
  -F, --gap-frac FLOAT     minimum fraction of gapped reads [0.002]
  -h, --tandem-qual INT    coefficient for homopolymer errors [100]
  -I, --skip-indels        do not perform indel calling
  -L, --max-idepth INT     maximum per-file depth for INDEL calling [250]
  -m, --min-ireads INT     minimum number gapped reads for indel candidates [1]
  -o, --open-prob INT      Phred-scaled gap open seq error probability [40]
  -p, --per-sample-mF      apply -m and -F per-sample for increased sensitivity
  -P, --platforms STR      comma separated list of platforms for indels [all]
      --input-fmt-option OPT[=VAL]
             Specify a single input file format option in the form
             of OPTION or OPTION=VALUE
      --reference FILE
             Reference sequence FASTA FILE [null]
```

If you are running this on datasets with large numbers of datasets with limited coverage where recombination is a factor, you can obtain increased sensitivity by passing all the BAM files to the variant caller simultaneously (hence the multiple BAM file options in samtools).

As the samtools mpileup command outputs an unfriendly output, we will pass it directly to the bcftools view command using the linux pipe ('|'). Type the following:

Part 2:   *Task 22: Identify SNPs and Indels using automated variant callers*

```
samtools mpileup -v -u -P Illumina --reference
~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna
E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam >  var.raw.vcf
```

This may take 10 minutes or so and will generate a VCF file containing the raw unfiltered variant calls for each position in the genome. Note that we are asking samtools mpileup to generate uncompressed VCF output with the -v and -u options. -P tells samtools it is dealing with Illumina data so that it can apply to the correct model to help account for mis-calls or indels.

This output by itself is not useful to us since it contains information on each position in the genome. So lets use the sister package of samtools, called bcftools to call what it thinks are the variant sites:

```
About:   SNP/indel variant calling from VCF/BCF. To be used in conjunction with samtools mpileup.
         This command replaces the former "bcftools view" caller. Some of the original
         functionality has been temporarily lost in the process of transition to htslib,
         but will be added back on popular demand. The original calling model can be
         invoked with the -c option.
Usage:   bcftools call [options] <in.vcf.gz>

File format options:
    --no-version                do not append version and command line to the header
  -o, --output <file>           write output to a file [standard output]
  -O, --output-type <b|u|z|v>   output type: 'b' compressed BCF; 'u' uncompressed BCF; 'z' compressed VCF; 'v' uncompressed VCF [v]
    --ploidy <assembly>[?]      predefined ploidy, 'list' to print available settings, append '?' for details
    --ploidy-file <file>        space/tab-delimited list of CHROM,FROM,TO,SEX,PLOIDY
  -r, --regions <region>        restrict to comma-separated list of regions
  -R, --regions-file <file>     restrict to regions listed in a file
  -s, --samples <list>          list of samples to include [all samples]
  -S, --samples-file <file>     PED file or a file with an optional column with sex (see man page for details) [all samples]
  -t, --targets <region>        similar to -r but streams rather than index-jumps
  -T, --targets-file <file>     similar to -R but streams rather than index-jumps
    --threads <int>             number of extra output compression threads [0]

Input/output options:
  -A, --keep-alts               keep all possible alternate alleles at variant sites
  -f, --format-fields <list>    output format fields: GQ,GP (lowercase allowed) []
  -g, --gvcf <int>,[...]        group non-variant sites into gVCF blocks by minimum per-sample DP
  -i, --insert-missed           output also sites missed by mpileup but present in -T
  -M, --keep-masked-ref         keep sites with masked reference allele (REF=N)
  -V, --skip-variants <type>    skip indels/snps
  -v, --variants-only           output variant sites only

Consensus/variant calling options:
  -c, --consensus-caller        the original calling method (conflicts with -m)
  -C, --constrain <str>         one of: alleles, trio (see manual)
  -m, --multiallelic-caller     alternative model for multiallelic and rare-variant calling (conflicts with -c)
  -n, --novel-rate <float>,[...] likelihood of novel mutation for constrained trio calling, see man page for details [1e-8,1e-9,1e-9]
  -p, --pval-threshold <float>  variant if P(ref|D)<FLOAT with -c [0.5]
  -P, --prior <float>           mutation rate (use bigger for greater sensitivity) [1.1e-3]
```

```
bcftools call -c -v --ploidy 1 -O v -o var.called.vcf var.raw.vcf
```

Note that we are asking bcftools to call using assuming a ploidy of 1 and to output only the variant sites in VCF format. Using grep we can count how many sites were identified as being variant sites (i.e. sites with a potential mutation). We ask grep not to count lines beginning with a comment (#).

```
grep -v -c  "^#" var.called.vcf
```

You should find 320 or so sites.

Now we just need to filter this a bit further to ensure we only retain regions where we have >90% allele frequency:

vcftools --minDP 10 --min-alleles 2 --max-alleles 2 --non-ref-af 0.9 --vcf var.called.vcf --recode --out var.called.filt.vcf

Once complete, view the file using the 'more' command. You should see something similar to: (lines beginning with # are just comment lines explaining the output)

```
#CHROM  POS      ID   REF   ALT   QUAL   FILTER  INFO
U00096.3  378700  .    A     C     222    .       DP=47;VDB=3.492280e-01;AF1=1;AC1=2;DP4=0,0,20,26;MQ=60;FQ=-165
U00096.3  566173  .    C     G     140    .       DP=74;VDB=1.335471e-01;RPB=-1.366788e+00;AF1=0.5;AC1=1;DP4=22,35,7,9;MQ=60;FQ=143;PV4=0.78,0.051,1,1
U00096.3  566205  .    T     C     152    .       DP=70;VDB=3.660676e-02;RPB=-2.810193e-01;AF1=0.5;AC1=1;DP4=22,31,6,9;MQ=60;FQ=155;PV4=1,1,1,1
U00096.3  566245  .    G     A     133    .       DP=67;VDB=1.726489e-02;RPB=7.739471e-01;AF1=0.5;AC1=1;DP4=22,29,5,9;MQ=60;FQ=136;PV4=0.76,1,1,0.35
U00096.3  566277  .    C     T     55     .       DP=63;VDB=3.921215e-03;RPB=2.597793e-01;AF1=0.5;AC1=1;DP4=25,28,3,6;MQ=60;FQ=58;PV4=0.49,1,1,1
U00096.3  566323  .    C     T     71     .       DP=58;VDB=6.304791e-03;RPB=2.418227e+00;AF1=0.5;AC1=1;DP4=25,23,3,6;MQ=60;FQ=74;PV4=0.47,1,1,1
U00096.3  566326  .    T     C     57     .       DP=57;VDB=5.476300e-03;RPB=2.654789e+00;AF1=0.5;AC1=1;DP4=24,23,3,6;MQ=60;FQ=60;PV4=0.47,1,1,1
U00096.3  566332  .    T     G     26     .       DP=57;VDB=3.998488e-03;RPB=2.444295e+00;AF1=0.5;AC1=1;DP4=25,22,3,7;MQ=60;FQ=29;PV4=0.3,0.32,1,1
U00096.3  566356  .    T     C     71     .       DP=60;VDB=3.343644e-02;RPB=3.626135e+00;AF1=0.5;AC1=1;DP4=25,21,3,7;MQ=60;FQ=74;PV4=0.3,0.49,1,0.13
```

You can see the chromosome, position, reference and alternate allele as well as a quality score for the SNP. This is a VCF file (Variant Call File). This is a standard developed for the 1000 genomes project. The full specification is given at http://samtools.github.io/hts-specs/VCFv4.2.pdf

The lines starting DP and INDEL contain various details concerning the variants. For haploid organisms, most of these details are not necessary.

This forms our definitive list of variants for this sample.

Take a look at some of the variants we just excluded, was it justified. Remember there is no filter that can keep all the correct variants and remove all the dubious!

You can load the VCF file to IGV:
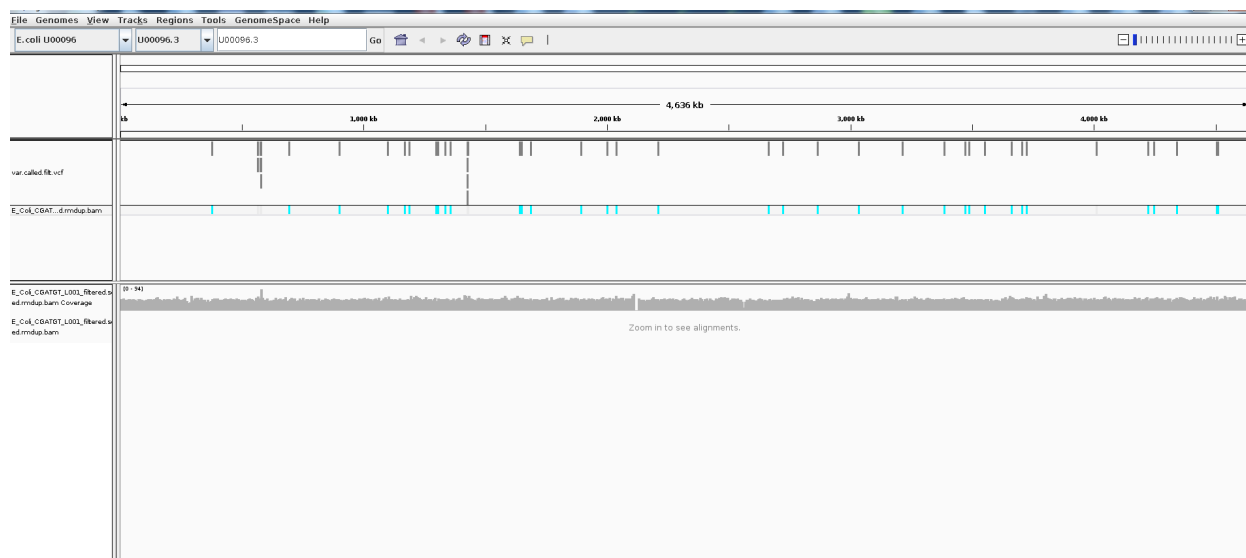
Task 23: Compare the variants found using this method to those you found in the manual section

Can you see any variants which may have been missed? Often variants within a few bp of indels are filtered out as they could be spurious SNPs thrown up by a poor alignment. This is especially the case if you use non-gapped aligners such as Bowtie.

Quickly locating genes which are missing compared to the reference

We can use a command from the BEDTools package (http://bedtools.readthedocs.org/en/latest/) to identify annotated genes which are not covered by reads across their full length.

Type the following on one line:

```
coverageBed -a ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.gff -b
E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam > gene_coverage.txt
```

This should only take a minute or so. The output contains one row per annotated gene - the 16th column contains the proportion of the gene that is covered but reads from our sequencing. 1.00 means the gene is 100% covered and 0.00 means no coverage.

If we sort by this column we can see which genes are missing
```
sort -t $'\t' -g -k 13 gene_coverage.txt | more
```

There is another region of about 10kb which is absent from out sequences - can you find it in IGV?

Evaluating the impact of variants

So far we have found a number of genes missing from this strain of *E.coli* which obviously could have a phenotypic effect. Let's now take a closer look at the variants. We'd like to obtain a list of genes in which these variants occur and whether they result in amino acid changes.

To do this we'll use a custom perl script developed by David Studholme and Konrad Paszkiewicz.

We'll just need the reference annotation, sequence and the VCF file containing the SNPs.

Task 24: Determine the effect of variants

Type (all on one line):
```
snp_comparator.pl 10
~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna
~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.gff
var.called.filt.recode.vcf  > snp_report.txt
```

You will see lots of warnings about 'Use of uninitialized value $gene_name - you can ignore these.

This program takes the information from the reference sequence and annotation, and the VCF SNP files and determines whether the variant occurs within a gene, and if so the effect of each mutation.

Once complete, view the snp_report.txt file using the more command:

```
## Table of SNP and Indel occurences between these samples. Note that any comma-separated values (e.g. A,C indicate potential heterozygosit
Chrom   Pos     Ref     out.snps.vcf4   Gene description        Status
U00096.3        1101543 T       A       curli production assembly%2Ftransport outer membrane lipoprotein    ,non-silent aaa -> Taa;
U00096.3        1169836 A       G       L%2CD-transpeptidase linking Lpp to murein    ,non-silent ctg -> cCg;
U00096.3        1189980 A       G       response regulator in two-component regulatory system with PhoQ    ,silent  act -> acC;
U00096.3        1299464 T       G
U00096.3        1301992 A       T       oligopeptide transporter subunit     ,non-silent aat -> Tat;
U00096.3        1301999 G       A       oligopeptide transporter subunit     ,non-silent agc -> aAc;
U00096.3        1302190 A       G       oligopeptide transporter subunit     ,non-silent aac -> Gac;
U00096.3        1305442 T       G       oligopeptide transporter subunit     ,non-silent gtc -> gGc;
```

In later workshops we will see how we can use this program to compare results between different strains.

You can also use tools such as SNPEff to evaluate the effect of variants (http://snpeff.sourceforge.net/index.html)

Task 25: Check each variant in IGV

N.B. If a variant doesn't seem to match what the snp_report file says, check the reverse reading frames.

That concludes the first part of the course. You have successfully, QC'd, filtered, remapped and analysed a whole bacterial genome! Well done!

In the next instalment we will be looking at how to extract and assemble unmapped reads. This will enable us to look at material which may be present in the strain of interest but not in the reference sequence.

Part 3:

Assembly of Unmapped Reads
Introduction

In this section of the workshop we will continue the analysis of a strain of *E.coli*. In the previous section we cleaned our data, checked QC metrics, mapped our data and obtained a list of variants and an overview of any missing regions.

Now, we will examine those reads which did not map to the reference genome. We want to know what these sequences represent. Are they novel genes, plasmids or just contamination?

To do this we will extract unmapped reads, evaluate their quality, prepare them for de novo assembly, assemble them using SPAdes, generate assembly statistics and then produce some annotation via Pfam, BLAST and RAST.

Extraction and QC of unmapped reads

Task 1: Extract the unmapped reads

First of all make sure you are in the ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter directory (hint: use the cd command). Then create a directory called unmapped_assembly in which we will do our de novo assembly and analysis.

    mkdir unmapped_assembly/
    cd unmapped_assembly/

Now we will use the bam2fastq program (http://gsl.hudsonalpha.org/information/software/bam2fastq) to extract from the BAM file just those reads which did NOT map to the reference genome. The bam2fastq program has a number of options, most of which are self-explanatory. Type (all on one line):

    bam2fastq --no-aligned -o una-
    ligned#.fastq ../remapping_to_reference/E_Coli_CGATGT_L001_filtered.sorted.rmdup.bam

The --no-aligned option means only extract reads which did not align. The -o unaligned\# means dump read 1 into a file called unaligned_1.fastq and read 2 into a file unaligned_2.fastq. Below we can see that the program has successfully created the two files.

```
[ec2-user@ip-10-171-67-183 unmapped_assembly]$ bam2fastq --no-aligned -o unaligned#.fastq ../rem
apping_to_reference/E_Coli_CGATGT_L001_filtered.sorted.fillmd.rmdup.bam
This looks like paired data from lane 8.
Output will be in unaligned_1.fastq and unaligned_2.fastq
1250574 sequences in the BAM file
351638 sequences exported
WARNING: 1414 reads could not be matched to a mate and were not exported
```

Note that some reads were singletons (i.e. one read mapped to the reference, but the other did not). These will not be included in this analysis.

Task 2
Check that the number of entries in both fastq files is the same. Also check that the last few entries in the read 1 and read 2 files have the same header (i.e. that they have been correctly paired).

Task 3: Evaluate QC of unmapped reads

Use the fastqc program to look at the statistics and QC for the unaligned_1.fastq and una-ligned_2.fastq files.

Do these look reasonably good? Remember, some reads will fail to map to the reference because they are poor quality, so the average scores will be lower than the initial fastqc report we did in the remapping workshop. The aim here is to see if it looks as though there are reads of reasonable quality which did not map.

Assuming these reads look ok, we will proceed with preparing them for de novo assembly.

De novo assembly

de novo is a Latin expression meaning "from the beginning," "afresh," "anew," "beginning again." when we perform a de novo assembly we try to reconstruct a genome or part of the genome from our reads without making any prior assumptions (in contrast to remapping where we compare out reads to what we think is a close reference sequence).

The advantage is that is that de novo assembly can reveal completely novel results, identify horizontal gene transfer events for example. The disadvantage is that it is difficult to get a good assembly from short reads and it can be prone to misleading results due to contamination and mis-assembly.

Task 4: Learn more about de novo assemblers

To understand more about de-novo assemblers, read the technical note at:
https://www.illumina.com/Documents/products/technotes/technote_denovo_assembly_ecoli.pdf

N.B. You will also learn more in the next section so don't worry if it doesn't all make sense immediately. You should however understand the idea of the k-mer and broadly how the assembly is built up from them.

Task 5: Generate the assembly.

We will be using an assembler called SPAdes (http://bioinf.spbau.ru/spades). It generally performs pretty well with a variety of genomes. It can also incorporate longer reads produced from PacBio sequencers that we will use later in the course.

One big advantage is that it is not just a pure assembler - it is a suite of programs that prepare the reads you have, assembles them and then refines the assembly.

SPAdes runs the modules that are required for a particular dataset and it produces the assembly with a minimum of preparation and parameter selection - making it very straightforward to produce a decent assembly. As with everything in bio-informatics you should try to assess the results critically and understand the implications for further analysis.

Let's start the assembler because it takes about 20 minutes to run (this might be a nice time to get coffee ☺):

> spades.py -k 21,33,55,77,99,127 --careful -o spades_assembly -1 unaligned_1.fastq -2 unaligned_2.fastq

We are telling it to run the SPAdes assembly pipeline with a range of k-mer sizes (-k); specifying --careful tells it to run a mismatch correction algorithm to reduce the number of errors; put the output in the spades_assembly directory and the reads to assemble.

Just because SPAdes does a lot for you does not mean you should not try to understand the process.

Have a read of this:
http://thegenomefactory.blogspot.co.uk/2013/08/how-spades-differs-from-velvet.html
It is a discussion of how SPAdes differs from Velvet another widely used assembler, it explains the overall process nicely:

"

1.   Read error correction based on k-mer frequencies using BayesHammer
2.   De Bruijn graph assembly at *multiple* k-mer sizes, not just a single fixed one.
3.   Merging of different k-mer assemblies (good for varying coverage)
4.   Scaffolding of contigs from paired end/mate pair reads
5.   Repeat resolution from paired end/mate pair data using rectangle graphs
6.   Contig error correction based on aligning the original reads with BWA back to contigs

"

Try to understand the steps in the context of the whole picture:
Can you explain why error correction of reads becomes more important as k-mer length increases?

Part 3:   Task 5: Generate the assembly.

When the assembly is complete:

```
===== Mismatch correction finished.

 * Corrected reads are in /home/genomics/workshop_data/genomics_tutorial/data/sequencing/ecol
i_exeter/unmapped_assembly/spades_assembly/corrected/
 * Assembled contigs are in /home/genomics/workshop_data/genomics_tutorial/data/sequencing/ec
oli_exeter/unmapped_assembly/spades_assembly/contigs.fasta
 * Assembled scaffolds are in /home/genomics/workshop_data/genomics_tutorial/data/sequencing/
ecoli_exeter/unmapped_assembly/spades_assembly/scaffolds.fasta
 * Assembly graph is in /home/genomics/workshop_data/genomics_tutorial/data/sequencing/ecoli_
exeter/unmapped_assembly/spades_assembly/assembly_graph.fastg
 * Paths in the assembly graph corresponding to the contigs are in /home/genomics/workshop_da
ta/genomics_tutorial/data/sequencing/ecoli_exeter/unmapped_assembly/spades_assembly/contigs.p
aths
 * Paths in the assembly graph corresponding to the scaffolds are in /home/genomics/workshop_
data/genomics_tutorial/data/sequencing/ecoli_exeter/unmapped_assembly/spades_assembly/scaffol
ds.paths

======= SPAdes pipeline finished.

SPAdes log can be found here: /home/genomics/workshop_data/genomics_tutorial/data/sequencing/
ecoli_exeter/unmapped_assembly/spades_assembly/spades.log

Thank you for using SPAdes!
```

Change to the spades_assembly directory (use cd) and look at the output.

```
genomics@genomics_2016:[~/workshop_data/genomics_tutorial/data/sequencing/ecoli_exeter/unmapp
ed_assembly/spades_assembly]$ ls -latr
total 2.1M
drwxrwxr-x  3 genomics genomics 4.0K Jan 10 10:59 ..
-rw-rw-r--  1 genomics genomics  294 Jan 10 10:59 input_dataset.yaml
-rw-rw-r--  1 genomics genomics 1.8K Jan 10 10:59 params.txt
drwxrwxr-x  3 genomics genomics 4.0K Jan 10 11:06 corrected
-rw-rw-r--  1 genomics genomics  185 Jan 10 11:06 dataset.info
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:06 K21
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:07 K33
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:08 K55
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:09 K77
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:10 K99
drwxrwxr-x  5 genomics genomics 4.0K Jan 10 11:12 K127
-rw-rw-r--  1 genomics genomics 353K Jan 10 11:12 before_rr.fasta
-rw-rw-r--  1 genomics genomics  36K Jan 10 11:12 scaffolds.paths
-rw-rw-r--  1 genomics genomics  36K Jan 10 11:12 contigs.paths
-rw-rw-r--  1 genomics genomics 708K Jan 10 11:12 assembly_graph.fastg
-rw-rw-r--  1 genomics genomics 354K Jan 10 11:15 contigs.fasta
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:15 tmp
drwxrwxr-x  4 genomics genomics 4.0K Jan 10 11:15 mismatch_corrector
drwxrwxr-x  2 genomics genomics 4.0K Jan 10 11:15 misc
-rw-rw-r--  1 genomics genomics 354K Jan 10 11:17 scaffolds.fasta
drwxrwxr-x 12 genomics genomics 4.0K Jan 10 11:17 .
-rw-rw-r--  1 genomics genomics 161K Jan 10 11:17 spades.log
```

Let's take a look at some of the more important content.

params.txt
This contains a summary of the parameters used for assembly - it is useful so you can repeat the exact analysis performed, or can remember you setting when you want to publish the genome.

contigs.fasta
This contains the final results of the assembly in fasta format.

Part 3:   Task 6: Assessment of the assembly

<u>scaffolds.fasta</u>

This contains the final results after scaffolding (which means using paired end information to join contigs together with gaps). In this case the files are identical, probably because the sum of the lengths of our paired reads is not much smaller than our insert size (there are very few large gaps between reads).

<u>assembly_graph.fastg</u>

Contains SPAdes assembly graph in FASTG format - this is a slightly different format that contains more information than fasta - for example it can contain alternative alleles in diploid assemblies. We don't need it here, but see http://fastg.sourceforge.net/FASTG_Spec_v1.00.pdf if you might be working with diploid organisms. You can use the Bandage (http://rrwick.github.io/Bandage/) to view this file.

Task 6: Assessment of the assembly

We will use QUAST (http://bioinf.spbau.ru/quast) to generate some statistics on the assembly (in the spades_assembly directory).

    quast.py --output-dir quast contigs.fasta

This will create a directory called quast and create some statistics on the assembly you produced.

    cat quast/report.txt

```
Assembly                     contigs
# contigs (>= 0 bp)          397
# contigs (>= 1000 bp)       14
# contigs (>= 5000 bp)       7
# contigs (>= 10000 bp)      2
# contigs (>= 25000 bp)      1
# contigs (>= 50000 bp)      1
Total length (>= 0 bp)       339380
Total length (>= 1000 bp)    131772
Total length (>= 5000 bp)    116713
Total length (>= 10000 bp)   86030
Total length (>= 25000 bp)   67492
Total length (>= 50000 bp)   67492
# contigs                    281
Largest contig               67492
Total length                 287109
GC (%)                       43.29
N50                          795
N75                          550
L50                          28
L75                          145
# N's per 100 kbp            0.00
```

Try to interpret the information in the light of what we were trying to do. Because we are assembling unaligned reads we are not expecting a whole chromosome to pop out. We are expecting bits of our

strain that does not exist in the reference we aligned against; possibly some contamination; various small contigs made up of reads that didn't quite align to our reference.
The N50 and L50 measures are very important in a normal assembly and we will visit them later, they are not really relevant to this assembly.

You will notice that we have 1 contig 30-60kb long - what do you think this might be? And 12 other contigs longer than 1kb. We need to find out what this is.

Analysing the de novo assembled reads

Now that we have assembled the reads and have a feel for how much (or in this case, how little) data we have, we can set about analysing it. By analysing, we mean identifying which genes are present, which organism they are from and whether they form part of the main chromosome or are an independent unit (e.g. plasmid).

We are going to take a 3-prong approach. The first will simply search the nucleotide sequences of the contigs against the NCBI non-redundant database. This will enable us to identify the species to which a given contig matches best (or most closely). The second will call open reading frames within the contigs and search those against the Swissprot database of manually curated (i.e. high quality) annotated protein sequences. Finally, we will search the open reading frames against the Pfam database of protein families (http://pfam.sanger.ac.uk).

Why not just search the NCBI blast database? Well, remember nearly all of our biological knowledge is based on homology – if two proteins are similar they probably share an evolutionary history and may thus share functional characteristics. Metrics to define whether two sequences are homologous are notoriously difficult to define accurately. If two sequences share 90% sequence identity over their length, you can be pretty sure they are homologous. If they share 2% they probably aren't. But what if they share 30%? This is the notorious twilight zone of 20-30% sequence identity where it is very difficult to judge whether two proteins are homologous based on sequence alone.

To help overcome this searching more subtle signatures may help – this is where Pfam comes in. Pfam is a database which contains protein families identified by particular signatures or patterns in their protein sequence. These signatures are modeled by Hidden Markov Models (HMMs) and used to search query sequences. These can provide a high level annotation where BLAST might otherwise fail. It also has the advantage of being much faster than BLAST.

Task 7: Search contigs against NCBI non-redundant database

Firstly we can filter out low coverage and very short contigs using a perl script:

> filter_low_coverage_contigs.pl < contigs.fasta > contigs.goodcov.fasta

The following command executes a nucleotide BLAST search (blastn) of the sequences in the contigs.fa file against the non-redundant database.
As this takes a long time to run the results have been precomputed and are available in ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/blast_precompute/unmapped_reads/

> blastn -db ~/workshop_materials/genomics_tutorial/db/blast/nt -query contigs.goodcov.fasta -evalue 1e-06 -num_threads 2 -show_gis -num_alignments 10 -num_descriptions 10 -out contigs.fasta.blastn

There are a lot of options in this command, let's go through them,

- **-db** is the prepared blast database to search
- **-evalue** apply an e-value (expectation value) cutoff (http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html) cutoff of 1e-06 to limit ourselves to statistically significant hits (i.e. in this case 1 in 1 million likelihood of a hit to a database of this size by a sequence of this length).
- **-num_alignments** and **-num_descriptions** flags tell blastn to only display the top 10 results for each hit,
- **-num_threads** that it should use 2 CPU cores
- **-show_gis** that it should include general identifier (GI) numbers in the output.
- **-out** file in which to place the output.

There is lots of information on running blast from the command line at
http://www.ncbi.nlm.nih.gov/books/NBK1763/

N.B. GI (GeneInfo Identifiers) are being phased out by NCBI so future versions of Blast and NCBI databases will not support the –show_gis option and may break some down-stream tools such as KronaTools and other databases.

Open the results file
> gedit contigs.fasta.blastn

```
BLASTN 2.2.30+

Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb
Miller (2000), "A greedy algorithm for aligning DNA sequences", J
Comput Biol 2000; 7(1-2):203-14.

Database: Nucleotide collection (nt)
          29,442,065 sequences; 84,823,117,434 total letters

Query= NODE_9_length_3631_cov_29.6618_ID_17
```

Part 3:   Task 7: Search contigs against NCBI non-redundant database

```
Length=3631
                                                             Score      E
Sequences producing significant alignments:                 (Bits)  Value

gi|549811571|gb|CP006698.1|   Escherichia coli C321.deltaA, comple...   6706   0.0
gi|383395315|gb|JQ086376.1|   Enterobacteria phage HK630, complete...   6685   0.0
gi|339305107|gb|JF340119.2|   Synthetic construct clone HO-HIS phy...   6680   0.0
gi|186702979|gb|EU421722.1|   Cloning vector lambdaS2775, complete...   6680   0.0
gi|215104|gb|J02459.1|LAMCG   Enterobacteria phage lambda, complet...   6680   0.0
gi|1066312|gb|U39286.1|CVU39286   Cloning vector TLF97-3, phage la...   6674   0.0
. . .
```

Search for our largest contig - SPAdes names the contigs by increasing size, so

> click on "Search" and then "Find" and enter NODE_1_
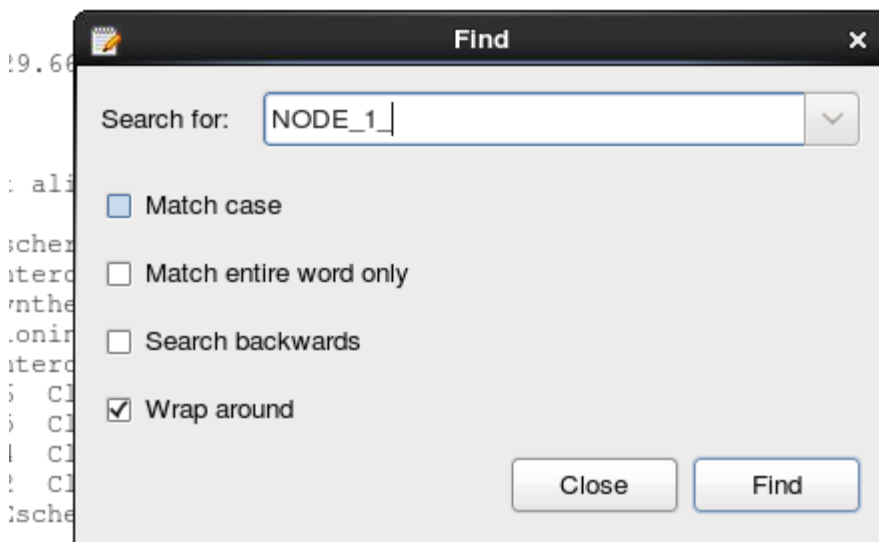

```
Query= NODE_1_length_67492_cov_565.407_ID_1

Length=67492
                                                             Score      E
Sequences producing significant alignments:                 (Bits)  Value

gi|664682453|gb|CP008801.1|   Escherichia coli KLY, complete genome   79013   0.0
gi|8918823|dbj|AP001918.1|   Escherichia coli K-12 plasmid F DNA, ...   78976   0.0
gi|619497957|gb|KJ170699.1|   Escherichia coli strain K-12 plasmid...   65330   0.0
gi|665821556|gb|KJ484626.1|   Escherichia coli plasmid pH2332-166,...   65302   0.0
gi|665821958|gb|KJ484628.1|   Escherichia coli plasmid pH2291-144,...   65213   0.0
gi|28629230|gb|AF550679.1|   Escherichia coli plasmid p1658/97, co...   64591   0.0
gi|4874241|gb|U01159.2|   Escherichia coli F sex factor transfer r...   61474   0.0
gi|665822931|gb|KJ484636.1|   Escherichia coli plasmid pC59-153, c...   41227   0.0
gi|301130432|gb|CP002090.1|   Salmonella enterica subsp. enterica ...   41026   0.0
gi|301130304|gb|CP002089.1|   Salmonella enterica subsp. enterica ...   41026   0.0
```

There are a number of good hits; notice from the contig header line that the average coverage is >500 and the coverage of our genome was around 50 - does this give you a clue to what it is?

Task 8: Obtain open reading frames

The first task is to call open reading frames within the contigs. These are designated by canonical start and stop codons and are usually identified by searching for regions free of stop codons. We will use the EMBOSS package program getorf to call these.

We will use codon table 11 which defines the bacterial codon usage table (http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi) and state that the sequences we are dealing with are not circular (they are nowhere near long enough!). We will also restrict the ORFs to just those sequences longer than 300 nucleotides (i.e. 100 amino acids). We will store the results in file contigs.orf.fa.

> getorf -table 11 -circular N -minsize 300 -sequence contigs.goodcov.fasta -outseq con-
> tigs.orf.fasta

If we look at the output file we can see that it is a FASTA formatted file containing the name of the contig on which the ORF occurs, followed by an underscore and a number (e.g. _1) to indicate the number of the ORF on that contig. The numbers in square brackets indicate the start and end position of the ORF on the contig (i.e. in nucleotide space). So the first ORF occurs on NODE 9 and is between position 934 and 1494. The third ORF occurs between positions 2400 and 2047 on the reverse strand. This is a relatively short peptide sequence and is unlikely to be a genuine peptide.

Also note that many ORFs do not start with a Methionine. This is because by default the getorf program calls ORFs between stop codons rather than start and stop codons. Primarily this is to avoid spurious ORFs due to Met residues within a protein sequence and to ensure untranslated regions are captured.

```
>NODE_9_length_3631_cov_29.6618_ID_17_1 [934 - 1494]
TERFEVSEINSQALREAAEQAMHDDWGFDADLFHELVTPSIVLELLDERERNQQYIKRRD
QENEDIALTVGKLRVELETAKSKLNEQREYYEGVISDGSKRIAKLESNEVREDGNQFLVV
RHPGKTPVIKHCTGDLEEFLRQLIEQDPLVTIDIITHRYYGVGGQWVQDAGEYLHMMSDA
GIRIKGE
>NODE_9_length_3631_cov_29.6618_ID_17_2 [2450 - 3529]
RGSEMGRRRSHERRDLPPNLYIRNNGYYCYRDPRTGKEFGLGRDRRIAITEAIQANIELF
SGHKHKPLTARINSDNSVTLHSWLDRYEKILASRGIKQKTLINYMSKIKAIRRGLPDAPL
EDITTKEIAAMLNGYIDEGKAASAKLIRSTLSDAFREAIAEGHITTNHVAATRAAKSEVR
RSRLTADEYLKIYQAAESSPCWLRLAMELAVVTGQRVGDLCEMKWSDIVDGYLYVEQSKT
GVKIAIPTALHIDALGISMKETLDKCKEILGGETIIASTRREPLSSGTVSRYFMRARKAS
GLSFEGDPPTFHELRSLSARLYEKQISDKFAQHLLGHKSDTMASQYRDDRGREWDKIEIK
>NODE_9_length_3631_cov_29.6618_ID_17_3 [2400 - 2047] (REVERSE SENSE)
FVEQILSSILNRRWEYPAFPNPSTNCFKASWTSLACVPLLKCQVHRKVSAITRKKKPPSG
GLVFFQFFNSNIGYVCMCYLRPYHPVVVAVVDVLRFDNSVEWLSIPFSCDSEVHLSSP
```

Task 9: Search open reading frames against NCBI non-redundant database

The first thing we can do with these open reading frames is to search them against the NCBI non-redundant database of protein sequences to see what they may match.

Here we will perform a BLAST search using the non-redundant (nr) database, using the blastp program and store the results in contigs.orf.blastp. We'll apply an e-value (expectation value) (http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html) cutoff of 1e-06 to limit ourselves to statistically significant hits (i.e. in this case 1 in 1 million likelihood of a hit to a database of this size by a sequence of this length). The –num_alignments and num_descriptions flags tell blastp to only display the top 10 results for each hit, the num_threads tells blastp to use 2 CPU cores and –show_gis tells blastp it should include general identifier (GI) numbers in the output.

First reduce the number of orfs so that we have a manageable number - this small perl program selects 10% of the orfs.

    reduce_fasta_10x.pl < contigs.orf.fasta > contigs.orf.small.fasta

Then you would type (all on one line). HOWEVER this takes several hours therefore the results have been precomputed in ~/workshop_materials/genomics_tutorial/data/sequencing/ecoli_exeter/blast_precompute/unmapped_reads/

    blastp -db ~/genomics_tutorial/db/blast/nr -query contigs.orf.small.fasta -evalue 1e-06 -num_threads 2 -show_gis -num_alignments 10 -num_descriptions 10 -out contigs.orf.blastp

Task 10: Review the BLAST format

Open the results file with gedit and search for plasmid in the text. You should find a number of hits to plasmid related proteins - one example is below - can you find any others? (Remember we only checked 10% of the orfs we found). This evidence is not conclusive, but combined with the high coverage over, it is starting to look like this contig is a plasmid.

```
Query= NODE_1_length_67492_cov_565.407_ID_1_32 [31455 - 31889]

Length=145
                                                                Score      E
Sequences producing significant alignments:                    (Bits)  Value

gi|446834068|ref|WP_000911324.1|  MULTISPECIES: pirin            275    3e-92
gi|446834058|ref|WP_000911314.1|  pirin                         273    1e-91
gi|446834061|ref|WP_000911317.1|  pirin                         271    1e-90
gi|446834059|ref|WP_000911315.1|  pirin                         269    6e-90
gi|545289568|ref|WP_021572485.1|  hypothetical protein          269    6e-90
gi|446834062|ref|WP_000911318.1|  MULTISPECIES: pirin           269    6e-90
gi|585223672|ref|WP_024168023.1|  plasmid maintenance protein   268    9e-90
gi|723058272|ref|WP_033552985.1|  plasmid maintenance protein   268    9e-90
gi|446834056|ref|WP_000911312.1|  plasmid maintenance protein   268    1e-89
gi|446834060|ref|WP_000911316.1|  pirin                         268    1e-89


>gi|446834068|ref|WP_000911324.1| MULTISPECIES: pirin [Escherichia]
 gi|32470009|ref|NP_862949.1| plasmid maintenance protein [Escherichia coli]
 gi|689926354|ref|YP_009060131.1| PIN domain protein [Escherichia coli]
 gi|691230621|ref|YP_009070585.1| VapC toxin protein [Escherichia coli]
 gi|28629266|gb|AAO49546.1| hypothetical protein [Escherichia coli]
 gi|323184064|gb|EFZ69443.1| PIN domain protein [Escherichia coli OK1357]
 gi|325495739|gb|EGC93600.1| plasmid maintenance protein [Escherichia fergusonii ECD227]
 gi|385154377|gb|EIF16391.1| plasmid maintenance protein [Escherichia coli O32:H37 str. P4]
```

Additional checks:
Task 11: Check that the contigs do not appear in the reference sequence

In theory, the unmapped reads used to generate the contigs should not assemble into something which will map against the genome. However, it is always possible (especially with more complex genomes), that this might happen. To double-check move back to the folder containing the contigs.goodcov.fasta:

> blastn -subject ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna -query contigs.goodcov.fasta | more

Here we use the BLAST+ package in a different mode to compare two sequences against each other. Unlike the previous examples where we have searched against a database of sequences, here we are doing a simple search of the contigs against the reference genome we are using. Scroll down a little…

```
Query= NODE_17_length_917_cov_10.3076_ID_33

Length=917

Subject= gi|545778205|gb|U00096.3| Escherichia coli str. K-12 substr. MG1655,
complete genome

Length=4641652


 Score =   193 bits (104),  Expect = 3e-49
 Identities = 186/227 (82%), Gaps = 0/227 (0%)
 Strand=Plus/Plus

Query  68       ACGGCATCCACGAAGGCGACAGAGGCTGCGGGAAGTGCGGTATCAGCATCGCAGAGCAAA  127
                |||||||||||||||||||||||||||||| || |||||||    | ||| | |||||||||
Sbjct  1430285  ACGGCATCCACGAAGGCGACAGAGGCTGCTGGCAGTGCGACGGCGGCAGCTCAGAGCAAA  1430344
```

You can see that some of the contigs that have been assembled hit the reference sequence. In the record above the evalue is 3e-49 which is massively significant; however, the evalue is calculated as the chance of a hit this close against a random sequence of the same size. Since our subject sequence is now very small and we know it is related to our strain it is not surprising that there are some hits. We are concerned about whole contigs that map closely to the reference genome.

Task 12: Run open reading frames through pfam_scan

Pfam is a database of protein families. They are grouped together using a number of criteria based on their function. For more information read http://en.wikipedia.org/wiki/Pfam. Pfam is grouped into several databases depending on the level of curation. Pfam-A is high-quality manual curation and consists of around 12,500 families. Pfam-B is full of automated predictions which may be informative but should not be relied upon without additional evidence. Pfam will also search for signatures of active-sites if you specify the correct flag.

Here we want to search the Pfam database of Hidden Markov Models to see which protein families are contained within this contig. You'll notice that this runs considerably faster than BLAST. Here we

Part 3: *Task 12: Run open reading frames through pfam_scan*

search using the contigs.orf.fa file against the Pfam databases ~/genomics_tutorial/db/pfam/ and output the results to contigs.orf.pfam. We'll use 2 CPU cores for the search and state that we want to search PfamB entries as well as active site residues.

This step might take about 30 minutes. So you can get a coffee in the meantime.

pfam_scan.pl -fasta contigs.orf.fasta -dir ~/workshop_materials/genomics_tutorial/db/pfam/ -outfile contigs.orf.pfam -cpu 2 -pfamB –as

View the output using gedit:

Search for NODE_9 (for example).

```
# <seq id> <alignment start> <alignment end> <envelope start> <envelope end> <hmm acc> <hmm name> <type> <hmm start> <hmm end> <hmm le
<bit score> <E-value> <significance> <clan> <predicted_active_site_residues>

NODE_9_length_3631_cov_29.6618_ID_17_1      7    106      7    106 PF13935.1   Ead_Ea22        Family   1   139   139    103.8
30   1 No_clan
NODE_9_length_3631_cov_29.6618_ID_17_1     77    113     77    126 PB009353    Pfam-B_9353     Pfam-B   1    37    82     53.4
14   NA NA
NODE_9_length_3631_cov_29.6618_ID_17_2      5     74      5     75 PF09003.5   Phage_integ_N   Domain   1    75    76     89.5
26   1 CL0081
NODE_9_length_3631_cov_29.6618_ID_17_2     85    162     84    162 PF02899.12  Phage_int_SAM_1 Domain   2    84    84     24.1
05   1 CL0469
NODE_9_length_3631_cov_29.6618_ID_17_2    183    349    182    353 PF00589.17  Phage_integrase Family   2   169   173    115.1
33   1 CL0382   predicted_active_site[239,312,216,346,337,315]
NODE_9_length_3631_cov_29.6618_ID_17_3     53    115     48    117 PB009641    Pfam-B_9641     Pfam-B  50   112   168     23.3
```

The 8th column shows the type of entry that was hit in the pfam database.

Go to http://pfam.sanger.ac.uk and enter the Pfam domain in the search box.

Let's take a look at Pfam domain Phage_integ_N

There are a lot of hits to phage domains and domains that manipulate DNA. You might expect this as these sequences have presumably been incorporated into our strain since it diverged from the reference.

Also look at domains (the most specific type of hit) from our large contig NODE_1_..... is there any evidence for it being a plasmid?
The Pfam-B matches do not tell you much that is useful.

Examine one or two more domains from your results file - is there anything interesting?

Analysing the results in RAST

By now you should be able to see that analysing results for de novo assembled reads of any sort can be difficult and time-consuming. Bear in mind that we have only been faced with a single contig of 3kb. Quite often you may find yourself dealing with hundreds, if not thousands of contigs. Some will be a few 100kb long. Others may only be 200-300bp. How should we go about analysing these in a more efficient manner? There are a number of options here. For eukaryotes I would suggest looking at MAKER (http://www.yandell-lab.org/software/maker.html). For prokaryotes the situation is somewhat easier and we can use a web-based service known as RAST. This is not the only service (Xbase is another), but it is one of the most common.

RAST is a website where you upload the results of your de novo assembly and RAST will attempt to provide annotation in commonly used GFF and Genbank formats. This can be used to load up the annotation in Artemis or Apollo. Alternatively RAST has its own in-built viewer.

Task 13 (Optional)
Log in to RAST

Within your instance, go to http://rast.nmpdr.org/ Log-in with the details RAST provided to you before you started this series of workshops. If you do not have one, you may need to wait several days for your login to be issued by RAST. Please skip ahead and come back to this section.

Task 14 (Optional)
Upload the assembled contigs and annotate using RAST

Click on Your jobs->Upload New Job

Upload the contigs.fasta file obtained by the de novo assembly of unmapped reads. Click on "Use this data and go to step 2".

Upload a Genome

Review genome data

We have analyzed your upload and have computed the following information.

Contig statistics

Statistic	As uploaded	After splitting into scaffolds
Sequence size	338383	338383
Number of contigs	394	394
GC content (%)	42.8	42.8
Shortest contig size	128	128
Median sequence size	530	530
Mean sequence size	858.8	858.8
Longest contig size	67492	67492

Please enter or verify the following information about this organism:

- RAST bases its genome identifiers on NCBI taxonomy-IDs.
- If you provide a valid taxonomy-ID, RAST will attempt to fill in the genome metadata for you.
- If you leave the taxonomy-ID field blank, RAST will assign a meaningless taxonomy-ID, and you will need to fill in the below genome metadata manually.
- If you plan on submitting this genome to PATRIC you will need to provide the most descriptive NCBI taxonomic grouping possible. If you leave the taxonomy-ID field blank, RAST will assign a meaningless taxonomic identifier and the genome will not be suitable for submission to PATRIC. We discuss the motivationand process for submitting your genome to PATRIC in this document.
- You may search for the taxonomy-ID of your organism using the search facilities at the NCBI taxonomy browser.

Genome information:

Taxonomy ID:	562	Look up taxonomy ID at NCBI.	• If you enter a valid NCBI taxonomy-ID and click "Fill in form based on NCBI taxonomy-ID," RAST will attempt to automatically fill in the form below. You may then edit any incorrect field values before going to the next step. • If you do not know the taxonomy-ID of your genome, please leave the taxonomy-ID field blank, and fill in the fields manually.
Taxonomy string:	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia		• If you leave this field blank, RAST will fill in a dummy taxonomy string of the form *"Domain; genus species strain.",* based on the form entries below.
Domain:	⦿ Bacteria ○ Archaea ○ Virus		
Genus:	Escherichia		• E.g., *"Escherichia".* If you do not know the genus, leave blank, and it will default to "Unknown".
Species:	coli		• E.g., *"coli".* If you do not know the species, leave blank, and it will default to "sp.".
Strain:			• E.g. "*str. K12 substr. MG1655".* This field is optional. (May also be used as a comment.)
Genetic Code:	⦿ 11 (Archaea, most Bacteria, most Virii, and some Mitochondria) ○ 4 (Mycoplasmaea, Spiroplasmaea, Ureoplasmaea, and Fungal Mitochondria)		

We know this is an *E.coli* genome so we can enter 562 as the Taxonomy ID and click on 'Fill in form based on NCBI taxonomy-ID'. If you're dealing with a different organism, be sure to change this number. RAST will automatically split any scaffolds (i.e. contigs with bits missing in the middle – denoted by Ns). Then click "Use this data and go to step 3".

Upload a Genome

Complete Upload

Please consider the following options for the RAST annotation pipeline:

RAST Annotation Settings:

Choose RAST annotation scheme	Classic RAST ▾	Choose "Classic RAST" for the current production RAST, or "RASTtk" for the new modular RAS
Select gene caller	RAST ▾	Please select which type of gene calling you would like RAST to perform. Note that using GLIM backfilling of gaps.
Select FIGfam version for this run	Release70 ▾	Choose the version of FIGfams to be used to process this genome.
Automatically fix errors?	☑ Yes	The automatic annotation process may run into problems, such as gene candidates overlappin these problems (even if that requires deleting some gene candidates), please check this box.
Fix frameshifts?	☐ Yes	If you wish for the pipeline to fix frameshifts, check this option. Otherwise frameshifts will not
Build metabolic model?	☑ Yes	If you wish RAST to build a metabolic model for this genome, check this option.
Backfill gaps?	☑ Yes	If you wish for the pipeline to blast large gaps for missing genes, check this option.
Turn on debug?	☐ Yes	If you wish debug statements to be printed for this job, check this box.
Set verbose level	0	Set this to the verbosity level of choice for error messages.
Disable replication	☐ Yes	Even if this job is identical to a previous job, run it from scratch.

Finish the upload

Replicate the settings above and click on 'Finish the upload'.
Your job may take several hours to run. In the meantime, proceed to the next workshop and come back to this later.

Part 3:   Task 14 (Optional)

Once complete, RAST should email you a message. You can then view the results or download them in standardized formats (e.g. GFF3, Genbank, EMBL etc).

On the start page click on view details for your annotation

Progress bar color key:

not started
queued for computation
in progress
requires user input
failed with an error
successfully completed

Jobs you have access to :

Job ▲▼	Owner ▲▼	ID ▲▼	Name ▲▼	Num contigs ▲▼	Size (bp) ▲▼	Creation Date	Annotation Progress	Status com ▼
205173	O'Neill, Paul	562.4461	Escherichia coli	15	129530	2014-12-04 10:33:46	[*view details*]	complete

You will get a summary of the sequence you uploaded and you have the ability to download the annotations to your computer

Job Details #205173

» Browse annotated genome in SEED Viewer

» View metabolic model

» Available downloads for this job: GFF3 ▼ | Download | | Update download files |

Download the GFF3 annotation and open it in a text editor

```
##gff-version 3
NODE_10_length_3324_cov_22.7003_ID_19   FIG    CDS    249    1163   .    -    0
    ID=fig|562.4461.peg.1;Name=FIG010773: NAD-dependent epimerase/dehydratase
NODE_10_length_3324_cov_22.7003_ID_19   FIG    CDS    1160   2782   .    -    2
    ID=fig|562.4461.peg.2;Name=FIG022758: Long-chain-fatty-acid--CoA ligase (EC
6.2.1.3);Ontology_term=KEGG_ENZYME:6.2.1.3
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    151    927    .    -    1
    ID=fig|562.4461.peg.3;Name=FIG00638373: hypothetical protein
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    973    1407   .    -    1
    ID=fig|562.4461.peg.4;Name=YcgB
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    1421   1642   .    -    2
    ID=fig|562.4461.peg.5;Name=putative cytoplasmic protein
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    1643   2326   .    -    2
    ID=fig|562.4461.peg.6;Name=Adenine-specific methyltransferase (EC
2.1.1.72);Ontology_term=KEGG_ENZYME:2.1.1.72
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    2434   2559   .    +    1
    ID=fig|562.4461.peg.7;Name=hypothetical protein
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    2573   2761   .    +    2
    ID=fig|562.4461.peg.8;Name=FIG00639560: hypothetical protein
NODE_1_length_67492_cov_565.407_ID_1    FIG    CDS    2712   3149   .    -    0
    ID=fig|562.4461.peg.9;Name=FIG01048508: hypothetical protein
```

note: your output may be different.
Scan down the list of annotations do any themes stand out?

Part 3:   Task 14 (Optional)

From the job details page:

Job Details #205173

» Browse annotated genome in SEED Viewer

» View metabolic model

» Available downloads for this job: | GFF3   ▼ | | Download | | Update download files |

Click on 'Browse annotated genome in SEED viewer'

| Subsystem Statistics | Features in Subsystems |

Subsystem Coverage

35%

65%

Subsystem Category Distribution

Subsystem Feature Counts

⊞ ■ Cofactors, Vitamins, Prosthetic Groups, Pigments (2)
⊞ ■ Cell Wall and Capsule (0)
⊞ ■ Virulence, Disease and Defense (1)
⊞ ■ Potassium metabolism (0)
⊞ ■ Photosynthesis (0)
⊞ ■ Miscellaneous (0)
⊞ ■ Phages, Prophages, Transposable elements, Plasmids (43)
⊞ ■ Membrane Transport (67)
⊞ ■ Iron acquisition and metabolism (0)
⊞ ■ RNA Metabolism (0)
⊞ ■ Nucleosides and Nucleotides (0)
⊞ ■ Protein Metabolism (2)
⊞ ■ Cell Division and Cell Cycle (0)
⊞ ■ Motility and Chemotaxis (0)
⊞ ■ Regulation and Cell signaling (4)
⊞ ■ Secondary Metabolism (0)
⊞ ■ DNA Metabolism (0)
⊞ ■ Fatty Acids, Lipids, and Isoprenoids (0)
⊞ ■ Nitrogen Metabolism (0)
⊞ ■ Dormancy and Sporulation (0)
⊞ ■ Respiration (0)
⊞ ■ Stress Response (0)
⊞ ■ Metabolism of Aromatic Compounds (0)
⊞ ■ Amino Acids and Derivatives (0)
⊞ ■ Sulfur Metabolism (0)
⊞ ■ Phosphorus Metabolism (0)
⊞ ■ Carbohydrates (0)

This gives you a hierarchical view of the subsystems.

Browse the rest of the RAST server and get a feel for the possibilities the platform may offer you.

When you're ready, move on to (or back to) the de novo assembly part of the workshop.

Part 4

Short read genomics: De-novo assembly

Introduction:

In this section of the workshop we will continue the analysis of a strain of *E.coli*. In the previous section we extracted those reads which did not map to the reference genome and assembled them. However, it is often necessary to be able to perform a de novo assembly of a genome. In this case, rather than doing any remapping, we will start with the filtered reads we obtained in part 3 of the workshop.

To do this we will a program called SPAdes to try to get the best possible assembly for a given genome. We will then generate assembly statistics and then produce some annotation via Pfam and BLAST.

Task 1: Start the Assembly

The assembly takes so the results have been **pre-computed** for you and are available in the directory **~/workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter/denovo_assembly**.

If you were to run the command it would be as follows:
```
spades.py -o denovo_assembly_rerun -1 E_Coli_CGATGT_L001_R1_001.filtered.fastq -2
E_Coli_CGATGT_L001_R2_001.filtered.fastq
```

This will create a directory called denovo_assembly_rerun to hold the results.

Assembly theory

We are using SPAdes (http://bioinf.spbau.ru/en/spades) to perform our assembly. It is a de Bruijn graph based assembler, similar to other short read assemblers like velvet (https://www.ebi.ac.uk/~zerbino/velvet/). The advantage of SPAdes is that it does lot of error correction and checking before and after the assembly which improve the final result. A downside of SPAdes is that it was designed for assembling reads from a single cell and although it does a good job with DNA prepared from a community it can leave in some low coverage sequences which are likely to be artifacts.

You can read more about the comparison here http://thegenomefactory.blogspot.co.uk/2013/08/how-spades-differs-from-velvet.html

SPAdes is also very easy to use - apart from telling it where your input files are the only parameter that you might want to choose is the length of k-mer.

K-mer length. Rather than store all reads individually which would be unfeasible for Illumina type datasets, de Bruijn assemblers convert each read to a series of k-mers and stores each k-mer once, along with information about how often it occurs and which other k-mers it links to. A short k-mer

length (e.g. 21) reduces the chance that data will be missed from an assembly (e.g. due to reads being shorter than the k-mer length or sequencing errors in the k-mer), but can result in shorter contigs as repeat regions cannot be resolved. .

When using the Velvet assembler it is necessary to try a large combination of parameters to ensure that you obtain the 'best' possible assembly for a given dataset. There is even a program called VelvetOptimiser which does it for you. However, what 'best' actually means in the context of genome assembly is ill-defined. For a genomic assembly you want to try to obtain the lowest number of contigs, with the longest length, with the fewest errors. However, although numbers of contigs and longest lengths are easy to evaluate, it is extremely difficult to know what is or isn't an error when sequencing a genome for the first time.

SPAdes allows you to choose more than one k-mer length - it then performs an assembly for each k-mer and merges the result - trying to get the best of both worlds. It actually has some pre-calculated k-mer settings based on the length of reads you have, so you don't even have to choose that.

Let's look at the assembly process in more detail:

Description of k-mers:

What are they? Let's say you have a single read:

AACTAACGACGCGCATCAAAA

The set of k-mers obtained from this read with length 6 (i.e. 6-mers) would be obtained by taking the first six bases, then moving the window along one base, taking the next 6 bases and so-on until the end of the read. E.g:

You may well ask, "So what? How does that help"? For a single read, it really doesn't help. However let's say that you have another read which is identical except for a single base:
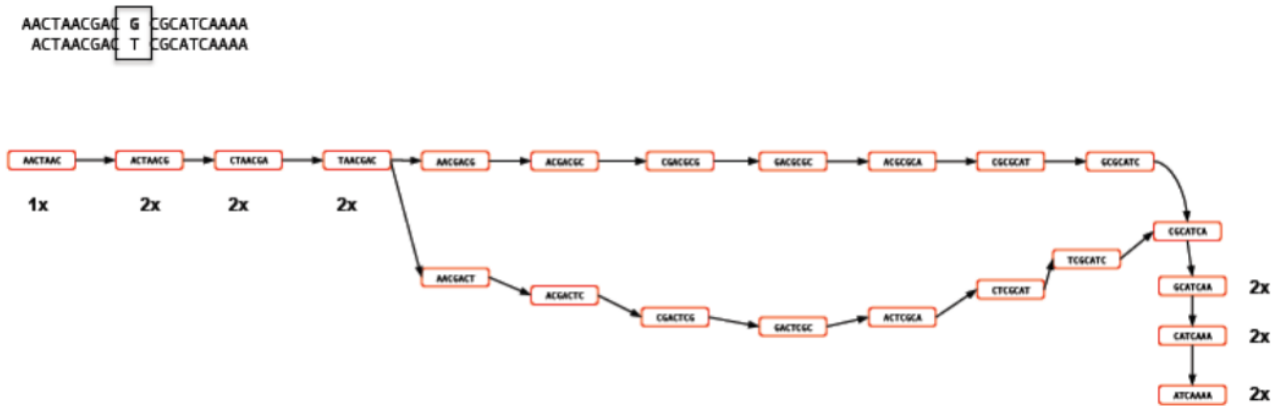
Rather than represent both reads separately, we need only store the k-mers which differ and the number of times they occur. Note the 'bubble' like structure which occurs when a single base-change occurs. This kind of representation of reads is called a 'k-mer graph' (sometimes inaccurately referred to as a de-bruijn graph).

Now let's see what happens when we add in a third read. This is identical to the first read except for a change at another location. This results in an extra dead-end being added to the path.
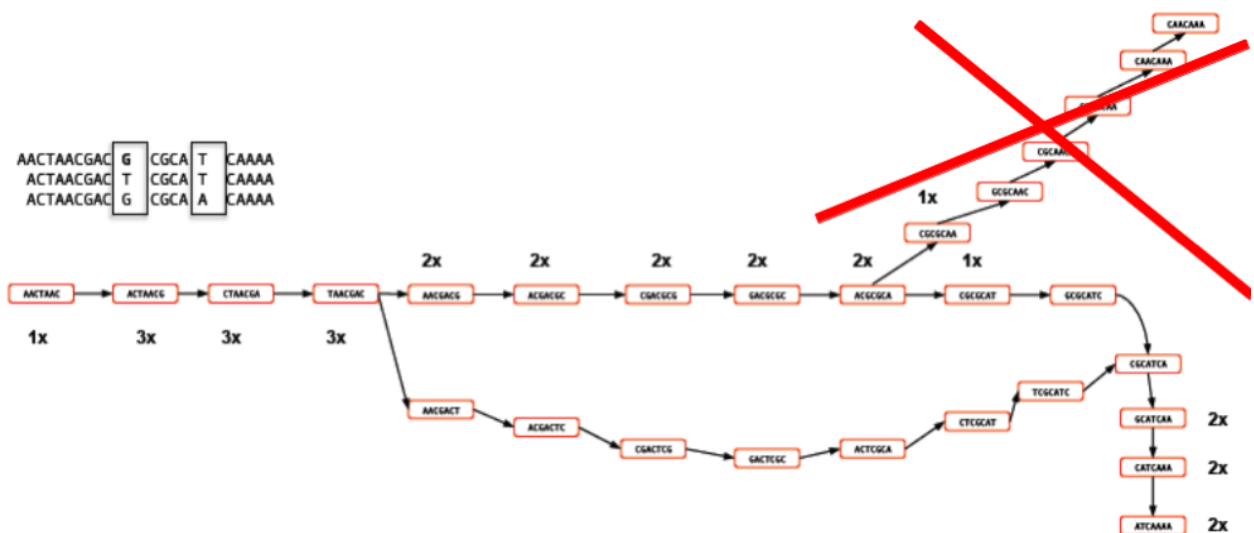
The job of any k-mer based assembler is to find a path through the k-mer graph which correctly represents the genome sequence.

Images courtesy of Mario Caccamo

Description of coverage cutoff:

In the figure above, you can see that the coverage of various k-mers varies between 1x and 3x. The question is which parts of the graph can be trimmed or removed so that we avoid any errors. As the graph stands, we could output three different contigs as there are three possible paths through the graph. However, we might wish to apply a coverage cutoff and remove the top right part of the graph because it has only 1x coverage and is more likely to be an error than a genuine variant.

In a real graph you would have millions of k-mers and thousands of possible paths to deal with. The best way to estimate the coverage cutoff in such cases is to look at the frequency plot of contig (node) coverage, weighted by length. In the example below you can see that contigs with a coverage below 7x or 8x occur very infrequently. As such it is probably a good idea to exclude those contigs which have coverage less than this – they are likely to be errors.

Description of expected coverage:
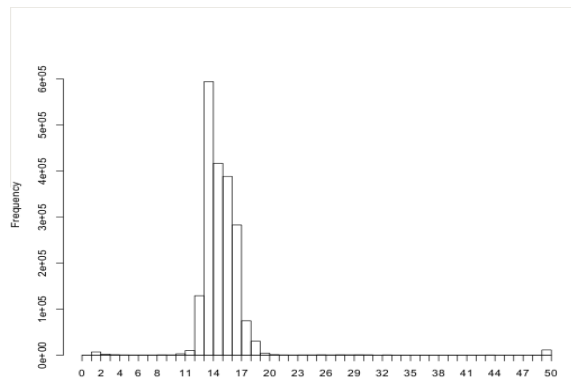
In the example below you can see a stretch of DNA with many reads mapping to it. There are two re-petitive regions A1 and A2 which have identical sequence. If we try to assemble the reads without any knowledge of the true DNA sequence, we will end up with an assembly that is split into two or more contigs rather than one.

One contig will contain all the reads which did not fall into A1 and A2. The other will contain reads from both A1 and A2. As such the coverage of the repetitive contig will be twice as high as that of the non-repetitive contig.

If we had 5 repeats we would expect 5x more coverage relative to the non-repetitive contig. As such, provided we know what level of coverage we expect for a given set of data, we can use this infor-mation to try and resolve the number of repeats we expect.

RPT A1

RPT A2

Can try to identify collapsed repeats by increased relative coverage
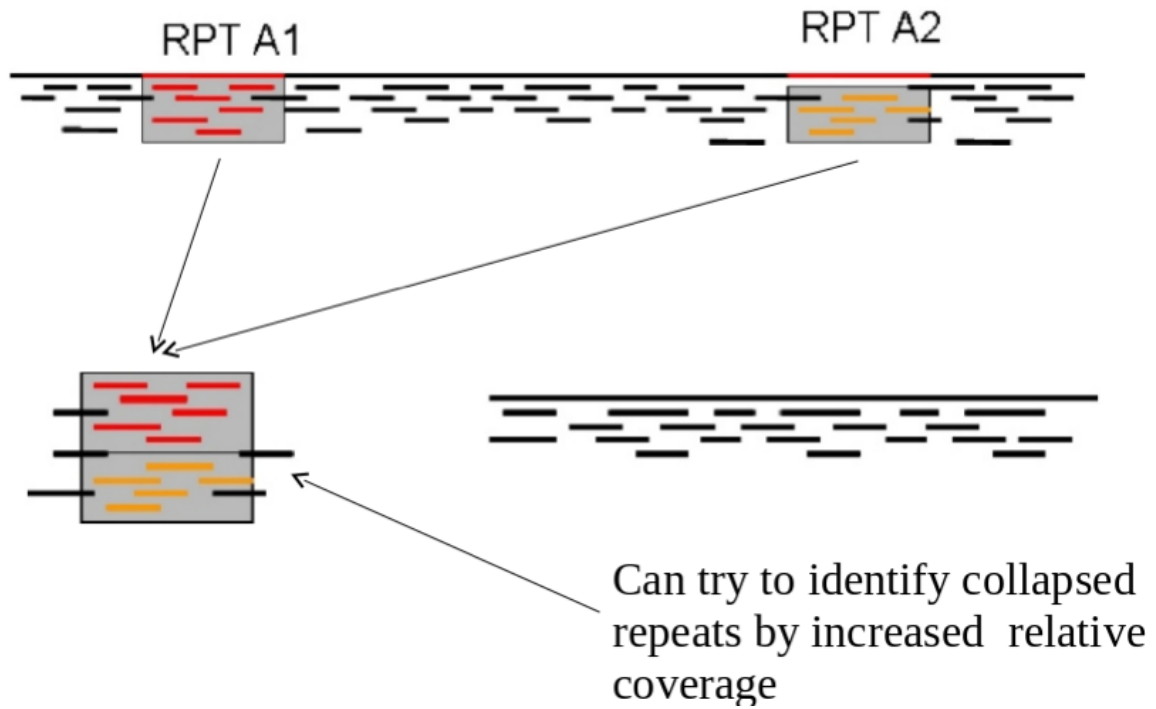
A commonly used metric to describe the effectiveness of the assembly is called N50 - see http://en.wikipedia.org/wiki/N50_statistic for details.

Task 2: Checking the assembly

Change into the denovo_assembly directory:

```
cd denovo_assembly
```

Firstly we can filter out low coverage and very short contigs using a perl script:

```
filter_low_coverage_contigs.pl < contigs.fasta > contigs.goodcov.fasta
```

We will use QUAST again (http://bioinf.spbau.ru/quast) to generate some statistics on the assembly.

```
quast.py --output-dir quast contigs.goodcov.fasta
```

This will create a directory called quast and create some statistics on the assembly you produced.

```
cat quast/report.txt
```

```
Assembly                    contigs.goodcov
# contigs (>= 0 bp)         81
# contigs (>= 1000 bp)      67
# contigs (>= 5000 bp)      49
# contigs (>= 10000 bp)     46
# contigs (>= 25000 bp)     42
# contigs (>= 50000 bp)     29
Total length (>= 0 bp)      4689514
Total length (>= 1000 bp)   4679794
Total length (>= 5000 bp)   4642981
Total length (>= 10000 bp)  4623085
Total length (>= 25000 bp)  4560613
Total length (>= 50000 bp)  4090836
# contigs                   81
Largest contig              293215
Total length                4689514
GC (%)                      50.72
N50                         136627
N75                         95318
L50                         12
L75                         21
# N's per 100 kbp           0.00
```

You can see that there are 81 contigs in the assembly - so it is still far from complete.
The N50 is 136K and the N75 is 95K so most of the assembly is in quite large contigs.

This is fairly normal for a short read assembly - don't expect complete chromosomes.

A good check at this point is to map the original reads back to the contigs.fasta file and check that all positions are covered by reads. Amazingly it is actually possible for de-novo assemblers to generate contigs to which the original reads will not map.

Task 3: Map reads back to assembly

Here we will use BWA again to index the contigs.fasta file and remap the reads. This is almost identical to the procedure we followed during the alignment section, the only difference is that instead of aligning to the reference genome, we are aligning to our newly created reference.

Make sure you are in the following directory:
~/workshop_meterials/genomics_tutorial/data/sequencing/ecoli_exeter/denovo_assembly/

Let's create a subdirectory to keep our work separate
       mkdir remapping_to_assembly
       cd remapping_to_assembly
       cp ../contigs.fasta .

Let's start by indexing the contigs.fasta file. Type:
       bwa index contigs.fasta

```
[ec2-user@ip-10-181-110-211 remapping_to_assembly]$ bwa index contigs.fasta
[bwa_index] Pack FASTA... 0.07 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 2.04 seconds elapse.
[bwa_index] Update BWT... 0.06 sec
[bwa_index] Pack forward-only FASTA... 0.04 sec
[bwa_index] Construct SA from BWT and Occ... 0.68 sec
[main] Version: 0.7.10-r789
[main] CMD: bwa index contigs.fasta
[main] Real time: 6.961 sec; CPU: 2.901 sec
[ec2-user@ip-10-181-110-211 remapping_to_assembly]$ █
```

Once complete we can start to align the reads back to the contigs. Type (all on one line):

       bwa mem -t 2 contigs.fasta ../../E_Coli_CGATGT_L001_R1_001.filtered.fastq ../../E_Coli_CGATGT_L001_R2_001.filtered.fastq > E_Coli_CGATGT_L001_filtered.sam

Once complete we can convert the SAM file to a BAM file:

       samtools view -bS E_Coli_CGATGT_L001_filtered.sam > E_Coli_CGATGT_L001_filtered.bam

Part 4   Task 3: Map reads back to assembly

And then we can sort the BAM file:

    samtools sort -o E_Coli_CGATGT_L001_filtered.sorted.bam
    E_Coli_CGATGT_L001_filtered.bam

Once completed, we can index the BAM file:

    samtools index E_Coli_CGATGT_L001_filtered.sorted.bam

We can then (at last!) obtain some basic summary statistics using the samtools flagstat command:

    samtools flagstat E_Coli_CGATGT_L001_filtered.sorted.bam

```
genomics@genomics_2016:[~/workshop_data/genomics_tutorial/data/sequencing/ecoli_
exeter/denovo_assembly/remapping_to_assembly]$samtools flagstat E_Coli_CGATGT_L0
01_filtered.sorted.bam
1269338 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 duplicates
1266061 + 0 mapped (99.74%:-nan%)
1269338 + 0 paired in sequencing
634685 + 0 read1
634653 + 0 read2
1252740 + 0 properly paired (98.69%:-nan%)
1264970 + 0 with itself and mate mapped
1091 + 0 singletons (0.09%:-nan%)
9276 + 0 with mate mapped to a different chr
7977 + 0 with mate mapped to a different chr (mapQ>=5)
```

We can see here that very few of the reads do not map back to the contigs. Importantly 98% of reads are properly paired which gives us some indication that there are not too many mis-assemblies.

Run qualimap to get some more detailed information (and some images)

    qualimap bamqc -outdir bamqc -bam E_Coli_CGATGT_L001_filtered.sorted.bam

    firefox bamqc/qualimapReport.html

Part 4   Task 3: Map reads back to assembly

In the Chromosome stats section:

Chromosome stats

Name	Length	Mapped bases	Mean coverage	Standard deviation
NODE_1_length_293215_cov_26.7248_ID_1	293215	15962208	54.44	11.39
NODE_2_length_235405_cov_25.9929_ID_3	235405	12493267	53.07	10.9
NODE_3_length_229124_cov_26.8329_ID_5	229124	11966638	52.23	10.41
NODE_4_length_227801_cov_26.3369_ID_7	227801	11934749	52.39	12.45

The larger of our contigs have a mean coverage of around 50 - which is what we would expect from our original alignment.

NODE_25_length_67492_cov_567.168_ID_49	67492	78055078	1,156.51	225.09

There is one contig which has the size of 67492 - this is exactly the same as the contig we found in the unmapped reads - that is pretty good indication that it is a separate sequence (remember we suspected a plasmid) and not integrated into the chromosome.

Let's double check that by blasting these contigs against the unmapped assembly contigs from part 4:
        blastn -subject ../contigs.goodcov.fasta -
        query ../../unmapped_assembly/spades_assembly/contigs.fasta > check_plasmid.blastn

Open the file in a text editor:
        gedit check_plasmid.blastn

and about 30% of the way down the file you should find: (hint use search/find)

```
Query= NODE_1_length_67492_cov_601.94_ID_2528

Length=67492

Subject= NODE_25_length_67492_cov_567.168_ID_49

Length=67492

 Score = 1.246e+05 bits (67474),  Expect = 0.0
 Identities = 67486/67492 (99%), Gaps = 0/67492 (0%)
 Strand=Plus/Plus
```
This shows us that this contig exactly almost matches that in the unmapped assembly, strongly supporting that this is a plasmid sequence and not integrated into the chromosomes.

Task 4: View assembly in IGV

Load up IGV
    `igv.sh`

Click Genomes -> Load Genome from File….
We are going to import the contigs we have assembled as the reference. Unlike the reference genome though, we have no annotation available. Make sure you select the contigs.goodcov.fasta file for the complete de novo assembly (not the unmapped reads assembly).

Once loaded, click on File->Load From File… select the E_Coli_CGATGT_L001_filtered.sorted.bam file. Again, make sure you load the file in the **remapping_to_assembly** directory.
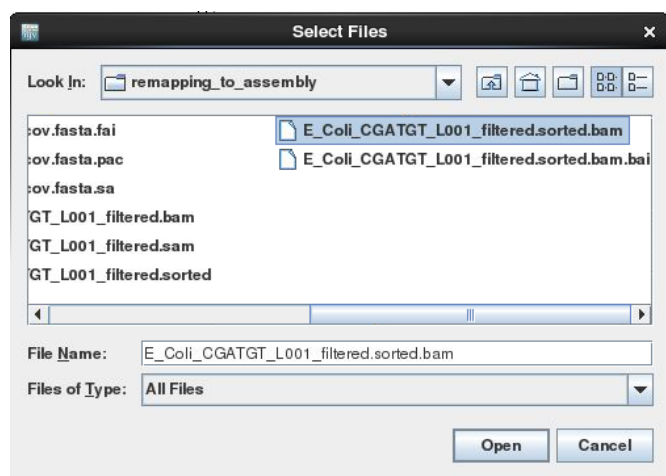
Once loaded, explore some of the contigs in IGV.
See if you can find anything unusual in any of the contigs.

Here is one to get you started.
Select **NODE_3**…
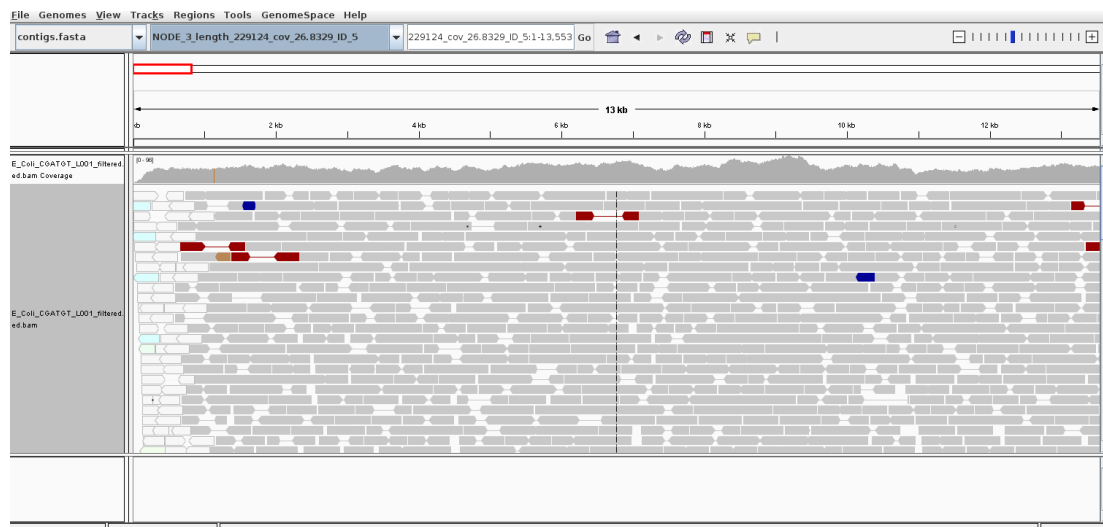
Why does the contig start and end in repetitive sequence (indicated by the white reads)? You may need to zoom in to see the details. Think about what an assembler will do if it cannot uniquely assign reads.

If an assembler cannot resolve these repetitive regions with paired-end reads or coverage information, it will generally be unable to assemble any further sequence for that contig. Therefore it is quite common to see contigs which start and end in sequence which is repeated elsewhere.

Here is another:

Select NODE_49…..

Right click on the reads and select view as pairs:

What do you think is going on here? Try blasting the contig sequence using BlastX at http://blast.ncbi.nlm.nih.gov/Blast.cgi to identify which genes the contig contains. To obtain the sequence you can right click and select 'Copy consensus sequence':

Part 4   Task 4: View assembly in IGV
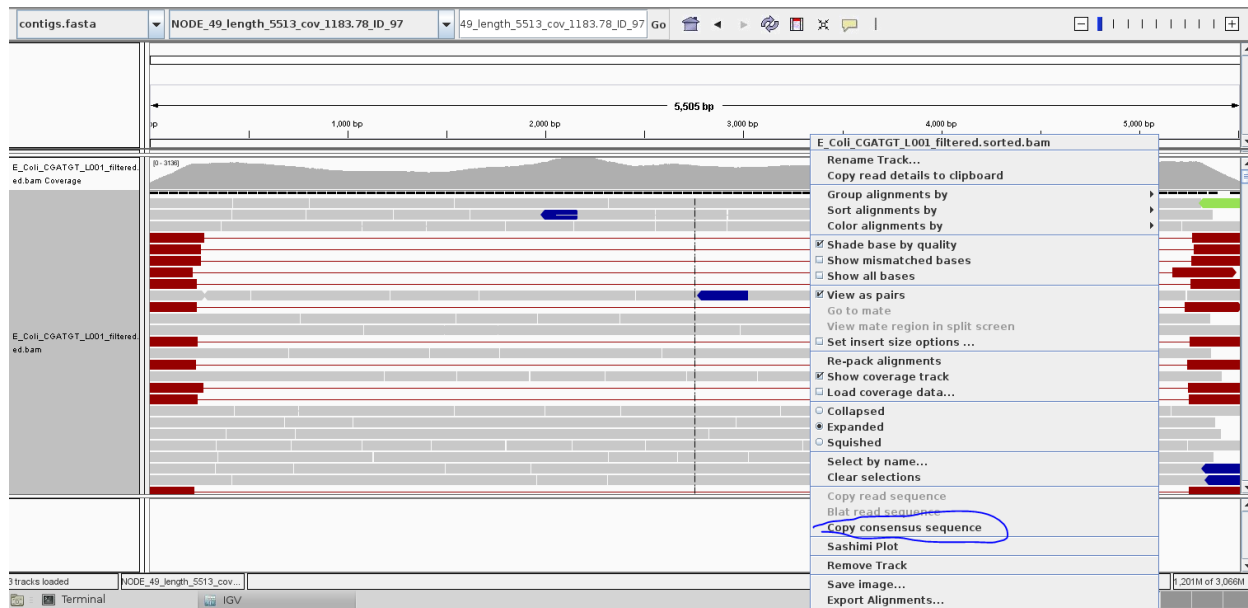
You can also do the same for individual reads, but you need to un-select 'View as pairs' before right clicking on a read. You may lose track of the paired-end reads and find it easier to copy the read name before un-selecting 'View as pairs' and then and then pasting it into the 'Select by name…' search box.

You should find that the contig contains at least two phage genes. There appear to be at least two phages present, one which seems to be the full contig, the other with the red read-pairs seems to be missing the sequence in the middle of the contig.

Annotation of de-novo assembled contigs

We will now annotate the contigs using BLAST, Pfam and RAST as with the unmapped contigs.

Task 5: Obtain open reading frames

As before, we'll call open reading frames within the de-novo assembly. Again, we will use codon table 11 which defines the bacterial codon usage table (http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi) and state that the sequences we are dealing with are not circular. We will also restrict the ORFs to just those sequences longer than 300 nucleotides (i.e. 100 amino acids). We will store the results in file contigs.orf.fasta.

Make sure you are in the denovo_assembly/ directory:

```
getorf -table 11  -circular N -minsize 300 -sequence contigs.goodcov.fasta -outseq con-
tigs.orf.fasta
```

The following two tasks are optional. Be warned - the BLAST searches and RAST will take several days! I recommend you skip these and proceed to Task 9.

Task 6 (Optional): Search open reading frames against NCBI non-redundant database

We can also search these open reading frames against the NCBI non-redundant database.

```
blastp -db ~/workshop_meterials/genomics_tutorial/db/blast/nr -query contigs.orf.fasta -evalue
1e-06 -num_threads 4 -show_gis -num_alignments 10 -num_descriptions 10 -out con-
tigs.orf.fasta.blastp
```

Task 7 (Optional): Search contigs against NCBI non-redundant database
The following command executes a nucleotide BLAST search (blastn) of the sequences in the contigs.fasta file against the non-redundant database. Again we restrict ourselves to 10 results per hit and an e-value cutoff of 1e-06.

```
blastn -db ~/workshop_meterials/genomics_tutorial/db/blast/nt -query contigs.fasta -evalue 1e-
06 -num_threads 4 -show_gis -num_alignments 10 -num_descriptions 10 -out con-
tigs.fasta.blastn
```

Task 8 (Optional)

Run the contigs through the RAST server and import the resulting GFF annotation into IGV (refer back to Part 4 for instructions).

Task 9: Run open reading frames through Pfam

As with the unmapped reads we will search the open reading frames against the Pfam HMM database of protein families. Later on we will be able to use these results to identify Pfam domains which are unique to a particular strain.

> pfam_scan.pl -fasta contigs.orf.fasta -dir ~/workshop_meterials/genomics_tutorial/db/pfam/ -outfile contigs.orf.pfam -cpu 2 -pfamB -as

This will take around 5 hours so it is recommended that you leave this running while continuing with the rest of the tutorial. If it is still running when you finish your session for today, leave your instance running overnight, but please be sure to turn it off in the morning!

Hybrid de-novo assembly

You will have seen that even with good coverage and a relatively long (300bp) paired end Illumina dataset - the assembly we get is still fairly fragmented. Our *E.coli* example assembles into 78 contigs and the largest contig is around 10% of the genome size.

Why is this?

One possible reason would be that regions of the original genome were not sequenced, or sequenced at too low coverage to assemble correctly. Regions of the genome will occur with different frequencies in the library that was sequenced - You can see this in the variation of coverage when you did the alignment. This can be due to inherent biases in the preparation and the random nature of the process.

However as coverage increases the chances of not sequencing a particular region of the genome reduces and the most significant factor becomes the resolution of repeats within the assembly process. If two regions contain the same or very similar sequences the assembler cannot reliably detect that they are actually two or more distinct sequences and incorrectly 'collapses' the repeat into a single sequence. The assembler is now effectively missing a sequence and therefore breaks in the assembly occur.

One resolution to this is to use a sequencing technology like PacBio or Sanger which can produce longer reads - the reads are then long enough to include the repeated sequence, plus some unique sequence, and the problem can be resolved. Unfortunately getting enough coverage using Sanger sequencing is expensive and PacBio - although relatively inexpensive has a high error rate.

An approach becoming more and more popular is to combine technologies. For example: high quality Illumina sequencing to get the accuracy of reads combined with low quality PacBio sequencing to enable the repeats to be spanned and correctly resolved.

Our exercise will be to use Illumina and PacBio datasets to assemble a species of pseudomonas. These are subsets of data used in "Evaluation and validation of de novo and hybrid assembly techniques to derive high-quality genome sequences" Utturkar et al., 2014. (http://www.ncbi.nlm.nih.gov/pubmed/24930142). This paper also contains a good explanation of the process and different approaches that are available.

Task 10: QC the data

It is always important to check and understand the quality of the data you are working with:
Change to the directory and run fastqc

```
cd ~/workshop_meterials/genomics_tutorial/data/sequencing/pseudomonas_gm41
```

```
fastqc
```

Part 4   Task 10: QC the data

Open the files SRR1042836a.fastq SRR491287a_1.fastq -2 SRR491287a_2.fastq and look at the reports generated.
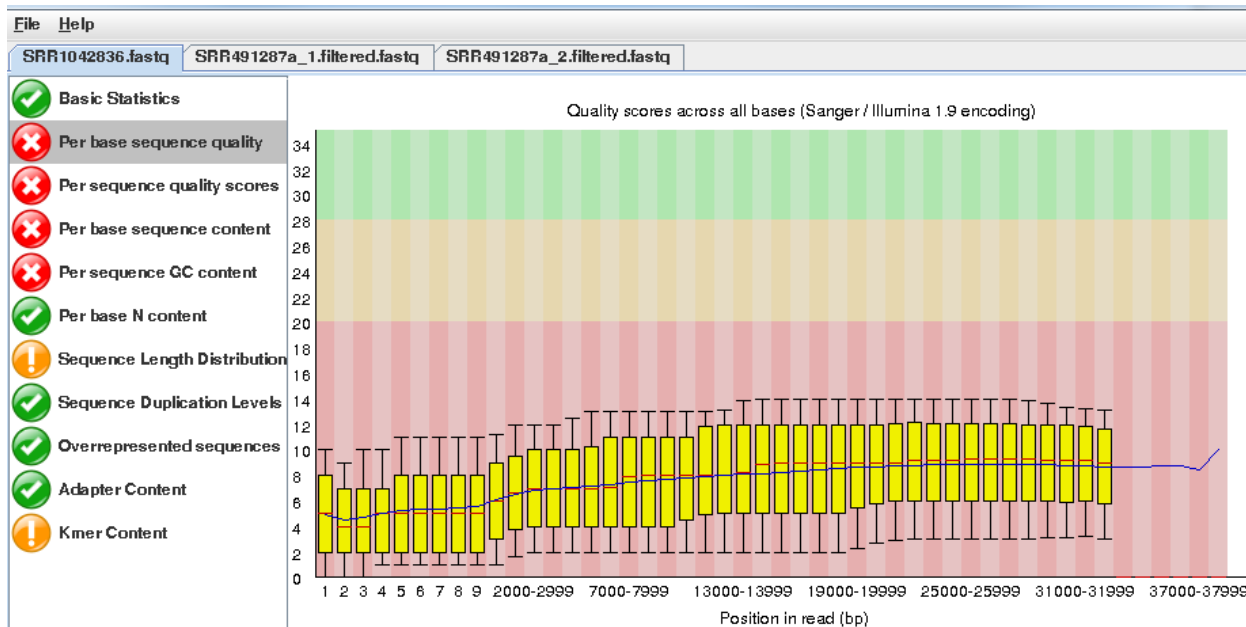
Note that the quality of the PacBio reads (SRR1042836a.fastq) is much lower than the Illumina reads with a greater than 1 chance in 10 of there being a mistake for most reads.
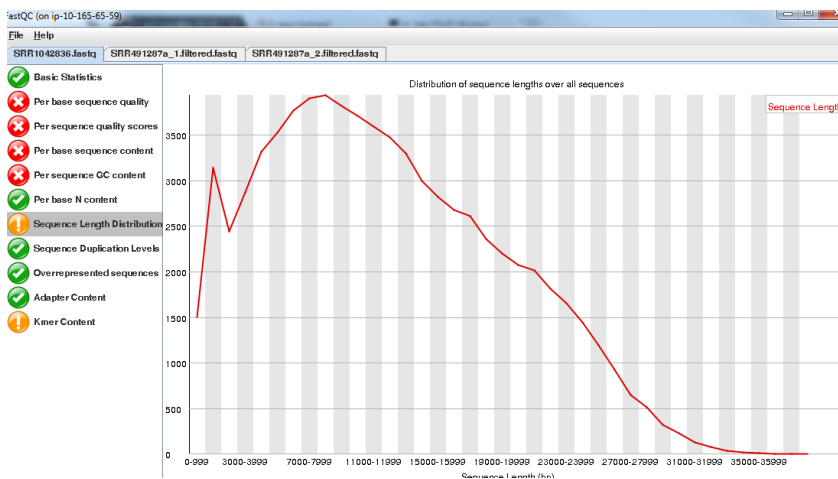
However, importantly, the length of the PacBio reads is much longer.

Trim the Illumina reads as before:

> fastq-mcf ../../reference/adaptors/adaptors.fasta SRR491287a_1.fastq SRR491287a_2.fastq -o SRR491287a_1.filtered.fastq -o SRR491287a_2.filtered.fastq -q 20 -p 10 -u -x 0.01

You can check the number of filtered reads using grep –c and the quality if trimmed reads with fastqc if you want.

For this exercise we want the long reads from PacBio even though they are low quality. We are relying on the assembler to use them appropriately.

Task 11: Illumina Only Assembly

Firstly let's construct an assembly using only the available Illumina data.
Make sure you are in the directory
~/workshop_meterials/genomics_tutorial/data/sequencing/pseudomonas_gm41
Run:

```
spades.py --threads 2 --careful -o illumina_only_assembly -1 SRR491287a_1.filtered.fastq -2
SRR491287a_2.filtered.fastq
```

(This may take some time so the data has been precomputed and is available in illumina_assembly/ if you are impatient!)

Change to the directory:

```
cd illumina_only_assembly
```

Filter out low coverage and very short contigs using a perl script:

```
filter_low_coverage_contigs.pl < contigs.fasta > contigs.goodcov.fasta
```

Let's look at the metrics for the assembly.

```
quast.py --output-dir quast contigs.goodcov.fasta
```

```
cat quast/report.txt
```

```
Assembly                      contigs.goodcov
# contigs (>= 0 bp)           117
# contigs (>= 1000 bp)        106
# contigs (>= 5000 bp)        83
# contigs (>= 10000 bp)       78
# contigs (>= 25000 bp)       63
# contigs (>= 50000 bp)       46
Total length (>= 0 bp)        6630828
Total length (>= 1000 bp)     6623034
Total length (>= 5000 bp)     6572670
Total length (>= 10000 bp)    6534104
Total length (>= 25000 bp)    6280222
Total length (>= 50000 bp)    5673771
# contigs                     117
Largest contig                358339
Total length                  6630828
GC (%)                        59.01
N50                           117617
N75                           72000
L50                           17
L75                           35
# N's per 100 kbp             0.00
```

(Your results may be slightly different. This is because spades uses a random seed that changes every time)

Task 12: Create Hybrid Assembly

Now will execute the same command, but this time include the longer PacBio reads to see the effect it has on our assembly.

Change back into the directory

~/workshop_meterials/genomics_tutorial/data/sequencing/pseudomonas_gm41

Run (This may take some time so the data has been precomputed and is available in hybrid_assembly/ if you are impatient!):

```
spades.py --threads 2 --careful -o hybrid_assembly --pacbio SRR1042836a.fastq -1
SRR491287a_1.filtered.fastq -2 SRR491287a_2.filtered.fastq
```

Change to the directory:
```
cd hybrid_assembly
```

Filter out low coverage and very short contigs using a perl script:

```
filter_low_coverage_contigs.pl < contigs.fasta > contigs.goodcov.fasta
```

Let's look at the metrics for the assembly - this time we will compare it with the illumina only assembly:
```
quast.py --output-dir quast con-
tigs.goodcov.fasta ../illumina_only_assembly/contigs.goodcov.fasta
```

```
cat quast/report.txt
```

Assembly	hybrid_assembly_contigs.goodcov	illumina_only_contigs.goodcov
# contigs (>= 0 bp)	90	117
# contigs (>= 1000 bp)	80	106
# contigs (>= 5000 bp)	74	83
# contigs (>= 10000 bp)	70	78
# contigs (>= 25000 bp)	57	63
# contigs (>= 50000 bp)	51	46
Total length (>= 0 bp)	6666636	6630828
Total length (>= 1000 bp)	6660077	6623034
Total length (>= 5000 bp)	6642135	6572670
Total length (>= 10000 bp)	6614046	6534104
Total length (>= 25000 bp)	6404993	6280222
Total length (>= 50000 bp)	6158714	5673771
# contigs	90	117
Largest contig	484701	358339
Total length	6666636	6630828
GC (%)	59.00	59.01
N50	122016	117617
N75	78853	72000
L50	16	17
L75	34	35
# N's per 100 kbp	0.00	0.00

You can also explore the interactive html report:
```
firefox quast/report.html
```

It seems that using the longer reads has improved the completeness of our assembly - reducing the number of contigs.

Task 13: Align reads back to reference

Let's realign our original reads back to the assembly and see what we have - refer to previous notes if you are unsure of the steps.

Start in the hybrid assembly directory
~/workshop_meterials/genomics_tutorial/data/sequencing/pseudomonas_gm41/hybrid_assembly

```
mkdir remapping_to_assembly

cd remapping_to_assembly

cp ../contigs.fasta .

bwa index contigs.fasta
```

First remap the Illumina reads. Type all on one line:
```
bwa mem -t 2 con-
tigs.fasta ../../SRR491287a_1.filtered.fastq ../../SRR491287a_2.filtered.fastq >
gm41.illumina.sam
```

Process the output so that it is viewable in igv:
```
samtools view -bS gm41.illumina.sam > gm41.illumina.bam

samtools sort –o gm41.illumina.sorted.bam
gm41.illumina.bam

samtools index gm41.illumina.sorted.bam

samtools flagstat gm41.illumina.sorted.bam
```

We can also map the PacBio reads, but we need to tell bwa we are using PacBio reads
```
bwa mem -t 2 -x pacbio contigs.fasta ../../SRR1042836a.fastq > gm41.pacbio.sam

samtools view -bS gm41.pacbio.sam > gm41.pacbio.bam

samtools sort -o gm41.pacbio.sorted  gm41.pacbio.bam

samtools index gm41.pacbio.sorted.bam

samtools flagstat gm41.pacbio.sorted.bam
```

```
23495 + 0 in total (QC-passed reads + QC-failed reads)
0 + 0 duplicates
19470 + 0 mapped (82.87%:-nan%)
0 + 0 paired in sequencing
0 + 0 read1
0 + 0 read2
0 + 0 properly paired (-nan%:-nan%)
0 + 0 with itself and mate mapped
0 + 0 singletons (-nan%:-nan%)
0 + 0 with mate mapped to a different chr
0 + 0 with mate mapped to a different chr (mapQ>=5)
```

You will notice that not such a high proportion of PacBio reads map back to the assembly.

Now start igv:
    igv.sh

Load your assembled genome -
    Click on genome - load from file

Make sure you get the assembly from the hybrid_assembly (igv remembers the previous directory which may contain similar files.)

Now load your 2 alignment files:
    click on load from File and then select gm41.pacbio.sorted.bam and gm41.illumina.sorted.bam

On the toolbar select - "Show Details on Click"
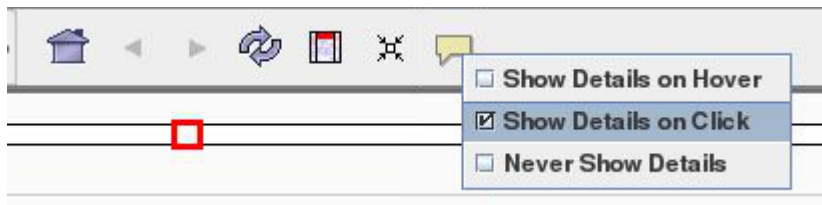
Find a region that has decent coverage of both reads and zoom in.
(Region shown here: NODE_79_length_15988_cov_20.944_ID_49:7,963-8,084)

You can see that the PacBio reads are much longer, but the error rate particularly insertions and deletions is much higher than for the Illumina reads.

Explore a few other contigs to see if you can find something that looks like an error or mis-assembly. Remember the assembly process is difficult and far from perfect.

Summary

You have seen that de-novo assembly of short reads is a challenging problem. Even for small genomes, the resulting assembly is fragmented into contigs and far from complete.

Incorporating longer reads to produce a hybrid assembly can be used to reduce the fragmentation of the genome. We have only used a single (perhaps the simplest) technique to incorporate long reads. You can read more about hybrid assembly techniques here:
http://www.ncbi.nlm.nih.gov/pubmed/24930142

Part 5 (Optional)

Short read genomics:
Comparison of results between different strains

Projects!

In the previous sections you have been taken through the steps required to:

1.   QC and filter Illumina data
2.   Remap Illumina short-read data to a reference sequence
3.   View the results in IGV
4.   Identify SNPs and Indels in an automated fashion using samtools and bcftools
5.   Determine whether SNPs result in synonymous or non-synonymous changes in the corresponding amino acid
6.   Extract unmapped reads
7.   Assemble unmapped reads and obtain assembly statistics
8.   Annotate unmapped reads using Pfam, RAST and/or BLAST
9.   Assemble a bacterial genome de-novo using SPAdes
10.   Obtain assembly statistics
11.   Annotate as per the unmapped reads (where computationally feasible).

Now we want you to do the same on a set of *Vibrio parahaemolyticus* strains which have been isolated and sequenced. There are six strains available depending on how much time is available and enthusiasm you have - choose a number of strains (at least 2) as we want to run some comparisons.

The strains can be found in:
~/workshop_meterials/genomics_tutorial/data/sequencing/Vibrio_parahaemolyticus

```
[ec2-user@ip-10-181-126-118 Vibrio_parahaemolyticus]$ ls -l
total 24
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:35 Sample_G35
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:44 Sample_PSU3384
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:37 Sample_T02347066
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:33 Sample_T024_47060
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:41 Sample_T0347070
drwxrwxr-x. 3 ec2-user ec2-user 4096 Dec  9 11:39 Sample_T0847053
```

Under each Sample directory is a subdirectory called raw_illumina_reads which contains the fastq files.

For remapping, the reference can be found in the folder:
~/workshop_meterials/genomics_tutorial/data/reference/Vibrio_parahaemolyticus_RIMD_2210633_uid 57969
(Remember, you will need to create an index first).

Part 5 (Optional)

 For each strain, make a list of:

1.   SNPs, Indels and their effects (from the remapping)
2.   Missing genes (from the remapping)
3.   Novel plasmids and/or genes (Pfam domains are the easiest way to do this via denovo assembly of unmapped reads - when performing the assembly - don't specify the k-mers SPAdes will choose appropriate ones.)

Once completed, see if you can predict what the phenotype of these bacteria might be. Then proceed to the final part of the tutorial where we will compare the results from all of these strains.

N.B.

It is recommended that you follow the same directory naming convention we have followed here (i.e. one for remapping to the reference, another for assembly of unmapped reads and a final one for the denovo assembly).

These tasks may take you several days. However, remember that all of the basic procedures are detailed in the previous sections – only the input FASTQ files will have changed. Feel free to refer to these previous tasks to remind yourself of the commands and parameters. By all means feel free to play around with different parameters if you wish, although remember that the results may differ from those you see here.

Just to give you some guidance:

You should find that strain Sample_T0347070 yields many more SNPs than other strains.

You may find that some scripts and programs run more slowly because of these extra differences and larger datasets.

Also, if you find the de novo assembly process causes your NX session to end, the chances are that SPAdes has caused your instance to run out of memory. If this happens, increase the minimum k-mer size in the spades.py command line.

Part 5 (Optional)

Comparing variants between several samples and a reference genome:

Here we will use a script to compare the variants called in each sample. Ensure you are in the ~/workshop_meterials/genomics_tutorial/data/sequencing/Vibrio_parahaemolyticus directory

First of all, let's make a directory to store the results of the comparison:

```
mkdir snp_comparison/
```

We need a copy of all of the vcf4 files we created here. This is a quick way to do it - paste this in as one command

```
for sample in Sample*
do
cp -v $sample/remapping_to_reference/out.snps.vcf4 snp_comparison/$sample.out.snps.vcf4
done

cd  snp_comparison/
```

Note that the copy commands may require modification depending on where you have saved the variant call results.

Our directory contents should look something like:

```
[ec2-user@ip-10-181-126-118 snp_comparison]$ ls -l
total 64388
-rw-rw-r--. 1 ec2-user ec2-user  7923228 Dec  9 17:16 Sample_G35.out.snps.vcf4
-rw-rw-r--. 1 ec2-user ec2-user  7377661 Dec  9 17:16 Sample_PSU3384.out.snps.vcf4
-rw-rw-r--. 1 ec2-user ec2-user  7766075 Dec  9 17:16 Sample_T02347066.out.snps.vcf4
-rw-rw-r--. 1 ec2-user ec2-user  7828043 Dec  9 17:16 Sample_T024_47060.out.snps.vcf4
-rw-rw-r--. 1 ec2-user ec2-user 27354227 Dec  9 17:16 Sample_T0347070.out.snps.vcf4
-rw-rw-r--. 1 ec2-user ec2-user  7666231 Dec _9 17:16 Sample_T0847053.out.snps.vcf4
```

We'll now set up some variables so we don't have to type long path names

```
ref=~/workshop_meterials/genomics_tutorial/data/reference/Vibrio_parahaemolyticus_RIMD_2210633_uid57969/Vibrio_parahaemolyticus_RIMD_2210633_uid57969.fasta

gff=~/workshop_meterials/genomics_tutorial/data/reference/Vibrio_parahaemolyticus_RIMD_2210633_uid57969/Vibrio_parahaemolyticus_RIMD_2210633_uid57969.gff

samples=`ls *.vcf4`
```

We can now use $ref instead of the long path to our reference and $gff for the feature file e.g.

```
head $ref
echo $samples
```

Part 5 (Optional)

When we are happy our variables are correct then run:

    snp_comparator.pl 10 $ref $gff $samples > snp_comparison.txt

Looking at the snp_comparison.txt file (either in a text editor, or in a spreadsheet):
If you have chosen different samples - you will get different results of course.

```
## Table of SNP and Indel occurences between these samples. Note that any comma-separated values (e.g. A,C indicate potential heterozygosity
 and/or sample heterogeneity
Chrom   Pos     Ref     Sample_G35.out.snps.vcf4        Sample_PSU3384.out.snps.vcf4    Gene description        Status
NC_004603       1000    G       A       G       VP0002 tRNA modification GTPase TrmE    ,silent  atc -> atT;
NC_004603       1000051 T       A       T       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,non-silent ctg -> cAg;
NC_004603       1000065 G       A       G       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,non-silent gtc -> Atc;
NC_004603       1000067 C       T       C       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,silent  gtc -> gtT;
NC_004603       1000080 C       C       A       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,non-silent ctc -> Atc;
NC_004603       100009  C       C       T       VP0092 hypothetical protein     ,silent  tcg -> tcA;
NC_004603       1000091 C       T       T       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,silent  gac -> gaT; ,silent  gac ->
 gaT;
NC_004603       1000100 C       T       C       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,silent  tgc -> tgT;
NC_004603       1000103 A       G       A       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,silent  cca -> ccG;
NC_004603       100020  A       G       G       VP0092 hypothetical protein     ,silent  ttg -> Ctg; ,silent  ttg -> Ctg;
NC_004603       1000219 T       C       C       VP0959 zinc/cadmium/mercury/lead-transporting ATPase    ,non-silent ttg -> tCg;,non-silent t
```

Here we can see the chromosome ID, the position in bp, the reference base and the base at each position as well as the gene (if any) the variant occurs in as well as the effect (silent, non-silent or indel).

Obtaining a phylogeny based on synonymous SNPs only:

How are the strains related on the basis of these variants? We can ask a number of questions, but if we are looking at the long-term evolutionary history of the strains we should only look at synonymous (i.e. silent) mutations as these should not confer a significant selective advantage to any strain. Using the data snp_comparison.txt file, we can form 'pseudo-sequences' using the script snp2tree_fullsequence.pl. These are concatenated bases consisting of only those positions which are silent across all strains. It is essentially the same as turning each column of each strain in the snp_comparison.txt file into a FASTA entry.

    snp2tree_fullsequence.pl snp_comparison.txt > synonymous_tree.fasta

Examine the contents of the tree.fasta file. We can then treat this file as an alignment (since each base in each sequence is at the same position on the chromosome) and pass it to a phylogeny program called FastTree. FastTree will take an input alignment and output a Newick formatted tree (http://en.wikipedia.org/wiki/Newick_format).

    FastTree -nt -gtr < synonymous_tree.fasta > synonymous_tree.newick

Now we can visually view this tree by using an online tool.

    firefox http://www.trex.uqam.ca/index.php?action=newick

Part 5 (Optional)

Either paste the contents of the .newick file into the window or select 'Sequences file' and load the file through the browser. Then select 'View Tree'.
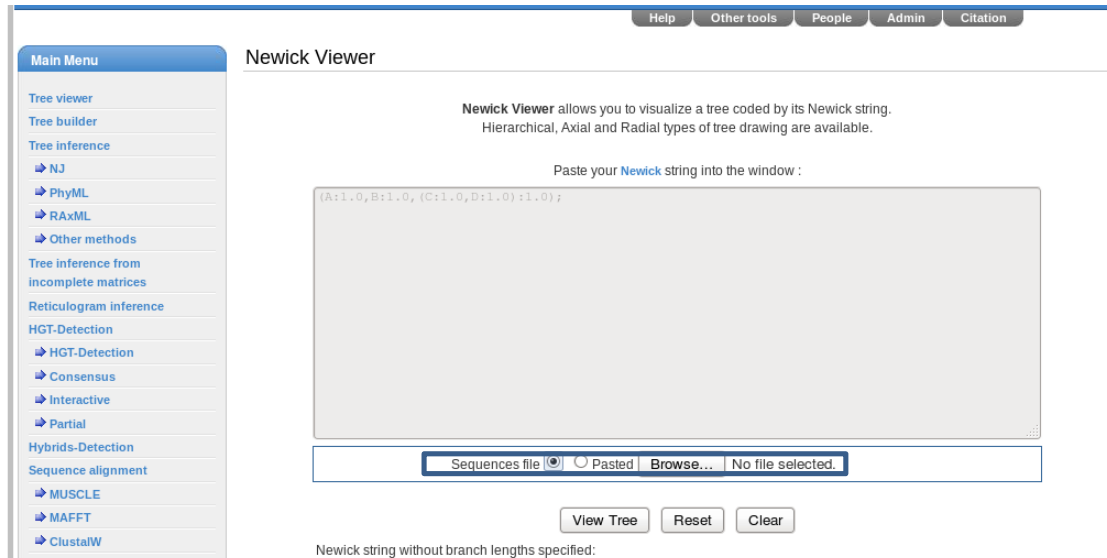
Advanced task (optional):

Copy the snp2tree_fullsequence.pl script to this directory (~/workshop_meterials/genomics_tutorial/data/sequencing/Vibrio_parahaemolyticus/snp_comparison) and modify it so that it selects positions containing only non-silent mutations (not indels as these modify the alignment). Generate a new alignment and compare the resulting tree against the silent mutations.

You will need to have some experience of programming in the Perl language to do this.

Part 5 (Optional)

Comparing Pfam domains found in each strain:

Here we will use a script to compare the various Pfam domains found in each sample. Ensure you are in the ~/workshop_meterials/genomics_tutorial/data/sequencing/Vibrio_parahaemolyticus/ directory

First of all, let's make a directory to store the results of the comparison:

mkdir pfam_comparison/

for sample in Sample*
do
cp -v $sample/unmapped_assembly/spades_assembly/contigs.orf.pfam
pfam_comparison/$sample.pfam
done

Note that the copy command may require modification depending on where you have saved the Pfam search results.

cd pfam_comparison/

Your directory should look something like this.

```
[ec2-user@ip-10-13-133-174 pfam_comparison]$ ls -l
total 216
-rw-rw-r--. 1 ec2-user ec2-user 61261 Dec 10 10:25 Sample_G35.pfam
-rw-rw-r--. 1 ec2-user ec2-user 52472 Dec 10 10:25 Sample_PSU3384.pfam
-rw-rw-r--. 1 ec2-user ec2-user 51979 Dec 10 10:25 Sample_T02347066.pfam
-rw-rw-r--. 1 ec2-user ec2-user 51691 Dec 10 10:25 Sample_T024_47060.pfam
```

To compare the pfam outputs for each strain, run:
compare_pfam.pl *.pfam > pfam_comparison.txt

Examining the pfam_comparison.txt file you should see something similar to:

```
PF13401      AAA_22 Sample_G35.pfam,
PF13402      M60-like      Sample_G35.pfam,
PF13414      TPR_11 Sample_T024_47060.pfam,Sample_T02347066.pfam,Sample_PSU3384.pfam,
PF13419      HAD_2  Sample_T024_47060.pfam,Sample_T02347066.pfam,
PF13420      Acetyltransf_4      Sample_PSU3384.pfam,Sample_G35.pfam,
PF13425      O-antigen_ligSample_T024_47060.pfam,Sample_T02347066.pfam,
PF13437      HlyD_3 Sample_G35.pfam,
PF13440      Polysacc_synt_3      Sample_PSU3384.pfam,
PF13443      HTH_26 Sample_G35.pfam,
PF13466      STAS_2 Sample_T024_47060.pfam,Sample_T02347066.pfam,
PF13476      AAA_23 Sample_T024_47060.pfam,Sample_T02347066.pfam,
PF13477      Glyco_trans_4_2      Sample_PSU3384.pfam,
PF13489      Methyltransf_23      Sample_PSU3384.pfam,
```

Search the Pfam database to see what some of these differences are (http://pfam.sanger.ac.uk).

Part 6

Genomics workshop:
De-novo assembly of long-reads

Introduction:

The latest third-generation sequencing platforms from Oxford Nanopore and Pacific Biosciences enable sequencing of *single DNA molecules.* Second generation sequencing platforms (e.g. Illumina) rely on sequencing a group of ~1000 mono-clonal molecules formed via PCR-like reactions. This has advantages in terms of reducing the quantity of DNA input required to enable sequencing and usually enables lower per-base costs, but has many shortcomings.

In the case of Illumina, although the sequencer is capable of generating large quantities of data (ter-abases in the case of a HiSeq run), the amplification step introduces GC-biases and also places a maximum limit on the size of the fragments which can be sequenced since the amplification becomes inefficient at larger fragments sizes and sequencing becomes de-phased within individual mono-clonal clusters due to polymerase errors. In the case of genome assembly, this introduces fragmentation of the assembly in areas of low coverage or repetitive regions which cannot be spanned.

Third generation sequencing of single molecules has the following features:

1.   Ability to sequence native genomic DNA without limitations on size (100s kilobases are possible)
2.   Higher single pass per-base error rates than second-generation sequencers
3.   Higher per-base cost than second-generation sequencing
4.   Full length cDNA or amplicon sequencing
5.   Some platforms such as the MinION from Oxford Nanopore hold the potential to enable portable sequencing and sequencing of other types of nucleic acid (e.g. RNA).

PacBio sequencing:

You can watch a video outlining PacBio sequencing at http://sequencing.exeter.ac.uk/pacific-biosciences-overview/. Pacific Biosciences have two sequencing platforms – RSII and Sequel. The RSII is an older platform capable of generating around 750Mbases of data in 4-6 hours. The Sequel platform is the latest platform and in theory can generate up to 7Gbases of data in a similar time frame. However the chemistry is still being refined and at present 4-5 Gbases is the maximum which should be expected. The Sequel platform is PacBio's focus for development at the moment, with optics included on the chip rather than the instrument, PacBio aims to increase the number of Zero Mode Waveguides (ZMWs) to increase throughput without significant instrument modifications.
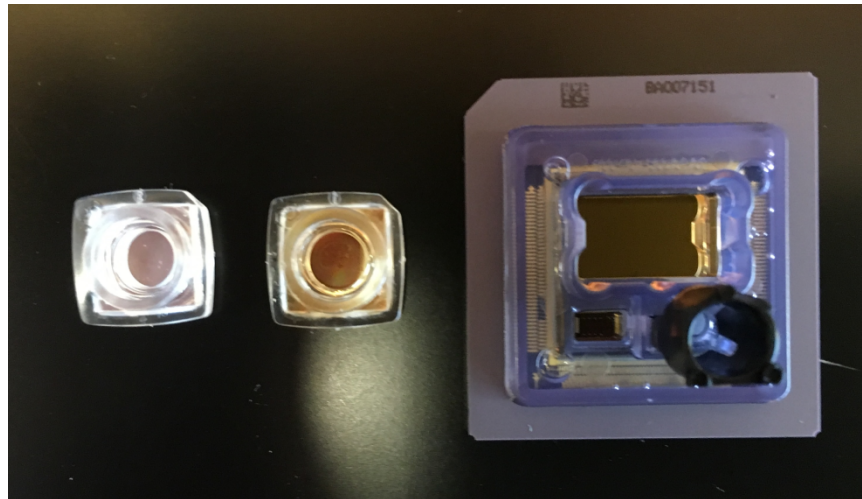
RSII SMRTcells (left), Sequel SMRT cell (right)

At present (Nov 2016), the Sequel platform chemistry is inferior to the RSII with shorter read lengths
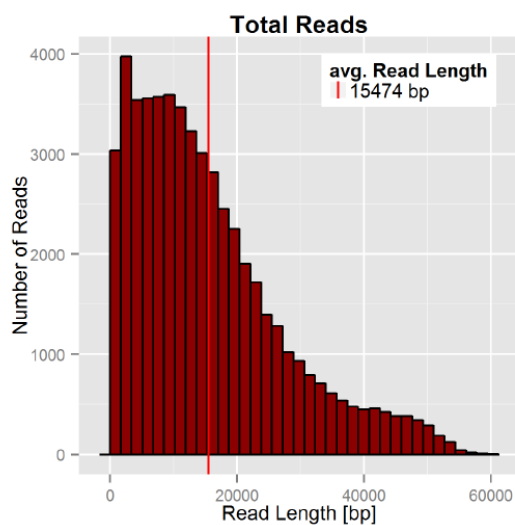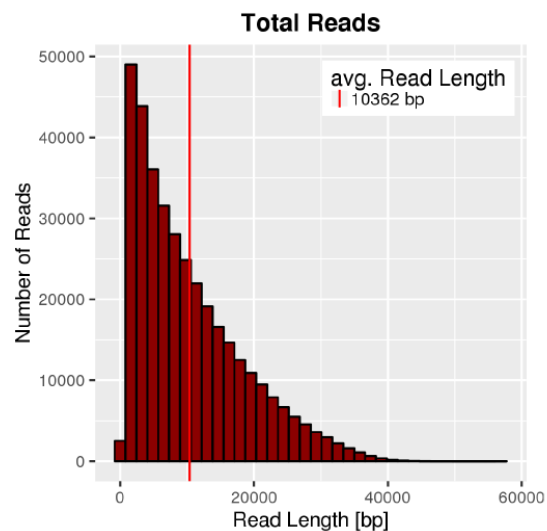
RSII (left), Sequel (right) read length distributions

A typical genomic library preparation workflow is similar to that of Illumina sequencing with the difference that much higher molecular weight DNA is used and dumb-bell shaped adaptors are attached:

Sample Preparation

DNA Sample

Fragment DNA

Repair Ends

Ligate Adapters

Purify DNA

Polymerase binding

Building of the SMRTbell™ template

Library sizes range from 250bp to >20kb
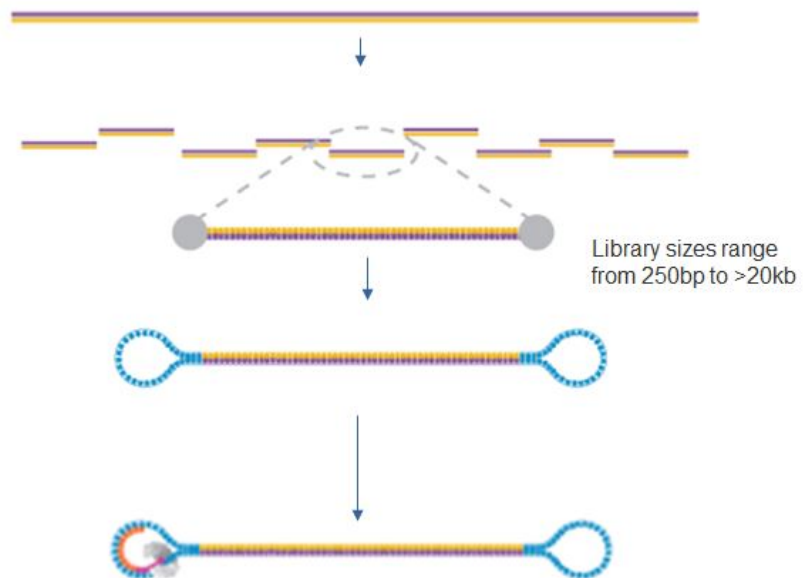
An important note regarding PacBio data quality:

PacBio libraries are circular. Because of this, a single polymerase can sequence the same piece of DNA several times. However, a balance exists between the DNA template length, polymerase life time (also known as polymerase read length) and read quality with PacBio data. The figure below illustrates this.

A polymerase which is able to read for 10kb, could read a single 10kb template or, for example, it could read a 2kb template 5 times. The 2kb fragment, having been read 5 times, would be of much higher quality than the 10kb fragment.
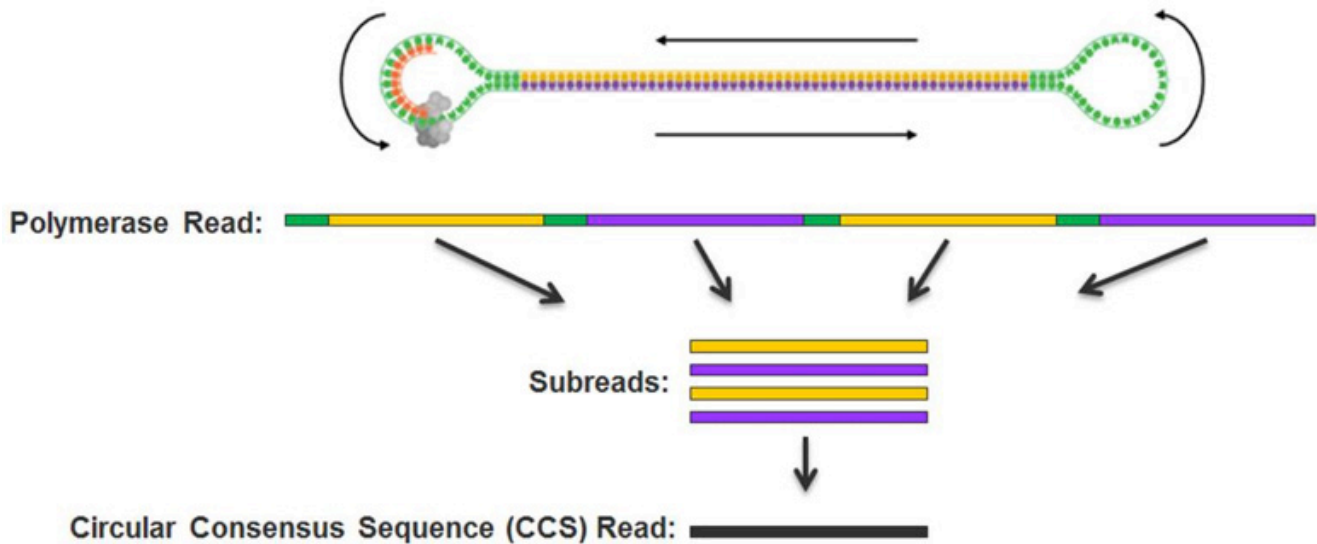
Terminology which is important to understand with PacBio data is highlighted in above. A polymerase read refers to the complete set of basecalls associated with the polymerase sequencing the forward strand, adaptor, and reverse strand. Internally, we remove the adaptor sequences and provide the subreads (just the forward and reverse sequences of the template). These can be further analysed to produce circular consensus reads (CCS) if the enzyme has made more than 1 pass of the molecule.

Most assembly programs require the subreads if utilizing PacBio data.

MinION sequencing technology:

This is a radically new sequencing technology based upon monitoring electrical current fluctuations associated with the translocation of single DNA molecules through nanopores embedded in a membrane. You can read more about the technology in detail here.

This is a remarkable technology which has the potential to supplant short-read sequencing, if the error rates can be brought down sufficiently. It has the advantage of being portable and of generating long fragment lengths.

A typical long read assembly pipeline:

1. Generate and evaluate QC metrics
2. Perform an initial assembly with Minimap/Miniasm to check whether we have sufficient data
3. Check for any potential contamination in the Miniasm assemblies using Blast and Krona
4. Correct long reads via consensus alignment and assemble
5. Polish the assembly with Illumina or other short-read data
6. If necessary, attempt to circularize the genome correctly
7. Compare assemblies against the reference using Quast
8. Generate gene predictions and/or functional annotation

Part 6

We have provided you with data generated from the same strain of *E.coli K12* MG1655 across three different platforms – Oxford Nanopore MinION, PacBio RSII and PacBio Sequel. The MinION data was generated and published by Nick Loman and Josh Quick whilst the PacBio data was generated at Exeter from the same material.

We have provided you with data from both 1D and 2D libraries. Remember these are two different types of library preparation. A 1D library enables just the template strand of the DNA molecule to be read, whilst a 2D library enables both the template and complement strand to be sequenced. This means that a 2D library will be sequenced

First of all let's create a directory to keep these analyses separate. Change into to the genomics_tutorial directory and then create a new directory:

cd long_read_tutorial

You can find these datasets in the directory:

~/genomics_tutorial/long_read_tutorial/raw_data

```
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial/raw_data$ ls
E_coli_K12_1D_R9.2.pass.fasta  E_coli_K12_2D_R9.0.pass.fasta  RSII_Ecoli_K12_subreads.fastq  Sequel_Ecoli_K12_subreads.fastq
```

Note that we have four datasets in total – Sequel, RSII, MinION 1D and MinION 2D the MinIon dataset consists of two fasta files. One is a 1D dataset and another is a 2D dataset. Note that typically MinION datasets are generated in a binary formatted file on a per-read basis which contain the current-levels and other data. However, for simplicity we have converted these into fasta files.

We'll follow a similar analysis protocol for all 4 datasets (some are pre-generated).

Task 1: Generate basic statistics for each of these datasets

You can use the fasta_summary.pl script to do this for both fasta-formatted MinION datasets. It might take a while to compute, therefore we have pre-computed the results for you:

~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_assemblies/fasta_summaries

Part 6

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/long_read_tutorial$ fasta_summary.pl

Usage:

    fasta_summary.pl -i infile.fasta  -o output_dir  -t ( read | contig | isotig ) [ -r 0 ] [ -n num_reads ] [ -c cutoff_length ] [ -l 1 ] [ -d 0 ] [ -f (w | t

where:

  -i or -infile  infile.fasta  :  input fatsa file of raeds, contigs or isotigs,

  -o or -output_dir output_directory : directory to put output stats and graphs into.

  -t or -type (read or contig or isotig) : for displaying the graph title, where type is 'read' or 'contig' or 'isotig'.

  -r or -repeats 0 or 1      :  1=count number of reads that contain over 70% simple mono-nucleotide and di-nucleotide repeat bases; 0=don't count.

  -n or -number num_reads    : For outputting specified number of randomly selected reads or contigs.

  -c or -cutoff cutoff_length : Give a number of reads to do extra analysis (calculating again the number of reads and number of bases in reads above this l

  -l or -longest 0 or 1      : 1=Output the longest read;  0= don't output the longest read

  -d or -doCommify 0 or 1    : Output numbers formatted with commas to make easier to read: 0=no commas, default=1

  -f or -format w or t       : w=wiki_format (ie. table with || and | for column dividers), t=tabs between column symbols for the wiki pages, default is sp

  -b or -bucket1             : To also output histogram file of exact read lengths (ie. bucket size of 1)

 eg: For 454 reads:     fasta_summary.pl  -i RawReads.fna -o read_stats -t read
     For 454 isotigs:   fasta_summary.pl  -i 454Isotigs.fna -o isotig_stats -t isotig

genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/long_read_tutorial$ fasta_summary.pl -i E_coli_K12_
E_coli_K12_1D_R9.2.pass.fasta  E_coli_K12_2D_R9.0.pass.fasta
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/long_read_tutorial$ fasta_summary.pl -i E_coli_K12_1D_R9.2.pass.fasta -o 1D_sum
  Directory '1D_summary' created
```

For the PacBio data you can use the fastqc program as per the Illumina datasets you have looked at previously. You can open precomputed results in firefox. You can find them in:

~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_asse mblies/fastqc_results

The fasta_summary.pl script will generate several output files:

```
genomics@genomics-build-2:/mnt/genomics_storage/workshop_data/genomics_tutorial/long_read_tutorial/1D_summary$
histogram_bins.dat       sorted_contigs.fa  summed_contig_lengths.dat      sum_reads_vs_read_len.dat
histogram_bins.dat.png   stats.txt          summed_contig_lengths.dat.png  sum_reads_vs_read_len.dat.png
```

The stats.txt file contains text-data on the number of bases, longest read and other useful statistics. The histograms_bins.dat.png file is a picture file with a histogram of read lengths (you can use firefox to see the file).

Task 2: Compare the datasets

Note that unlike Illumina sequencers, the read lengths produced by these platforms are highly varia- ble. The MinION datasets for example vary between 100bp and 140,000bp.

	Max read length (bp)	Total number of reads	Median read length (bp)	Genome coverage
MinION 1D	131,969	164,472	5,945	315x
MinION 2D	144,661	132,412	6,780	194x
PacBio Sequel	53,516	452,240	~7000	436x
PacBio RSII	59,184	260,074	~7000	372x

GC content of E.coli K12 MG1655 is approx. 51%.

Note the relatively poor quality scores in the fastqc report for the PacBio sequences. These reflect the 10-12% raw single-pass per-base error rate of the polymerase. Similar error rates are observed in the MinION datasets. Note that *unlike* Illumina datasets, this error rate does not appreciably increase over

the length of the read (although at very short or very long read lengths there are far fewer reads present which increase the variance estimates).

Quality scores across all bases (Sanger / Illumina 1.9 encoding)
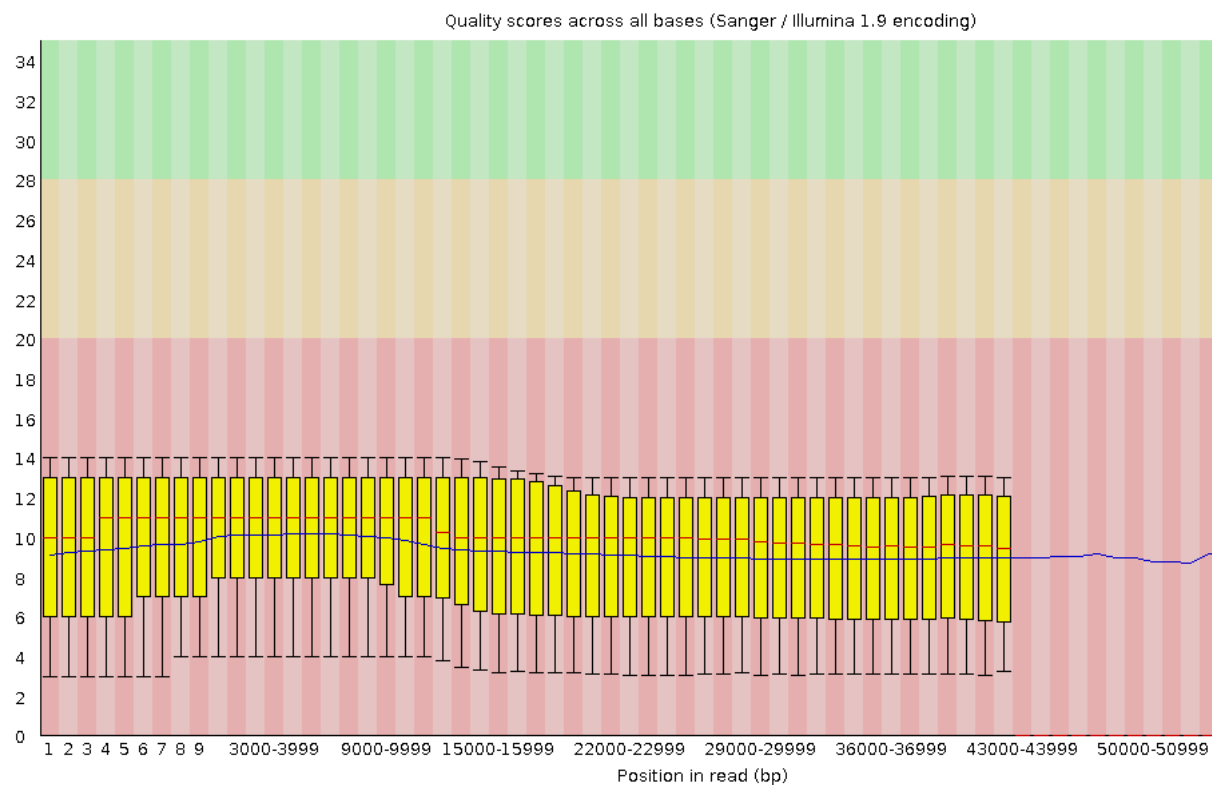
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Task 3: Evaluate the likelihood of obtaining a successful assembly using Minimap/Miniasm

This very useful assembler tries to assemble long-reads *without* trying to correct the reads. Although you would not want to use such an assembly without further correction, it provides a useful yardstick to determine whether a more computationally intensive assembly is likely to yield good results. You can read more here https://github.com/lh3/miniasm.

Because of limitations on the memory available to us, we'll need to subset the data first, selecting a random 100,000 reads to use (note that this is not the best approach since we should really pick the longest 100,000 reads).

The syntax can be a bit confusing, so here is an example using the RSII dataset to get you started. Each assembly should take about 5-10 mins.

Create a new working directory in ~/workshop_materials/genomics_tutorial/long_read_tutorial called miniasm_assemblies:

> cd ~/workshop_materials/long_read_tutorial
> mkdir miniasm_assemblies

```
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial$ mkdir miniasm_assemblies
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial$ cd miniasm_assemblies/
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial/miniasm_assemblies$ ls
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial/miniasm_assemblies$ pwd
/home/genomics/workshop_materials/genomics_tutorial/long_read_tutorial/miniasm_assemblies
genomics@genomics:~/workshop_materials/genomics_tutorial/long_read_tutorial/miniasm_assemblies$ 
```

We'll use the *seqtk* package from the same author as samtools to randomly select 100,000 reads from the raw dataset:

seqtk sample
~/workshop_materials/genomics_tutorial/long_read_tutorial/raw_data/RSII_Ecoli_K12_subreads.fastq
100000 > RSII_Ecoli_K12_subreads.subsampled.fastq

Now we can ask minimap to calculate all the overlaps between those 100,000 reads and output the results in a compressed gzip file. Note that you will have to put the input reads (RSII_Ecoli_K12_subreads.subsampled.fastq) in *twice* since we are asking minimap to calculate the overlaps between *all* reads in the dataset:

minimap -Sw5 -L100 -m0 -t 2 RSII_Ecoli_K12_subreads.subsampled.fastq
RSII_Ecoli_K12_subreads.subsampled.fastq | gzip -1 > overlaps_RSII.paf.gz

Once complete, we can ask miniasm to create an assembly graph and find an assembly path through it:

miniasm -f RSII_Ecoli_K12_subreads.fastq overlaps_RSII.paf.gz > overlaps_RSII.gfa

awk '/^S/{print ">"$2"\n"$3}' overlaps_RSII.gfa | fold > miniasm.PacBio_RSII.contigs.fasta

Repeat this for the Sequel and MinION datasets and then use the QUAST package (quast.py) to compare the assemblies against the reference genome. You should use the same E.coli reference as you used for the Illumina assemblies.

You should be able to open the report.html file in firefox or other web-browser to compare the assemblies. It should look somewhat similar to:

Genome statistics	miniasm.MinION_1D.contigs	miniasm.MinION_2D.contigs	miniasm.PacBio_RSII.contigs
Genome fraction (%)	0.002	0.68	0.003
Duplication ratio	0.975	0.999	1.013
Largest alignment	77	10 663	154
Total aligned length	77	31 526	154
NGA50	-	-	-
Misassemblies			
# misassemblies	0	0	0
Misassembled contigs length	0	0	0
Mismatches			
# mismatches per 100 kbp	0	1147.57	1973.68
# indels per 100 kbp	2531.65	1981.3	2631.58
# N's per 100 kbp	0	0	0
Statistics without reference			
# contigs	1	4	1
Largest contig	4 478 239	3 697 194	4 768 901
Total length	4 478 239	4 628 860	4 768 901
Total length (>= 1000 bp)	4 478 239	4 628 860	4 768 901
Total length (>= 10000 bp)	4 478 239	4 628 860	4 768 901
Total length (>= 50000 bp)	4 478 239	4 581 011	4 768 901

Extended report

Note that your results will differ since you will have a different subset of reads. Minimap/miniasm does not correct reads prior to assembly. This means that the alignments to the reference are likely to be error prone. Note that even without this correction, the assembler is able to reconstruct the genome in (more or less) a single contig of 4.7Mb with just 100,000 reads! This bodes well for a more computationally intensive assembly which first corrects reads and highlights the power of long reads for denovo assembly.

If you wish you can subset the data with the *seqtk sample* and compare how the datasets assemble at different levels of coverage.

Task 4: Generate a corrected assembly with Canu (results pre-computed)

Canu is derived from the original Celera assembler used to assemble the human genome from Sanger data. It has been optimized for long-read PacBio and Nanopore data. You can read more at http://canu.readthedocs.io/en/stable/tutorial.html. A number of long read assemblers are available (including Nanopore-specific assemblers- a good review paper on this is:

Chu J, Mohamadi H, Warren RL, Yang C, Bi-Rol I. Innovations and challenges in detecting long read overlaps: an evaluation of the state-of- the-art. Bioinformatics. 2016

Part 6

For now we will just use Canu as it is relatively straightforward to use. Here is an example command to assemble the RSII E.coli data. We have pre-computed the results for you for the MinION 2D, RSII and Sequel data.

~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_assemblies

To save time, and provide a fairer comparison between platforms we will not use the MinION 1D data.

```
canu -p canu_RSII -d canu_RSII genomeSize=4.7m  useGrid=False  -pacbio-raw
RSII_Ecoli_K12_subreads.fastq
```

Note that Canu will:
      1.   Correct reads by performing all-against-all alignments and taking a consensus
      2.   Overlap and trim reads
      3.   Generate an assembly

Task 5: Polish Canu assemblies with Illumina data using Pilon (results pre-computed)

Oxford Nanopore and MinION data suffer a tendency to introduce insertions or deletions into a sequence (even after read correction). This means that to obtain the highest per-base quality it is desirable to polish assemblies by aligning short reads using BWA and then using a tool such as Pilon (https://github.com/broadinstitute/pilon/wiki) to polish the assemblies.

We have supplied you with pre-computed results. We'll use just the RSII data to illustrate an example set of commands. First let's align the Illumina reads from the short-read section of the workshop using BWA.

We need to create a reference for the PacBio RSII contigs:

```
bwa index RSII_canu_contigs.fasta
```

Now we need to align the Illumina reads against the contigs:

```
bwa mem -x pacbio -t 2
RSII_canu_contigs.fasta ../../../data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R1_001.fastq ../.
./../data/sequencing/ecoli_exeter/E_Coli_CGATGT_L001_R2_001.fastq  |  samtools sort -@ 2 -O
bam -o RSII_canu_contigs_illumina_aligned.bam
```

```
samtools index RSII_canu_contigs_illumina_aligned.bam
```

Now that we have aligned the Illumina data against the contigs we can run Pilon to correct the contigs where they differ from the Illumina reads.

```
pilon --genome RSII_canu_contigs.fasta --frags RSII_canu_contigs_illumina_aligned.bam  --changes -
-outdir RSII_canu_pilon_polished
```

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You can view the corrected contigs in RSII_canu_pilon_polished/pilon.fasta and the see a list of the changes which have been made in RSII_canu_pilon_polished/pilon.changes. Note that most changes correct indels and the much higher number of corrections made for the MinION assembly vs the Pac-Bio assemblies (46558 for MinION 2D vs 387 for RSII and 2247 for Sequel).

We need to interpret these polished results with care. Remember we are using Illumina sequencing which contains all sorts of biases of its own thanks to amplification biases introduced by PCR and other artefacts. As such whilst we might be correcting some errors, we could be introducing Illumina biases into these assemblies. We're also using Illumina reads which are not quite identical to the reference or the PacBio material which is also not ideal (but often the case in the real world!). We'll see the effect of this in the final task.

Task 6: Use Blast and Krona to confirm species present in the assembly (results pre-computed)

We can use BLAST to identify taxonomic hits to ensure that we have the correct species present and filter out any contigs resulting from control spike-in DNA or other contaminants. You can also use other tools to do this such as Kraken or Centrifuge.

As the blast searches take some time, the results have been pre-computed for you in

~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/krona_plot/blast/results

but we have included the commands used below:

blastn -db ../../../db/blast/nt -query RSII_canu_pilon_polished.fasta -outfmt 7 -evalue 1e-06 -out RSII_canu_pilon_polished.fasta.blastn.outfmt7 -num_threads 2

Once this has completed, we can import the results into Krona which is a neat little visualization tool for BLAST results.

ktImportBLAST -i RSII_canu_pilon_polished.fasta.blastn.outfmt7 -o krona_blast_results.html

These results can be visualized in a web-browser such as firefox.

Note that the MinION data contains Lambda virus DNA which is used as a spike-in to some MinION runs. You can identify which contigs hit species of interest by clicking on the area of the pie chart you are interested in and then clicking on the 'Count' in the top right corner. This will give you the contig names which match to the virus as opposed to the bacteria.

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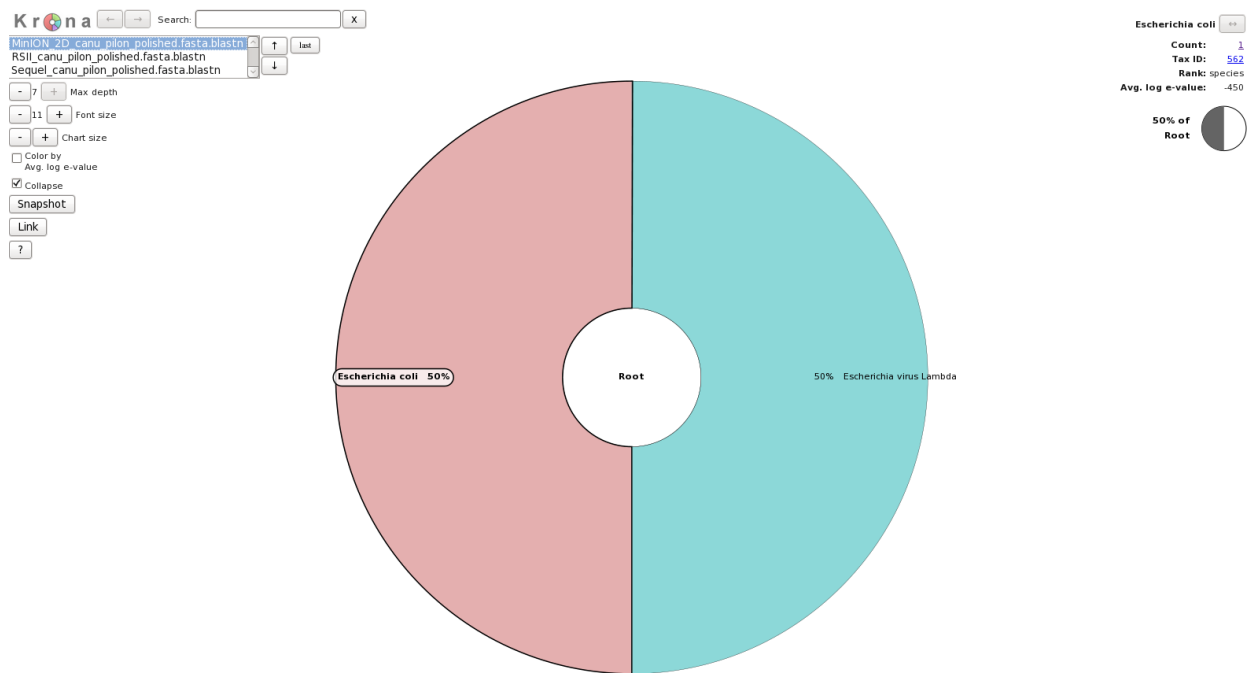
Task 6: Circularise assemblies using Circlator (results pre-computed)

Note that bacterial genomes are circular. As such we may end up mis-assembling the genome because it is circular. The circlator package (https://github.com/sanger-pathogens/circlator/wiki/Minimus2-circularization-pipeline) attempts to correct this.

In the circularized_results/ directory you will find the results of the circularization pipeline.

Task 7: Compare polished assemblies using Quast

Use quast.py to generate a Quast report for the original Canu results (i.e. pre Illumina correction and circularization) and these corrected and circularized results. E.g (all on one line):

```
quast.py -R ~/workshop_materials/genomics_tutorial/data/reference/U00096/U00096.fna MinION_2D_canu_pilon_polished.circularise.fasta
~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_assemblies/MinION_2D.contigs.fasta RSII_canu_pilon_polished_circularise.fasta
~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_assemblies/RSII_canu_contigs.fasta Sequel_canu_pilon_polished.circularise.fasta
~/workshop_materials/genomics_tutorial/long_read_tutorial/pre_computed_results/canu_assemblies/Sequel_canu_contigs.fasta
```

Genome statistics	MinION_2D_canu_pilon_polished...	MinION_2D.contigs	RSII_canu_pilon_polished_circ...	RSII_canu_contigs	Sequel_canu_pilon_polished.ci...	Sequel.canu.contigs
Genome fraction (%)	99.548	99.989	99.348	99.998	98.954	99.601
Duplication ratio	1.004	0.992	1.009	1.011	1	1
Largest alignment	2 936 866	3 932 583	2 003 968	3 975 034	1 999 977	2 666 330
Total aligned length	4 639 267	4 602 545	4 653 997	4 691 092	4 594 197	4 622 022
NG50	4 635 362	4 598 803	4 636 005	4 650 265	2 657 046	2 666 705
NG75	4 635 362	4 598 803	4 636 005	4 650 265	1 231 069	1 249 068
NA50	2 936 866	3 932 583	900 693	3 975 034	787 802	2 666 330
NA75	809 610	3 932 583	787 005	3 975 034	442 121	1 043 489
NGA50	2 936 866	3 932 583	900 693	3 975 034	787 802	2 666 330
NGA75	809 610	3 932 583	787 005	3 975 034	442 121	1 043 489
LG50	1	1	1	1	1	1
LG75	1	1	1	1	2	2
LA50	1	1	2	1	2	1
LA75	2	1	3	1	4	2
LGA50	1	1	2	1	2	1
LGA75	2	1	3	1	4	2
Misassemblies						
# misassemblies	5	2	9	4	5	2
# relocations	5	2	7	4	5	2
# translocations	0	0	0	0	0	0
# inversions	0	0	2	0	0	0
# misassembled contigs	1	1	1	1	2	1
Misassembled contigs length	4 635 362	4 598 803	4 636 005	4 650 265	3 888 115	1 249 068
# local misassemblies	4	6	5	3	7	8
Unaligned						
# fully unaligned contigs	0	0	0	0	0	0
Fully unaligned length	0	0	0	0	0	0
# partially unaligned contigs	1	1	0	0	0	0
# with misassembly	1	1	0	0	0	0
# both parts are significant	1	1	0	0	0	0
Partially unaligned length	47 385	47 451	0	0	0	0
Mismatches						
# mismatches	193	1612	81	1	107	152
# indels	598	43 655	34	139	97	1964
Indels length	846	54 537	47	145	610	3491
# mismatches per 100 kbp	4.18	34.73	1.76	0.02	2.33	3.29
# indels per 100 kbp	12.94	940.61	0.74	2.99	2.11	42.48
# short indels	596	43 582	34	138	81	1913
# long indels	2	73	0	1	16	51
# N's	0	0	8	0	0	0
# N's per 100 kbp	0	0	0.17	0	0	0
Statistics without reference						
# contigs	2	2	2	3	6	6
# contigs (>= 0 bp)	2	2	2	3	6	6
# contigs (>= 1000 bp)	2	2	2	3	6	6
# contigs (>= 5000 bp)	2	2	2	3	6	6
# contigs (>= 10000 bp)	2	2	2	3	6	6
# contigs (>= 25000 bp)	2	2	1	1	6	6
# contigs (>= 50000 bp)	2	2	1	1	6	6
Largest contig	4 635 362	4 598 803	4 636 005	4 650 265	2 657 046	2 666 705
Total length	4 687 265	4 650 066	4 654 672	4 691 401	4 595 324	4 622 743
Total length (>= 0 bp)	4 687 265	4 650 066	4 654 672	4 691 401	4 595 324	4 622 743
Total length (>= 1000 bp)	4 687 265	4 650 066	4 654 672	4 691 401	4 595 324	4 622 743
Total length (>= 5000 bp)	4 687 265	4 650 066	4 654 672	4 691 401	4 595 324	4 622 743
Total length (>= 10000 bp)	4 687 265	4 650 066	4 654 672	4 691 401	4 595 324	4 622 743
Total length (>= 25000 bp)	4 687 265	4 650 066	4 636 005	4 650 265	4 595 324	4 622 743
Total length (>= 50000 bp)	4 687 265	4 650 066	4 636 005	4 650 265	4 595 324	4 622 743
N50	4 635 362	4 598 803	4 636 005	4 650 265	2 657 046	2 666 705
N75	4 635 362	4 598 803	4 636 005	4 650 265	1 231 069	1 249 068
L50	1	1	1	1	1	1

Part 6

Look at the effect of the polishing step for each technology. Have a think about the following:

1.   The PacBio reads have a higher mismatch error after polishing but a lower indel rate? (think about the possible effects of PCR amplification on GC-bias)
2.   What about the total number of contigs for each platform?
3.   How does the mis-match rate to the reference genome compare between the MinION and the PacBio platforms?
4.   The Sequel platform used very early chemistry – how does it perform compared to the other platforms?
5.   Which platform would you select if you wanted to use the assembly as a reference to perform variant calling against?

Part 6

Concluding remarks:

Well done! If you have reached this far, you deserve a round of applause. You have completed some of the most common tasks in genomics. You can use the same machine and the same scripts to perform analysis of any dataset!

If you need to transfer data to/from the instance a tutorial can be found at
http://www.siteground.com/tutorials/ssh/ssh_winscp.htm