RNAseq Analysis: A Practical Walkthrough (part 1/n)

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Overview

1. Workflow
2. Data Quality
   - fastqc
   - trimmomatic
3. Genomes
   - Downloading
   - Indexing
4. Data Prep
5. Alignment
   - tophat

Today's Goals

- Clean Data
- Prepare genome files
- Run tophat2 (?)
    - (at least give you the tools to run it...)

Workflow

1. Get in your groups
2. Fill in the missing blanks on my diagram
    - Fill in a description and software
    - Should take about 10 minutes

slogin OR sbatch script

- What is the difference?
- Make sure you're making a conscious choice between the two
- Today we'll be working on slogin with SMALL files
- (why does this matter?)
- When you analyze your files, make sure to use an sbatch script

Data Quality

- So far you’ve downloaded data
- Next step is to check the quality
- And if the qulity is poor, “trim” the data

Data Quality

- This will simultaneously accomplish two goals:
    1. Check that the data is as expected (length, format)
        - Important because you otherwise can't see the data
    2. Confirm that the data is a good quality across samples
- We'll be using fastqc

fastqc

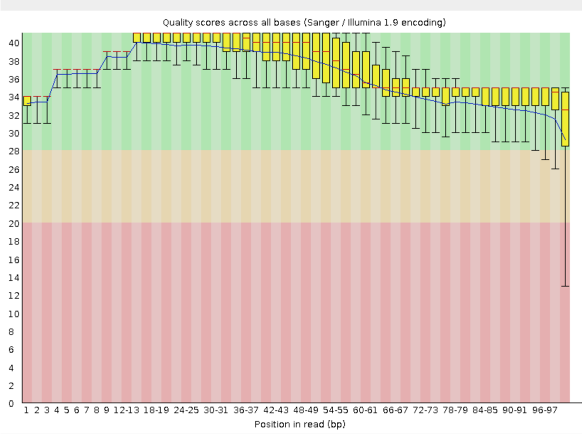
- Runs on the cluster - (small files on slogin, large as an sbatch)
- Output html file describes the fastq file (length, base composition, phred score quality, etc)
- To run fastqc:

```
cd /work/cc216/490S/<your netid>
export PATH=/work/cc216/490S/software/FastQC/:$PATH
EXAMPLE: fastqc [-f fastq|bam|sam] seqfile1 seqfile2 ..
seqfileN
fastqc -f fastq /work/cc216/490S/cc216/test_data/RNAseq_r1.fq
/work/cc216/490S/cc216/test_data/RNAseq_r2.fq
```

- The html output is generated but on the cluster, what is the last step?

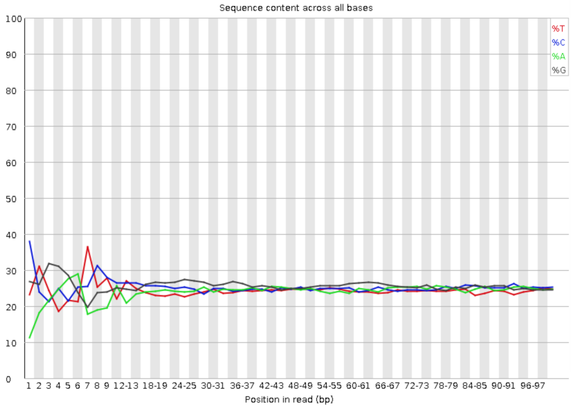
fastqc output

- Run Quality
- Base Content
- Run Length



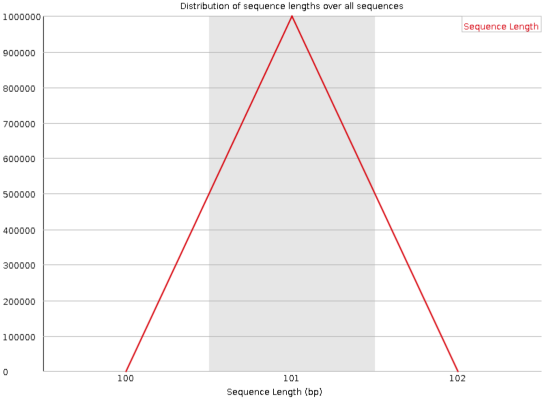
fastqc output

- Run Quality
- <u>Base Content</u>
- Run Length



fastqc output

- Run Quality
- Base Content
- Run Length



Trimming Data

- Remove data that is low quality
  - You have TONS of data, taking out 5% is OK
- We will set a couple of parameters:
  - Minimum quality at the end of the read
  - Average quality along a sliding window
  - Overall read quality
- If the read doesn't meet some, or all, of these the whole read is tossed
- We'll be using trimmomatic

trimmomatic

- Runs on the cluster (sbatch script)
- Writes a new fastq file (or pair, R1 & R2)
- To run trimmomatic:

```
cd /work/cc216/490S/<your netid>

EXAMPLE:

java -jar /work/cc216/490S/software/Trimmomatic-0.36/trimmomatic-0.36.jar PE -phred33 -trimlog
RNAseq.trimlog RNAseq_r1.fastq RNAseq_r2.fastq RNAseq_r1.trimm.5.20.fastq
RNAseq_r1.trimm.unpaired.5.20.fastq RNAseq_r2.trimm.5.20.fastq RNAseq_r2.trimm.unpaired.5.20.fastq
LEADING:3 TRAILING:3 SLIDINGWINDOW:5:20 MINLEN:50
```

- trimmomatic is a java file, you’re essentially running the command “java” then pointing it to the .jar file for instructions
- Do you need to move this file down to your laptop?

trimmomatic example

- To run trimmomatic:

```
EXAMPLE:
java -jar
/work/cc216/490S/software/Trimmomatic-0.36/trimmomatic-0.36.jar
<PE or SE> -phred33 -trimlog <output log> <Read 1.fq> <Read
2.fq> <Read 1 output> <Read 1 output unpaired> <Read 2 output>
<Read 2 output unpaired> LEADING:3 TRAILING:3 SLIDINGWINDOW:5:20
MINLEN:50
```

- Remove (leading/trailing) low quality or N bases (below quality 3)
- Scan the read with a 5-base wide sliding window, cutting when the average quality per base drops below 20
- Drop reads which are less than 50 bases long after these steps

trimmomatic example

- Output:

```
Input Read Pairs:  1000000 Both Surviving:  955447 (95.54%)
Forward Only Surviving:  29029 (2.90%) Reverse Only Surviving:
9577 (0.96%) Dropped:  5947 (0.59%)

>head RNAseq.log

SRR848963.63 ILLUMINA:322:D0UFKACXX:3:1101:11445:2184 length=101
101 0 101 0

SRR848963.64 ILLUMINA:322:D0UFKACXX:3:1101:11909:2032 length=101
97 1 98 3

SRR848963.64 ILLUMINA:322:D0UFKACXX:3:1101:11909:2032 length=101
98 0 98 3
```

trimmomatic

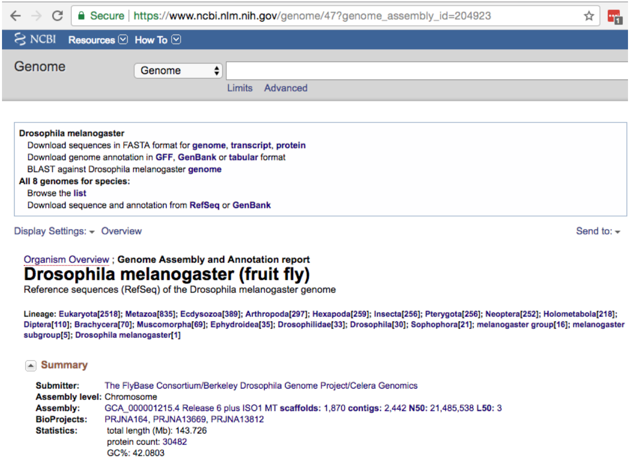
- At this point, you should run fastqc again
- Why?
- Check that trimmomatic improved the quality
- Check that trimmomatic fixed specific problems
- Always want to assess your final product

So you say you need a genome...

- Why do we need a genome?
- Where do we get it?
- And if you don’t know...

Downloading genomes

- Hopefully your googling led you to NCBI
- Model species have a page like this
- Download from the “FASTA format for genome” and “annotation in GFF” links


Secure
https://www.ncbi.nlm.nih.gov/genome/47?genome_assembly_id=204923
NCBI
Resources
How To
Genome
Genome
Limits
Advanced

Drosophila melanogaster
Download sequences in FASTA format for **genome**, **transcript**, **protein**
Download genome annotation in **GFF**, **GenBank** or **tabular** format
BLAST against Drosophila melanogaster **genome**
All 8 genomes for species:
Browse the **list**
Download sequence and annotation from **RefSeq** or **GenBank**

Organism Overview ; **Genome Assembly and Annotation report**

Drosophila melanogaster (fruit fly)

Reference sequences (RefSeq) of the Drosophila melanogaster genome

Lineage: Eukaryota[2518]; Metazoa[835]; Ecdysozoa[389]; Arthropoda[297]; Hexapoda[259]; Insecta[256]; Pterygota[256]; Neoptera[252]; Holometabola[218]; Diptera[110]; Brachycera[70]; Muscomorpha[69]; Ephydroidea[35]; Drosophilidae[33]; Drosophila[30]; Sophophora[21]; melanogaster group[16]; melanogaster subgroup[5]; Drosophila melanogaster[1]

Summary

Submitter: The FlyBase Consortium/Berkeley Drosophila Genome Project/Celera Genomics
Assembly level: Chromosome
Assembly: GCA_000001215.4 Release 6 plus ISO1 MT **scaffolds:** 1,870 **contigs:** 2,442 **N50:** 21,485,538 **L50:** 3
BioProjects: PRJNA164, PRJNA13669, PRJNA13812
Statistics: total length (Mb): 143.726
protein count: 30482
GC%: 42.0803

Indexing

- Most aligners require their genome to be indexed
- What do you think this means?
- You'll need to index using bowtie2 (the aligner tophat2 uses)

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bowtie index

- This is going to be a computationally intensive process
- So write a submission script to do it
- You can start with my template:

```
export PATH=/opt/apps/tophat-bowtie/:$PATH


cat /work/cc216/490S/cc216/genome_index.submit
cp /work/cc216/490S/cc216/genome_index.submit
/work/cc216/490S/duke-bio490s/projects/<your_project>/
```

- Now, edit the file to index the genome you downloaded, in your folder

Preparing your data files

- We're now ready to align the RNAseq reads to the genome
- At this point, you're going to start generating a lot of different files
- So this is a good point to plan out file names with your group
- So that everyone knows which files are which, it is best to rename files:
    - e.g. change SRR1201401_1.fastq to adultSample1_r1.fastq
    - Keep a file with a record of these changes
    - (The same file can be a for loop to make the changes)

tophat

- tophat2 is the command/software that aligns the reads to the genome
- This (also) is going to be a computationally intensive process
- So write a submission script to do it:

```
export PATH=/opt/apps/tophat-bowtie/:$PATH


tophat2
```

- No template this time!
- Run tophat2 command and read over the manual
- The next slide has an example command – be careful, the defaults are suitable for mammals, if you're working with non-mammalian data check your paper to for suggestions

tophat

- tophat2 example (all one line):

```
tophat -p 4 -o RNAseq1 -G
/work/cc216/490S/cc216/genomes/dmel_20171011.gff dmel
/work/cc216/490S/cc216/test_data/RNAseq_r1.trimm.5.20.fastq
/work/cc216/490S/cc216/test_data/RNAseq_r2.trimm.5.20.fastq
```

translated:

```
tophat -p <number of threads> -o <output dir> -G <gff file,
annotations> <bowtie2 index> <R1 fastq> <R2 fastq>
```

- Help can be found by running “tophat2”
- On in the tophat2 manual online
- http://ccb.jhu.edu/software/tophat/manual.shtml

The End