

## BIMM-143: INTRODUCTION TO BIOINFORMATICS (Lecture 13)

### Genome Informatics (Part 1)

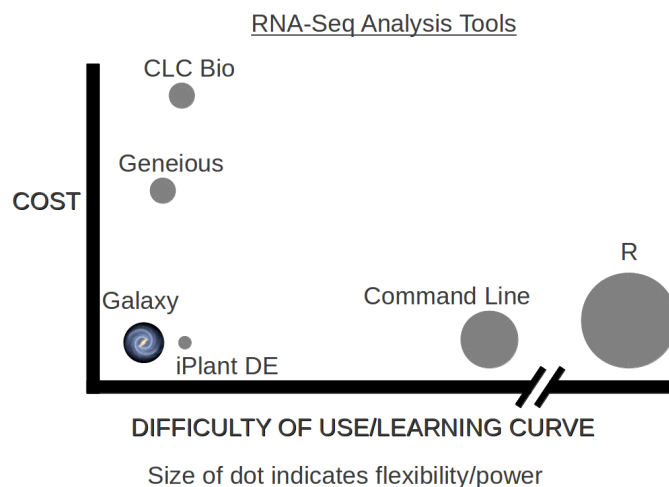
<http://thegrantlab.org/bimm143/>

Dr. Barry Grant

**Overview:** The purpose of this lab session is to introduce a set of tools used in high-throughput sequencing and the process of investigating interesting gene variance in Genomics. High-throughput sequencing is now routinely applied to gain insight into a wide range of important topics in biology and medicine [see: [Soon et al. EMBO 2013](#)].

In this lab we will use the **Galaxy** web-based interface to a suite of bioinformatics tools for genomic sequence analysis. Galaxy is free and comparatively easy to use (see Figure 1 for a schematic comparison of some common bioinformatics RNA-Seq analysis methods).

Galaxy was originally written for genomic data analysis. However, the set of available tools has been greatly expanded over the years and Galaxy is now also used for gene expression, genome assembly, epigenomics, transcriptomics and host of other sub-disciplines in bioinformatics.



### **Section 1: Identify genetic variants of interest**

There are a number of gene variants associated with childhood asthma. A study from Verlaan *et al.* (2009) shows that 4 candidate SNPs demonstrate significant evidence for association. You want to find what they are by visiting OMIM (<http://www.omim.org>) and locating the Verlaan et al. paper description.

**Q1: What are those 4 candidate SNPs?**

[HINT, you will may want to check the first few links of search result]

rs12936231, rs8067378, rs9303277, and rs7216389

**Q2: What three genes do these variants overlap or effect?**

[HINT, you can find the information from the ENSEMBL page as shown in the image below with red rectangles]

ZPBP2, GSDMB, and ORMDL3

Ensembl BLAST/BLAT | BioMart | Tools | Downloads | Help & Documentation | More | Search Human...

Human (GRCh38.p10) Variant: rs12936231

Location: 17:39,872,367-39,873,367

**Variant displays**

- Explore this variant
- Genomic context
- Genes and regulation**
- Flanking sequence
- Population genetics
- Phenotype data
- Sample genotypes
- Linkage disequilibrium
- Phylogenetic context
- Citations

Configure this page | Custom tracks | Export data | Share this page | Bookmark this page

**rs12936231 SNP**

Most severe consequence: **Intron variant** | See all predicted consequences

Alleles: C/G | Ancestral: G | MAF: 0.43 (G) | Highest population MAF: 0.50

Location: **Chromosome 17:39872867** (forward strand) | VCF: 17 39872867 rs12936231 C G

Co-located variant: HGMD-PUBLIC CR095665

Evidence status: [Icons]

HGVS names: This variant has 7 HGVS names - Show

Synonyms: Archive dbSNP rs60433922, rs56578515, rs60243308

Genotyping chips: This variant has assays on 4 chips - Show

Original source: Variants (including SNPs and indels) imported from dbSNP (release 150) | View in dbSNP

About this variant: This variant overlaps 4 transcripts, 1 regulatory feature, has 3972 sample genotypes, is associated with 1 phenotype and is mentioned in 22 citations.

Description from SNPPedia: Description not available | More information from SNPPedia

**Genes and regulation**

Gene and Transcript consequences

Gene	Transcript (strand)	Allele (transcript allele)	Consequence Type	Position in transcript	Position in CDS	Position in protein
ENSG0000018607	ENST00000348931.8 (+)	G (G)	Intron variant	-	-	-

HGNC: ZPBP2 biotype: protein\_coding

Now, you want to know the location of SNPs and genes in the genome. You can find the coordinates for the SNP itself on the Ensembl page along with overlapping genes or whether it is intergenic (i.e. between genes). However, to explore the surrounding regions and neighboring SNPs you will need to visit the linked Ensembl genome browser by clicking on the **Location** tab (highlighted with a yellow rectangle above).

**Q3: What is the location of rs8067378 and what are the different alleles for rs8067378?**

[HINT, alleles and location are listed at the top of the the Ensembl page. You may search in a genome browser to find this information]

Chromosome 17: 39895095 (forward strand) A/G variants (43% G)

**Q4: What are the downstream genes for rs8067378? Any genes named ZPBP2, GSDMB, and ORMDL3?**

You are interested in the genotypes of these SNPs in a particular sample. Click on the “**Sample genotypes**” navigation link of of SNPs ensemble variant display page to look up their genotypes in the “Mexican Ancestry in Los Angeles, California” population.

Variant: rs8067378

### rs8067378 SNP

Most severe consequence

intergenic variant

Alleles

A/G | Ancestral: G | MAF: 0.43 (G) | Highest population MAF: 0.50

Location

Chromosome 17:39895095 (forward strand) | VCF: 17 39895095 rs8067378 A G

Co-located variant

HGMD-PUBLIC CR095668

Evidence status ⓘ

HGVS name

NC\_000017.11:g.39895095A>G

Synonyms

Archive dbSNP rs17676953, rs58640242

Genotyping chips

This variant has assays on 12 chips - Show

Original source

Variants (including SNPs and indels) imported from dbSNP (release 150) | View in dbSNP

About this variant

This variant has 3763 sample genotypes, is associated with 2 phenotypes and is mentioned in 25 citations.

### Explore this variant ⓘ

Genomic context

Genes and regulation

Flanking sequence

Population genetics
67

Phenotype data
2

Sample genotypes
3763

Linkage disequilibrium

Phylogenetic context

Citations
25

**Q5:** What proportion of the Mexican Ancestry in Los Angeles sample population (MXL) are homozygous for the asthma associated SNP (G|G)?  
[HINT: You can download a CVS file for this population from ENSEMBLE and use the R functions `read.csv()`, and `table()` to answer this question]  
14%

**Q6.** Back on the ENSEMBLE page, search for the particular sample **HG00109**. This is a male from the GBR population group. What is the genotype for this sample?  
G|G

## **Section 2: Initial RNA-Seq analysis**

Now, you want to understand whether the SNP will affect gene expression. You can find the raw RNA-Seq data of this one sample on the class webpage:

[https://bioboot.github.io/bimm143\\_F18/class-material/HG00109\\_1.fastq](https://bioboot.github.io/bimm143_F18/class-material/HG00109_1.fastq)

[https://bioboot.github.io/bimm143\\_F18/class-material/HG00109\\_2.fastq](https://bioboot.github.io/bimm143_F18/class-material/HG00109_2.fastq)

Download and examine these files with your favorite UNIX utilities such as **head**, **tail** and **less**. You can use your RStudio Terminal tab to issue these commands.

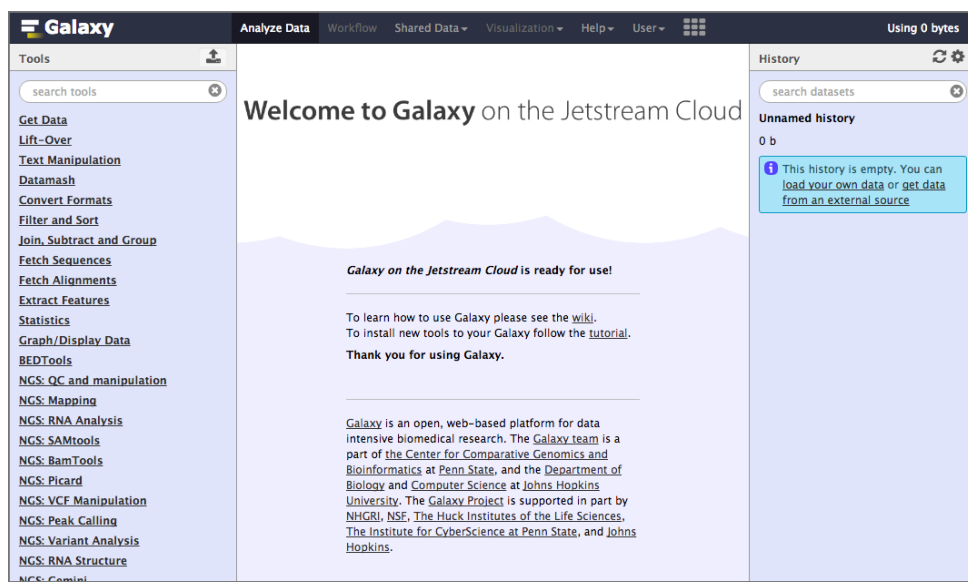
**Note:** For more details about the ubiquitous fastq format see ([http://en.wikipedia.org/wiki/FASTQ\\_format](http://en.wikipedia.org/wiki/FASTQ_format) ). You can read about this while you are waiting for your **Galaxy server** to become available (see below). **Let Barry know when you are at this point so we can discuss common fastq formats further.**

To begin our analysis of this data we will use Galaxy on Jetstream.

### **Using Galaxy for NGS analyses**

Follow Barry's instructions for accessing and logging into our very-own **Galaxy Server**. To find out more about Galaxy see: <https://galaxyproject.org/tutorials/g101/>

Once you are ready you should be able to type (or copy/paste) your assigned instance IP address into your web browser to see your very own Galaxy server. Under the **User** tab at the top of the page, select the **Register** link and follow the instructions on that page.



## Upload our **fastqsanger** sequences

In the left side **Tools** list, click the **Get Data > Upload File** link to upload our sequence files for analysis. You can load them from your own local laptop (with **chose local file** option) or more simply upload them via the URL from above (with the **paste/fetch data** option i.e. No need to download them to your computer first - this is often useful when dealing with very large files).

Be careful of the file type you upload. Tophat2 only takes **fastqsanger** file format. So, You need to choose **fastqsanger** for the upload *Type*.

Download data directly from web or upload files from your disk




Name	Size	Type	Genome	Settings	Status
HG00109_1.fastq	0.8 MB	fastqsan...	----- Additional Sp...	⚙	
HG00109_2.fastq	0.8 MB	fastqsan...	----- Additional Sp...	⚙	

You added 2 file(s) to the queue. Add more files or click 'Start' to proceed.

**Choose local file** Choose FTP file Paste/Fetch data **Start** Pause Reset Close






Now, you can check the data on the right panel. When they are colored gray they are still uploading and when they are green they are uploaded. Clicking in the name and various icons will provide more information to help you answer question 7 below.

**Q7: How many sequences are there in the first file?**  
**What is the file size and format of the data? Make sure the format is **fastqsanger** here!**  
[HINT, you can check the fastq format wiki for more information]

**3: adrenal 2.fastq**   

7.8 Mb  
format: **fastqsanger**, database: ?

uploaded fastq file

```
@ERR030881.107 HWI-BRUNOP16X_0001:2:
CGGATTTTCAGCTACTGCAAGCTCAGTACCACAGCCT
+
HH;HHHHHHHHHHHHHHHHHHGHDHEHHHHHEHHHHB
@ERR030881.311 HWI-BRUNOP16X_0001:2:
GAGTGCGAGGGAAGTCAGGGGAGGATCGCGAGGGA/
```

**Q8: Does the first sequence have good quality?**

[HINT, what is the quality score for each nucleotide? You can use the R functions `gtools::asc()` together with the `seqinr::s2c()` to return the values corresponding to the specified ASCII codes.

```
asc( s2c("DDDDCDEDCDDDDBBDDDDCC@") )
```

## Quality Control

You should understand the reads a bit before analyzing them in detail. Run a quality control check with the **FastQC** tool on your data using the “**NGS: QC and manipulation**” > **FastQC Read Quality reports**.

FastQC Read Quality reports (Galaxy Version 0.65) Options

Short read data from your current history

2: HG00109\_1.fastq

Contaminant list

Nothing selected

tab delimited file with 2 columns: name and sequence. For example: Illumina Small RNA RT Primer  
CAAGCAGAAGACGGCATACGA

Submodule and Limit specifying file

Nothing selected

a file that specifies which submodules are to be executed (default=all) and also specifies the thresholds for the each submodules warning parameter

FastQC performs several quality control checks on raw sequence data coming from high throughput sequencing pipelines. It provides a modular set of analyses which you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis. For example, it is often useful to trim reads to remove base positions that have a low median (or bottom quartile) score.

After running the FastQC program, you will get a FastQC Report both as a **Webpage** and **Raw Data**. Click on eye icon to view each version.

**Note:** You can find more about each analysis section (or module) here:

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

**Q9: What is the GC content and sequence length of the second fastq file?**

[HINT, you may check “Basic Statistics”]

**Q10:** How about per base sequence quality? Does any base have a mean quality score below 20?

[HINT, blue line is the mean quality score and for this exercise, assume a median quality score of below 20 to be unusable. Given this criterion, is trimming needed for the dataset?]

### Section 3: Mapping RNA-Seq reads to genome

The next step is mapping the processed reads to the genome. The major challenge when mapping RNA-seq reads is that the reads, because they come from RNA, often cross splice junction boundaries; splice junctions are not present in a genome's sequence, and hence typical NGS mappers such as **Bowtie** (<http://bowtie-bio.sourceforge.net/index.shtml>) and **BWA** (<http://bio-bwa.sourceforge.net/>) are not ideal without modifying the genome sequence. Instead, it is better to use a mapper such as **Tophat** (<http://ccb.jhu.edu/software/tophat>) that is designed to map RNA-seq reads.

Use the **NGS: RNA Analysis > Tophat** tool to map RNA-seq reads to the **hg19** build of the Human reference genome.

TopHat Gapped-read mapper for RNA-seq data (Galaxy Version 2.1.0) Options

Is this single-end or paired-end data?  
Paired-end (as individual datasets)

RNA-Seq FASTQ file, forward reads  
7: FASTQ Groomer on data 3  
Must have Sanger-scaled quality values with ASCII offset 33

RNA-Seq FASTQ file, reverse reads  
8: FASTQ Groomer on data 4  
Must have Sanger-scaled quality values with ASCII offset 33

Mean Inner Distance between Mate Pairs  
150  
-r/--mate-inner-dist; This is the expected (mean) inner distance between mate pairs. For, example, for paired end runs with fragments selected at 300bp, where each end is 50bp, you should set -r to be 200. The default is 50bp.

Std. Dev for Distance between Mate Pairs  
20  
--mate-std-dev; The standard deviation for the distribution on inner distances between mate pairs. The default is 20bp.

Report discordant pair alignments?  
Yes  
--no-discordant

Use a built in reference genome or own from your history  
Use a built-in genome  
Built-ins genomes were created using default options

Select a reference genome  
Human (Homo sapiens) (b37): hg19  
If your genome of interest is not listed, contact the Galaxy team

**Note:** Our input data is pair-end data. For Tophat in Galaxy, you need to set **paired-end** as your input type and then provide the forward read file and reverse read file. Because the reads are paired, you'll also need to set **mean inner distance between pairs**; this is the average distance in basepairs between reads. Use a mean inner distance of 150 for our data as this was the fragment length from the experimental library preparation step. See the red rectangles in the image below for details of the settings to change.

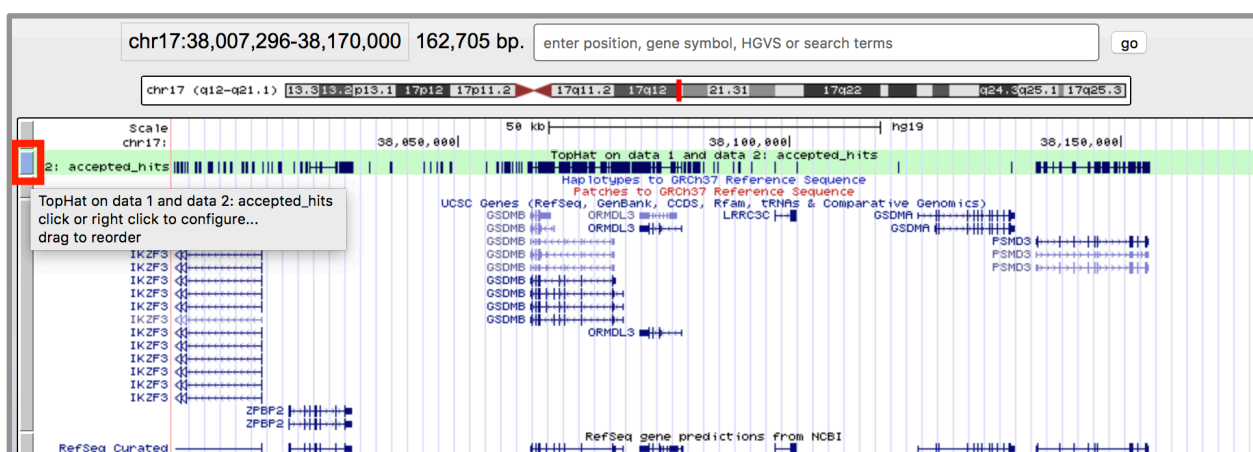
The calculation may take some time. There will eventually be five outputs: *accepted\_hits*, *insertions*, *deletions*, *splice junctions* and an *alignment summary*.

We will focus only on the alignment **summary** and the **accepted hits** files for this exercise, but the other files can be of interest depending upon the goal of any other analysis.

The *accepted\_hits* file is in BAM format, which is binary version of the human readable SAM format. To inspect these results we will convert the BAM file to SAM format using **NGS: SAMtools > BAM-to-SAM** tool. Once converted click the eye icon to view within galaxy. Note there is lots of metadata in the SAM file (lines beginning with @). After this is our alignment section, which includes details of the chromosome locations that our reads have been aligned to. See: [https://bioboot.github.io/bimm143\\_F18/class-material/sam\\_format/](https://bioboot.github.io/bimm143_F18/class-material/sam_format/)

## Display at UCSC

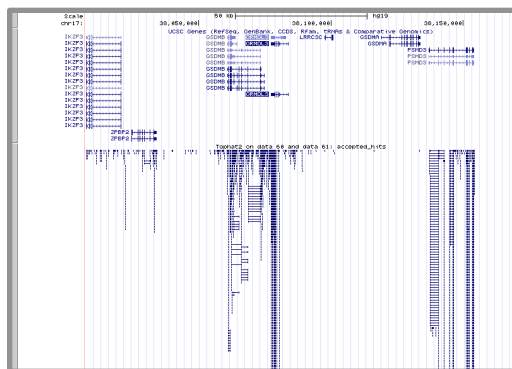
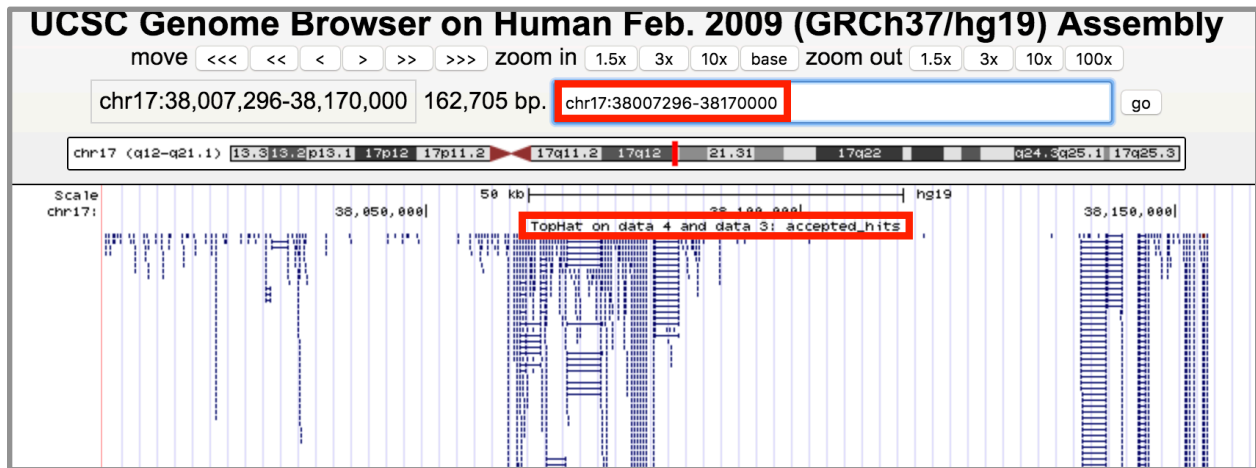
Once complete select and expand the **accepted\_hits** file in your history sidebar. Then Click on the “display at UCSC main” link.



This will load your TopHat results as a custom track on the **UCSC Genome Browser**. You can then click on the custom track (see above image) and change the display mode from **Dense** to **Full** and enter the region chr17:38007296-38170000 into

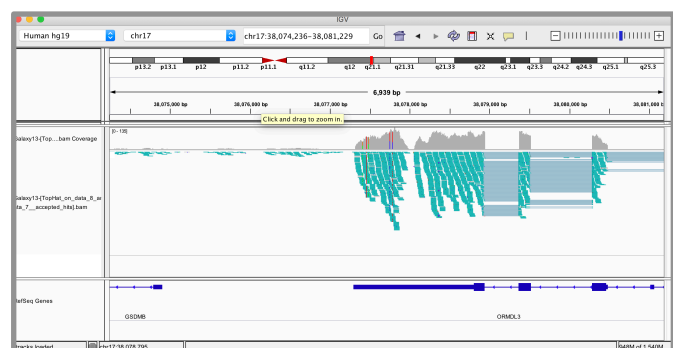


the text box to see the pile-up of aligned sequence reads in this location. See figure below for an example:



**Q11:** Where are most the accepted hits located?  
[HINT, you can view the SAM version of your accepted hits file in galaxy and also use the **UCSC Genome Browser** via following the galaxy provided link and focusing on particular regions as described above]

You may also want to view your results in the stand-alone IGV browser available from: <https://software.broadinstitute.org/software/igv/download> You can download both your accepted hits bam file and the corresponding index file from clicking the disc save icon in galaxy.



**Q12:** Following Q13, is there any interesting gene around that area?  
[HINT, you can find genes around accepted hits in either the UCSC Genome Browser or IGV - depending on which browser you prefer]

With alignment result from TopHat, we can now calculate gene expression with the **NGS: RNA Analysis > Cufflinks** tool. Before running Cufflinks, you should upload the reference annotation file “**gene.chr17.gtf**” (available from the course website: [https://bioboot.github.io/bimm143\\_W18/class-material/genes.chr17.gtf](https://bioboot.github.io/bimm143_W18/class-material/genes.chr17.gtf)) into the workspace of Galaxy first. This is a tab-delimited text file obtained from UCSC describing genomic features (locations of exons, stop\_codons, CDS, etc for our region of chromosome 17). Examine this file in galaxy before use).

The following figure shows the parameters you need to change when running cufflinks.

Cufflinks transcript assembly and FPKM (RPKM) estimates for RNA-Seq data (Galaxy Version 2.2.1.0) Options

**SAM or BAM file of aligned RNA-Seq reads**  
   16: TopHat on data 4 and data 3: accepted\_hits

**Max Intron Length**  
   
 ignore alignments with gaps longer than this

**Min Isoform Fraction**  
   
 suppress transcripts below this abundance level

**Pre mRNA Fraction**  
   
 suppress intra-intronic transcripts below this level

**Use Reference Annotation**  
   Use reference annotation

**Reference Annotation**  
   18: genes.chr17.gtf  
 Gene annotation dataset in GTF or GFF3 format.

**Q13:** Cufflinks again produces multiple output files that you can inspect from your right-hand-side galaxy history. From the “**gene expression**” output, what is the FPKM for the **ORMDL3** gene? What are the other genes with above zero FPKM values?

136853

**Note.** In later classes we will discuss some shortfalls of the FPKM metric and why some more recent normalization schemes (that also attempt to normalize for sequencing depth and gene length) are preferred. Note that genes will have more reads mapped in a sample with high coverage than one with low read coverage –  $2\times$  depth  $\approx 2\times$  expression. Also longer genes will have more reads mapped than shorter genes –  $2\times$  length  $\approx 2\times$  more reads. Normalization allows us to compare across genes within a sample and between samples (e.g. WT and Mutant etc.)

If you have time you can run a separate **htseq-count** analysis for your “accepted hits” file. Open htseq-count, and set it up using default parameters other than “Aligned SAM/BAM File” and “GFF File” which you need to select from your history as in the above example.

Subsequent steps in a typical RNA-Seq analysis would use a tool such as **DESeq2** (an R package) to set up a differential expression analysis to essentially compare the counts of each transcript/gene between different samples (including replicates) to assign a probability to the observed counts being generated if the gene is NOT differentially expressed between conditions. The DESeq2 package will be the subject of a separate class. For now we will skip this step and move onto a population scale analysis to complete the circle back to our childhood asthma associated SNPs.

#### **Section 4: Population Scale Analysis**

One sample is obviously not enough to know what is happening in a population. You are interested in assessing genetic differences on a population scale. So, you processed about ~230 samples and did the normalization on a genome level. Now, you want to find whether there is any association of the 4 asthma-associated SNPs (**rs8067378...**) on **ORMDL3** expression.

This is the final file you got ( [https://bioboot.github.io/bimm143\\_S18/class-material/rs8067378\\_ENSG00000172057.6.txt](https://bioboot.github.io/bimm143_S18/class-material/rs8067378_ENSG00000172057.6.txt) ). The first column is sample name, the second column is genotype and the third column is the expression value.

**Q14:** Read this file into R and determine the sample size for each genotype and their corresponding median expression levels for each of these genotypes. **Hint:** The **read.table()**, **summary()** and **boxplot()** functions will likely be useful here. There is an example R script online to be used ONLY if you are struggling in vein. Note that you can find the medium value from saving the output of the **boxplot()** function to an R object and examining this object. There is also the **medium()** and **summary()** function that you can use to check your understanding.

**Q15:** Generate a boxplot with a box per genotype, what could you infer from the relative expression value between A/A and G/G displayed in this plot? Does the SNP effect the expression of **ORMDL3**?

**Q17:** What one part of this lab or associated lecture material is still confusing? If appropriate please also indicate the question number from this lab instruction pdf and answer the question in the following anonymous form: <https://forms.gle/Qx4UTSc4zkpafRvq9>

**Reference:**

- All data files can also be found at: [https://bioboot.github.io/bimm143\\_S18/lectures/#13](https://bioboot.github.io/bimm143_S18/lectures/#13)  
Section 2 was adapted from <https://usegalaxy.org/u/jeremy/p/galaxy-rna-seq-analysis-exercise>.
- **Verlaan, et al.** Allele-specific chromatin remodeling in the ZBP2/ GSDMB/ORMDL3 locus associated with the risk of asthma and autoimmune disease. **Am. J. Hum. Genet.** 85: 377-393, 2009.