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A short tutorial on how to use RSEM

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README.md

A Short Tutorial for RSEM

Bo Li

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Introduction

RSEM[1,2] is an RNA-Seq transcript quantification program developed in 2009. In this tutorial, we will use some single cell RNA-Seq data from Shalek *et al.* to demonstrate the common uses of RSEM.

The Shalek *et al.* study contains thousands of single cell RNA-Seq experiments from bone-marrow-derived mouse dendritic cells. These data are available at GSE48968. In this tutorial, we first analyze one paired-end RNA-Seq data set from a single dendritic cell sequenced 6 hours after lipopolysaccharide (LPS) stimulation. Then we demonstrate how to perform differential expression analysis on two groups of single cells. Lastly, we show how we can use the RSEM simulator to help us design sequencing experiments.

Please note that this tutorial cannot replace the RSEM manual. In order to make full use of RSEM, we still recommend you to go over the manual.

Prerequisites

You need a server with Linux/Mac OS. To run RSEM, your server should have C++, Perl and R installed. In addition, you need at least one aligner to align RNA-Seq reads for you. RSEM can call Bowtie, Bowtie 2 or STAR for you if you have them installed. Last but not least, you need to install the latest version of RSEM.

In this tutorial, we align reads with Bowtie 2. We will show you how to install Bowtie 2 and RSEM step by step.

First, clone the following git repository to your favorite location:

```
git clone git@github.com:bli25ucb/RSEM_tutorial.git $(your_favorite_location)
cd $(your_favorite_location)
```

You will find source codes for Bowtie 2 and RSEM in the `software` subdirectory. Type commands below to install them:

```
cd software
unzip bowtie2-2.2.6-source.zip
cd bowtie2-2.2.6
make -j 8
cd ..
tar -xzf RSEM-1.2.25.tar.gz
cd RSEM-1.2.25
make -j 8
make ebseq
cd ..
cd ..
```

In the above commands, `make -j 8` asks for 8 threads to compile the software. We always use 8 threads for parallelization through this tutorial. However, you should adjust this number according to your needs. `make ebseq` compiles EBSeq and is only required if you want to use EBSeq for differential expression analysis.

Build References

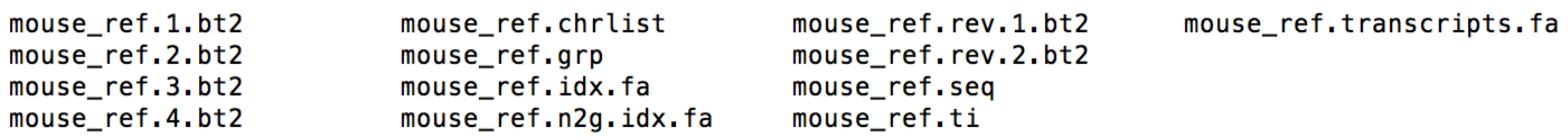
RSEM works with a set of transcripts, instead of a genome. We have two ways to build RSEM transcript references: building references from a genome, or buidling references from a set of transcripts. The RSEM command to build references is `rsem-prepare-reference`.

1. Building references from a genome. RSEM can extract transcript sequences from the genome based on a given GTF file. Optionally, RSEM can also call Bowtie/Bowtie2/STAR to build their own indices.

Download the Ensembl mouse genome (ftp://ftp.ensembl.org/pub/release-82/fasta/mus_musculus/dna/Mus_musculus.GRCm38.dna.toplevel.fa.gz) and GTF (ftp://ftp.ensembl.org/pub/release-82/gtf/mus_musculus/Mus_musculus.GRCm38.82.chr.gtf.gz) files to the `ref` subdirectory. Then type the following commands to build the references and create Bowtie2 indices:

```
gunzip ref/Mus_musculus.GRCm38.dna.toplevel.fa.gz
gunzip ref/Mus_musculus.GRCm38.82.chr.gtf.gz
software/RSEM-1.2.25/rsem-prepare-reference --gtf ref/Mus_musculus.GRCm38.82.chr.gtf \
                                             --bowtie2 --bowtie2-path software/bowtie2-2.2.6 \
                                     ref/Mus_musculus.GRCm38.dna.toplevel.fa ref/mouse_ref
```

The figure below lists files generated by the above commands. `mouse_ref.transcripts.fa` contains all extracted transcript sequences, and `*.bt2` are Bowtie2 indices.



```
mouse_ref.1.bt2        mouse_ref.chrlist       mouse_ref.rev.1.bt2     mouse_ref.transcripts.fa
mouse_ref.2.bt2        mouse_ref.grp           mouse_ref.rev.2.bt2
mouse_ref.3.bt2        mouse_ref.idx.fa        mouse_ref.seq
mouse_ref.4.bt2        mouse_ref.n2g.idx.fa    mouse_ref.ti
```

Let us take a closer look at `mouse_ref.transcripts.fa` (a snippet is shown below). It is a multi-FASTA file containing extracted sequences. For each sequence, the first line provides its identifier and the second line provides its sequence.

```
>ENSMUST00000000001
CACACATCCGGTTCTTCCGGGAGCTAGGGGAGCTGACGGAGAAGGCCACCGCCCAGCAGAAGACCCGTCTCCGCCGGTGTGTGGCGATTCCCGCGGTGTGTG
TGAGTGAGCCCGGGCCCGGTCCCCTCTCCCGCCGCCGCCATGGCTGCACGTTGAGCGCCGAGGACAAGGCGGCGGTGGAGCGGAGCAAGATGATCGACCGC
AACTTGCGGGAGGACGGGGAGAAAGCGGCCAAAGAAGTGAAGCTGCTGCTGCTCGGCGCTGGAGAATCTGGTAAAAGTACCATCGTGAAACAGATGAAAATC
ATTCATGAGGACGGCTATTCAGAGGACGAATGTAAACAGTATAAAGTAGTTGTCTACAGCAATACTATTCAGTCCATCATTGCAATCATACGAGCCATGGGA
CGGTTGAAGATTGATTTTGGGGAATCTGCCAGAGCAGATGATGCCCGACAGTTATTTGTTTTAGCTGGGAGTGCTGAAGAAGGAGTCATGACTTCAGAACTA
GCAGGCGTGATTAAACGTTTATGGCGAGATGGCGGGGTACAGGCATGCTTTAGCAGGTCCAGGGAATATCAGCTCAATGATTCTGCTTCATACTACCTAAAT
GATTTGGATAGAATATCCCAGACCAACTACATTCCAACTCAGCAAGATGTTCTTCGGACAAGAGTGAAGACTACAGGCATTGTGGAGACCCACTTCACCTTC
AAGGAACTCTACTTCAAAATGTTTGATGTAGGTGGCCAAAGATCCGAACGAAAAAAGTGGATTCACTGTTTTGAGGGAGTGACAGCAATTATCTTTTGTGTG
```

If you want to skip the reference building step, you can download the prebuilt references to `ref` and untar it by typing:

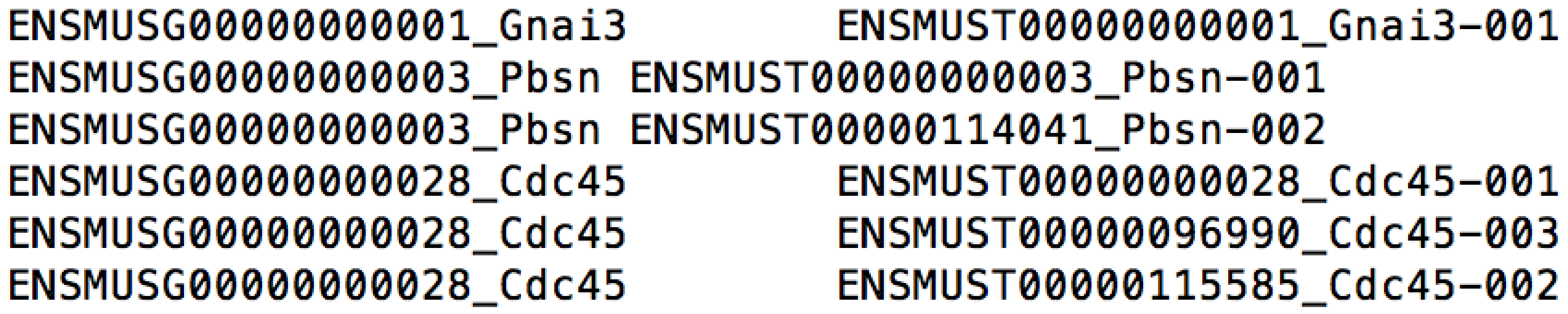
```
tar -C ref -xzf ref/mouse_ref.tar.gz
```

2. Building references from a set of transcripts. If we only have a *de novo* assembled transcriptome, we have to build references directly from transcripts. In this case, if we want to quantify gene-level expression, we need to provide RSEM a map from isoforms to genes using the `--transcript-to-gene-map` option. RSEM is also able to quantify allele-specific expression. To quantify allele-specific expression, we need to build RSEM references from phased sequences and provide RSEM a map from phased haplotypes to isoforms and then to genes using the `--allele-to-gene-map` option.

Download mouse transcripts and the associated mapping file to `ref`, and untar the tarball by

```
tar -C ref -xzf ref/mouse_ref_building_from_transcripts.tar.gz
```

You should see two files: `mouse_ref.fa` and `mouse_ref_mapping.txt`. `mouse_ref.fa` is a multi-FASTA file containing all Ensembl mouse transcripts. Each transcript's identifier is a concatenation of its transcript_id and transcript_name (separated by a '_' sign). `mouse_ref_mapping.txt` is a mapping from transcripts to genes. As shown in the snippet below, each line of the mapping file contains a gene identifier and a transcript identifier.



```
ENSMUSG00000000001_Gnai3        ENSMUST00000000001_Gnai3-001
ENSMUSG00000000003_Pbsn ENSMUST00000000003_Pbsn-001
ENSMUSG00000000003_Pbsn ENSMUST00000114041_Pbsn-002
ENSMUSG00000000028_Cdc45        ENSMUST00000000028_Cdc45-001
ENSMUSG00000000028_Cdc45        ENSMUST00000096990_Cdc45-003
ENSMUSG00000000028_Cdc45        ENSMUST00000115585_Cdc45-002
```

Type the following command to build references from the Ensembl mouse transcripts:

```
software/RSEM-1.2.25/rsem-prepare-reference \
                                             --transcript-to-gene-map ref/mouse_ref_mapping.txt \
                                             --bowtie2 --bowtie2-path software/bowtie2-2.2.6 \
                                     ref/mouse_ref.fa ref/mouse_ref
```

Single Sample Analysis

Run RSEM on a single cell RNA-Seq data set

Great. Now we are ready to play with some real data!

First, please download this file to your `data` subdirectory and unzip it:

```
unzip -d data data/SRR937564.zip
```

It contains around 1 million 101bp-long paired-end reads sequenced from a single dendritic cell at 6h post-stimulation with LPS (Note: the original data file can be found at here).

Then type the following command to run RSEM on this data set. I'll explain the meaning of each option/parameter later.

```
software/RSEM-1.2.25/rsem-calculate-expression -p 8 --paired-end \
                                        --bowtie2 --bowtie2-path software/bowtie2-2.2.6 \
                                        --estimate-rspd \
                                        --append-names \
                                        --output-genome-bam \
                                        data/SRR937564_1.fastq data/SRR937564_2.fastq \
                                        ref/mouse_ref exp/LPS_6h
```

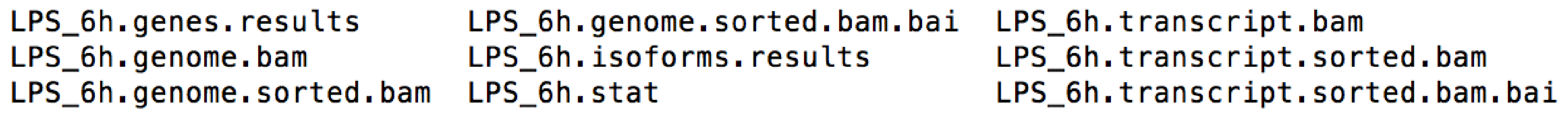
In the above command, `-p 8` tells RSEM to use 8 threads and `--paired-end` indicates the input reads are paired-end. In the second line, we tell RSEM to align reads using Bowtie 2, which is located at `software/bowtie2-2.2.6`. In the third line, we turn on the `--estimate-rspd` option because we wonder if there is any sequencing bias in the data. `--estimate-rspd` enabls RSEM to learn from data how the reads are distributed across a transcript. The learned statistics can help us assess if any positional biases are shown in the data. In the fourth line, `--append-names` tells RSEM to append gene_name/transcript_name to the result files. By default, RSEM generates an annotated BAM file in transcript coordinates. Sometimes, we want the alignments in genomic coordinates instead. Thus, we turn on the `--output-genome-bam` option in the fifth line. Note that this option is only available when you build references from a genome. Then in the sixth line, we provide RSEM with two FASTQ files, which contain the first and second mates of the paired-end reads. In the last line, we tell RSEM where the references locate and where to output the results.

Because the reads are long (101bp), it might take a while for Bowtie 2 to align all reads. If you do not want to wait, you can download the Bowtie 2 alignments in BAM format to subdirectory `exp`, and then type the following command to run RSEM:

```
software/RSEM-1.2.25/rsem-calculate-expression -p 8 --paired-end \
                                        --bam \
                                        --estimate-rspd \
                                        --append-names \
                                        --output-genome-bam \
                                        exp/LPS_6h.bam \
                                        ref/mouse_ref exp/LPS_6h
```

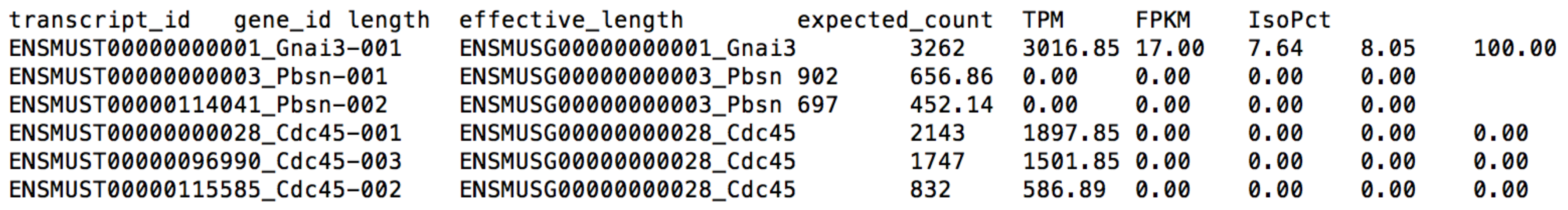
Note that the `--bam` option tells RSEM the input is a BAM file, instead of a pair of FASTQ files.

When RSEM finishes, You can find its outputs at the `exp` subdirectory. As shown in the snapshot below, all files share the prefix 'LPS_6h' in their names. Among the files, `LPS_6h.genes.results` and `LPS_6h.isoforms.results` contain the estimated gene and isoform level expressions. `LPS_6h.transcript.bam`, `LPS_6h.transcript.sorted.bam` and `LPS_6h.transcript.sorted.bam.bai` describe the annotated alignments in transcript coordinates. `LPS_6h.genome.bam`, `LPS_6h.genome.sorted.bam` and `LPS_6h.genome.sorted.bam.bai` describe the annotated alignments in genomic coordinates. `LPS_6h.stat` is a folder that contains model parameters learned from the real data.



```
LPS_6h.genes.results        LPS_6h.genome.sorted.bam.bai  LPS_6h.transcript.bam
LPS_6h.genome.bam           LPS_6h.isoforms.results       LPS_6h.transcript.sorted.bam
LPS_6h.genome.sorted.bam    LPS_6h.stat                   LPS_6h.transcript.sorted.bam.bai
```

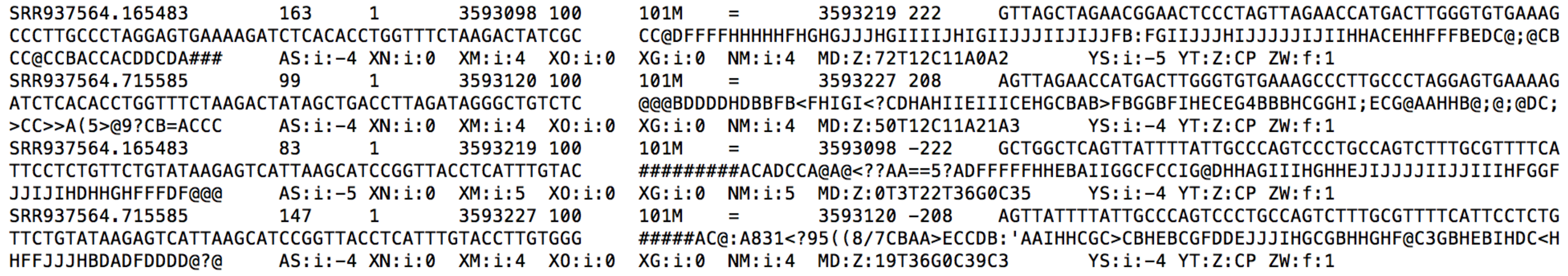
You can find a detailed explanation of each output file at here. Let us look at `LPS_6h.isoforms.results` and `LPS_6h.genome.sorted.bam` more closely. Below is a snippet of `LPS_6h.isoforms.results`:



```
transcript_id   gene_id length  effective_length        expected_count  TPM     FPKM    IsoPct
ENSMUST00000000001_Gnai3-001    ENSMUSG00000000001_Gnai3        3262    3016.85 17.00   7.64    8.05    100.00
ENSMUST00000000003_Pbsn-001     ENSMUSG00000000003_Pbsn 902     656.86  0.00    0.00    0.00    0.00
ENSMUST00000114041_Pbsn-002     ENSMUSG00000000003_Pbsn 697     452.14  0.00    0.00    0.00    0.00
ENSMUST00000000028_Cdc45-001    ENSMUSG00000000028_Cdc45        2143    1897.85 0.00    0.00    0.00    0.00
ENSMUST00000096990_Cdc45-003    ENSMUSG00000000028_Cdc45        1747    1501.85 0.00    0.00    0.00    0.00
ENSMUST00000115585_Cdc45-002    ENSMUSG00000000028_Cdc45        832     586.89  0.00    0.00    0.00    0.00
```

The first two columns of this file give the transcript ID and its parent gene's ID for each transcript. Note that you can find the transcript/gene name at the end of each ID. The sixth column gives the expression level for each isoform in TPM (Transcript per Million). TPM is a relative measure of expression levels. It represents the number of copies each isoform should have supposing the whole transcriptome contains exactly 1 million transcripts. The fifth column provides the expected read count in each transcript, which can be utilized by tools like EBSeq, DESeq and edgeR for differential expression analysis. The format of gene-level result file, `LPS_6h.genes.results`, is very similar.

`LPS_6h.genome.sorted.bam` is mainly used for visualization. Each alignment in this file is sorted in ascending order by its chromosome name and genomic coordinate. Look at the snippet below, you can find each alignment is also annotated with a `ZW:f:value` field. The ZW field gives the posterior probability that this alignment is true. In addition, the MAPQ field (5th field) is re-calculated based on the ZW value to reflect RSEM's confidence on each alignment.



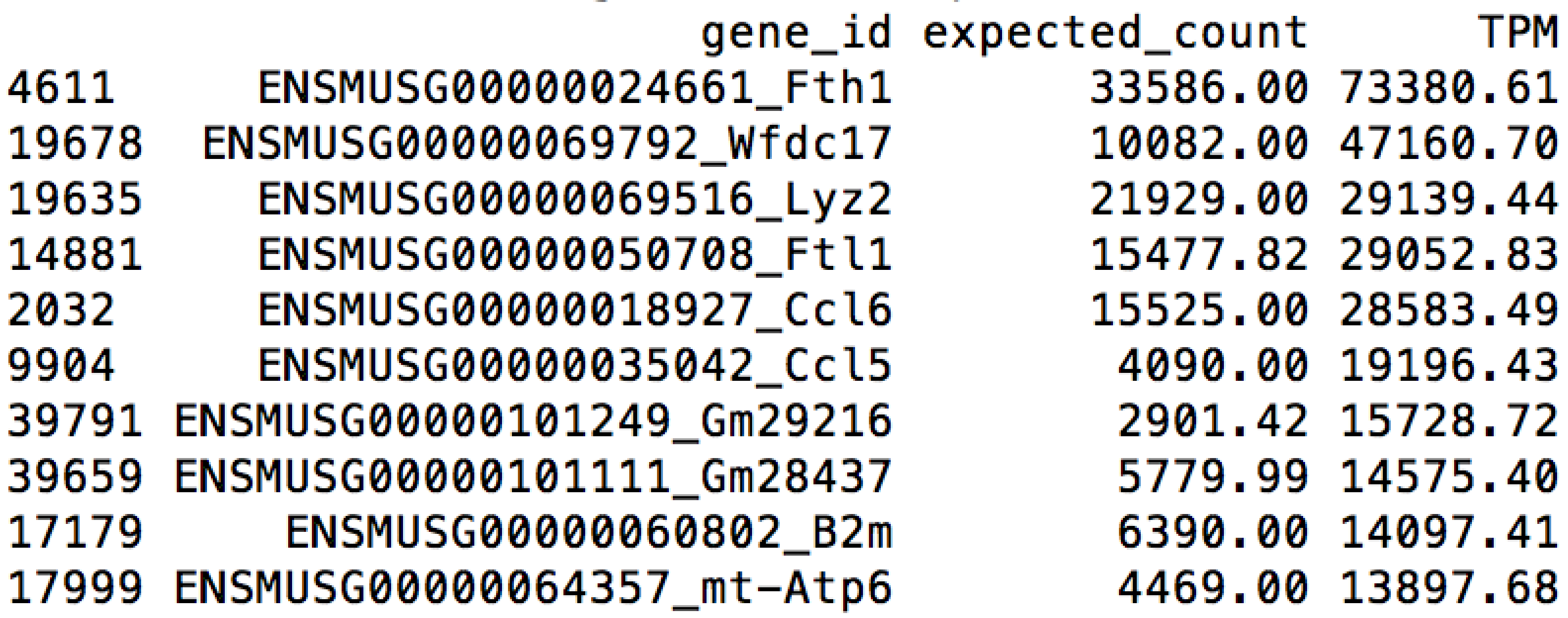
```
SRR937564.165483        163     1       3593098 100     101M    =       3593219 222     GTTAGCTAGAACGGAACTCCCTAGTTAGAACCATGACTTGGGTGTGAAAG
CCCTTGCCCTAGGAGTGAAAAGATCTCACACCTGGTTTCTAAGACTATCGC     CC@DFFFFHHHHHFHGHGJJJHGIIIIJHIGIIJJJIIJIJJFB:FGIIJJJHIJJJJJIJIIHHACEHHFFFBEDC@;@CB
CC@CCBACCACDDCDA###     AS:i:-4 XN:i:0  XM:i:4  XO:i:0  XG:i:0  NM:i:4  MD:Z:72T12C11A0A2       YS:i:-5 YT:Z:CP ZW:f:1
SRR937564.715585        99      1       3593120 100     101M    =       3593227 208     AGTTAGAACCATGACTTGGGTGTGAAAGCCCTTGCCCTAGGAGTGAAAAG
ATCTCACACCTGGTTTCTAAGACTATAGCTGACCTTAGATAGGGCTGTCTC     @@@BDDDDHDBBFB<FHIGI<?CDHAHIIEIIICEHGCBAB>FBGGBFIHECEG4BBBHCGGHI;ECG@AAHHB@;@;@DC;
>CC>>A(5>@9?CB=ACCC     AS:i:-4 XN:i:0  XM:i:4  XO:i:0  XG:i:0  NM:i:4  MD:Z:50T12C11A21A3      YS:i:-4 YT:Z:CP ZW:f:1
SRR937564.165483        83      1       3593219 100     101M    =       3593098 -222    GCTGGCTCAGTTATTTTATTGCCCAGTCCCTGCCAGTCTTTGCGTTTTCA
TTCCTCTGTTCTGTATAAGAGTCATTAAGCATCCGGTTACCTCATTTGTAC     #########ACADCCA@A@<??AA==5?ADFFFFFHHEBAIIGGCFCCIG@DHHAGIIIHGHHEJIJJJJIIJJIIIHFGGF
JJIJIHDHHGHFFFDF@@@     AS:i:-5 XN:i:0  XM:i:5  XO:i:0  XG:i:0  NM:i:5  MD:Z:0T3T22T36G0C35     YS:i:-4 YT:Z:CP ZW:f:1
SRR937564.715585        147     1       3593227 100     101M    =       3593120 -208    AGTTATTTTATTGCCCAGTCCCTGCCAGTCTTTGCGTTTTCATTCCTCTG
TTCTGTATAAGAGTCATTAAGCATCCGGTTACCTCATTTGTACCTTGTGGG     #####AC@:A831<?95((8/7CBAA>ECCDB:'AAIHHCGC>CBHEBCGFDDEJJJIHGCGBHHGHF@C3GBHEBIHDC<H
HFFJJJHBDADFDDDD@?@     AS:i:-4 XN:i:0  XM:i:4  XO:i:0  XG:i:0  NM:i:4  MD:Z:19T36G0C39C3       YS:i:-4 YT:Z:CP ZW:f:1
```

Explore the data

First, let us take a look at the highest expressed genes. Please switch to the `exp` subdirectory (by command `cd exp`) and type the following `R` commands:

```
data = read.table("LPS_6h.genes.results", header=T, stringsAsFactors=F)
idx = order(data[,"TPM"], decreasing=T)
data[idx[1:10], c("gene_id", "expected_count", "TPM")]
```

The above `R` commands list the top 10 highest expressed genes (shown below). Among the list, we can find several immune system related genes, such as Lyz2, Ccl6, Ccl5, which reassures us that the data are produced from a bone-marrow-derived dendritic cell.

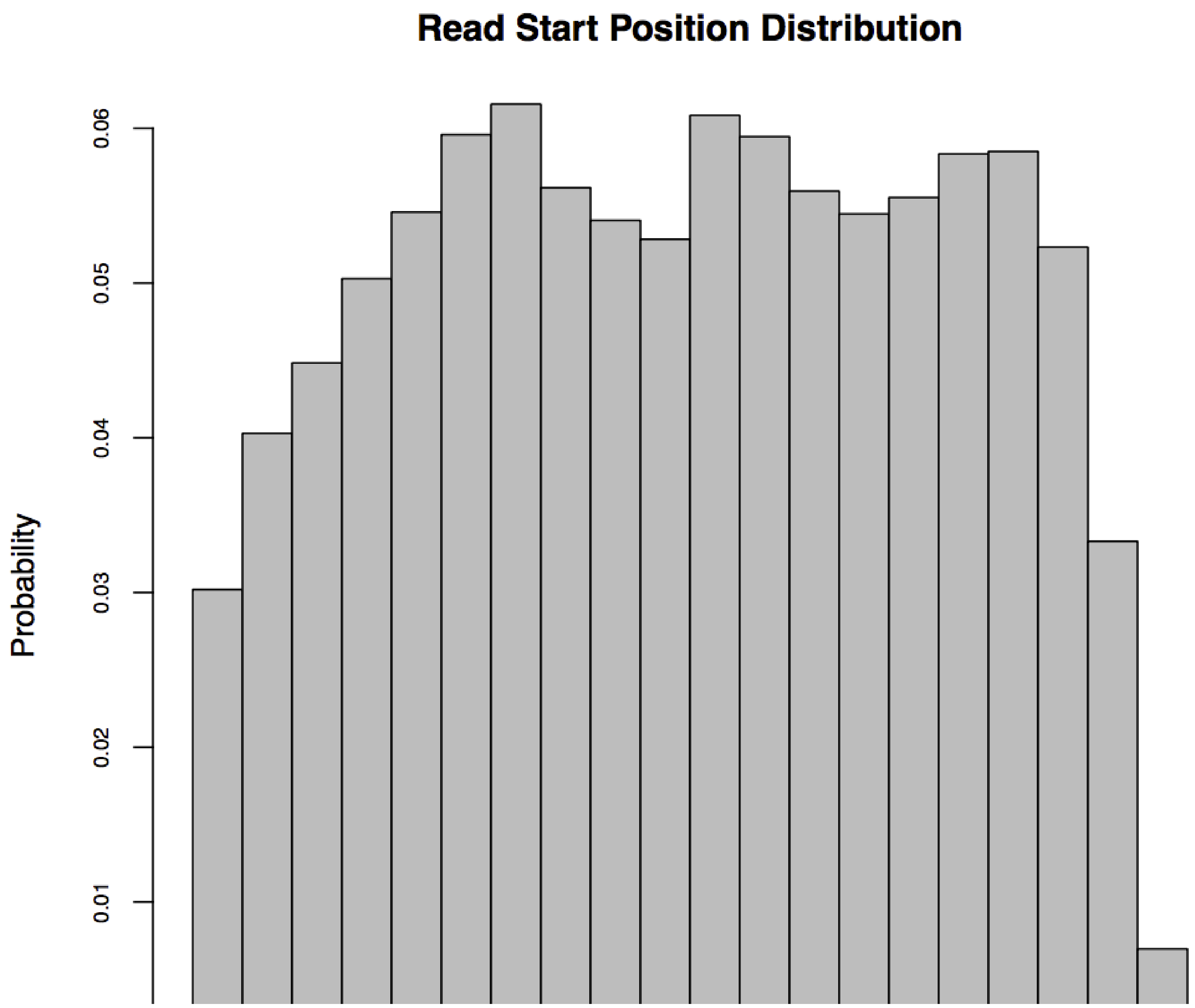


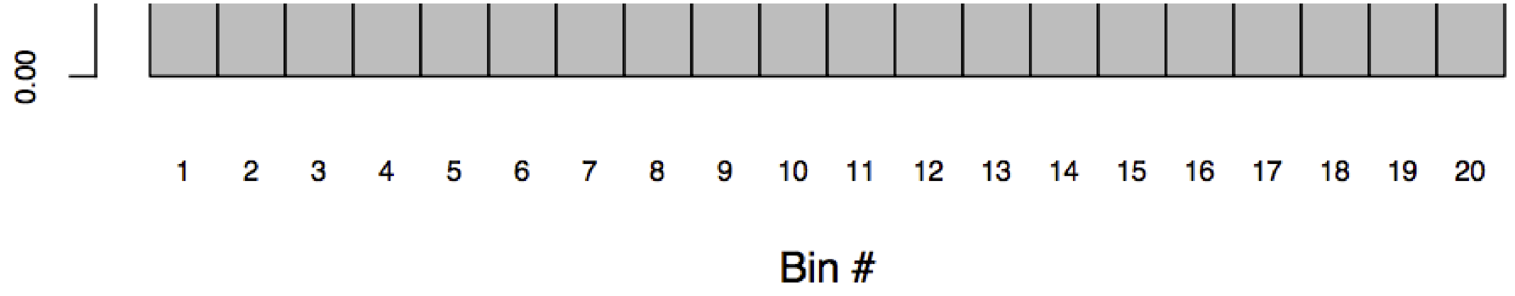
```
                               gene_id expected_count      TPM
4611      ENSMUSG00000024661_Fth1       33586.00 73380.61
19678   ENSMUSG00000069792_Wfdc17       10082.00 47160.70
19635     ENSMUSG00000069516_Lyz2       21929.00 29139.44
14881     ENSMUSG00000050708_Ftl1       15477.82 29052.83
2032      ENSMUSG00000018927_Ccl6       15525.00 28583.49
9904      ENSMUSG00000035042_Ccl5        4090.00 19196.43
39791 ENSMUSG00000101249_Gm29216        2901.42 15728.72
39659 ENSMUSG00000101111_Gm28437        5779.99 14575.40
17179      ENSMUSG00000060802_B2m        6390.00 14097.41
17999 ENSMUSG00000064357_mt-Atp6        4469.00 13897.68
```

Then let us focus on the data statistics learned by RSEM. Quit `R` and type the following command in the terminal:

```
../software/RSEM-1.2.25/rsem-plot-model LPS_6h LPS_6h_diagnostic.pdf
```

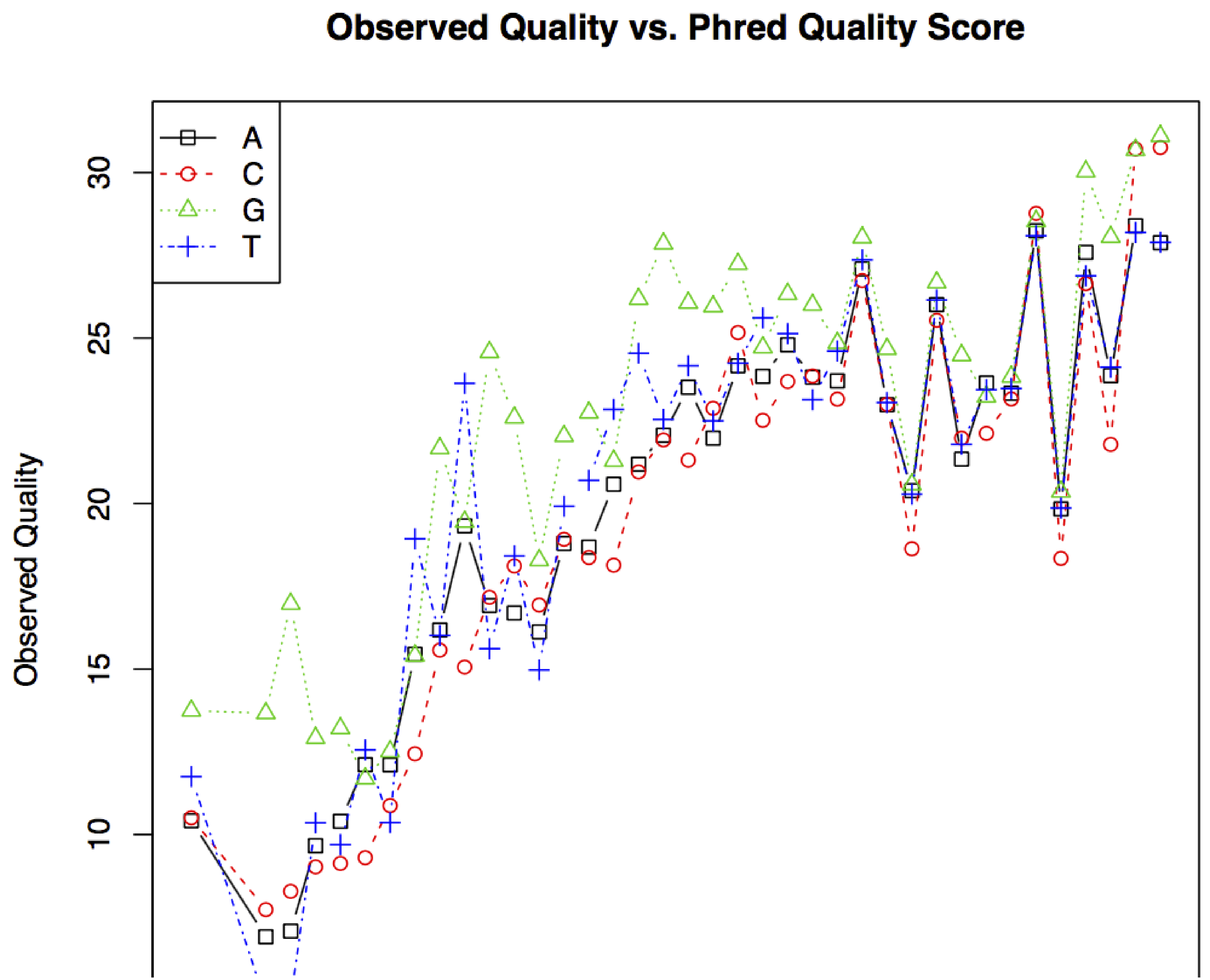
Command `rsem-plot-model` plots the model statistics RSEM learned from the data. The resulting file, `LPS_6h_diagnostic.pdf` contains plots of learned fragment length distribution, read length distribution, read start position distribution, quality score information and alignment statistics. For example, the below figure shows the read start position distribution (RSPD) learned from the single cell data.

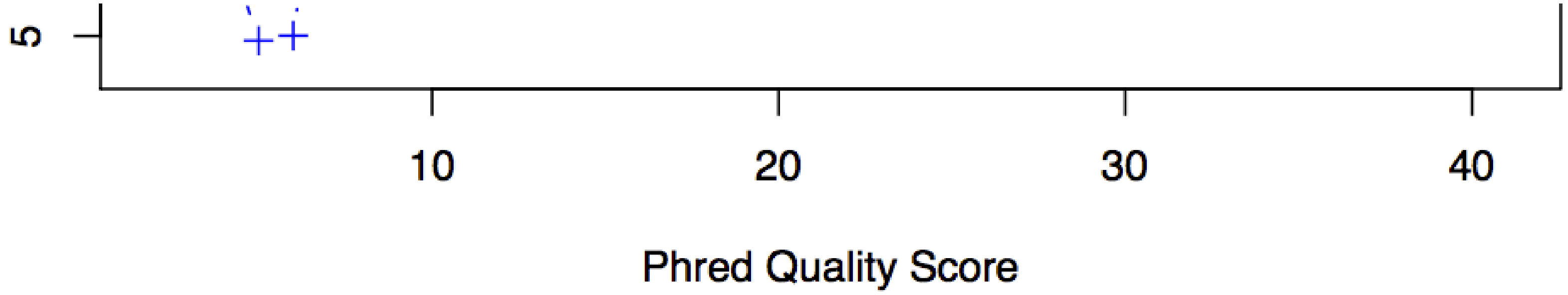

Read Start Position Distribution
Probability
0.01
0.02
0.03
0.04
0.05
0.06



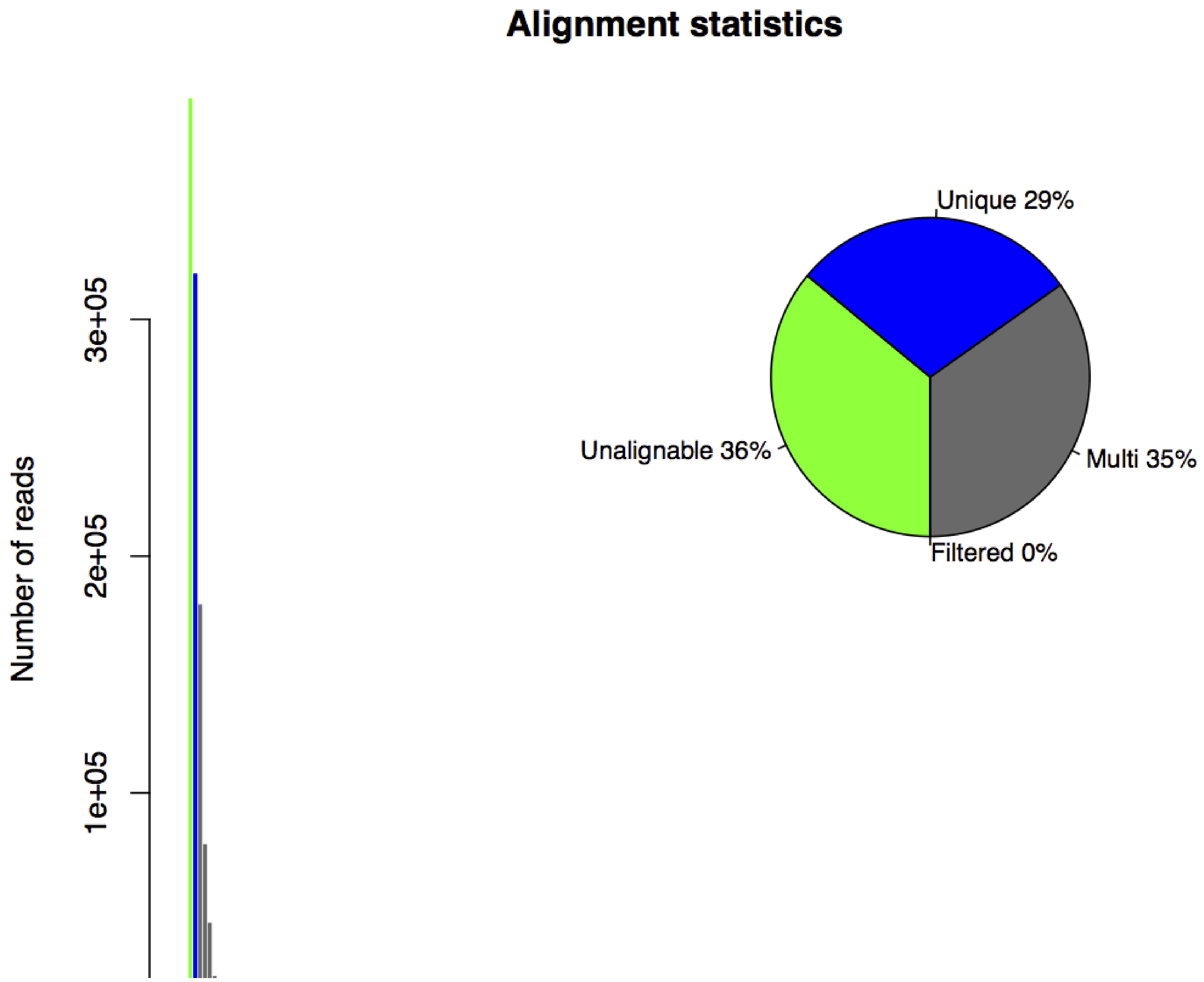
RSPD partitions each transcript into 20 bins and counts the frequency of reads starting at each bin. By examing the above RSPD, we notice that there are biases toward the ends of transcripts. This is reasonable because the sample was prepared using Nextera^{TM} transposons and it is known that these transposons are hard to reach the ends.

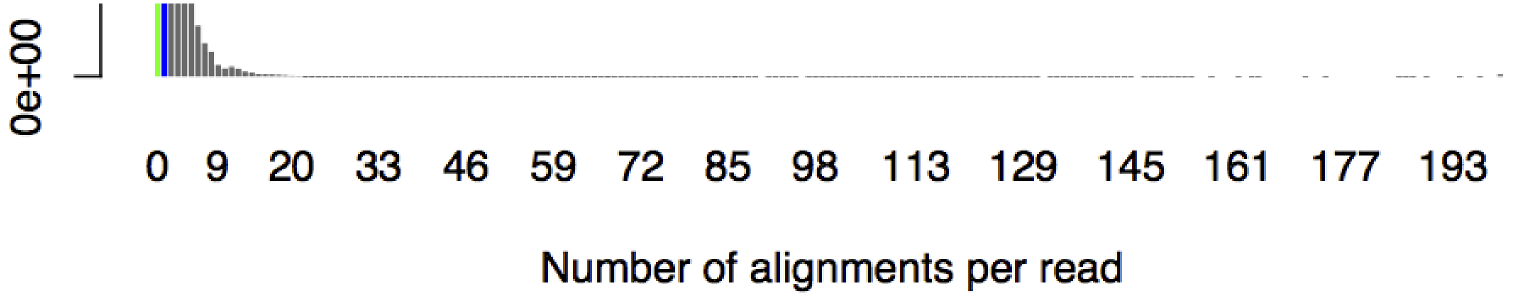
The quality score plot (shown below) plots the observed quality against the theoretical quality score for each nucleotide. In general, sequencing error decreases as the quality score increases. However, the theoretical quality score is more optimistic than the observed quality learned from the data.


Observed Quality vs. Phred Quality Score
Observed Quality
30
25
20
15
10
A
C
G
T



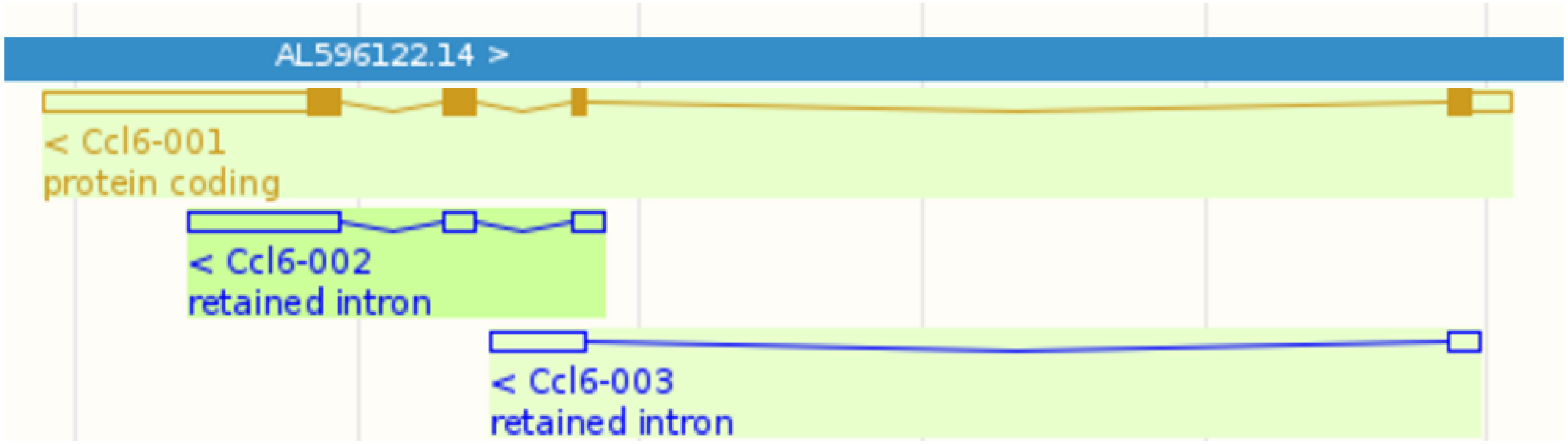
The alignment statistics plot (shown below) is another interesting figure to look at. In this plot, unalignable reads, uniquely mapped reads, multi-mapping reads and reads filtered due to too many alignments are represented by colors of green, blue, grey and red. The x-axis of the histogram bins reads by their number of transcript alignments. The y-axis counts the number of reads belonging to each bin. We also have a pie chart at the top-right corner showing the percentage of each type of read in the data. Based on the pie chart, there are much more multi-mapping reads (35%) than uniquely mapped reads (29%).


Alignment statistics
Number of reads
3e+05
2e+05
1e+05
Unique 29%
Multi 35%
Filtered 0%
Unalignable 36%



Visualize the alignments

Let us look at Ccl6, one of the top 10 highest expressed genes. This gene contains 3 isoforms: Ccl6-001, Ccl6-002, and Ccl6-003. Their splicing graphs are shown below (adopted from here):



It is very challenging to estimate isoform level expressions accurately for Ccl6 because Ccl6-002 is almost a subsequence of Ccl6-001 and Ccl6-003 also shares a significant portion of its sequence with Ccl6-001. We can find the following RSEM estimated isoform expression levels from `LPS_6h.isoforms.results`:

```
transcript_id   gene_id length  effective_length        expected_count  TPM     FPKM    IsoPct
ENSMUST00000019071_Ccl6-001     ENSMUSG00000018927_Ccl6 1440    1194.85 7805.95 8862.10 9334.46 31.00
ENSMUST00000138145_Ccl6-002     ENSMUSG00000018927_Ccl6 776     530.94  7719.05 19721.39        20772.55        69.00
ENSMUST00000150243_Ccl6-003     ENSMUSG00000018927_Ccl6 442     202.64  0.00    0.00    0.00    0.00
```

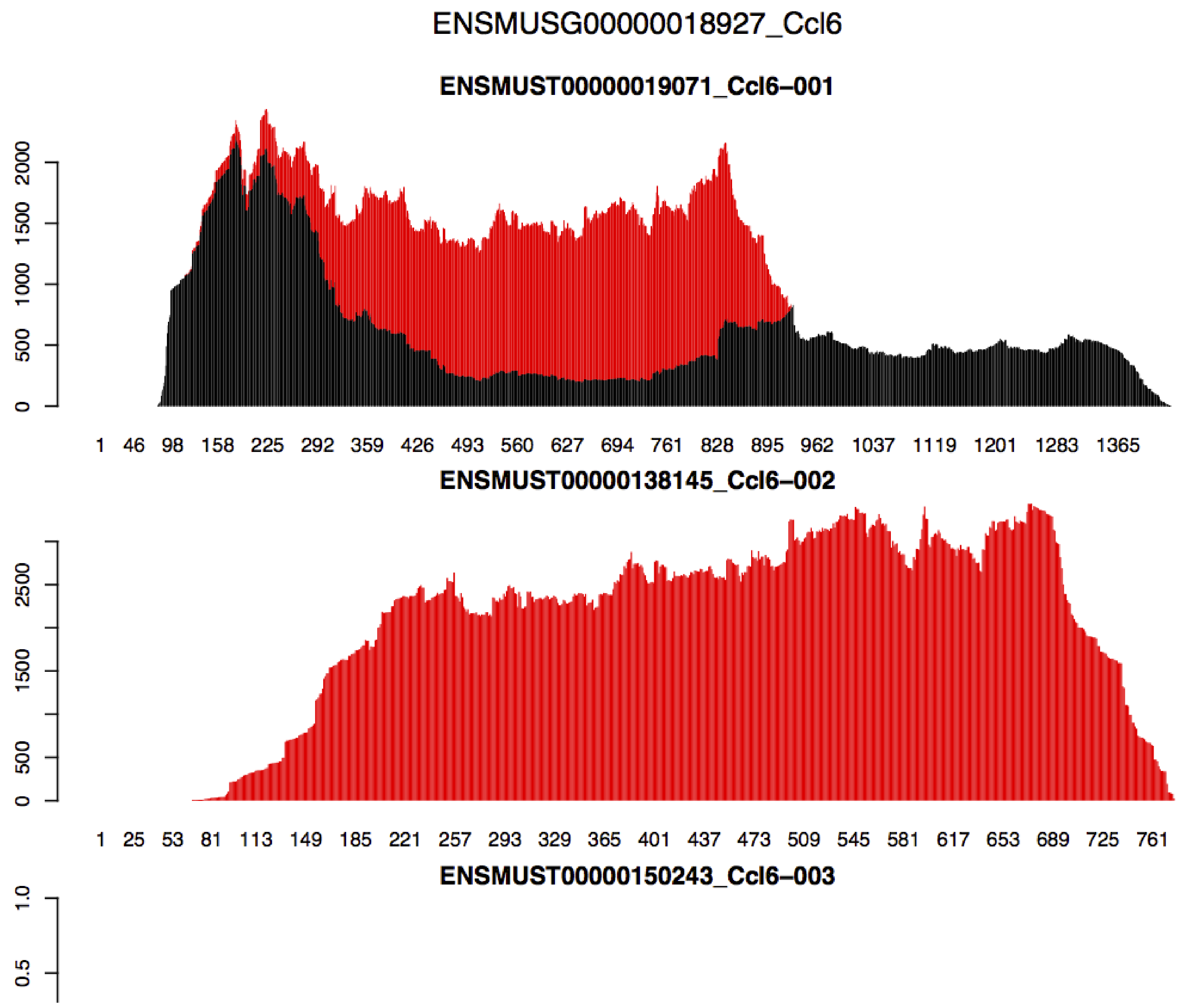
RSEM inferred that Ccl6-001 composes 31% of the Ccl6 gene in the transcriptome, Ccl6-002 composes 69%, and Ccl6-003 is not expressed. But how did RSEM make these conclusions? The stacked transcript wiggle plots for gene Ccl6 can provide us some hints. Wiggle plot is a way of displaying read depth at each transcript/genomic position. You can find a detailed description of its format from here. The stacked wiggle plots RSEM generate stack the expected multi-mapping read depth (shown in red) over the uniquely aligned read depth (shown in black).

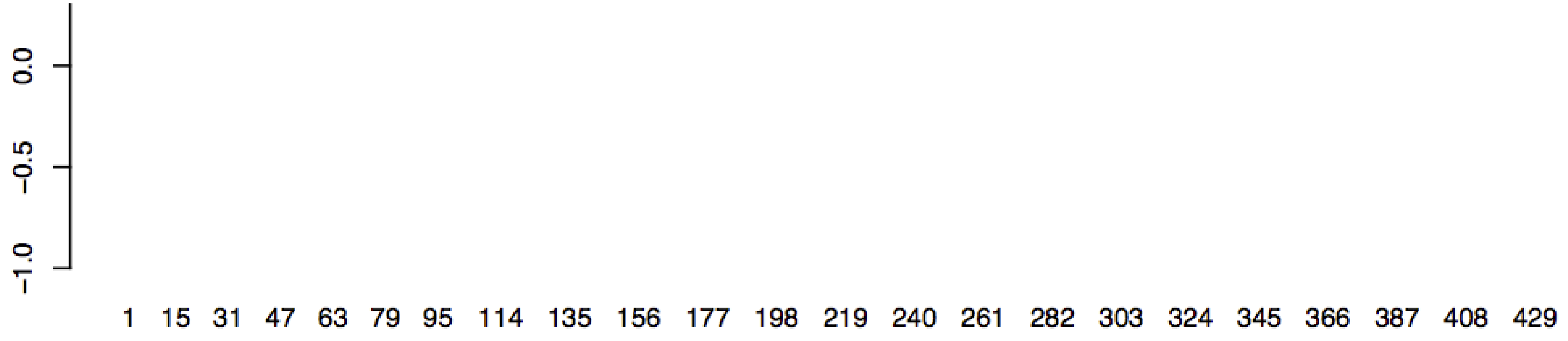
To generate the stacked transcript wiggle plots for Ccl6, type the following command:

```
../software/RSEM-1.2.25/rsem-plot-transcript-wiggles --gene-list --show-unique \
                                                       LPS_6h gene_ids.txt Ccl6_transcript_wiggle.pdf
```

Note that `gene_ids.txt` is already in the `exp` subdirectory and it contains the gene identifier of Ccl6.

In the generated figure (`Ccl6_transcript_wiggle.pdf`) shown below, black refers to uniquely aligned reads and red refers to the expected depth from multi-mapping reads. We can easily see that there is no uniquely mapped reads in Ccl6-002. Then why did RSEM assign more weights to Ccl6-002 than Ccl6-001 in the shared region? The reason is that RSEM tries to match read depth between different regions of isoform Ccl6-001. Thus, it needs to move extra weights to Ccl6-002. Ccl6-003 is marked as not expressed because there is no reads aligned to it.

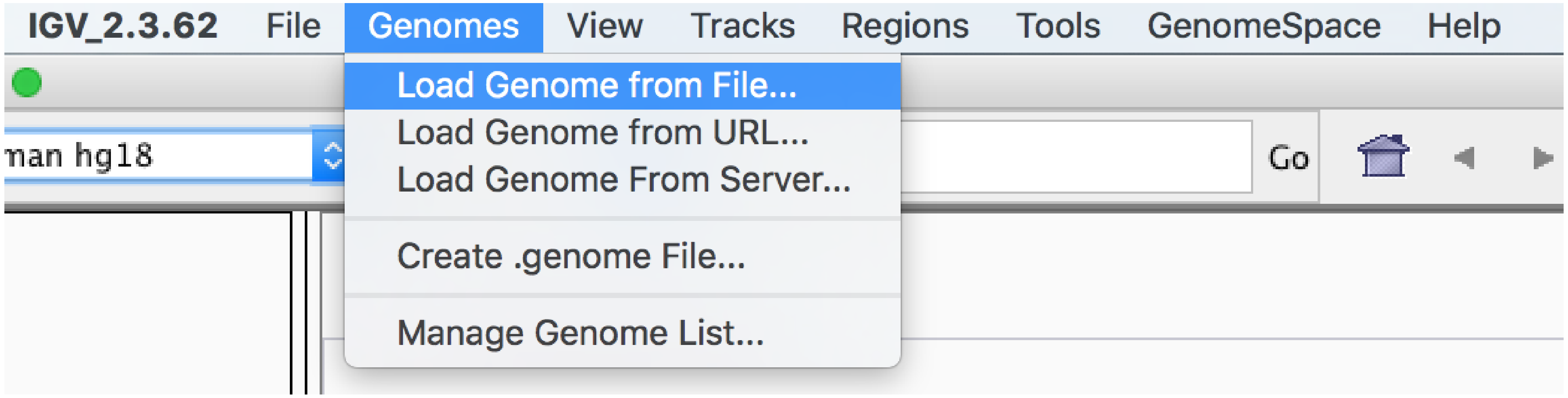

ENSMUSG00000018927_Ccl6
ENSMUST00000019071_Ccl6–001
0
500
1000
1500
2000
1 46 98 158 225 292 359 426 493 560 627 694 761 828 895 962 1037 1119 1201 1283 1365
ENSMUST00000138145_Ccl6–002
0
500
1500
2500
1 25 53 81 113 149 185 221 257 293 329 365 401 437 473 509 545 581 617 653 689 725 761
ENSMUST00000150243_Ccl6–003
0.5
1.0



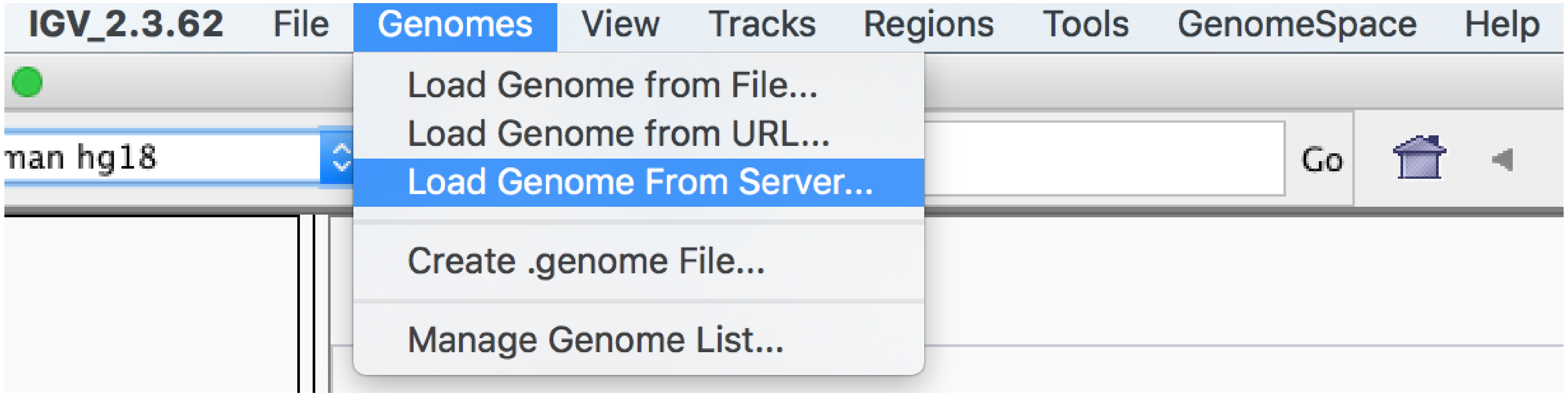
We can also visualize the read coverage and alignments information for gene Ccl6 in the genome. Let us first generate a genome-wide wiggle plot from the sorted RSEM genomic BAM file:

```
../software/RSEM-1.2.25/rsem-bam2wig LPS_6h.genome.sorted.bam LPS_6h.wig LPS_6h
```

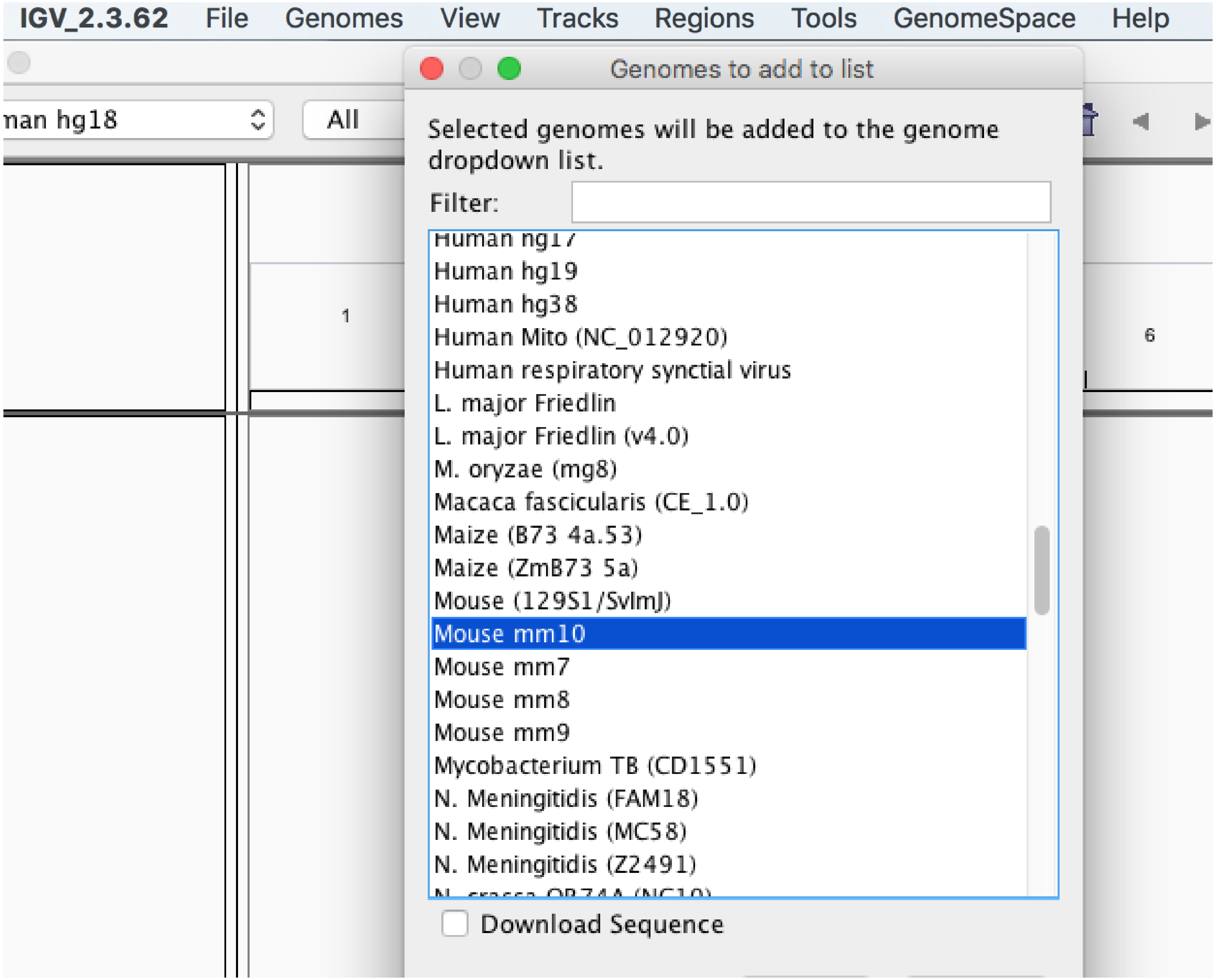
Then you need to install the Integrative Genomic Viewer (IGV) from here. Once IGV is installed, we can load the mouse reference genome from the file `Mus_musculus.GRCm38.dna.toplevel.fa`, which locates at `ref` (shown below).



If you haven't downloaded the mouse genome to `ref`, you can choose to load genome from server instead: Click 'Genomes' and then 'Load Genome From Server'.



After that choose `Mouse mm10`.

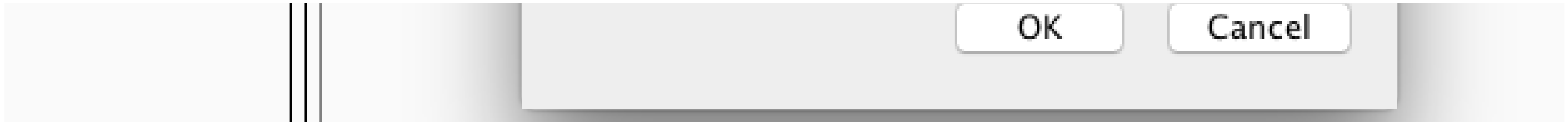


Selected genomes will be added to the genome dropdown list.

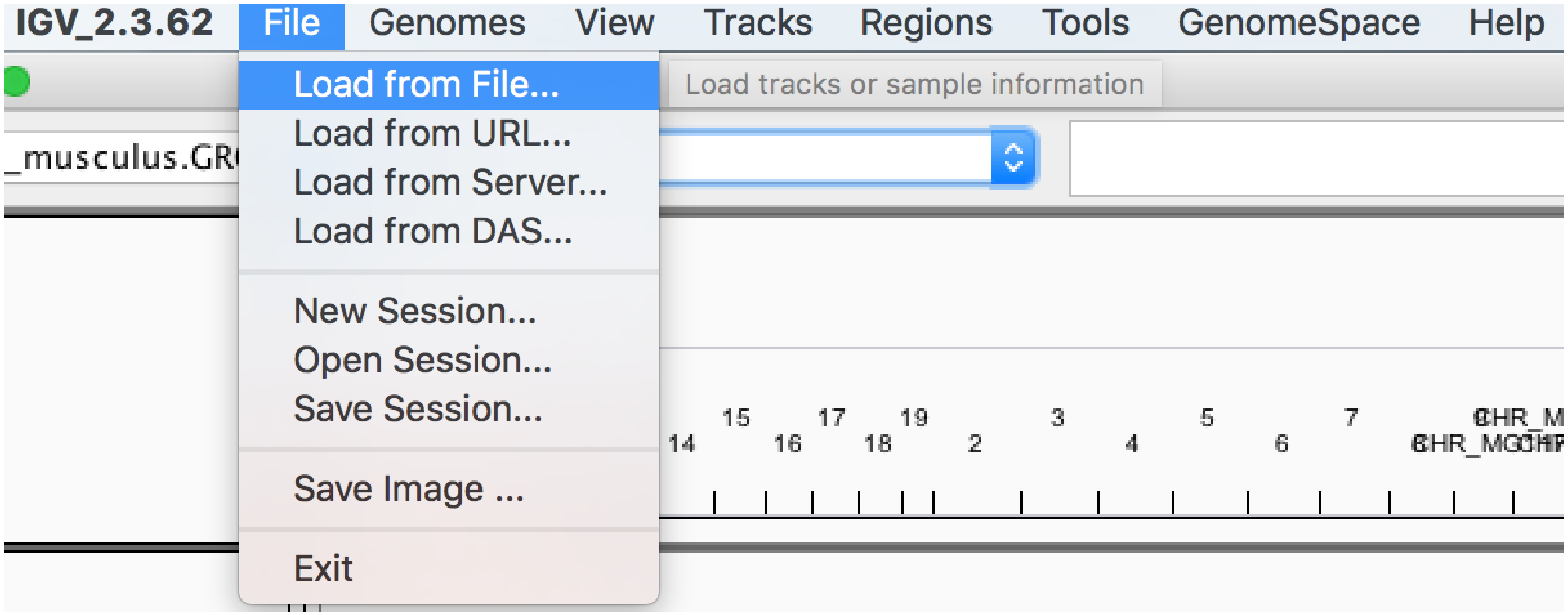
Filter:

Human hg17
Human hg19
Human hg38
Human Mito (NC_012920)
Human respiratory synctial virus
L. major Friedlin
L. major Friedlin (v4.0)
M. oryzae (mg8)
Macaca fascicularis (CE_1.0)
Maize (B73 4a.53)
Maize (ZmB73 5a)
Mouse (129S1/SvImJ)
Mouse mm10
Mouse mm7
Mouse mm8
Mouse mm9
Mycobacterium TB (CD1551)
N. Meningitidis (FAM18)
N. Meningitidis (MC58)
N. Meningitidis (Z2491)

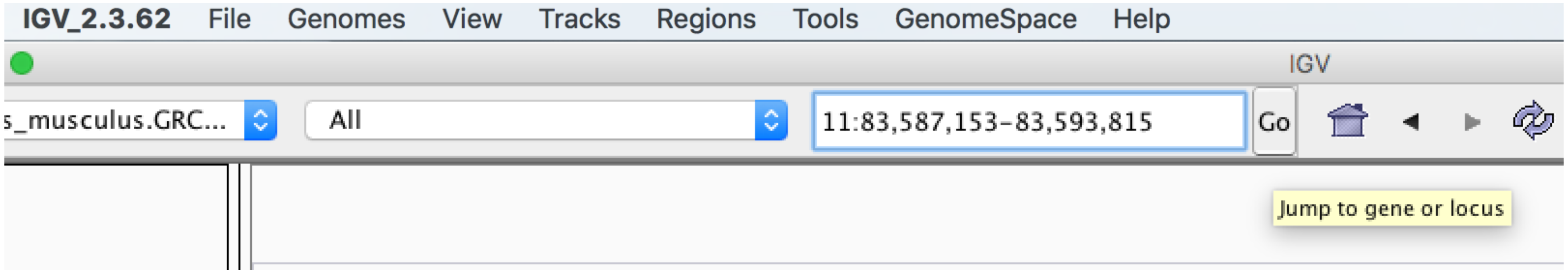
Download Sequence



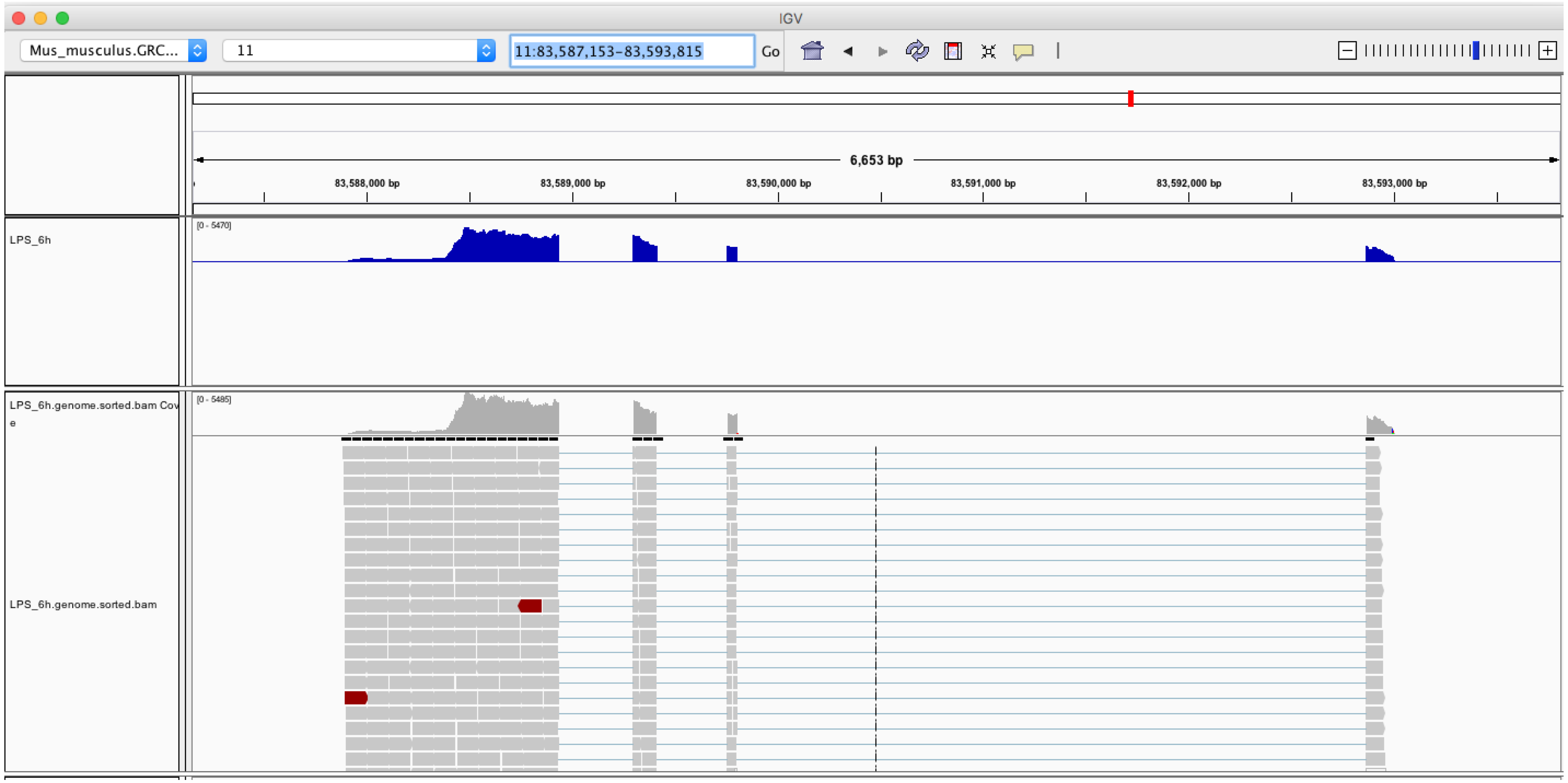
The next step is to load the wiggle and BAM files: Click 'File', 'Load from File', and then choose `LPS_6h.wig` to upload the wiggle file. Then repeat it and choose `LPS_6h.genome.sorted.bam` to load the sorted BAM file.



The last step is to locate the Ccl6 gene. Type `11:83,587,153-83,593,815` to IGV's position input box as shown below and then click 'Go'.



Now we can see the wiggle plot and BAM alignments at Ccl6 (shown below).



We can find that the shape of genomic wiggle plot is reversed. This is because Ccl6 locates at the reverse strand of chromosome 11.

Differential Expression Analysis using EBSeq

Differential expression analysis is one of the most common tasks biologists perform. The RSEM pipeline includes EBSeq for downstream differential expression analysis. Let us try the RSEM-EBSeq pipeline on some real single cell data sets.

Detecting differentially expressed genes

Sample Name	SRA Run ID	Number of Reads	Condition
LPS_6h	SRR937564	1,093,275	6h post-stimuation with LPS
LPS_6h_2	SRR937558	1,029,972	6h post-stimuation with LPS
LPS_6h_3	SRR937568	1,339,876	6h post-stimuation with LPS
Unstimulated	SRR937920	1,409,495	unstimulated
Unstimulated_2	SRR937927	1,526,289	unstimulated
Unstimulated_3	SRR937946	1,548,816	unstimulated

The single cell data listed in above consist of 6 samples in two conditions: 6h post-stimulation with LPS and unstimulated. Each condition contains 3 biological replicates. The first sample 'LPS_6h' is the one we have used in our previous analyses. Please download the RSEM-estimated expression levels for these 6 samples to subdirectory `exp`. Then type the following commands to detect differentially expressed genes:

```
unzip -u expression_levels_for_DE_analysis.zip
../software/RSEM-1.2.25/rsem-generate-data-matrix LPS_6h.genes.results \
                                                  LPS_6h_2.genes.results LPS_6h_3.genes.results \
                                                   Unstimulated.genes.results Unstimulated_2.genes.results \
                                                   Unstimulated_3.genes.results > GeneMat.txt
../software/RSEM-1.2.25/rsem-run-ebseq GeneMat.txt 3,3 GeneMat.results
../software/RSEM-1.2.25/rsem-control-fdr GeneMat.results 0.05 GeneMat.de.txt
```

In the above commands, `rsem-generate-data-matrix` extracts the estimated expected counts from each sample and then generates a count matrix `GeneMat.txt` that can be used by `EBSeq`. Then `rsem-run-ebseq` runs `EBSeq`. It takes as inputs the gene count matrix (`GeneMat.txt`), a comma-separated list of values representing the number of biological replicates each condition has (`3,3`), and outputs the results to `GeneMat.results`. Lastly, `rsem-control-fdr` selects a list of genes from `GeneMat.results` by controlling the false discovery rate (FDR) at level `0.05` and outputs them to `GeneMat.de.txt`.

`GeneMat.de.txt` contains 2,148 differentially expressed genes. The first several lines of this file are shown below.

```
"PPEE"  "PPDE"  "PostFC"        "RealFC"        "C1Mean"        "C2Mean"
"ENSMUSG00000000058_Cav2"       0       1       234.619078003079        21316.8486294564        213.158486294564        0
"ENSMUSG00000000290_Itgb2"      0       1       125.838714677226        147.215602534968        775.82056022441 5.26002944569089
"ENSMUSG00000000561_Wdr77"      0       1       48.5636211519093        4340.7951802958 43.397951802958 0
"ENSMUSG00000000805_Car4"       0       1       81.3050355752257        7328.18405164694        73.2718405164694        0
"ENSMUSG00000000959_Oxa1l"      0       1       82.1343315939511        7403.8505963284 74.028505963284 0
"ENSMUSG00000001228_Uhrf1"      0       1       0.0114695477375495      0.000127147169358197    0       78.6390179095389
"ENSMUSG00000001334_Fndc5"      0       1       0.0118649010871355      0.000131581952116991    0       75.9882644968579
"ENSMUSG00000001348_Acp5"       0       1       167.279046946416        15172.6162278209        151.716162278209        0
"ENSMUSG00000002983_Relb"       0       1       0.00435765887855449     4.79661343036252e-05    0       208.470423640148
"ENSMUSG00000003032_Klf4"       0       1       52.9109251501516        4737.45145839113        47.3645145839113        0
```

Each line describes a gene and contains 7 fields: the gene name, posterior probability of being equally expressed (PPEE), posterior probability of being differentially expressed (PPDE), posterior fold change of condition 1 over condition 2 (PostFC), real fold change of condition 1 over condition 2 (RealFC), mean count of condition 1 (C1Mean) and mean count of condition 2 (C2Mean). For fold changes, PostFC is recommended over the RealFC and you can find the definition of these two fold changes in the description of `PostFC` function of EBSeq vignette. Please also note that PostFC, RealFC, C1Mean and C2Mean are calculated based on normalized read counts.

These detected genes actually make sense to us. For example, there are evidence that the top three genes, Cav2, Itgb2, and Wdr77, are associated with the LPS stimulation.

Detecting differentially expressed isoforms

`EBSeq` can also detect differentially expressed isoforms. Why are we interested in differentially expressed isoforms? Let us consider this conceptual example: Suppose we have a genes with two isoforms. Compared to the first condition, the first isoform is up-regulated and the second isoform is down-regulated. Thus the net effect might be 0 but in fact both isoforms are differentially expressed. In this case, only conducting gene-level differential expression analyses will be misleading.

To produce a list of differentially expressed isoforms by controlling the FDR at level 0.05, type the following commands:

```
../software/RSEM-1.2.25/rsem-generate-ngvector ../ref/mouse_ref.transcripts.fa mouse_ref
../software/RSEM-1.2.25/rsem-generate-data-matrix LPS_6h.isoforms.results \
                                                  LPS_6h_2.isoforms.results LPS_6h_3.isoforms.results \
                                                  Unstimulated.isoforms.results Unstimulated_2.isoforms.results \
                                                  Unstimulated_3.isoforms.results > IsoMat.txt
../software/RSEM-1.2.25/rsem-run-ebseq --ngvector mouse_ref.ngvec \
                                       IsoMat.txt 3,3 IsoMat.results
../software/RSEM-1.2.25/rsem-control-fdr IsoMat.results 0.05 IsoMat.de.txt
```

Because isoforms of a same gene normally share a significant portion of their sequences, the read mapping uncertainty increases dramatically. Thus, the first command, `rsem-generate-ngvector` clusters isoform sequences into 3 clusters according to each isoform's hardness of being mapped uniquely. Then `EBSeq` estimates the mean and variance parameters separately for each cluster. You can find more details from the EBSeq paper. The rest of commands are similar to those used in the gene-level analysis.

Let us look at the first 10 differentially expressed isoforms in `IsoMat.de.txt`:

```
"PPEE"  "PPDE"  "PostFC"        "RealFC"        "C1Mean"        "C2Mean"
"ENSMUST00000000058_Cav2-001"   0       1       316.515067002259        21503.1705365843        215.021705365843        0
"ENSMUST00000000153_Gna12-001"  0       1       0.0146375529436093      0.000217929296751652    0       45.876441837124
"ENSMUST00000000299_Itgb2-001"  0       1       578.410019299716        45702.6219856393        457.016219856393        0
"ENSMUST00000000430_Galnt1-001" 0       1       0.0163058910464105      0.000209385316569498    0       47.7488408004764
"ENSMUST00000010278_Wdr77-001"  0       1       54.9435707771855        4270.59802880532        42.6959802880532        0
"ENSMUST00000080085_Krit1-001"  0       1       0.00816469957823659     0.000103993976169697    0       96.1494158461833
"ENSMUST00000197611_Krit1-010"  0       1       0.0162770153008303      0.000204843533142525    0       48.8077480957735
"ENSMUST00000199845_Krit1-002"  0       1       0.0145197864299237      0.000186116203278527    0       53.719873185919
"ENSMUST00000103194_Car4-001"   0       1       94.282739257009 7384.26725344871        73.8326725344871        0
"ENSMUST00000000985_Oxa1l-001"  0       1       93.7853483939529        7494.24834168224        74.9324834168224        0
```

Note that `Galnt1-001` is differentially expressed. However, its parent gene, `Galnt1` is not included in the list of differentially expressed genes. `Galnt1` has 8 isoforms and 4 of them are expressed in at least one sample. The `EBSeq` outputs for these 4 isoforms are:

```
                              "PPEE"             "PPDE"                 "PostFC"            "RealFC"             "C1Mean"          "C2Mean"
"ENSMUST00000000430_Galnt1-001" 0                1                      0.0163058910464105  0.000209385316569498 0                 47.7488408004764
"ENSMUST00000178605_Galnt1-201" 0.643051312626731 0.356948687373269     0.657133558468301   0.645292279785867    14.5918593938538  22.6182877562076
"ENSMUST00000170243_Galnt1-002" 1                1.7625519182148e-22    0.700757959020524   0.0287365854350868   0                 0.337988456129871
"ENSMUST00000164066_Galnt1-007" 1                5.20548250115874e-23   0.357023409998205   0.00696655563479249  0                 1.42542957585207
```

In addition, the `EBSeq` output for the gene is:

```
                            "PPEE"             "PPDE"             "PostFC"           "RealFC"           "C1Mean"          "C2Mean"
"ENSMUSG00000000420_Galnt1"   0.191211801098033  0.808788198901967  0.216338362067182  0.206170296749745  14.3291889772544  69.5402174819086
```

It's easy to see that isoform `Galnt1-201` blurs the effect of isoform `Galnt1-001` and therefore makes gene `Galnt1` not identified as differentially expressed.

Simulation

In this section we will show you how to use RSEM's simulator to guide your design of sequencing experiments. Suppose that sample *LPS_6h* is from a pilot project we have conducted and we are interested in gene `Cav2` because it is differentially expressed and supported by existing literature. We want to ask the following two questions:

1. Have we sequenced deeply enough for `Cav2`?
2. If not, how many reads do we need to sequence?

To answer the first question, we need to assess the variablility of our expression estimates. Fortunately, by enabling the `--calc-ci` option, RSEM can provide us the 95% credibility intervals for each isoform / gene. In addition, RSEM will provides the coefficient of quartile variation (CQV), which is a robust way to measure the ratio between standard deviation and mean, for each isoform / gene. If `Cav2` has a small CQV, it means that we have enough reads to produce a good estimate. In this tutorial, we will set a threshold of `0.05` on the CQV value.

Type the following command to produce credibility intervals and CQV values for sample `LPS_6h`:

```
../software/RSEM-1.2.25/rsem-calculate-expression -p 8 --paired-end \
                                                   --bam --no-bam-output \
                                                   --estimate-rspd \
                                                   --append-names \
                                                   --calc-ci --single-cell-prior \
                                                   LPS_6h.bam ../ref/mouse_ref LPS_6h_ci
```

In the above command, `--calc-ci` tells RSEM to calculate credibility intervals and CQV values. `--single-cell-prior` tells RSEM to use priors that are suitable for single cell RNA-Seq data. `--no-bam-output` tells RSEM that we do not need the BAM outputs, which can take some time to generate.

From `LPS_6h_ci.genes.results`, we can extract the following numbers for gene `Cav2`:

```
      gene_id                                      transcript_id(s)                                      length        effective_length
ENSMUSG00000000058_Cav2      ENSMUST00000000058_Cav2-001,ENSMUST00000115459_Cav2-002,ENSMUST00000115462_Cav2-003   2733.00            2487.85

expected_count  TPM    FPKM    posterior_mean_count   posterior_standard_deviation_of_count   pme_TPM                pme_FPKM
    112.00      61.07  64.32          112.00                         0.00                     54.23                  63.96

TPM_ci_lower_bound     TPM_ci_upper_bound     TPM_coefficient_of_quartile_variation
44.1394                64.5581                0.0644385

FPKM_ci_lower_bound    FPKM_ci_upper_bound    FPKM_coefficient_of_quartile_variation
51.9479                75.8885                0.0642524
```

We can find that `Cav2`'s CQV on the TPM estimate is `0.0644385 > 0.05`, which means that we may need to sequence deeper.

To answer the second question, we need to use RSEM's simulator. The simulator simulate RNA-Seq data based on parameters learned from the real data. Thus we may use the simulated data as a good surrogate. Our strategy is to simulate data at increasing depth and calculate the CQV using RSEM. Once the CQV is below 0.05, we find the right sequencing depth.

As a first try, let us simulate 2 million reads using the following command:

```
../software/RSEM-1.2.25/rsem-simulate-reads ../ref/mouse_ref LPS_6h.stat/LPS_6h.model \
                                            LPS_6h.isoforms.results 0.36 2000000 LPS_6h_sim_2M \
                                            --seed 0
```

The first argument of `rsem-simulate-reads`, `../ref/mouse_ref`, tells RSEM where the reference is. Then the second and third arguments, `LPS_6h.stat/LPS_6h.model` and `LPS_6h.isoforms.results`, provide the learned sequencing error model and estimated expression levels from real data. The next argument, `0.36`, specifies portion of reads that come from background noise. The first item at the third line of `LPS_6h.stat/LPS_6h.theta` provides an estimate of this portion. Then you specify the number of reads to be simulated, `2000000`, and the output name, `LPS_6h_sim_2M`. Lastly, option `--seed 0` provides RSEM a seed to initialize the random number generator. We set this option to make sure the simulation is replicable.

When `rsem-simulate-reads` finishes, you should be able to find two FASTQ files containing the simulated reads: `LPS_6h_sim_2M_1.fq` and `LPS_6h_sim_2M_2.fq`.

Then we can run RSEM on the simulated data set by typing the following command:

```
../software/RSEM-1.2.25/rsem-calculate-expression -p 8 --paired-end \
                                                  --bowtie2 --bowtie2-path ../software/bowtie2-2.2.6 \
                                                  --estimate-rspd \
                                                  --append-names \
                                                  --no-bam-output \
                                                  --calc-ci --single-cell-prior \
                                                  LPS_6h_sim_2M_1.fq LPS_6h_sim_2M_2.fq \
                                                  ../ref/mouse_ref LPS_6h_sim_2M
```

Again, we extract the following numbers for gene `Cav2`, from `LPS_6h_sim_2M.genes.results`:

```
        gene_id                                                   transcript_id(s)                                             length          effective_length
ENSMUSG00000000058_Cav2         ENSMUST00000000058_Cav2-001,ENSMUST00000115459_Cav2-002,ENSMUST00000115462_Cav2-003     2733.00              2488.93

expected_count  TPM     FPKM    posterior_mean_count    posterior_standard_deviation_of_count   pme_TPM                 pme_FPKM
    205.00      62.58   66.09           205.00                          0.00                    58.44                   65.99

TPM_ci_lower_bound      TPM_ci_upper_bound      TPM_coefficient_of_quartile_variation
50.276                  66.6582                 0.0476313

FPKM_ci_lower_bound     FPKM_ci_upper_bound     FPKM_coefficient_of_quartile_variation
56.8448                 75.2815                 0.0473059
```

We can find now `Cav2`'s CQV becomes `0.0476313 < 0.05`, which means 2M reads is a good sequencing depth for us.