Integrated Assignment : April 2015

Day1: Parts 1-2
Day2: Parts 3-4

Background: PCA3 gene plays a role in Prostate Cancer detection due to its localized expression in prostate tissues and its over-expression in tumour tissues. This gene's expression profile makes it a useful marker that can complement the most frequently used biomarker for prostate cancer, PSA. There are cancer assays available that tests the presence of PCA3 in urine.

Objectives: In this assignment, we will be using a subset of the GSE22260 dataset, which consists of 30 RNA-seq tumour normal pairs, to assess the prostate cancer specific expression of the PCA3 gene.

Things to keep in mind:

- The libraries are polyA selected.
- The libraries are prepared as paired end.
- The samples are sequenced on Illumina's Genome Analyzer II.
- Each read is 36 bp long
- The average insert size is 150 bp with standard deviation of 38bp.
- We will only look at chromosome 9 in this exercise.
- Dataset is located here: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22260
- 20 tumour and 10 normal samples are available
- For this exercise we will pick 3 matched pairs (C02,C03,C06 for tumour and N02,N03,N06 for normal). We can do more if we have time.

PART 1 ------Obtainning Data and References --------

Goals:
- Obtain the files necessary for data processing
- Familiarize yourself with reference and annotation file format
- Familiarize yourself with sequence FASTQ format

#set your working directory

```
mkdir -p ~/workspace/rnaseq/integrated_assignment/
export RNA_HOME=~/workspace/rnaseq/integrated_assignment
```

#copy the necessary reference and annotation files. Note, when initiating an environment variable, we don't need the $; however, everytime we call the variable, it needs to be preceeded by a $.

#make sure that the environment variable is set correctly

```
echo $RNA_HOME
cp -r ~/CourseData/RNA_data/integrated_assignment_files/* $RNA_HOME
cd $RNA_HOME
```

Q1) How many directories are there under the “refs” directory?

```
ubuntu@ip-10-182-231-187:~/workspace/rnaseq/integrated_assignment/refs$ tree
.
└── hg19
    ├── bwt
    │   └── 9
    │       ├── 9.1.bt2
    │       ├── 9.2.bt2
    │       ├── 9.3.bt2
    │       ├── 9.4.bt2
    │       ├── 9.fa
    │       ├── 9.rev.1.bt2
    │       └── 9.rev.2.bt2
    ├── fasta
    │   └── 9
    │       └── 9.fa
    └── genes
        └── genes_chr9.gtf

6 directories, 9 files
```

Q2) How many exons does the gene PCA3 have?

```
ubuntu@ip-10-182-231-187:~/workspace/rnaseq/integrated_assignment/refs/hg19/genes$ grep PCA3 genes_chr9.gtf
9       antisense       exon    79379352        79379471        .       +       .       exon_id "ENSE00001600928"; exon_number "1"; gene_biotype "antisense"; gene_id "ENSG00000225937"; gene_name "PCA3"; tr
anscript_id "ENST00000412654"; transcript_name "PCA3-001"; tss_id "TSS5481";
9       antisense       exon    79397584        79397748        .       +       .       exon_id "ENSE00001597304"; exon_number "2"; gene_biotype "antisense"; gene_id "ENSG00000225937"; gene_name "PCA3"; tr
anscript_id "ENST00000412654"; transcript_name "PCA3-001"; tss_id "TSS5481";
9       antisense       exon    79398621        79398803        .       +       .       exon_id "ENSE00001693743"; exon_number "3"; gene_biotype "antisense"; gene_id "ENSG00000225937"; gene_name "PCA3"; tr
anscript_id "ENST00000412654"; transcript_name "PCA3-001"; tss_id "TSS5481";
9       antisense       exon    79399032        79402485        .       +       .       exon_id "ENSE00001664394"; exon_number "4"; gene_biotype "antisense"; gene_id "ENSG00000225937"; gene_name "PCA3"; tr
anscript_id "ENST00000412654"; transcript_name "PCA3-001"; tss_id "TSS5481";
```

Q3) How many cancer/normal samples do you see under the data directory?

```
ubuntu@ip-10-182-231-187:~/workspace/rnaseq/integrated_assignment/data$ tree
.
├── carcinoma_C02_read1.fasta
├── carcinoma_C02_read2.fasta
├── carcinoma_C03_read1.fasta
├── carcinoma_C03_read2.fasta
├── carcinoma_C06_read1.fasta
├── carcinoma_C06_read2.fasta
├── normal_N02_read1.fasta
├── normal_N02_read2.fasta
├── normal_N03_read1.fasta
├── normal_N03_read2.fasta
├── normal_N06_read1.fasta
└── normal_N06_read2.fasta

0 directories, 12 files
```

NOTE: The fasta files you have copied above contain sequences for chr9 only. I have pre-processed those fasta files to obtain chr9 and also matched read1/read2 sequences for each of the samples. You do not need to redo this; However, I will explain below the process I went through to get them to this point.

############################## FYI ###################################
- Access the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22260. Scroll down to select the files you want to download.

-The raw data in GEO is provided as _map.txt. After you download the files, you can run the following command to convert them to FASTA:

```
cat GSM554076_C02_read1_map.txt | grep chr9 | cut -f1,2 | awk '{print ">"$1"\n"$2}' > GSM554076_C02_read1_map.chr9.fasta

cat GSM554076_C02_read2_map.txt | grep chr9 | cut -f1,2 | awk '{print ">"$1"\n"$2}' > GSM554076_C02_read2_map.chr9.fasta
```

-The second challenge was to match the reads for both read1 and read2, since the two FASTA files have different number of records.

```
for i in `cat GSM554076_C02_read2_map.chr9.fasta | grep ">"`;do R1=`echo ${i} | sed 's/0\/2/0\/1/g'`; grep -A1 $R1 GSM554076_C02_read1_map.chr9.fasta >> carcinoma_C02_read1.fasta;done;

for i in `cat carcinoma_C02._read1.fasta | grep ">"`;do R2=`echo ${i} | sed 's/0\/1/0\/2/g'`; grep -A1 $R2 GSM554076_C02_read2_map.chr9.fasta >> carcinoma_C02_read2.fasta;done;
```

- Now you have two FASTA files with the same number of reads at the each end
############################## FYI ###################################

Q4) What sample has the highest number of reads?

PART 2 ----- Data alignment -----

Goals:
- Familiarize yourself with Tophat/Bowtie alignment options
- Perform alignments
- Obtain alignment summary

Q5) What is the value of --mate-inner-dist? What calculation did you do to get that answer?

Q6) Considering that the read length in this exercise is 36bp, what should you set the --segment-length to (default is 25bp)?

```
cd $RNA_HOME/
export RNA_DATA_DIR=$RNA_HOME/data/
echo $RNA_DATA_DIR
mkdir -p alignments/tophat/trans_idx
cd alignments/tophat
export TRANS_IDX_DIR=$RNA_HOME/alignments/tophat/trans_idx/
echo $TRANS_IDX_DIR
```

#take a minute and try to figure out what each parameter means and how we go the numbers.

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=normal
--rg-sample=normal_N02 -o normal_N02 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/normal_N02_read1.fasta $RNA_DATA_DIR/normal_N02_read2.fasta

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=normal
--rg-sample=normal_N03 -o normal_N03 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/normal_N03_read1.fasta $RNA_DATA_DIR/normal_N03_read2.fasta

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=normal
--rg-sample=normal_N06 -o normal_N06 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/normal_N06_read1.fasta $RNA_DATA_DIR/normal_N06_read2.fasta

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=carcinoma
--rg-sample=carcinoma_C02 -o carcinoma_C02 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/carcinoma_C02_read1.fasta $RNA_DATA_DIR/carcinoma_C02_read2.fasta

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=carcinoma
--rg-sample=carcinoma_C03 -o carcinoma_C03 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/carcinoma_C03_read1.fasta $RNA_DATA_DIR/carcinoma_C03_read2.fasta

tophat2 -p 8 --mate-inner-dist 80 --mate-std-dev 38 --segment-length 18 --rg-id=carcinoma
--rg-sample=carcinoma_C06 -o carcinoma_C06 -G $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--transcriptome-index $TRANS_IDX_DIR/ENSG_Genes $RNA_HOME/refs/hg19/bwt/9/9
$RNA_DATA_DIR/carcinoma_C06_read1.fasta $RNA_DATA_DIR/carcinoma_C06_read2.fasta

At this point, each one of your samples should have the following files:

```
ubuntu@ip-10-182-231-187:~/workspace/rnaseq/integrated_assignment/alignments/tophat/carcinoma_C02$ tree
.
├── accepted_hits.bam
├── align_summary.txt
├── deletions.bed
├── insertions.bed
├── junctions.bed
├── logs
│   ├── bam_merge_um.log
│   ├── bowtie_build.log
│   ├── bowtie.left_kept_reads.log
│   ├── bowtie.left_kept_reads.m2g_um.log
│   ├── bowtie.left_kept_reads.m2g_um_seg1.log
│   ├── bowtie.left_kept_reads.m2g_um_seg2.log
│   ├── bowtie.right_kept_reads.log
│   ├── bowtie.right_kept_reads.m2g_um.log
│   ├── bowtie.right_kept_reads.m2g_um_seg1.log
│   ├── bowtie.right_kept_reads.m2g_um_seg2.log
│   ├── gtf_juncs.log
│   ├── juncs_db.log
│   ├── long_spanning_reads.segs.log
│   ├── m2g_left_kept_reads.err
│   ├── m2g_left_kept_reads.out
│   ├── m2g_right_kept_reads.err
│   ├── m2g_right_kept_reads.out
│   ├── prep_reads.log
│   ├── reports.log
│   ├── reports.samtools_sort.log0
│   ├── run.log
│   ├── segment_juncs.log
│   └── tophat.log
├── prep_reads.info
└── unmapped.bam

1 directory, 30 files
```

Q7) How would you obtain summary statistics for each aligned file?

PART 3 ---- Expression Estimation ---------

Goals:
- Familiarize yourself with Cufflinks options
- Run Cufflinks to obtain expression values
- Obtain expression values for the gene PCA3

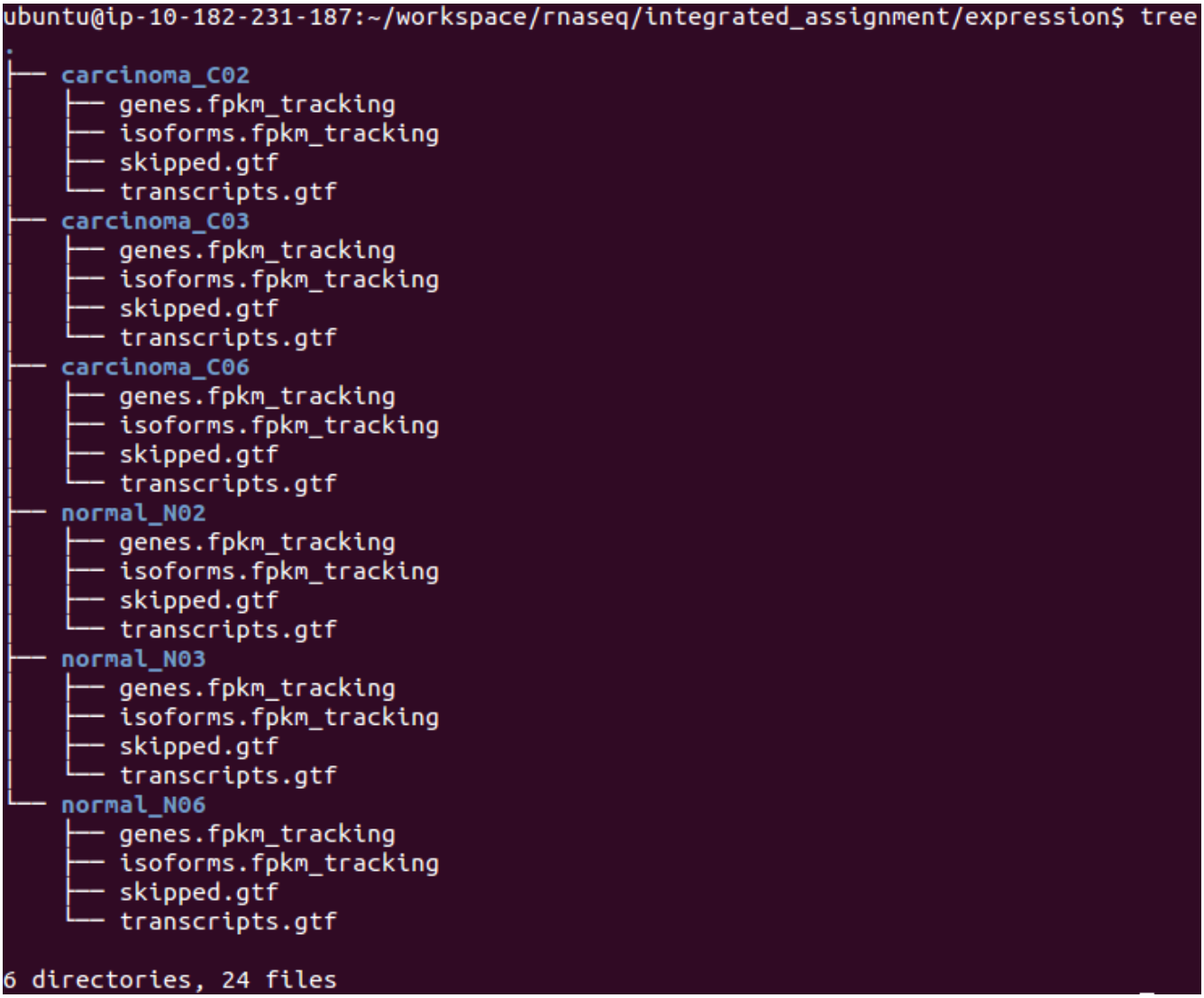
```
cd $RNA_HOME/
mkdir expression
cd expression
```

example (how to run cufflinks for one sample):

```
cufflinks -p 8 -o normal_N02 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf --no-update-check
$RNA_HOME/alignments/tophat/normal_N02/accepted_hits.bam
cufflinks -p 8 -o normal_N03 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf --no-update-check
$RNA_HOME/alignments/tophat/normal_N03/accepted_hits.bam
cufflinks -p 8 -o normal_N06 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf --no-update-check
$RNA_HOME/alignments/tophat/normal_N06/accepted_hits.bam

cufflinks -p 8 -o carcinoma_C02 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--no-update-check $RNA_HOME/alignments/tophat/carcinoma_C02/accepted_hits.bam
cufflinks -p 8 -o carcinoma_C03 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--no-update-check $RNA_HOME/alignments/tophat/carcinoma_C03/accepted_hits.bam
cufflinks -p 8 -o carcinoma_C06 --GTF $RNA_HOME/refs/hg19/genes/genes_chr9.gtf
--no-update-check $RNA_HOME/alignments/tophat/carcinoma_C06/accepted_hits.bam
```

At this point, you should have the following files in your “expression” directory:



Q8) How do you get the expression of PCA3 across the normal and carcinoma samples?

PART 4 -- Differential Expression Analysis ---

Goals:
- Perform differential analysis between tumor and normal samples
- Check if PCA3 is differentially expressed

cd $RNA_HOME/expression

ls -1 */transcripts.gtf > assembly_GTF_list.txt

cuffmerge -p 8 -o merged -g $RNA_HOME/refs/hg19/genes/genes_chr9.gtf -s $RNA_HOME/refs/hg19/bwt/9/ assembly_GTF_list.txt

cd $RNA_HOME/
mkdir de
mkdir de/reference_only
cd $RNA_HOME/alignments/tophat

#run cuffdiff to perform comparison

cuffdiff -p 8 -L Normal,Carcinoma -o $RNA_HOME/de/reference_only/ --no-update-check $RNA_HOME/expression/merged/merged.gtf normal_N02/accepted_hits.bam,normal_N03/accepted_hits.bam,normal_N06/accepted_hits.bam carcinoma_C02/accepted_hits.bam,carcinoma_C03/accepted_hits.bam,carcinoma_C06/accepted_hits.bam

At this point, you should have the following files under your “de” directory:

```
ubuntu@ip-10-182-231-187:~/workspace/rnaseq/integrated_assignment/de$ tree
.
└── reference_only
    ├── bias_params.info
    ├── cds.count_tracking
    ├── cds.diff
    ├── cds_exp.diff
    ├── cds.fpkm_tracking
    ├── cds.read_group_tracking
    ├── gene_exp.diff
    ├── genes.count_tracking
    ├── genes.fpkm_tracking
    ├── genes.read_group_tracking
    ├── isoform_exp.diff
    ├── isoforms.count_tracking
    ├── isoforms.fpkm_tracking
    ├── isoforms.read_group_tracking
    ├── promoters.diff
    ├── read_groups.info
    ├── run.info
    ├── splicing.diff
    ├── tss_group_exp.diff
    ├── tss_groups.count_tracking
    ├── tss_groups.fpkm_tracking
    ├── tss_groups.read_group_tracking
    └── var_model.info

1 directory, 23 files
```

Q9) any significant genes that are differentially expressed? what about PCA3?

NOTE: Make a copy of the data to use in generateCummerbund plots generation

```
cd $RNA_HOME/
mkdir final_results
cd $RNA_HOME/final_results
mkdir reference_only
cp $RNA_HOME/de/reference_only/isoform* reference_only/
cp $RNA_HOME/de/reference_only/read_groups.info reference_only/
```

NOTE: Rerun Obi's CummerBund Script focusing on PCA3 genes.

Q10) What plots can you generate to help you visualize this gene's expression profile?