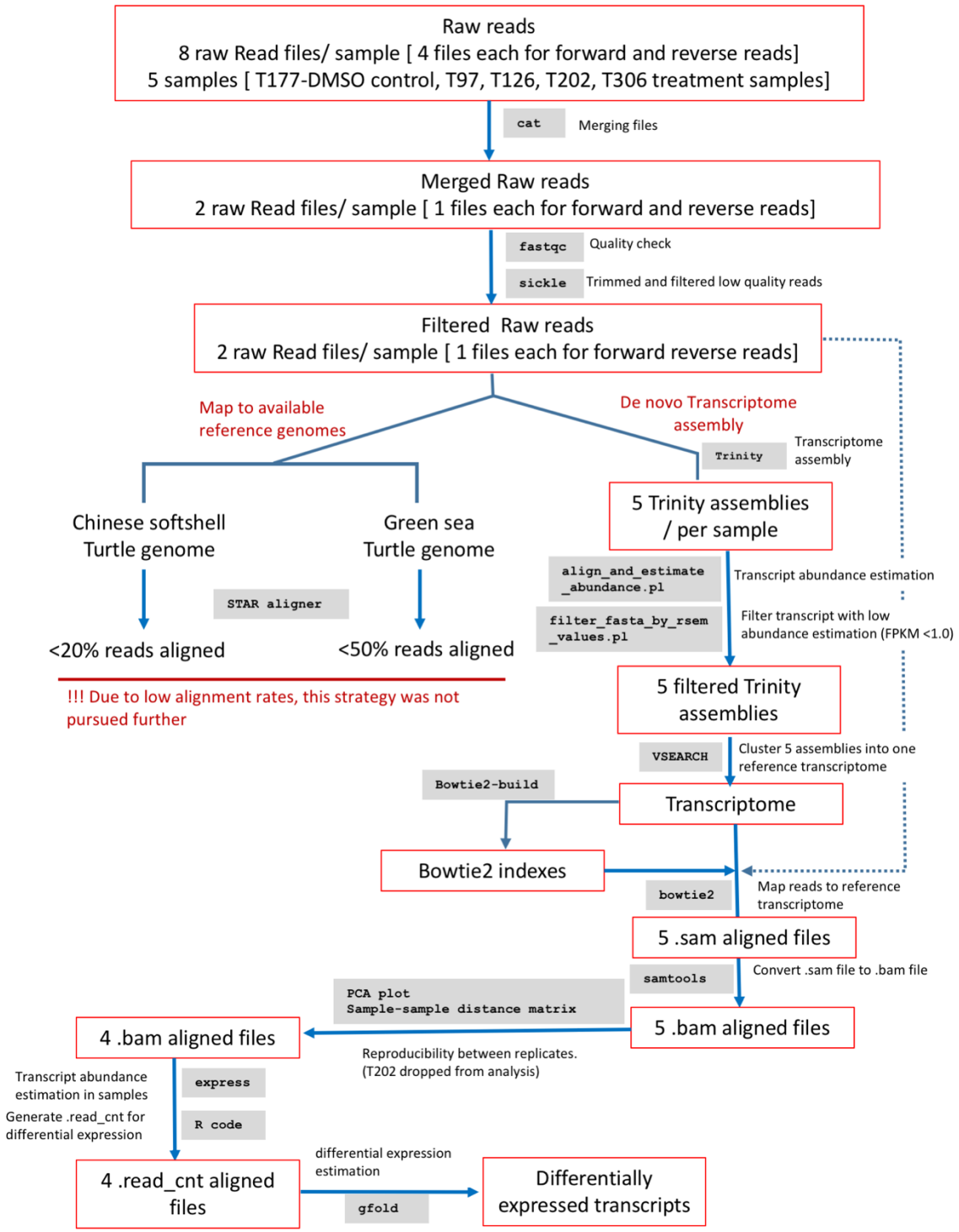
Analysis Outline:



RNA seq Analysis: Tutle yolk tissues following pesticide (Dieldrin) treatment

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Goal

The aim is to identify transcripts that are differentially expressed in tutle yolk tissues following pesticide (Dieldrin) treatment. In total 5 samples were collected, the control sample was treated with DMSO(T177) and the four treatment samples were treated with pesticide dieldrin (T97, T126, 202 and T306). There is variation in the pesticide crossing (or not crossing) the eggshell and getting to the yolk (so there is some unreliability in this regard). We will be interested in the largest fold-changes at target genes that are being differentially regulated in response

RAW READ Processing

Input Data

Raw Data : Format of file `.fastq`
Loc: `$WORK_DIR/original_fastq`

For each sample the raw sequence reads were split into 8 total files of which 4 corresponds to forward reads(R1) and other 4 for reverse reads(R2).

```
# T126 Sample
# T126_S2_L001_R1_001.fastq  T126_S2_L001_R2_001.fastq  T126_S2_L002_R1_001.f
astq  T126_S2_L002_R2_001.fastq
# T126_S2_L003_R1_001.fastq  T126_S2_L003_R2_001.fastq  T126_S2_L004_R1_001.f
astq  T126_S2_L004_R2_001.fastq
# T177 Sample
# T177_S1_L001_R1_001.fastq  T177_S1_L001_R2_001.fastq  T177_S1_L002_R1_001.f
astq  T177_S1_L002_R2_001.fastq
# T177_S1_L003_R1_001.fastq  T177_S1_L003_R2_001.fastq  T177_S1_L004_R1_001.f
astq  T177_S1_L004_R2_001.fastq
# T202 Sample
# T202_S5_L001_R1_001.fastq  T202_S5_L001_R2_001.fastq  T202_S5_L002_R1_001.f
astq  T202_S5_L002_R2_001.fastq
# T202_S5_L003_R1_001.fastq  T202_S5_L003_R2_001.fastq  T202_S5_L004_R1_001.f
astq  T202_S5_L004_R2_001.fastq
# T306 Sample
# T306_S3_L001_R1_001.fastq  T306_S3_L001_R2_001.fastq  T306_S3_L002_R1_001.f
astq  T306_S3_L002_R2_001.fastq
# T306_S3_L003_R1_001.fastq  T306_S3_L003_R2_001.fastq  T306_S3_L004_R1_001.f
astq  T306_S3_L004_R2_001.fastq
# T97 Sample
# T97_S4_L001_R1_001.fastq  T97_S4_L001_R2_001.fastq  T97_S4_L002_R1_001.fast
```

```
q  T97_S4_L002_R2_001.fastq
# T97_S4_L003_R1_001.fastq  T97_S4_L003_R2_001.fastq  T97_S4_L004_R1_001.fast
q  T97_S4_L004_R2_001.fastq
```

cat commands were used to put the reads from four files into a single file.

```
# cat TXXX_S5_L001_R1_001.fastq TXXX_S5_L002_R1_001.fastq TXXX_S5_L003_R1_001
.fastq TXXX_S5_L004_R1_001.fastq > $WORK_DIR/qc/TXXX_SX.R1.fastq
# cat TXXX_S5_L001_R2_001.fastq TXXX_S5_L002_R2_001.fastq TXXX_S5_L003_R2_001
.fastq TXXX_S5_L004_R2_001.fastq > $WORK_DIR/qc/TXXX_SX.R2.fastq
#
# XXX = 97, 126, 177, 202, 306
```

This leads to two raw reads files per sample, one for the forward reads and other for reverse reads. The new fastq files are located at **$WORK_DIR/qc**. The list of new files is

```
# T126_S2.R1.fastq  T126_S2.R2.fastq
# T177_S1.R1.fastq  T177_S1.R2.fastq
# T202_S5.R1.fastq  T202_S5.R2.fastq
# T306_S3.R1.fastq  T306_S3.R2.fastq
# T97_S4.R1.fastq  T97_S4.R2.fastq
```

The quality of the raw reads is assessed with fastqc tool. The results from fastqc are located at **$WORK_DIR/qc/fastqc_raw**.

```
# fastqc outputs
# multiqc_data
# multiqc_report.html
```

Firefox is ideal to visualise the **multiqc_report.html** result file. If you have X11 (refer : http://bioinformatics.uconn.edu/understanding-the-bbc-bioinformatics-facility-cluster-and-sge/)forwarding enabled then command **$ firefox multiqc_report.html** in your cluster terminal will open the file for inspection.The avearge quality scores at each base over the reads has a PHRED score >=30. However there will be reads in which the quality of bases is below acceptable level, to filter-out such reads **Sickle** was used. The general command that was used to filter the reads was

```
sickle pe -f TXXX_SX.R1.fastq -r TXXX_SX.R2.fastq -t sanger -o TXXX_SX.R1.tri
mmed.fastq -p TXXX_SX.R2.trimmed.fastq -s TXXX_SX.s.fastq -q 30 -l 40 -n
```

code explanation

command | *explanation*
sickle | *execute sickle command*
pe | *paired-end sequence trimming*
-f TXXX_SX.R1.fastq | *paired-end forward fastq file*
-r TXXX_SX.R2.fastq | *paired-end reverse fastq file*
-t sanger | *quality type*
-o TXXX_SX.R1.trimmed.fastq | *trimmed output forward file name*

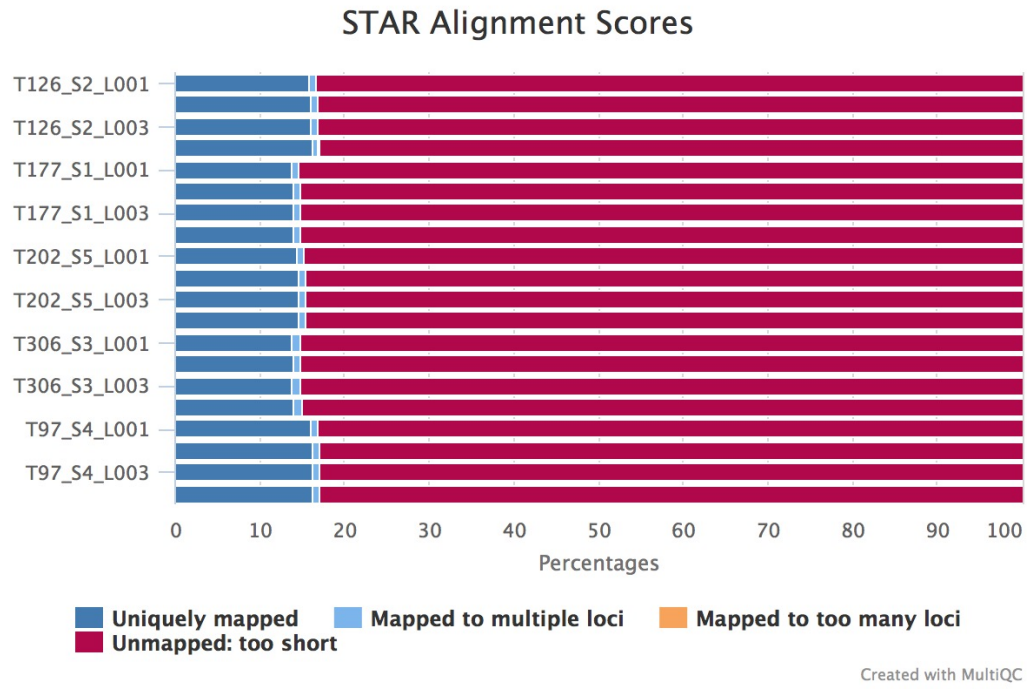
`-p TXXX_SX.R2.trimmed.fastq` | *trimmed output reverse file name*
`-s TXXX_SX.s.fastq` | *trimmed singles file.*
`-q 25` | *minimum quality score cutoff here it is 25.*
`-l 40` | *minimum read length to keep the read (here 40bps)*
`-n` | *Truncate sequences at position of first N*

The trimmed reads from the sickle are stored at **`$WORK_DIR/qc`** and the new files have suffix **`trimmed.fastq`**.

Aligning to existing turtle Genomes.

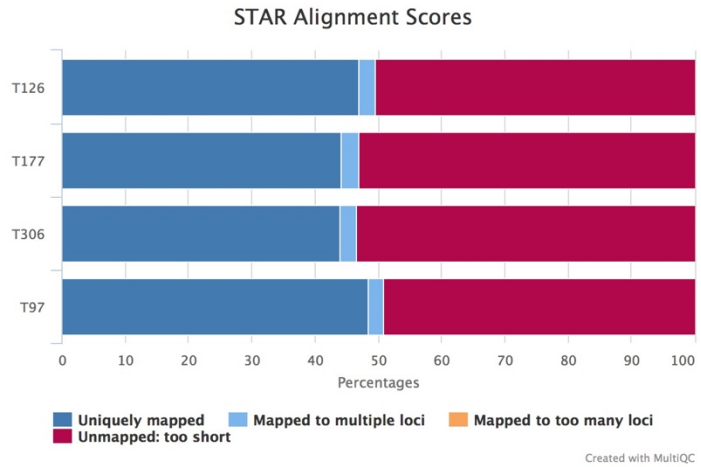
Two turtle genomes, Chinese softshell turtle and green shell turtle, were available which could have potentially served as reference genome provided there is a good alignmnt rate.

The reads were aligned to chinese softshell turtle (*Pelodiscus sinensis*) genome using STAR aligner. The alignment results are projected in the graph below::



As can be seen from the graph that less that 20% of reads alligned to the chinese softshell turtle. Beacuse of this poor allignment rate we did not use chinese softshell turtle genome as reference.

The other turtle genome that was available was green sea turtle (*Chelonia mydas*). A similar mapping approach with STAR aligner gave a better alignment around 50%, but even this is not good enough to serve as reference. The results of alignment from green sea turtle are shown in the graph below:



As the alignment rates of reads with available turtle genommes were below satisfactory levels we decided to do denovo transcriptome assembly using **Trinity**.

Trinity: denovo Transcriptome assembly

The trimmed and quality checked reads were used in Trinity. The Script is located at **$WORK_DIR/Trinity_result/Trinity/scripts/trinity_122116.sh**

```
#!/bin/bash
#$ -N tinity_turtle
#$ -M vijender.singh@uconn.edu
#$ -q highmem.q
#$ -m bea
#$ -S /bin/bash
#$ -cwd
#$ -pe smp 16
#$ -o /home/vsingh/jobsub_out_err/turtle_trinity_$JOB_ID.out
#$ -e /home/vsingh/jobsub_out_err/turtle_trinity_$JOB_ID.err


module load trinity/2.2.0

cp $WORK_DIR/qc/$1_*R1.trimmed.fastq /tempdata3/vjsingh/turtle/
cp $WORK_DIR/qc/$1_*R2.trimmed.fastq /tempdata3/vjsingh/turtle/
#/tempdata3/vjsingh/turtle
mkdir -p /tempdata3/vjsingh/turtle/Trinity/$1_trinity/Trinity

Trinity --seqType fq --max_memory 256G --left /tempdata3/vjsingh/turtle/$1_*.
R1.trimmed.fastq --right /tempdata3/vjsingh/turtle/$1_*.R2.trimmed.fast --CPU
16 --min_contig_length 300 --output /tempdata3/vjsingh/turtle/Trinity/$1_trin
ity --normalize_reads
```

Code explanation

In above script **$1** is a varibale that is sequentialy replaced by T97, T126, T177, T202 and T136. The first 10 lines beginning with **#** refers to resources and environment avriables that are requested at the cluster for Trinity job.

module load trinity/2.2.0 | *Loads Trinity*
cp $WORK_DIR/qc/$1_*R1.trimmed.fastq /tempdata3/vjsingh/turtle/ |
cp $WORK_DIR/qc/$1_*R2.trimmed.fastq /tempdata3/vjsingh/turtle/ | *Copies fastq file form /archive to /tempdata3 for effecient execution of trinity.*
mkdir -p /tempdata3/vjsingh/turtle/Trinity/$1_trinity/Trinity | *Makes directory for each sample to hold output.*
Trinity | *Runs Trinity*
--seqType fq | *input sequence type, fq=fastq*
--max_memory 256G | *Specifies the RAM for running the job*
--left /tempdata3/vjsingh/turtle/$1_*.R1.trimmed.fastq | *Forward read file*
--right /tempdata3/vjsingh/turtle/$1_*.R2.trimmed.fast | *Reverse read file*
--CPU 16 | *no of CPUs requested*
--min_contig_length 300 | *minimum length of contigous sequence* **--output /tempdata3/vjsingh/turtle/Trinity/$1_trinity** | *Output directory*
--normalize_reads | *run in silico normalization of reads.*

The results **Trinity.fasta** from the Trinity runs were saved at **$WORK_DIR/Trinity_result/Trinity/TXXX_trinity/Trinity_assembly**. **XXX= 97, 126, 177, 206 and 306** .
Trinity.fasta contains the Trinity assembled contig in .fasta format. Each of the assemblies were evaluated with help fo perlscript **TrinityStats.pl**. The script is part of Trinity pacakge.

```
perl /opt/bioinformatics/trinity2/util/TrinityStats.pl $WORK_DIR/Trinity_resu
lt/Trinity/TXXX_trinity/Trinity_assembly/Trinity.fasta
```

The results are summarised in the google_document in the sheet Trinity de novo assembly.

	Trinity assembly results				
Trinity	**T126**	**T177**	**T202**	**T306**	**T97**
Total transcripts	459525	455342	451602	366388	398661
Total genes	391824	381534	381737	314217	342182
Average length	908	873	904	845	846
N50	1278	1190	1272	1135	1136
GC%	42.38	42.87	42.27	42.57	42.64

Since the number of contigs were very high (>365000) so the contigs were filtered based on their expression levels. RSEM was used to estimate the abundance of the transcripts.

```
/opt/bioinformatics/trinity2/util/align_and_estimate_abundance.pl \
        --transcripts /tempdata3/vjsingh/turtle/Trinity/$1_trinity/Trinity.fa
sta \
        --seqType fq \
        --left $WORK_DIR/qc/$1_*R1.trimmed.fastq \
        --right $WORK_DIR/qc/$1_*R2.trimmed.fastq \
        --est_method RSEM \
        --aln_method bowtie \
        --thread_count 4 \
        --trinity_mode \
        --output_dir $WORK_DIR/Trinity_result/Trinity/$1_trinity/Trinity/ \
        --prep_reference \
        --output_prefix $1
```

Transcripts whose abundance was filter with threshold value of FPKM value as 1. All Transcripts with FPKM abundance values less than 1 are filtered out.

```
/opt/bioinformatics/trinity2/util/filter_fasta_by_rsem_values.pl \
        --rsem_output=$WORK_DIR/Trinity_result/Trinity/TXXX_trinity/RSEM_abun
dance_results/TXXX.isoforms.results \
        --fasta=$WORK_DIR/Trinity_result/Trinity/TXXX_trinity/Trinity_assembl
y/Trinity.fasta \
        --output=$WORK_DIR/Trinity_result/Trinity/TXXX_trinity/Trinity_filter
edcd /TXXX_Trinity_fpkm_filtered.fasta  \
        --fpkm_cutoff=1
        --isopct_cutoff=1
```

The stats of the filtered Trinity.fasta files is summarised in the google_documnet in the sheet Trinity de novo assembly with coloumns grouped under heading "After Filtering fpkm_cutoff=1,isopct_cutoff=1".

	After Filtering fpkm_cutoff=1,isopct_cutoff=1				
Trinity	**T126**	**T177**	**T202**	**T306**	**T97**
Total transcripts	101141	132948	105427	121913	144012
Total genes	79178	109390	84034	102961	119888
Average length	1333	1115	1268	1066	1076
N50	2066	1671	1948	1598	1648
GC%	42.79	43.26	42.67	42.9	42.92

The number of contigs/transcripts in each sample ranges from 101141 to 144012. In order to get a representative transcriptome for differential expression analysis the filtered fasta files are clustered using vsearch. This was achieved in 2 steps, step first all filtered fasta files are merged into single fasta file and step two. Vsearch programm was run with merged fasta file.

STEP1

```
cat T306_Trinity_fpkm_filtered.fasta T202_Trinity_fpkm_filtered.fasta T177_Tr
inity_fpkm_filtered.fasta T126_Trinity_fpkm_filtered.fasta T97_Trinity_fpkm_f
iltered.fasta >> Trinity_merged.fasta
```

STEP2

```
vsearch --cluster_fast Trinity_merged.fasta \
    --threads 16 \
    --id 0.9 \
    --centroids vsearch_mergered_cluster.fasta \
    --uc clusters.uc
```

The output file is located at **/archive/OBrien_Turtle_RNASeq/Trinity_result/Trinity/cluster_results/vsearch_mergered_cluster.fasta**.

In order to calculate differential expression of transcripts from denovo assembled reference transcriptome generated from last step following steps were followed,

1. **STEP1** : Generate bowtie2 index of transcriptome.
2. **STEP2** : Align reads to reference genome in each sample with **bowtie2**.
3. **STEP3** : Evaluate reproduciblity/variation between samples using PCA and distance-matrix.
4. **STEP4** : Measure expression level of each transcript with express.
5. **STEP5** : Format express output for use in gfold for fold change estimation. R code**.**
6. **STEP6** : Find differentially expressed transcripts with gfold.
7. **STEP7** : Blast and identify the differentially expressed transcripts.

Codes used in each step are given below.

STEP1

```
module load bowtie2
bowtie2-build -f /archive/OBrien_Turtle_RNASeq/Trinity_result/gfold/Trinity_r
esult/Trinity/cluster_results/vsearch_mergered_cluster.fasta turtle_bowtie2_i
ndex
```

STEP2

```
module load bowtie2
bowtie2 -x turtle_bowtie2_index  \
  -1 $WORK_DIR/qc/$1_*R1.trimmed.fastq/TXXX_S2.R1.trimmed.fastq \
  -2 $WORK_DIR/qc/$1_*R1.trimmed.fastq/TXXX_S2.R2.trimmed.fastq  \
```

```
  -S T1XXX_bowtie2.sam

module load samtools/1.3.1
samtools view -bhS TXXX_bowtie2.sam | samtools sort - TXXX_sorted
```

STEP3

```
directory <- "/Users/vijendersingh/Documents/Data_analysis_projects/turtle"
outputPrefix <- "Turtle"
setwd(directory)

express_file_1 <- data.frame(read.table("T97_results.xprs",sep="\t", header =
TRUE))
express_file_2 <- data.frame(read.table("T126_results.xprs",sep="\t", header
= TRUE))
express_file_3 <- data.frame(read.table("T177_results.xprs",sep="\t", header
= TRUE))
express_file_4 <- data.frame(read.table("T202_results.xprs",sep="\t", header
= TRUE))
express_file_5 <- data.frame(read.table("T306_results.xprs",sep="\t", header
= TRUE))

subset1 <- data.frame(express_file_1$target_id, express_file_1$tot_counts)
subset2 <- data.frame(express_file_2$target_id, express_file_2$tot_counts)
subset3 <- data.frame(express_file_3$target_id, express_file_3$tot_counts)
subset4 <- data.frame(express_file_4$target_id, express_file_4$tot_counts)
subset5 <- data.frame(express_file_5$target_id, express_file_5$tot_counts)

sorted1 <- subset1[order(express_file_1$target_id),]
sorted2 <- subset2[order(express_file_2$target_id),]
sorted3 <- subset3[order(express_file_3$target_id),]
sorted4 <- subset4[order(express_file_4$target_id),]
sorted5 <- subset5[order(express_file_5$target_id),]

write.table(sorted1, "T97.txt", sep = "\t", row.names = FALSE, col.names = FA
LSE, quote = FALSE)
write.table(sorted2, "T126.txt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
write.table(sorted3, "T177.txt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
write.table(sorted4, "T202.txt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
write.table(sorted5, "T306.txt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)

sampleFiles<- c("T97.txt","T126.txt","T177.txt","T202.txt","T306.txt" )
sampleNames <- c("T97_RNAseq","T126_RNAseq","T177_RNAseq","T202_RNAseq","T306
_RNAseq")
sampleCondition <- c("T97","T126","T177","T202","T306")
sampleTable <- data.frame(sampleName = sampleNames, fileName = sampleFiles, c
```

```
ondition = sampleCondition)

treatments= c("T97","T126","T177","T202","T306")

suppressMessages(library("DESeq2"))
ddsHTSeq <- DESeqDataSetFromHTSeqCount(sampleTable = sampleTable,
                                       directory = directory,
                                       design = ~ condition)
colData(ddsHTSeq)$condition <- factor(colData(ddsHTSeq)$condition,
                                      levels = treatments)
dds <- DESeq(ddsHTSeq)


vsd <- varianceStabilizingTransformation(dds, blind=T)
rld <- rlog(dds, blind=T)

#PCA Plot
suppressMessages(library("genefilter"))
suppressMessages(library("ggplot2"))
suppressMessages(library("grDevices"))

rv <- rowVars(assay(rld))
select <- order(rv, decreasing=T)[seq_len(min(500,length(rv)))]
pc <- prcomp(t(assay(vsd)[select,]))

condition <- treatments
scores <- data.frame(pc$x, condition)
(pcaplot <- ggplot(scores, aes(x = PC1, y = PC2, col = (factor(condition))))
  + geom_point(size = 5)
  + ggtitle("Principal Components")
  + scale_colour_brewer(name = " ", palette = "Set1")
  + theme(
    plot.title = element_text(face = 'bold'),
    legend.position = c(.9,.2),
    legend.key = element_rect(fill = 'NA'),
    legend.text = element_text(size = 10, face = "bold"),
    axis.text.y = element_text(colour = "Black"),
    axis.text.x = element_text(colour = "Black"),
    axis.title.x = element_text(face = "bold"),
    axis.title.y = element_text(face = 'bold'),
    panel.grid.major.x = element_blank(),
    panel.grid.major.y = element_blank(),
    panel.grid.minor.x = element_blank(),
    panel.grid.minor.y = element_blank(),
    panel.background = element_rect(color = 'black',fill = NA)
  ))


# Heat map of sample-sample distance
```

```
sampleDists <- dist(t(assay(rld)))
suppressMessages(library("RColorBrewer"))
sampleDistMatrix <- as.matrix(sampleDists)
rownames(sampleDistMatrix) <- paste(rld$condition, rld$type, sep="-")
colnames(sampleDistMatrix) <- NULL
colors <- colorRampPalette( rev(brewer.pal(8, "Blues")) )(255)
heatmap(sampleDistMatrix,
         clustering_distance_rows=sampleDists,
         clustering_distance_cols=sampleDists,
         col=colors)
```

Based on Sample-sample distance matrix and PCA plot it appears that the treatment in sample T202 is not very effective. Sample-sample distance matrix T202 failed to group with other treatment samples and in PCA plot it is closer to T177 in PC1 of variation. PCA plot also shows that the reproducibility between samples was low and may be acounted to uneven treatmenty. Based on which T202 was not considered further in differential expression ananlysis.

FIGURE1

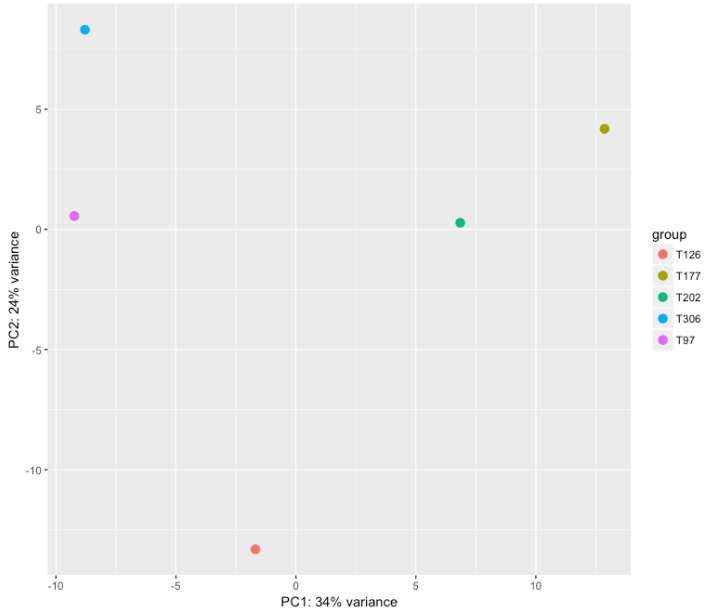
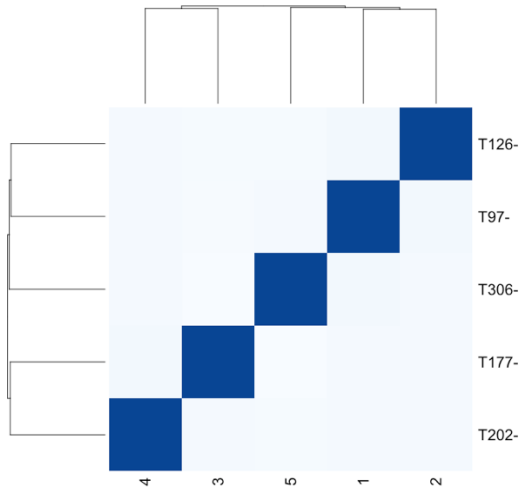


FIGURE2



STEP4

```
express $WORK_DIR/Trinity_result/Trinity/cluster_results/vsearch_mergered_clu
ster.fasta TXXX_sorted.bam -o TXXX_express
```

STEP5

```
express_file_1 <- data.frame(read.table("T177_results.xprs",sep="\t", header
= TRUE))
express_file_2 <- data.frame(read.table("T97_results.xprs",sep="\t", header =
TRUE))
express_file_3 <- data.frame(read.table("T126_results.xprs",sep="\t", header
= TRUE))
express_file_4 <- data.frame(read.table("T306_results.xprs",sep="\t", header
= TRUE))

subset1 <- data.frame(express_file_1$target_id, express_file_1$target_id, exp
ress_file_1$tot_counts, express_file_1$length,express_file_1$fpkm)
subset1 <- data.frame(express_file_1$target_id, express_file_1$target_id, exp
ress_file_1$tot_counts, express_file_1$length,express_file_1$fpkm)
subset1 <- data.frame(express_file_1$target_id, express_file_1$target_id, exp
ress_file_1$tot_counts, express_file_1$length,express_file_1$fpkm)
subset1 <- data.frame(express_file_1$target_id, express_file_1$target_id, exp
ress_file_1$tot_counts, express_file_1$length,express_file_1$fpkm)



subset1 <- rename(subset1,c("express_file_1.target_id"="GeneSymbol","express_
file_1.target_id.1"="GeneName","express_file_1.tot_counts"="Read Count","expr
ess_file_1.length" ="Gene exonlength","express_file_1.fpkm"="RPKM"))

sorted1 <- subset1[order(express_file_1$target_id),]
sorted2 <- subset2[order(express_file_2$target_id),]
```

```
sorted3 <- subset3[order(express_file_3$target_id),]
sorted4 <- subset4[order(express_file_4$target_id),]

write.table(sorted1, "T177.cnt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
write.table(sorted2, "T97.cnt", sep = "\t", row.names = FALSE, col.names = FA
LSE, quote = FALSE)
write.table(sorted3, "T126.cnt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
write.table(sorted4, "T306.cnt", sep = "\t", row.names = FALSE, col.names = F
ALSE, quote = FALSE)
```

STEP6

```
gfold diff -s1 T97,T126,T306 -s2 T177 -suf .read_cnt -o $WORK_DIR/Trinity_res
ult/gfold/DEseqNormalisation/T97_126_306_VS_T177_DEseqNorm.diff

gfold diff -s1 T97,T126,T306 -s2 T177 -suf .read_cnt -norm count -o $WORK_DIR
/Trinity_result/gfold/countNormalisation/T97_126_306_VS_T177_countNorm.diff
```

Sample output

```
# This file is generated by gfold V1.1.4 on Thu Mar 16 18:10:02 2017
# Normalization constants :
#    T97    484518  1.22474
#    T126   403897  1
#    T306   438018  1.10668
#    T177   412156  1.02988
# The GFOLD value could be considered as a reliable log2 fold change.
# It is positive/negative if the gene is up/down regulated.
# A gene with zero GFOLD value should never be considered as
# differentially expressed. For a comprehensive description of
# GFOLD, please refer to the manual.
#GeneSymbol                      GeneName                GFOLD(0.01)   E-FDR
log2fdc 1stRPKM 2ndRPKM
T126_TRINITY_DN100084_c0_g1_i1  T126_TRINITY_DN100084_c0_g1_i1  0   1   -0.44
1601   7.53901 0
T126_TRINITY_DN100158_c0_g1_i1  T126_TRINITY_DN100158_c0_g1_i1  0   1   0.344
063    4.0825  4.37953
T126_TRINITY_DN100177_c0_g1_i1  T126_TRINITY_DN100177_c0_g1_i1  0   1   -0.03
91536  4.63939 0
T126_TRINITY_DN100177_c1_g1_i1  T126_TRINITY_DN100177_c1_g1_i1  0   1   0.004
15352  3.49838 0
T126_TRINITY_DN100232_c0_g1_i1  T126_TRINITY_DN100232_c0_g1_i1  0   1   0.165
359    6.25644 5.03373
T126_TRINITY_DN100238_c0_g1_i1  T126_TRINITY_DN100238_c0_g1_i1  0   1   -0.44
4182   7.31943 0
T126_TRINITY_DN100263_c0_g1_i1  T126_TRINITY_DN100263_c0_g1_i1  0   1   0.312
583    6.21347 6.66555
```

```
T126_TRINITY_DN100331_c0_g1_i1  T126_TRINITY_DN100331_c0_g1_i1  0   1   0.519
429    2.3197  7.46542
```

The first output file contains 7 columns:

GeneSymbol:
Gene symbols. The order of gene symbol is the same as what appearing in the read count file.

GeneName:
Gene name. The order of gene name is the same as what appearing in the read count file.

GFOLD(value):
GFOLD value for every gene. The GFOLD value could be considered as a reliable log2 fold change. It is positive/negative if the gene is up/down regulated. The main usefulness of GFOLD is to provide a biological meanlingful ranking of the genes. The GFOLD value is zero if the gene doesn't show differential expression. If the log2 fold change is treated as a random variable, a positive GFOLD value x means that the probability of the log2 fold change (2nd/1st) being larger than x is (1 - the parameter specified by -sc); A negative GFOLD value x means that the probability of the log2 fold change (2st/1nd) being smaller than x is (1 - the parameter specified by -sc). If this file is sorted by this column in descending order then genes ranked at the top are differentially up-regulated and genes ranked at the bottom are differentially down-regulated. Note that a gene with GFOLD value 0 should never be considered differentially expressed. However, it doesn't mean that all genes with non-negative GFOLD value are differentially expressed. For taking top differentially expressed genes, the user is responsible for selecting the cutoff. Value in the bracket indicates the significant cutoff for fold change. Default 0.01.

E-FDR:
Empirical FDR based on replicates. It is always 1 when no replicates are available.

log2fdc:
log2 fold change. If no replicate is available, and -acc is T, log2 fold change is based on read counts and normalization constants. Otherwise, log2 fold change is based on the sampled expression level from the posterior distribution.

1stRPKM:
The RPKM for the first condition. It is available only if gene length is available. If multiple replicates are available, the RPKM is calculated simply by summing over replicates. Because RPKM is acturally using sequencing depth as the normalization constant, log2 fold change based on RPKM could be different from the log2fdc field.

2ndRPKM:
The RPKM for the second condition. It is available only if gene length is available. Please refer to 1stRPKM for more information.

STEP7 The Transcripts which had GFOLD value of >1 or <-1 (2 fold change in expression) were identified. The list of such transcripts are beow. The transcripts were blasted to RefSeq data base and link to the top hit is provided in the table below.

```
#GeneSymbol GeneName    GFOLD(0.01) E-FDR   log2fdc 1stRPKM 2ndRPKM BLAST mat
ch
T306_TRINITY_DN182495_c5_g2_i1  T306_TRINITY_DN182495_c5_g2_i1  1.164   0   2
.59571 0.941592    5.30302 https://www.ncbi.nlm.nih.gov/protein/gi%7C11026164
71
T202_TRINITY_DN201895_c12_g2_i6 T202_TRINITY_DN201895_c12_g2_i6 1.11337 0   2
.69724 0.840202    6.18057 https://www.ncbi.nlm.nih.gov/protein/gi%7C10693677
18
T177_TRINITY_DN214142_c4_g1_i3  T177_TRINITY_DN214142_c4_g1_i3  1.03659 0   2
.71875 3.85956 49.6845
T306_TRINITY_DN185419_c0_g4_i2  T306_TRINITY_DN185419_c0_g4_i2  1.02746 0   2
.58503 1.18818 8.03017 https://www.ncbi.nlm.nih.gov/protein/gi%7C1072289864
T306_TRINITY_DN181469_c0_g1_i1  T306_TRINITY_DN181469_c0_g1_i1  1.02281 0   2
.57231 1.4096  11.3412 https://www.ncbi.nlm.nih.gov/protein/gi%7C946605930
```

Transcript sequemces:

```
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```

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