# Class 14: RNASeq mini-project

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Here we will perform a complete RNASeq analysis from counts to pathways and biological interpretation.

The data for for hands-on session comes from GEO entry: GSE37704, which is associated with the following publication:

- Trapnell C, Hendrickson DG, Sauvageau M, Goff L et al. "Differential analysis of gene regulation at transcript resolution with RNA-seq". Nat Biotechnol 2013 Jan;31(1):46-53. PMID: 23222703

The authors report on differential analysis of lung fibroblasts in response to loss of the developmental transcription factor HOXA1.

## Required packages

```
library(DESeq2)
library(AnnotationDbi)
library(org.Hs.eg.db)
library(pathview)
library(gage)
library(gageData)
```

## Data import

```
colData <- read.csv("GSE37704_metadata.csv",row.names=1)
countData <- read.csv("GSE37704_featurecounts.csv",row.names=1)
```

```
head(colData)
```

```
             condition
SRR493366 control_sirna
SRR493367 control_sirna
SRR493368 control_sirna
SRR493369      hoxa1_kd
SRR493370      hoxa1_kd
SRR493371      hoxa1_kd
```

```
head(countData)
```

```
                length SRR493366 SRR493367 SRR493368 SRR493369 SRR493370
ENSG00000186092    918         0         0         0         0         0
ENSG00000279928    718         0         0         0         0         0
ENSG00000279457   1982        23        28        29        29        28
ENSG00000278566    939         0         0         0         0         0
ENSG00000273547    939         0         0         0         0         0
ENSG00000187634   3214       124       123       205       207       212
                SRR493371
ENSG00000186092         0
ENSG00000279928         0
ENSG00000279457        46
```

```
ENSG00000278566          0
ENSG00000273547          0
ENSG00000187634        258
```

## Tidy the metadata

Check the correspondance of colData row and countData columns

```
rownames(colData)
```

```
[1] "SRR493366" "SRR493367" "SRR493368" "SRR493369" "SRR493370" "SRR493371"
```

```
colnames(countData)
```

```
[1] "length"    "SRR493366" "SRR493367" "SRR493368" "SRR493369" "SRR493370"
[7] "SRR493371"
```

```
## cannot just match due to "length" must remove or else returns false data
```

Remove the troublesome first column so we match the metadata

```
counts <- countData[,-1]
```

```
all(rownames(colData)==colnames(counts))
```

```
[1] TRUE
```

## Remove zero count genes

We will have rows in `counts` for genes that we can not say anything about because they have zero expression in the particular tissue we are looking at.

```
head(counts)
```

| | SRR493366 | SRR493367 | SRR493368 | SRR493369 | SRR493370 | SRR493371 |
|---|---|---|---|---|---|---|
| ENSG00000186092 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENSG00000279928 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENSG00000279457 | 23 | 28 | 29 | 29 | 28 | 46 |
| ENSG00000278566 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENSG00000273547 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENSG00000187634 | 124 | 123 | 205 | 207 | 212 | 258 |

If the `rowSums()` is zero than a given gene (i.e. row) has no count data and we should exlude these genes from further consideration

```
to.keep <-rowSums(counts) !=0
cleancounts <- counts[to.keep,]
```

Q. How many genes do we have left?

```
nrow(cleancounts)
```

```
[1] 15975
```

#Setup DEQSeq object for analysis

```
dds <- DESeqDataSetFromMatrix(countData=cleancounts,
                              colData=colData,
                              design=~condition)
```

```
Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
design formula are characters, converting to factors
```

#Run DeSeq analysis

```
dds <-DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

#Extract the results

```
res <-results(dds)
head(res)
```

```
log2 fold change (MLE): condition hoxa1 kd vs control sirna
Wald test p-value: condition hoxa1 kd vs control sirna
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE       stat      pvalue
                <numeric>      <numeric> <numeric>  <numeric>   <numeric>
ENSG00000279457   29.9136      0.1792571 0.3248216   0.551863 5.81042e-01
ENSG00000187634  183.2296      0.4264571 0.1402658   3.040350 2.36304e-03
ENSG00000188976 1651.1881     -0.6927205 0.0548465 -12.630158 1.43990e-36
ENSG00000187961  209.6379      0.7297556 0.1318599   5.534326 3.12428e-08
ENSG00000187583   47.2551      0.0405765 0.2718928   0.149237 8.81366e-01
ENSG00000187642   11.9798      0.5428105 0.5215598   1.040744 2.97994e-01
                      padj
                 <numeric>
ENSG00000279457 6.86555e-01
ENSG00000187634 5.15718e-03
ENSG00000188976 1.76549e-35
ENSG00000187961 1.13413e-07
ENSG00000187583 9.19031e-01
ENSG00000187642 4.03379e-01
```

## Add Gene Annotation

```
res$symbol<- mapIds(x=org.Hs.eg.db,
             keys=rownames(res),
             keytype="ENSEMBL",
             column="SYMBOL")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$name<- mapIds(x=org.Hs.eg.db,
             keys=rownames(res),
             keytype="ENSEMBL",
             column="GENENAME")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$entrez<- mapIds(x=org.Hs.eg.db,
             keys=rownames(res),
             keytype="ENSEMBL",
             column="ENTREZID")
```

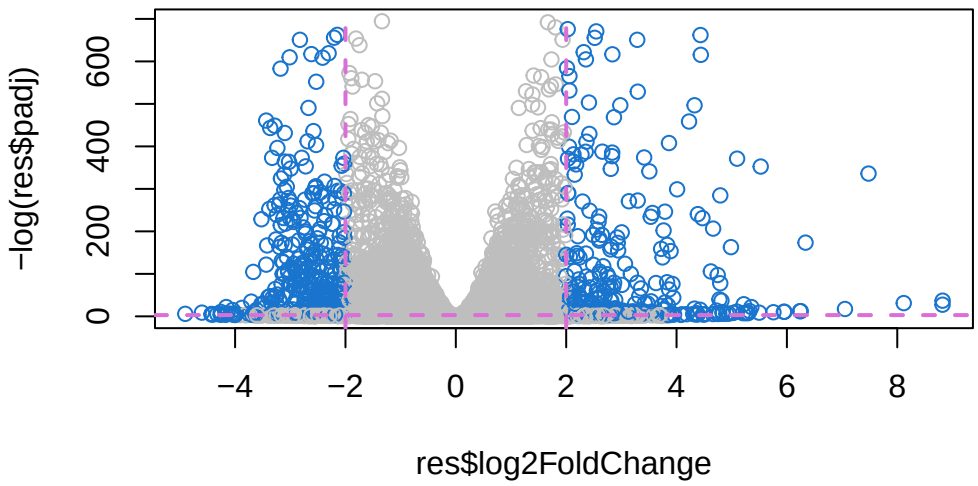
```
'select()' returned 1:many mapping between keys and columns
```

## Save my results to a CSV file

```
write.csv(res, file="results.csv")
```

## Result Visualization

```
mycols <-rep("gray",nrow(res))
mycols[res$log2FoldChange <= -2] <-"dodgerblue3"
mycols[res$log2FoldChange>=2] <- "dodgerblue3"
mycols[res$padj >=0.05] <- "gray"

# make the plot
plot(res$log2FoldChange,-log(res$padj),col=mycols)

# adds the guidelines
abline(v=-2,col="orchid",lty=2,lwd=2)
abline(v=2, col="orchid",lty=2,lwd=2)
abline(h=-log(0.05),col="orchid",lty=2,lwd=2)
```



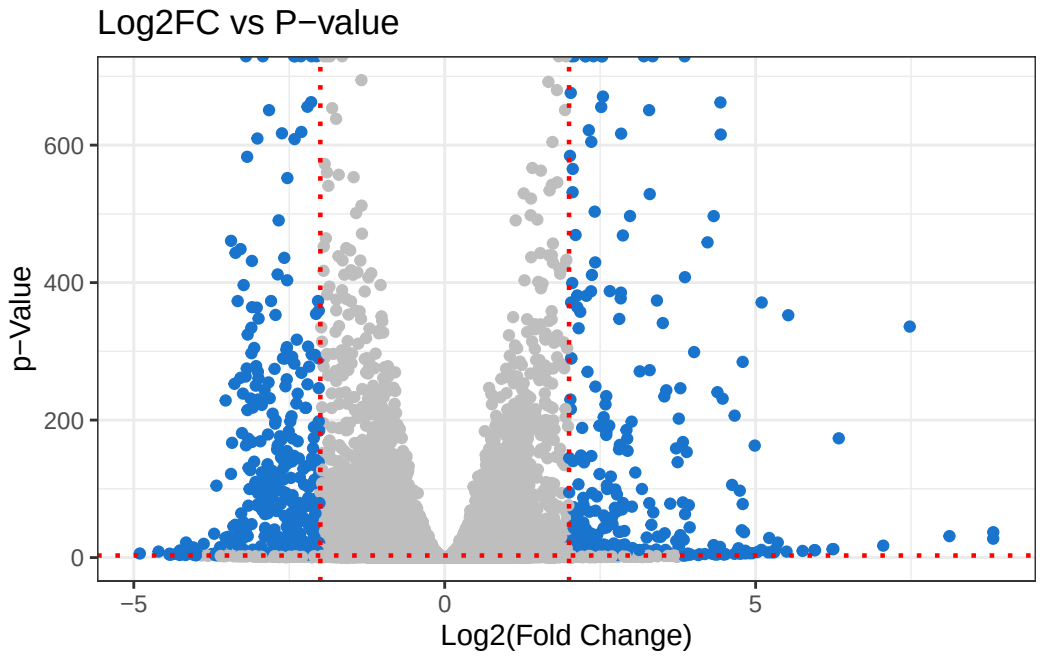

```
library(ggplot2)
library(ggrepel)

ggplot(as.data.frame(res))+
  aes(log2FoldChange,-log(padj),label=name)+
  geom_text_repel(max.overlaps=)+
  geom_point(col=mycols)+
  geom_vline(xintercept=2,
             col="red",
             linetype="dotted",
             size=0.8)+
  geom_vline(xintercept=-2,
             col="red",
             linetype="dotted",
             size=0.8)+
  geom_hline(yintercept=-log(0.05),
             col="red",
             linetype="dotted",
             size=0.8)+
  theme_bw()+
  labs(title="Log2FC vs P-value",
       x= "Log2(Fold Change)",
       y="p-Value")
```

```
Warning: Using `size` aesthetic for lines was deprecated in ggplot2 3.4.0.
i Please use `linewidth` instead.
```

```
Warning: Removed 1409 rows containing missing values or values outside the scale range
(`geom_text_repel()`).
```

```
Warning: Removed 1237 rows containing missing values or values outside the scale range
(`geom_point()`).
```

```
Warning: ggrepel: 14566 unlabeled data points (too many overlaps). Consider
increasing max.overlaps
```




#Pathway Analysis

```
data(kegg.sets.hs)
data(sigmet.idx.hs)

# Focus on signaling and metabolic pathways only
kegg.sets.hs = kegg.sets.hs[sigmet.idx.hs]

# Examine the first 3 pathways
head(kegg.sets.hs, 3)
```

```
$`hsa00232 Caffeine metabolism`
[1] "10"   "1544" "1548" "1549" "1553" "7498" "9"

$`hsa00983 Drug metabolism - other enzymes`
 [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
 [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
[17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
[25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
[33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
[41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
[49] "8824"   "8833"   "9"      "978"

$`hsa00230 Purine metabolism`
  [1] "100"    "10201"  "10606"  "10621"  "10622"  "10623"  "107"    "10714"
  [9] "108"    "10846"  "109"    "111"    "11128"  "11164"  "112"    "113"
 [17] "114"    "115"    "122481" "122622" "124583" "132"    "158"    "159"
 [25] "1633"   "171568" "1716"   "196883" "203"    "204"    "205"    "221823"
 [33] "2272"   "22978"  "23649"  "246721" "25885"  "2618"   "26289"  "270"
 [41] "271"    "27115"  "272"    "2766"   "2977"   "2982"   "2983"   "2984"
 [49] "2986"   "2987"   "29922"  "3000"   "30833"  "30834"  "318"    "3251"
 [57] "353"    "3614"   "3615"   "3704"   "377841" "471"    "4830"   "4831"
 [65] "4832"   "4833"   "4860"   "4881"   "4882"   "4907"   "50484"  "50940"
 [73] "51082"  "51251"  "51292"  "5136"   "5137"   "5138"   "5139"   "5140"
 [81] "5141"   "5142"   "5143"   "5144"   "5145"   "5146"   "5147"   "5148"
 [89] "5149"   "5150"   "5151"   "5152"   "5153"   "5158"   "5167"   "5169"
 [97] "51728"  "5198"   "5236"   "5313"   "5315"   "53343"  "54107"  "5422"
[105] "5424"   "5425"   "5426"   "5427"   "5430"   "5431"   "5432"   "5433"
[113] "5434"   "5435"   "5436"   "5437"   "5438"   "5439"   "5440"   "5441"
[121] "5471"   "548644" "55276"  "5557"   "5558"   "55703"  "55811"  "55821"
[129] "5631"   "5634"   "56655"  "56953"  "56985"  "57804"  "58497"  "6240"
[137] "6241"   "64425"  "646625" "654364" "661"    "7498"   "8382"   "84172"
[145] "84265"  "84284"  "84618"  "8622"   "8654"   "87178"  "8833"   "9060"
[153] "9061"   "93034"  "953"    "9533"   "954"    "955"    "956"    "957"
[161] "9583"   "9615"
```

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
head(foldchanges)
```

```
       <NA>      148398       26155      339451       84069       84808
 0.17925708  0.42645712 -0.69272046  0.72975561  0.04057653  0.54281049
```

Run gage pathway analysis

```
keggres = gage(foldchanges, gsets=kegg.sets.hs)
attributes(keggres)
```

```
$names
[1] "greater" "less"    "stats"
```

```
head(keggres$less)
```

```
                                       p.geomean stat.mean        p.val
hsa04110 Cell cycle                    8.995727e-06 -4.378644 8.995727e-06
hsa03030 DNA replication               9.424076e-05 -3.951803 9.424076e-05
hsa03013 RNA transport                 1.246882e-03 -3.059466 1.246882e-03
hsa03440 Homologous recombination      3.066756e-03 -2.852899 3.066756e-03
hsa04114 Oocyte meiosis                3.784520e-03 -2.698128 3.784520e-03
hsa00010 Glycolysis / Gluconeogenesis  8.961413e-03 -2.405398 8.961413e-03
                                            q.val set.size         exp1
hsa04110 Cell cycle                    0.001448312      121 8.995727e-06
hsa03030 DNA replication               0.007586381       36 9.424076e-05
hsa03013 RNA transport                 0.066915974      144 1.246882e-03
hsa03440 Homologous recombination      0.121861535       28 3.066756e-03
hsa04114 Oocyte meiosis                0.121861535      102 3.784520e-03
hsa00010 Glycolysis / Gluconeogenesis  0.212222694       53 8.961413e-03
```

```
pathview(gene.data=foldchanges, pathway.id="hsa04110", kegg.native=FALSE)
```

```
'select()' returned 1:1 mapping between keys and columns
```

```
Warning: reconcile groups sharing member nodes!
```

```
     [,1] [,2]
[1,] "9"  "300"
[2,] "9"  "306"
```

```
Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14
```

```
Info: Writing image file hsa04110.pathview.pdf
```

### Plots for Top 5 (Up Regulated) Pathways

```
keggresgreater <- rownames(keggres$greater)[1:5]
keggresids = substr(keggresgreater, start=1, stop=8)
keggresids
```

```
[1] "hsa04640" "hsa04630" "hsa00140" "hsa04142" "hsa04330"
```

```
pathview(gene.data=foldchanges, pathway.id=keggresids, species="hsa")
```

```
'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04640.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04630.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa00140.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04142.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04330.pathview.png
```

### Plots for Top 5 (Down-Regulated) Pathways

```
keggresless <- rownames(keggres$less)[1:5]
keggreslessids = substr(keggresless, start=1, stop=8)
keggreslessids
```

```
[1] "hsa04110" "hsa03030" "hsa03013" "hsa03440" "hsa04114"
```

```
pathview(gene.data=foldchanges, pathway.id=keggreslessids, species="hsa")
```

```
'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04110.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa03030.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa03013.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa03440.pathview.png

'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/16dph/OneDrive/Documents/BIMM 143/class14

Info: Writing image file hsa04114.pathview.png
```

## Gene Ontology

```
data(go.sets.hs)
data(go.subs.hs)

# Focus on Biological Process subset of GO
gobpsets = go.sets.hs[go.subs.hs$BP]

gobpres = gage(foldchanges, gsets=gobpsets, same.dir=TRUE)

lapply(gobpres, head)
```

```
$greater
                                           p.geomean stat.mean        p.val
GO:0007156 homophilic cell adhesion       8.519724e-05  3.824205 8.519724e-05
GO:0002009 morphogenesis of an epithelium 1.396681e-04  3.653886 1.396681e-04
GO:0048729 tissue morphogenesis           1.432451e-04  3.643242 1.432451e-04
GO:0007610 behavior                       1.925222e-04  3.565432 1.925222e-04
GO:0060562 epithelial tube morphogenesis  5.932837e-04  3.261376 5.932837e-04
GO:0035295 tube development               5.953254e-04  3.253665 5.953254e-04
                                               q.val set.size         exp1
GO:0007156 homophilic cell adhesion       0.1951953      113 8.519724e-05
GO:0002009 morphogenesis of an epithelium 0.1951953      339 1.396681e-04
GO:0048729 tissue morphogenesis           0.1951953      424 1.432451e-04
GO:0007610 behavior                       0.1967577      426 1.925222e-04
GO:0060562 epithelial tube morphogenesis  0.3565320      257 5.932837e-04
GO:0035295 tube development               0.3565320      391 5.953254e-04

$less
                                             p.geomean stat.mean        p.val
GO:0048285 organelle fission              1.536227e-15 -8.063910 1.536227e-15
GO:0000280 nuclear division               4.286961e-15 -7.939217 4.286961e-15
GO:0007067 mitosis                        4.286961e-15 -7.939217 4.286961e-15
GO:0000087 M phase of mitotic cell cycle  1.169934e-14 -7.797496 1.169934e-14
GO:0007059 chromosome segregation         2.028624e-11 -6.878340 2.028624e-11
GO:0000236 mitotic prometaphase           1.729553e-10 -6.695966 1.729553e-10
                                                 q.val set.size         exp1
GO:0048285 organelle fission              5.841698e-12      376 1.536227e-15
GO:0000280 nuclear division               5.841698e-12      352 4.286961e-15
GO:0007067 mitosis                        5.841698e-12      352 4.286961e-15
GO:0000087 M phase of mitotic cell cycle  1.195672e-11      362 1.169934e-14
```

```
GO:0007059 chromosome segregation        1.658603e-08      142 2.028624e-11
GO:0000236 mitotic prometaphase          1.178402e-07       84 1.729553e-10

$stats
                                          stat.mean     exp1
GO:0007156 homophilic cell adhesion        3.824205 3.824205
GO:0002009 morphogenesis of an epithelium  3.653886 3.653886
GO:0048729 tissue morphogenesis            3.643242 3.643242
GO:0007610 behavior                        3.565432 3.565432
GO:0060562 epithelial tube morphogenesis   3.261376 3.261376
GO:0035295 tube development                3.253665 3.253665
```

## Reactome Analysis

Let's now conduct over-representation enrichment analysis and pathway-topology analysis with Reactome using the previous list of significant genes generated from our differential expression results above.

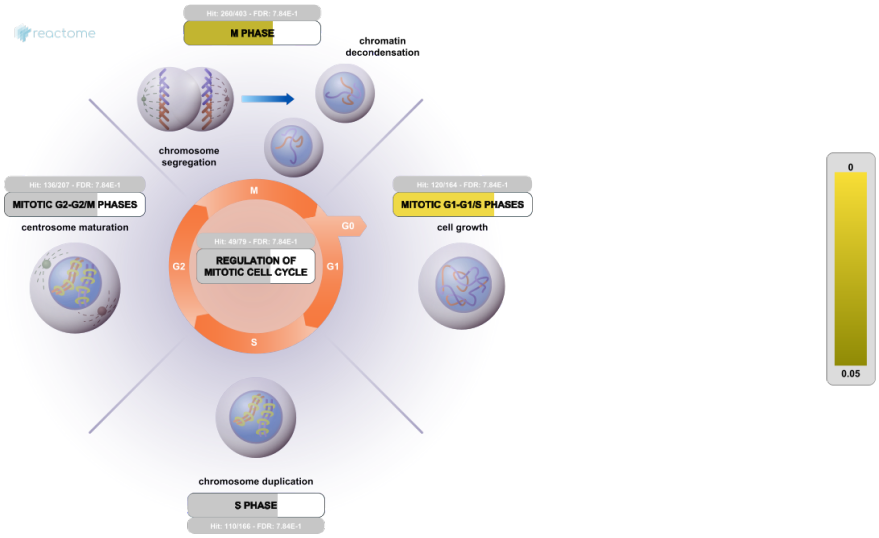
First, Using R, output the list of significant genes at the 0.05 level as a plain text file:

```
sig_genes <- res[res$padj <= 0.05 & !is.na(res$padj), "symbol"]
print(paste("Total number of significant genes:", length(sig_genes)))
```

```
[1] "Total number of significant genes: 8147"
```

```
write.table(sig_genes, file="significant_genes.txt", row.names=FALSE, col.names=FALSE, quote=
```

Then, to perform pathway analysis online go to the Reactome website (https://reactome.org/PathwayBrowser/# Select "choose file" to upload your significant gene list. Then, select the parameters "Project to Humans", then click "Analyze".


reactome
Hit: 260/403 - FDR: 7.84E-1
M PHASE
chromatin decondensation
chromosome segregation
Hit: 136/207 - FDR: 7.84E-1
MITOTIC G2-G2/M PHASES
centrosome maturation
Hit: 120/164 - FDR: 7.84E-1
MITOTIC G1-G1/S PHASES
cell growth
Hit: 49/79 - FDR: 7.84E-1
REGULATION OF MITOTIC CELL CYCLE
M
G0
G1
S
G2
chromosome duplication
S PHASE
Hit: 110/166 - FDR: 7.84E-1
0
0.05