BIOS 7659 Homework 3

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# 1. T-statistics

Read in the data:

array <- read.table("./hw3data/hw3arraydata.txt")  
gene\_names <- read.table("./hw3data/hw3genenames.txt",  
 blank.lines.skip = FALSE)

## a) Fold change

For each gene (row), find the mean expression among controls and among the knock out group. Then calculate fold change using :

fc <- apply(array,1,function(x){  
 control = mean(as.numeric(x[1:8]))  
 knockout = mean(as.numeric(x[9:16]))  
 return(c(control,knockout,control-knockout))  
})  
fc <- t(fc)  
fc\_results <- as.data.frame(cbind(gene\_names,fc))  
colnames(fc\_results) <-   
 c("Gene","Control mean","Knockout mean","log2FC")  
kable(head(fc\_results[order(abs(fc\_results$log2FC),  
 decreasing = T),],10),  
 caption = "Top 10 genes with largest absolute value of fold change",  
 row.names = F)

Top 10 genes with largest absolute value of fold change

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 |
| EST,HighlysimilartoA | 8.245822 | 3.672996 | 4.572826 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 |
| ESTs,Highlysimilarto | 7.835267 | 6.320549 | 1.514718 |
| est | 7.704867 | 6.238731 | 1.466135 |
| similartoyeaststerol | 7.356597 | 5.924143 | 1.432454 |
| ApoCIII,lipid-Img | 7.781127 | 6.382253 | 1.398874 |
| psoriasis-associated | 7.742241 | 6.485528 | 1.256714 |
| Cy3RT | 6.889612 | 8.082898 | -1.193286 |

## b) Standard t test

For each gene, calculate the two-sample independent t-statistic (not assuming equal variances) for the comparison between controls and knockouts:

# Tests  
tp <- apply(array,1,function(x){  
 control = as.numeric(x[1:8])  
 knockout = as.numeric(x[9:16])  
 t <- t.test(control,knockout)  
 return(c(t$statistic,t$p.value))  
})  
# Format results  
tp <- as.data.frame(t(tp))  
colnames(tp) <- c("T","p value")  
fc\_results <- as.data.frame(cbind(fc\_results,tp))  
kable(head(fc\_results[order(abs(fc\_results$T),decreasing = T),],10),  
 caption = "Top 10 genes with largest t-statistic",  
 row.names = F)

Top 10 genes with largest t-statistic

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.7492467 | 23.104347 | 0.0000000 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.5404305 | 12.982368 | 0.0000000 |
| EST,HighlysimilartoA | 8.245822 | 3.672996 | 4.5728257 | 11.762486 | 0.0000019 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.7722489 | 11.759068 | 0.0000000 |
| ApoCIII,lipid-Img | 7.781127 | 6.382253 | 1.3988735 | 10.430072 | 0.0000020 |
| est | 7.704867 | 6.238731 | 1.4661354 | 9.087422 | 0.0000031 |
| ESTs,Highlysimilarto | 7.835267 | 6.320549 | 1.5147176 | 9.018613 | 0.0000061 |
| similartoyeaststerol | 7.356597 | 5.924143 | 1.4324539 | 7.208906 | 0.0000123 |
| Caspase7,heart-Img | 8.011684 | 7.558373 | 0.4533114 | 4.578842 | 0.0005343 |
| EST,WeaklysimilartoF | 7.945457 | 7.089572 | 0.8558850 | 4.434296 | 0.0007886 |

Out of the 6384 genes, 75 were significant at the p < 0.01 level.

## c) Alternative t-statistics

### i) Modified t-statistic (using the samr package)

y <- ifelse(grepl("c",colnames(array)),1,2)  
x <- as.matrix(array)  
data=list(x=x,y=y)  
samr\_obj <- samr(data)

samr\_pvalues <- samr.pvalues.from.perms(samr\_obj$tt,samr\_obj$ttstar)  
samr\_results <- cbind(gene\_names,samr\_obj$tt,samr\_pvalues)  
colnames(samr\_results) <- c("Gene","Modified t-statistic","p value")  
# P values  
kable(head(samr\_results[order(abs(samr\_results[,2]),  
 decreasing = T),],10),  
 caption = "Top 10 genes with largest modified t-statistic",  
 row.names = F)

Top 10 genes with largest modified t-statistic

|  |  |  |
| --- | --- | --- |
| Gene | Modified t-statistic | p value |
| ApoAI,lipid-Img | -20.592874 | 0.0001566 |
| EST,HighlysimilartoA | -11.049934 | 0.0001566 |
| EST,WeaklysimilartoC | -10.717909 | 0.0001566 |
| CATECHOLO-METHYLTRAN | -10.628833 | 0.0001566 |
| ApoCIII,lipid-Img | -8.787524 | 0.0001566 |
| est | -7.865276 | 0.0001566 |
| ESTs,Highlysimilarto | -7.847305 | 0.0001566 |
| similartoyeaststerol | -6.401300 | 0.0001598 |
| EST,WeaklysimilartoF | -3.924562 | 0.0004464 |
| Caspase7,heart-Img | -3.653656 | 0.0006986 |

Based on the modified t-statistic, there are 94 genes that are significantly different at the 0.01 level.

### ii) Moderated t-statistic (using the limma package)

First, create the design matrix for limma:

design <- matrix(ncol = 2,nrow = ncol(array))  
colnames(design) <- c("Control","Knockout")  
rownames(design) <- colnames(array)  
design[,1] <- rep(1,nrow(design))  
design[,2] <- ifelse(grepl("k",rownames(design)),1,0)

Fit the model with limma:

fit <- lmFit(array, design)  
eb <- eBayes(fit)  
limma\_res <- topTable(eb,coef = 2,number = 10)  
rownames(limma\_res) <- gene\_names$V1[as.numeric(rownames(limma\_res))]  
kable(limma\_res,  
 caption = "Top 10 differentially expressed genes (based on the moderated t-statistic)")

Top 10 differentially expressed genes (based on the moderated t-statistic)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | logFC | AveExpr | t | P.Value | adj.P.Val | B |
| ApoAI,lipid-Img | -4.749247 | 5.773086 | -23.976817 | 0.0000000 | 0.0000000 | 14.9269328 |
| EST,HighlysimilartoA | -4.572826 | 5.959409 | -12.963071 | 0.0000000 | 0.0000005 | 10.8150265 |
| CATECHOLO-METHYLTRAN | -2.772249 | 6.617134 | -12.439908 | 0.0000000 | 0.0000006 | 10.4483231 |
| EST,WeaklysimilartoC | -1.540431 | 6.817930 | -11.749992 | 0.0000000 | 0.0000012 | 9.9246200 |
| ApoCIII,lipid-Img | -1.398874 | 7.081690 | -9.831229 | 0.0000000 | 0.0000157 | 8.1890866 |
| ESTs,Highlysimilarto | -1.514718 | 7.077908 | -9.012972 | 0.0000000 | 0.0000423 | 7.3031534 |
| est | -1.466135 | 6.971799 | -8.999811 | 0.0000000 | 0.0000423 | 7.2881051 |
| similartoyeaststerol | -1.432454 | 6.640370 | -7.440210 | 0.0000007 | 0.0005617 | 5.3097967 |
| EST,WeaklysimilartoF | -0.855885 | 7.517514 | -4.553948 | 0.0002495 | 0.1769590 | 0.5618636 |
|  | -0.549536 | 7.325818 | -3.961031 | 0.0009254 | 0.5284860 | -0.5563623 |

Based on the moderated t-statistic, there are 93 genes that are significantly different at the 0.01 level (without additional adjustment for multiple comparisons).

## d) Method comparisons

Generally speaking, the four methods are pretty similar, at least in terms of ranking the top ten differentially expressed genes. The ranked order is not exactly the same for each method, but the same 10 genes are chosen regardless. However, the modified and moderated t-statistic approaches reject more null hypotheses than the standard t-statistic. One potential downside to the samr approach is that the number of significant genes changes slightly depending on the random seed and the number of permutations.

The standard t-statistic (assuming independent samples with unequal variances) for a gene is calculated using the formula:

where , , and represent the mean, sample variance, and number of samples, respectively, for groups 1 and 2. This generally works well for larger sample sizes, but the standard error (SE) estimates are unreliable with smaller samples as is often the case in gene expression studies. This can lead to artificially inflated t-statistics. The modified and moderated t-statistics try to address this issue in two different ways.

The modified t-statistic from the samr package adds a constant amount to the SE estimate (based on pooled variance) for each gene. This is often the percentile of SE across all genes, but this can be altered. So, the formula is:

Where represents the standard error estimate for gene and is the added constant. This approach is not grounded in distributional theory, and is considered more of an ad-hoc approach.

The moderated t-statistic calculated by the limma package also aims to estimate a more stable SE, but uses a more complex Bayesian approach instead of simply adding a constant. The statistic is calculated as:

Where is a shrunken variance estimate that depends on hyperparameters chosen by sharing information across all genes. There is some complex theory behind this method, but the essential idea is that all of the information across genes contributes to the calculation of the t-statistic, which results in more stable SE estimates.

# P Values and Multiple Testing

## a) Permutation tests

First, find all the possible combinations of group labels (in this case control vs. knockout) using the combinations() functions. Then, for each gene calculate a t-statistic for each possible combination and count the number of permuted t-statistics that are larger than the “true” statistic. The proportion of permuted t-statistics greater than or equal to the “true” statistic is the permutation-based p value.

combos <- combinations(16,8,colnames(array))  
cores <- detectCores()  
cl <- makeCluster(cores,type = "FORK")  
pvalues <- parApply(cl,array,1,function(g){  
 maxt <- t.test(g[grep("c",names(array))],  
 g[grep("k",names(array))])  
 perms <- apply(combos,1,function(c){  
 control <- g[c]  
 knockout <- g[setdiff(names(g),c)]  
 t <- t.test(control,knockout)  
 return(t$statistic)  
 })  
 return(sum(abs(perms)>=abs(maxt$statistic))/length(perms))  
})  
stopCluster(cl)

Using the permutation test approach, there are 117 genes significant at the 0.01 level.

\*Note: I know that the homework sheet says not to use parallel computing methods, but I was curious about why this is and decided to test it. Using parApply() from the parallel package produced exactly the same results as using apply() and was approximately four times faster.

## b) P value adjustment methods

### i) Bonferroni

The Bonferroni correction rejects the null hypothesis for each when , where is the total number of null hypotheses. We have a total of 6384 tests, so will reject the null hypothesis when .

a\_bonf <- 0.01 / nrow(array)  
kable(fc\_results[fc\_results$`p value`<=a\_bonf,],  
 caption = "Significant genes after Bonferroni correction",  
 row.names = F)

Significant genes after Bonferroni correction

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 | 23.10435 | 0 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 | 12.98237 | 0 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 | 11.75907 | 0 |

After Bonferroni correction, we reject the null hypothesis for 3 genes.

### ii) Sidak

The Sidak correction rejects the null hypothesis for each when , where again is the total number of null hypotheses. We have a total of 6384 tests, so will reject the null hypothesis when .

a\_sid <- 1-(1-0.01)^(1/nrow(array))  
kable(fc\_results[fc\_results$`p value`<=a\_sid,],  
 caption = "Significant genes after Sidak correction",  
 row.names = F)

Significant genes after Sidak correction

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 | 23.10435 | 0 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 | 12.98237 | 0 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 | 11.75907 | 0 |

After Sidak correction, we reject the null hypothesis for 3 genes.

### iii) Holm step-down

The Holm step-down procedure is as follows:

1. Rank p-values: .
2. Find the first such that .
3. Reject all null hypotheses up to .

ordered <- fc\_results[order(fc\_results$`p value`),]  
m <- nrow(ordered)  
j <- 1:m  
jstar <- min(which((ordered$`p value` > 0.01/(m+1-j))==T))  
kable(ordered[1:(jstar-1),],  
 caption = "Significant genes after Holm correction",  
 row.names = F)

Significant genes after Holm correction

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 | 23.10435 | 0 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 | 12.98237 | 0 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 | 11.75907 | 0 |

After Holm step-down correction, we reject the null hypothesis for 3 genes.

### iv) Benjamini-Hochberg

The Benjamini-Hochberg step-up procedure is as follows:

1. Rank p-values: .
2. Find the maximum such that where is the desired false discovery rate.
3. Reject all null hypotheses through .

q <- 0.01  
jstar <- max(which((ordered$`p value` <= (j/m)\*q)==T))  
kable(ordered[1:jstar,],  
 caption = "Significant genes after Benjamini-Hochberg correction",  
 row.names = F)

Significant genes after Benjamini-Hochberg correction

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 | 23.104347 | 0.00e+00 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 | 12.982368 | 0.00e+00 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 | 11.759068 | 0.00e+00 |
| EST,HighlysimilartoA | 8.245822 | 3.672996 | 4.572826 | 11.762486 | 1.90e-06 |
| ApoCIII,lipid-Img | 7.781127 | 6.382253 | 1.398874 | 10.430072 | 2.00e-06 |
| est | 7.704867 | 6.238731 | 1.466135 | 9.087422 | 3.10e-06 |
| ESTs,Highlysimilarto | 7.835267 | 6.320549 | 1.514718 | 9.018613 | 6.10e-06 |
| similartoyeaststerol | 7.356597 | 5.924143 | 1.432454 | 7.208906 | 1.23e-05 |

After Benjamini-Hochberg step-up correction, we reject the null hypothesis for 8 genes.

### Comparison

The Bonferroni, Sidak, and Holm methods above are more conservative than the Benjamini-Hochberg approach, because they aim to control the family-wise error rate (FWER), or the probability of making at least 1 type 1 error. In other words, these methods ensure that . Of these methods, the Bonferroni approach is the most conservative, because it is a single step procedure and all tests are subject to the same stringent bound.

On the other hand, the Benjamini-Hochberg approach aims to control the false discovery rat (FDR), or the expected proportion of false positives among rejected hypotheses. Because FDR-based approaches focus on limiting type 1 error among “discoveries” (significant p values) as opposed to across all tests, they tend to be less conservative. This is why the Benjamini-Hochberg step-up correction rejects 8 null hypotheses compared to 3 for the more conservative methods.

## c) Q-values

Calculate q-values using the qvalue package (without a pre-specified parameter):

q <- qvalue(fc\_results$`p value`)  
fc\_results$qvalue <- q$qvalues  
kable(fc\_results[fc\_results$qvalue<=0.01,],  
 caption = "Significant genes based on q-value",  
 row.names = F)

Significant genes based on q-value

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Gene | Control mean | Knockout mean | log2FC | T | p value | qvalue |
| EST,HighlysimilartoA | 8.245822 | 3.672996 | 4.572826 | 11.762486 | 1.90e-06 | 0.0021906 |
| est | 7.704867 | 6.238731 | 1.466135 | 9.087422 | 3.10e-06 | 0.0027982 |
| ApoCIII,lipid-Img | 7.781127 | 6.382253 | 1.398874 | 10.430072 | 2.00e-06 | 0.0021906 |
| ApoAI,lipid-Img | 8.147709 | 3.398463 | 4.749247 | 23.104347 | 0.00e+00 | 0.0000020 |
| ESTs,Highlysimilarto | 7.835267 | 6.320549 | 1.514718 | 9.018613 | 6.10e-06 | 0.0047561 |
| EST,WeaklysimilartoC | 7.588145 | 6.047714 | 1.540431 | 12.982368 | 0.00e+00 | 0.0000197 |
| similartoyeaststerol | 7.356597 | 5.924143 | 1.432454 | 7.208906 | 1.23e-05 | 0.0084502 |
| CATECHOLO-METHYLTRAN | 8.003259 | 5.231010 | 2.772249 | 11.759068 | 0.00e+00 | 0.0000222 |

There are 8 genes with a q-value 0.01. represents the proportion of truly null hypotheses, and this package estimates it at approximately 0.859.