Differential Gene Expression 3 – quantifying differences with Bioconductor

Biol4230 Thurs, April 6, 2018
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- Bioconductor: a comprehensive 'R' package for expression and genome analysis
  - Obtaining/installing
  - Datasets
  - Vignettes
  - Major packages (affy, edgeR)
- Using Bioconductor/EdgeR for RNAseq
  - reading in data (what to look for)
  - removing genes with low/no signal
  - normalization
  - finding differentially expressed genes

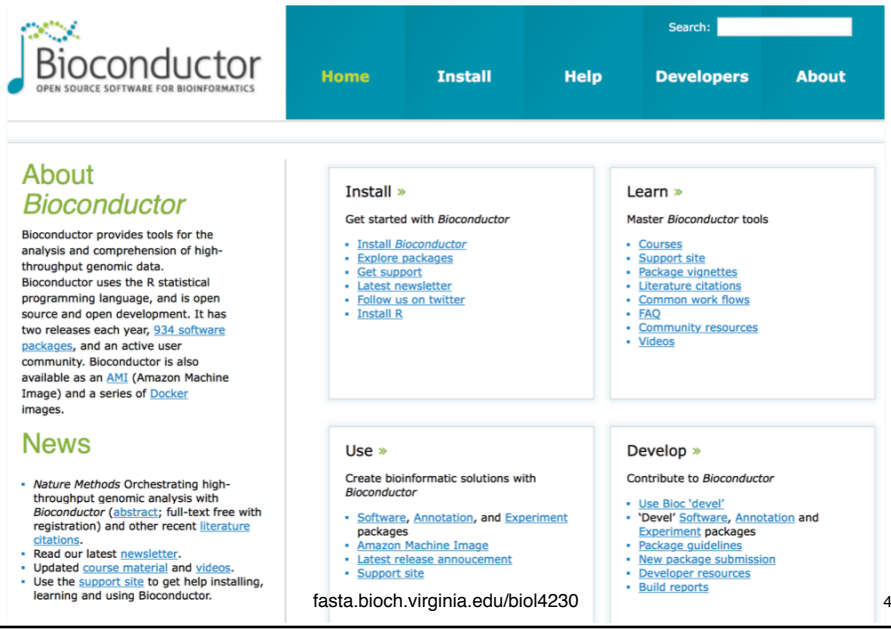
To learn more:

1. Pevsner, Chapter 8 pp. 331-373
2. Draghici, Soren (2012) "Statistics and data analysis for microarrays using R and Bioconductor" Chapman and Hall
3. Anders, S. *et al.* Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nature protocols* **8,** 1765–1786 (2013).
4. http://www.bioconductor.org/help/workflows/rnaseq Gene/

bioconductor.org

- More than 600 packages of functions for genome and expression analysis
  - expression analysis: *affy*
  - RNA-seq: *edgeR, DESeq2*
  - ChIP-seq (interaction of protein with DNA in chromatin)
  - extracting genomic features
- "Vignettes" that come with data for research problems
- Must be installed (often individually)
- Work with 'R' objects typically much more abstract than data.frames()
- Use the common 'R' logic for selecting rows and columns from data

bioconductor.org



bioconductor installation

```
>R ...
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

> source("http://bioconductor.org/biocLite.R")
trying URL
'http://www.bioconductor.org/packages/3.0/bioc/bin/macosx/mavericks/contrib/3.1/Bi
ocInstaller_1.16.2.tgz'
Content type 'application/x-gzip' length 49063 bytes (47 KB)
opened URL
==================================================
downloaded 47 KB
The downloaded binary packages are in
          /var/folders/cd/56p6y3_x1lq_pr5lm1dqqngc0000jc/T//Rtmp8pZlmn/downloaded_
packages
Bioconductor version 3.0 (BiocInstaller 1.16.2), ?biocLite for help
> biocLite()
BioC_mirror: http://bioconductor.org
Using Bioconductor version 3.0 (BiocInstaller 1.16.2), R version 3.1.3.
Installing package(s) 'Biobase' 'IRanges' 'AnnotationDbi'
also installing the dependencies 'BiocGenerics', 'S4Vectors', 'GenomeInfoDb',
'DBI', 'RSQLite'
```

Bioconductor installs packages incrementally

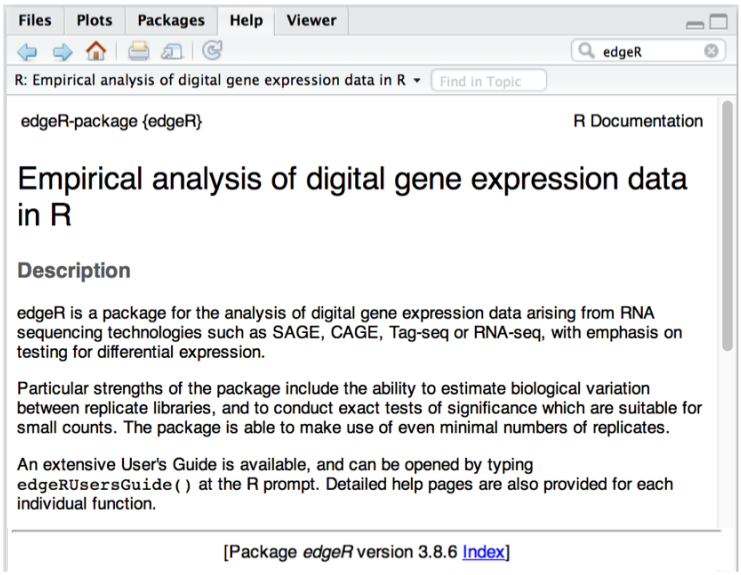
Bioconductor installs packages incrementally

```
> library(affy)
Error in library(affy) : there is no package called 'affy'
> biocLite('affy')
BioC_mirror: http://bioconductor.org
Using Bioconductor version 3.0 (BiocInstaller 1.16.2), R version 3.1.3.
Installing package(s) 'affy'
also installing the dependencies 'affyio', 'preprocessCore', 'zlibbioc'
trying URL
'http://bioconductor.org/packages/3.0/bioc/bin/macosx/mavericks/contrib/3.1/affyio
_1.34.0.tgz'
Content type 'application/x-gzip' length 89679 bytes (87 KB)
opened URL
==================================================
downloaded 87 KB
trying URL
'http://bioconductor.org/packages/3.0/bioc/bin/macosx/mavericks/contrib/3.1/prepro
cessCore_1.28.0.tgz'
Content type 'application/x-gzip' length 137216 bytes (134 KB)
opened URL
==================================================
The downloaded binary packages are in
 /var/folders/cd/56p6y3_x1lq_pr5lm1dqqngc0000jc/T//Rtmp8pZlmn/downloaded_packages
```

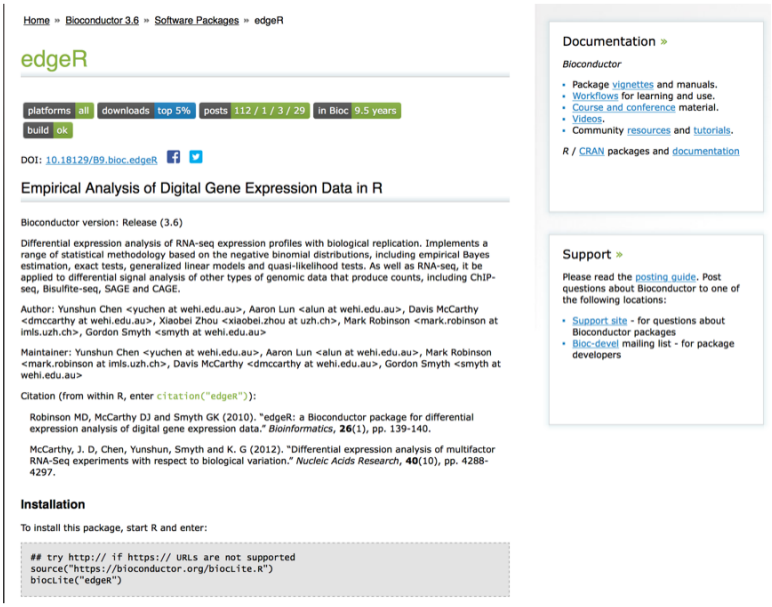
Bioconductor installs packages incrementally

```
> library(affy)
Loading required package: BiocGenerics
Loading required package: parallel
Attaching package: 'BiocGenerics'
The following objects are masked from 'package:parallel':
...
    parLapplyLB, parRapply, parSapply, parSapplyLB
The following object is masked from 'package:stats':
    xtabs
The following objects are masked from 'package:base':
    Filter, Find, Map, Position, Reduce, anyDuplicated, append,
 ...
    unique, unlist, unsplit

Loading required package: Biobase
Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.
```


Bioconductor: getting help



Bioconductor: getting help



Home » Bioconductor 3.6 » Software Packages » edgeR

edgeR

platforms all | downloads top 5% | posts 112 / 1 / 3 / 29 | in Bioc 9.5 years | build ok

DOI: 10.18129/B9.bioc.edgeR

Empirical Analysis of Digital Gene Expression Data in R

Bioconductor version: Release (3.6)

Differential expression analysis of RNA-seq expression profiles with biological replication. Implements a range of statistical methodology based on the negative binomial distributions, including empirical Bayes estimation, exact tests, generalized linear models and quasi-likelihood tests. As well as RNA-seq, it be applied to differential signal analysis of other types of genomic data that produce counts, including ChIP-seq, Bisulfite-seq, SAGE and CAGE.

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Citation (from within R, enter `citation("edgeR")`):

Robinson MD, McCarthy DJ and Smyth GK (2010). "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data." *Bioinformatics*, **26**(1), pp. 139-140.

McCarthy, J. D, Chen, Yunshun, Smyth and K. G (2012). "Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation." *Nucleic Acids Research*, **40**(10), pp. 4288-4297.

Installation

To install this package, start R and enter:

```
## try http:// if https:// URLs are not supported
source("https://bioconductor.org/biocLite.R")
biocLite("edgeR")
```

Documentation »

Bioconductor

- Package vignettes and manuals.
- Workflows for learning and use.
- Course and conference material.
- Videos.
- Community resources and tutorials.

R / CRAN packages and documentation

Support »

Please read the posting guide. Post questions about Bioconductor to one of the following locations:

- Support site - for questions about Bioconductor packages
- Bioc-devel mailing list - for package developers

Bioconductor: getting help

Documentation

To view documentation for the version of this package installed in your system, start R and enter:

```
browseVignettes("edgeR")
```

PDF	edgeR Vignette
PDF	edgeRUsersGuide.pdf
PDF	Reference Manual
Text	NEWS

Details

biocViews	AlternativeSplicing, BatchEffect, Bayesian, ChIPSeq, Clustering, Coverage, DNAMethylation, DifferentialExpression, DifferentialMethylation, DifferentialSplicing, GeneExpression, GeneSetEnrichment, Genetics, MultipleComparison, Normalization, Pathways, QualityControl, RNASeq, Regression, SAGE, Sequencing, Software, TimeCourse, Transcription
Version	3.20.9
In Bioconductor since	BioC 2.3 (R-2.8) (9.5 years)
License	GPL (>=2)
Depends	R (>= 2.15.0), limma(>= 3.34.5)
Imports	graphics, stats, utils, methods, locfit, Rcpp
LinkingTo	Rcpp
Suggests	AnnotationDbi, org.Hs.eg.db, splines
SystemRequirements	C++11
Enhances	
URL	http://bioinf.wehi.edu.au/edgeR
Depends On Me	ASpli, DBChIP, EDDA, IntEREst, manta, methylMnM, MLSeq, RnaSeqGeneEdgeRQL, RnaSeqSampleSizeData, RUVSeq, samExploreR, TCC, tRanslatome
Imports Me	affycoretools, ampliQueso, anota2seq, ArrayExpressHTS, baySeq, compcodeR, coseq, csaw, debrowser, DEFormats, DEGreport, DEsubs, DiffBind, diffHic, diffloop, DRIMSeq, easyRNASeq, EBSEA, EDDA, eegc, EGSEA, EnrichmentBrowser, erccdashboard, Glimma, HTSFilter, IsoformSwitchAnalyzeR, MEDIPS, metaseqR, MIGSA, msgbsR, msmsTests, PathoStat, PROPER, psichomics, PureCN, regsplice, Repitools, ReportingTools, rnaSeqMap, RnaSeqSampleSize, scater, scde, scone, scran, splatter, STATegRa, SVAPLSseq, systemPipeR, TCGAbiolinks, TCseq, ToPASeq, tweeDEseq, yarn, zinbwave
Suggests Me	ABSSeq, biobroom, BitSeq, ClassifyR, clonotypeR, cqn, cydar, EDASeq, gage, gCrisprTools, GenomicAlignments, GenomicRanges, goseq, groHMM, GSAR, GSVA, ideal, JunctionSeqData, leeBamViews, missMethyl, multiMiR, oneChannelGUI, regionReport, SSPA, stageR, subSeq, tximport, variancePartition, zFPKM

Build Report

Bioconductor: the installation loop

- Initial intall:

```
source("http://www.bioconductor.org/biocLite.R")
biocLite()
```

- When you need something:

```
> library(simpleaffy)
Error in library(simpleaffy) : there is no package
called 'simpleaffy'
> biocLite("simpleaffy")
BioC_mirror: http://bioconductor.org
Using Bioconductor version 3.0 (BiocInstaller 1.16.2),
Installing package(s) 'simpleaffy'
also installing the dependencies 'Biostrings', 'gcrma'
...
> library(simpleaffy)
Loading required package: genefilter
Attaching package: 'genefilter'
```


Differential Gene Expression

- Large quantity of data (>20,000 genes)
  - Affychip data has ?20 replicates per gene
  - RNAseq has counts (FPKM: Fragments per Kilobase per Million mapped reads)
  - but a small number of biological replicates
- Ideally, identify modest change (1.5x or larger) for modest levels of transcription
  - 10 or fewer transcripts may account for 90% of reads, so 5,000 – 10,000 transcripts for < 10% of reads
  - If technical replicates vary more than 2x, how do you measure 1.5x change?
- Large numbers of tests: how to correct?
  - Family-wide-error-rate (FWER) Bonferroni correction (used for similarity search E()-values)
  - False-discovery-rate (FDR, qvalue)

Identifying differentially expressed genes

1. convert to FPKM (probably not done properly in my example) (cpm)
2. With RNA-seq data, make sure counts > 1
3. Normalize, adjust medians, quantile normalization
4. Look at bulk properties:
   - PCA analysis should group replicates
   - variance should be relatively linear
5. Calculate pair-wise differential expression with t-tests
6. Use topTags to do FDR correction, identify largest changes
   - go back and compare topTags results to actual counts
7. Log2(FC) vs Log10(abundance)
8. Volcano plots show fold-change, q-value tradeoff

Measuring differences – sources of variation

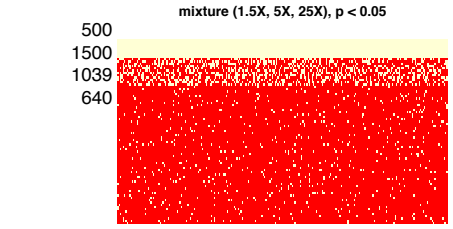
Technical

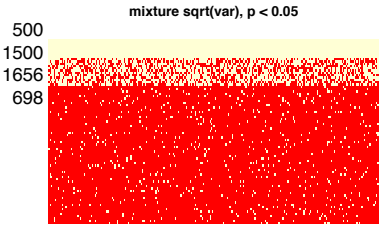
- RNA isolation
- cDNA synthesis
- hybridization (AffyChip)
- PCR amplification
- G+C content
- sequencing depth
- location on AffyChip/ sequencing "lane"

Biological

- genetic background
- sex
- last meal/sleep/exercise
- dividing/quiescent
- cell type within tissue type
- …

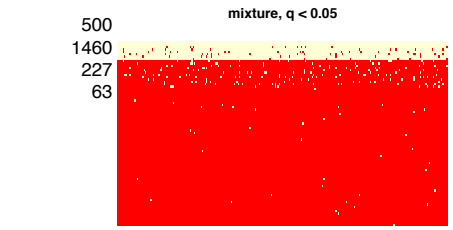
Reducing variance improves detection

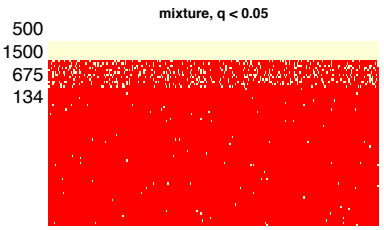





```
summary(mix_pvals_a_qv)
 Cumm       <1e-04 <0.001 <0.01 <0.025 <0.05 <0.1
p-value        937   1582  2372   2915  3679 4945
q-value         86    708  1597   1952  2250 2664
```

```
qvalue(mix_pvals_b
 <1e-04 <0.001 <0.01 <0.025 <0.05 <0.1
p  1853   2121  2826   3529  4354 5599
q  1381   1906  2176   2420  2809 3496
```





Identifying differentially expressed genes

- Differences in expression in the presence of noise:
  - are the differences significant? statistical model
    - t-test (normally distributed, array data)
    - negative binomial (variance increases with mean)
  - are the differences biological?
    - batch effects from experiment
    - gene effects (length, G+C)
- Analysis packages (edgeR, deSeq2) visualize batch effects, normalize data, apply statistical model

edgeR vs DESeq

Box 2 Differences between DESeq and edgeR

The two packages described in this protocol, DESeq and edgeR, have similar strategies to perform differential analysis for count data. However, they differ in a few important areas.

First, their look and feel differs. For users of the widely used limma package (for analysis of microarray data), the data structures and steps in edgeR follow analogously.

The packages differ in their default normalization: edgeR uses the trimmed mean of M values, whereas DESeq uses a relative log expression approach by creating a virtual library that every sample is compared against; in practice, the normalization factors are often similar.

Perhaps most crucially, the tools differ in the choices made to estimate the dispersion. edgeR moderates feature-level dispersion estimates toward a trended mean according to the dispersion-mean relationship. In contrast, DESeq takes the maximum of the individual dispersion estimates and the dispersion-mean trend.

In practice, this means DESeq is less powerful, whereas edgeR is more sensitive to outliers.

Recent comparison studies have highlighted that no single method dominates another across all settings.

Rapaport, F. *et al.* Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. *Genome Biol* **14,** R95 (2013).

Differential Gene Expression with edgeR

- Starting data: HTS counts (not FPKM)

```
> GSE_HTSeq <- read.table("GSE_ENCODE_HTSeq.txt",
+                         row.names=1,sep='\t',header=T)
# row.names=1 uses gene names
> summary(GSE_HTSeq)
  GM12892_Rep1      GM12892_Rep2       GM12892_Rep3       H1.hESC_Rep1     H1.hESC_Rep2
 Min.   :      0   Min.   :     0.0   Min.   :     0.0   Min.   :     0   Min.   :     0
 1st Qu.:      1   1st Qu.:     1.0   1st Qu.:     1.0   1st Qu.:     4   1st Qu.:     4
 Median :    103   Median :    56.0   Median :    47.0   Median :   200   Median :   208
 Mean   :   1830   Mean   :   814.2   Mean   :   765.1   Mean   :  1418   Mean   :  1460
 3rd Qu.:   1246   3rd Qu.:   630.0   3rd Qu.:   567.5   3rd Qu.:  1159   3rd Qu.:  1164
 Max.   :1045434   Max.   :482679.0   Max.   :426204.0   Max.   :646940   Max.   :628301
```

  - Do the replicates look similar?
  - Approx how many genes have <= 1 count?
  - Why is the Max 1000X the 3rd quartile?
  - how much data?

```
> dim(GSE_HTSeq)
[1] 21711    10
```

Rapaport, F. *et al.* Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. *Genome Biol* **14,** R95 (2013).

Differential Gene Expression with edgeR

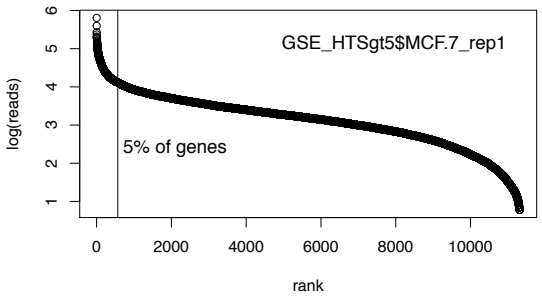
- Wide distribution of abundance

```
> summary(GSE_HTS)
  GM12892_Rep1       GM12892_Rep2       GM12892_Rep3       H1.hESC_Rep1      H1.hESC_Rep2
 Min.   :      0   Min.   :     0.0   Min.   :     0.0   Min.   :     0   Min.   :     0
 1st Qu.:      1   1st Qu.:     1.0   1st Qu.:     1.0   1st Qu.:     4   1st Qu.:     4
 Median :    103   Median :    56.0   Median :    47.0   Median :   200   Median :   208
 Mean   :   1830   Mean   :   814.2   Mean   :   765.1   Mean   :  1418   Mean   :  1460
 3rd Qu.:   1246   3rd Qu.:   630.0   3rd Qu.:   567.5   3rd Qu.:  1159   3rd Qu.:  1164
 Max.   :1045434   Max.   :482679.0   Max.   :426204.0   Max.   :646940   Max.   :628301

  H1.hESC_Rep3       H1.hESC_Rep4        MCF.7_Rep1        MCF.7_Rep2        MCF.7_Rep3
 Min.   :     0   Min.   :     0.0   Min.   :     0   Min.   :     0   Min.   :     0
 1st Qu.:     4   1st Qu.:     2.0   1st Qu.:     2   1st Qu.:     1   1st Qu.:     2
 Median :   206   Median :   120.0   Median :   215   Median :   173   Median :   187
 Mean   :  1385   Mean   :   867.5   Mean   :  2160   Mean   :  2450   Mean   :  2400
 3rd Qu.:  1130   3rd Qu.:   713.0   3rd Qu.:  1796   3rd Qu.:  1733   3rd Qu.:  1628
 Max.   :597077   Max.   :406388.0   Max.   :639987   Max.   :816273   Max.   :885833
```


Differential Gene Expression with edgeR

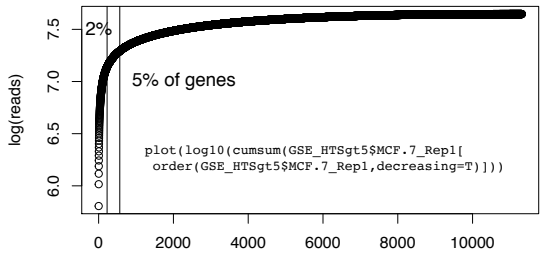
```
> min_cnt<-GSE_HTS$MCF.7_Rep1 > 5 & GSE_HTS$MCF.7_Rep2 > 5 & GSE_HTS$MCF.7_Rep3>5 &
GSE_HTS$H1.hESC_Rep1 > 5 & GSE_HTS$H1.hESC_Rep2 > 5 & GSE_HTS$H1.hESC_Rep3 > 5 &
GSE_HTS$H1.hESC_Rep4 > 5 & GSE_HTS$GM12892_Rep1 > 5 & GSE_HTS$GM12892_Rep2 > 5 &
GSE_HTS$GM12892_Rep3 > 5
> GSE_HTSgt5 <- GSE_HTS[min_cnt,]
> summary(GSE_HTSgt5)
GM12892_Rep1       GM12892_Rep2      GM12892_Rep3      H1.hESC_Rep1      H1.hESC_Rep2
 Min.   :      6   Min.   :     6   Min.   :     6   Min.   :     6   Min.   :     6
 1st Qu.:    347   1st Qu.:   182   1st Qu.:   152   1st Qu.:   341   1st Qu.:   345
 Median :   1083   Median :   537   Median :   487   Median :   936   Median :   942
 Mean   :   3294   Mean   :  1458   Mean   :  1380   Mean   :  2486   Mean   :  2559
 3rd Qu.:   2613   3rd Qu.:  1235   3rd Qu.:  1133   3rd Qu.:  2257   3rd Qu.:  2256
 Max.   :1045434   Max.   :482679   Max.   :426204   Max.   :646940   Max.   :628301

  H1.hESC_Rep3      H1.hESC_Rep4       MCF.7_Rep1        MCF.7_Rep2          MCF.7_Rep3
 Min.   :     6   Min.   :     6   Min.   :     6   Min.   :     6.0   Min.   :     6
 1st Qu.:   342   1st Qu.:   213   1st Qu.:   530   1st Qu.:   470.8   1st Qu.:   417
 Median :   922   Median :   585   Median :  1542   Median :  1412.0   Median :  1352
 Mean   :  2420   Mean   :  1528   Mean   :  3918   Mean   :  4072.6   Mean   :  4294
 3rd Qu.:  2195   3rd Qu.:  1393   3rd Qu.:  3642   3rd Qu.:  3441.2   3rd Qu.:  3656
 Max.   :597077   Max.   :406388   Max.   :639987   Max.   :808982.0   Max.   :885833
> colSums(GSE_HTSgt5)
GM12892_Rep1 GM12892_Rep2 GM12892_Rep3 H1.hESC_Rep1 H1.hESC_Rep2 H1.hESC_Rep3 H1.hESC_Rep4
    37278141     16498913     15621942     28137254     28956711     27384301     17293143
 MCF.7_Rep1   MCF.7_Rep2   MCF.7_Rep3
    44331343     46086020     48589601
```

Abundance differences in MCF.7 mRNA




```
plot(log10(GSE_HTSgt5$MCF.7_Rep1[
 order(GSE_HTSgt5$MCF.7_Rep1,
decreasing=T)]))
```

>90% of counts come from < 2% of genes (200)



Differential Gene Expression with edgeR

- Convert to a dge (edgeR) structure:

```
>GSE_dge<-DGEList(counts=GSE_HTSeq,lib.size=colSums(GSE_HTSeq),
+   group=c(rep("GM128",3),rep("H1",4),rep("MCF7",3)))
```

- Select genes with at least n counts

```
>GSE_cpms<-cpm(GSE_dge)  # cpm, counts per kb per million (FPKM),
                    # needs gene lengths
>keep2 <- rowSums(GSE_cpms[,1:3])>5 & rowSums(GSE_cpms[,4:7]) > 5 &
rowSums(GSE_cpms[,8:10])>5
> length(GSE_cpms[keep2,1])
[1] 10147
```

- Set up groups of factors so replicates can be combined:

```
> GSE_groups<-c(rep("GM128",3),rep("H1",4),rep("MCF7",3))
> GSE_groups
 [1] "GM128" "GM128" "GM128" "H1"    "H1"    "H1"    "H1"    "MCF7"
"MCF7"  "MCF7"
```

Differential Gene Expression with edgeR

- build new dge (edgeR) structure for genes with counts:

```
>GSE_d2<-DGEList(counts=GSE_counts2,lib.size=colSums(GSE_counts2),
+    group=GSE_groups)
> summary(GSE_counts2[,c(1,2,4,5,8)])
  GM12892_Rep1      GM12892_Rep2       H1.hESC_Rep1     H1.hESC_Rep2        MCF.7_Rep1
 Min.   :      0   Min.   :     0.0   Min.   :    18   Min.   :    31.0   Min.   :     0
 1st Qu.:    522   1st Qu.:   266.5   1st Qu.:   472   1st Qu.:   475.5   1st Qu.:   741
 Median :   1274   Median :   635.0   Median :  1097   Median :  1103.0   Median :  1798
 Mean   :   3678   Mean   :  1624.4   Mean   :  2736   Mean   :  2816.0   Mean   :  4259
 3rd Qu.:   2884   3rd Qu.:  1353.5   3rd Qu.:  2496   3rd Qu.:  2472.5   3rd Qu.:  3998
 Max.   :1045434   Max.   :482679.0   Max.   :646940   Max.   :628301.0   Max.   :639987
```

  - still see differences in bulk properties, but what is 1st quartile now?
  - notice that mean is > 3rd quartile. Why?

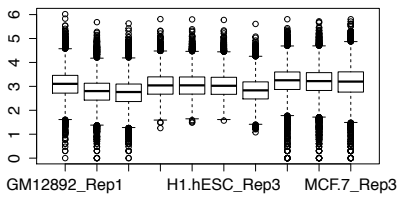
```
> summary(GSE_HTSeq)
  GM12892_Rep1      GM12892_Rep2       H1.hESC_Rep1     H1.hESC_Rep2
 Min.   :      0   Min.   :     0.0   Min.   :     0   Min.   :     0
 1st Qu.:      1   1st Qu.:     1.0   1st Qu.:     4   1st Qu.:     4
 Median :    103   Median :    56.0   Median :   200   Median :   208
 Mean   :   1830   Mean   :   814.2   Mean   :  1418   Mean   :  1460
 3rd Qu.:   1246   3rd Qu.:   630.0   3rd Qu.:  1159   3rd Qu.:  1164
 Max.   :1045434   Max.   :482679.0   Max.   :646940   Max.   :628301
```


Differential Gene Expression with edgeR

- build new dge (edgeR) structure for genes with counts:

```
>GSE_d2<-DGEList(counts=GSE_counts2,lib.size=colSums(GSE_counts2),
+    group=GSE_groups)
> summary(GSE_counts2[,c(1,2,4,5,8)])
  GM12892_Rep1      GM12892_Rep2       H1.hESC_Rep1     H1.hESC_Rep2        MCF.7_Rep1
 Min.   :      0   Min.   :     0.0   Min.   :    18   Min.   :    31.0   Min.   :     0
 1st Qu.:    522   1st Qu.:   266.5   1st Qu.:   472   1st Qu.:   475.5   1st Qu.:   741
 Median :   1274   Median :   635.0   Median :  1097   Median :  1103.0   Median :  1798
 Mean   :   3678   Mean   :  1624.4   Mean   :  2736   Mean   :  2816.0   Mean   :  4259
 3rd Qu.:   2884   3rd Qu.:  1353.5   3rd Qu.:  2496   3rd Qu.:  2472.5   3rd Qu.:  3998
 Max.   :1045434   Max.   :482679.0   Max.   :646940   Max.   :628301.0   Max.   :639987
```

  - still see differences in bulk properties, but what is 1st quartile now?
  - notice that mean is > 3rd quartile. Why?
  - Are the bulk properties similar?



How to compare relative mRNA expression?

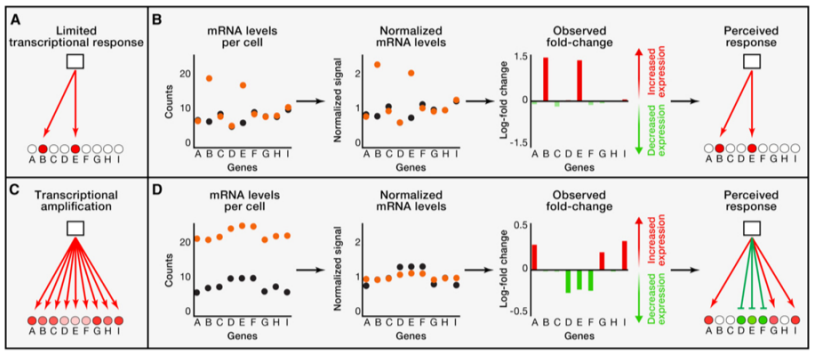


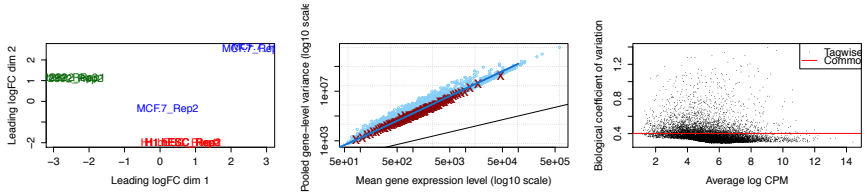
Figure 1. Normalization and Interpretation of Expression Data

Lovén, J. *et al. Cell* **151,** 476–482 (2012).

Differential Gene Expression with edgeR

- do some simple normalization, evaluate data quality:

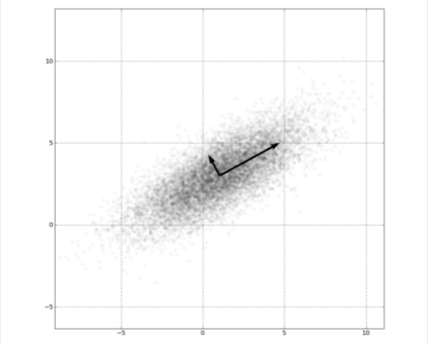
```
> GSE_d2<-calcNormFactors(GSE_d2)
# plot Principal Components Analysis (PCA) of fold-changes
> plotMDS(GSE_d2,labels=colnames(GSE_counts2),
+  col=c("darkgreen", "red", "blue")[factor(GSE_groups)])
> GSE_d2<-estimateCommonDisp(GSE_d2)
> GSE_d2<-estimateTagwiseDisp(GSE_d2)
> plotMeanVar(GSE_d2,show.tagwise.vars=TRUE,NBline=TRUE)
> plotBCV(GSE_d2)   # BCV = Biological Coefficient of Variation
```



Diversion: Principal Components Analysis (PCA)

- We are interested in the differences, and similarities, between biological samples (replicate treated and controls), from the perspective of expression levels of 10,000 – 20,000 genes.
  - imagine that in the treated sample (e.g. a BHA-treated liver vs normal), only ONE gene has increased expression: (GSTM1), all the rest are the same.
  - to find this gene, we might plot our 6 samples (3 treated, 3 controls) in n=20,000 dimensional space (one axis for every gene), and look so see which point has moved between treated and controls.
  - but if only ONE gene has changed expression level, then all the other genes will be highly correlated, so we do not need 20,000 dimensions, we only need ONE (or possibly two, the second for random noise)
- Principal Components Analysis (PCA) examines the correlation between the datasets, and reduces the dimensionality to the mininum number of "axes" (Principal Components) to explain the variation in the data.
  - first component has most variance – shows a weighting of a gene set that with internal expression correlation, but different from genes not in the set
  - replicate samples should be similar; different samples should be different
  - check for outliers

Diversion: Principal Components Analysis (PCA)

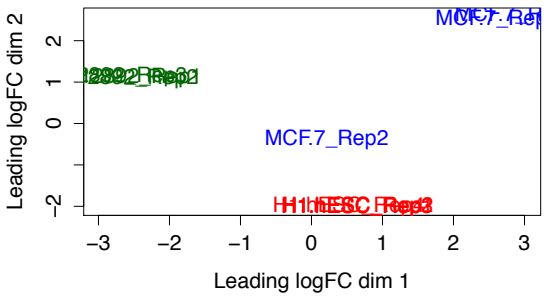


A scatter plot of samples that are distributed according a multivariate (bivariate) Gaussian distribution centered at (1,3) with a standard deviation of 3 in roughly the (0.878, 0.478) direction and of 1 in the orthogonal direction. The directions represent the Principal Components (PC) associated with the sample

en.wikipedia.org/wiki/Principal_component_analysis#/media/File:GaussianScatterPCA.png
"GaussianScatterPCA" by —Ben FrantzDale (talk)

Diversion: Principal Components Analysis (PCA)

```
> GSE_c2_prin<-princomp(GSE_counts2,cor=T)
> summary(GSE_c2_prin)
Importance of components:
                          Comp.1     Comp.2     Comp.3     Comp.4      Comp.5      Comp.6
Standard deviation     2.9115294 0.78123223 0.66206994 0.55342497 0.301356842 0.200813419
Proportion of Variance 0.8477003 0.06103238 0.04383366 0.03062792 0.009081595 0.004032603
Cumulative Proportion  0.8477003 0.90873271 0.95256637 0.98319429 0.992275889 0.996308492
```



Differential Gene Expression with edgeR

- Normalization done: do t-tests on gene groups

```
> GSE_de2 <- exactTest(GSE_d,pair=c("GM128","H1"))  # two-way compare
> GSE_tt2<-topTags(GSE_de2,n=nrow(GSE_de2))
> head(GSE_tt2$table,n=10)
             logFC    logCPM       PValue          FDR
LCP1    -10.077901 10.155089 8.290374e-57 8.412243e-53
RASSF5   -6.159025  5.542985 5.657588e-51 2.870377e-47
TIFA     -5.809657  5.629944 1.409506e-44 4.767419e-41
SMARCA2  -5.407642  7.584283 3.756685e-43 9.529771e-40
DOCK10   -5.948203  5.386774 3.129221e-41 6.350442e-38
CD58     -5.876585  5.406012 1.194831e-40 2.020658e-37
SMAP2    -5.036339  6.867746 3.039079e-40 4.405362e-37
NEAT1    -5.451577  9.251840 1.435513e-38 1.820769e-35
EPHB4     5.930016  7.329484 5.557970e-38 6.266302e-35
BCL2     -5.237690  5.635830 1.829338e-37 1.856230e-34
```

- what direction are the fold changes? all the same?
- why are PValues < FDR?

Differential Gene Expression with edgeR

- look at "top tags"

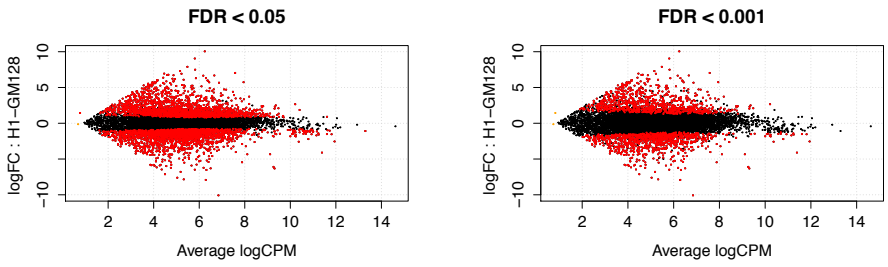
```
> GSE_counts2[row.names(head(GSE_tt2$table,n=10)),c(1:3,4:5)]
        GM12892_Rep1 GM12892_Rep2 GM12892_Rep3 H1.hESC_Rep1 H1.hESC_Rep2
LCP1          153736        50863        58206          107           89
RASSF5          5356         2659         1983           63           66
TIFA            3788         2800         2543           91           63
SMARCA2        21764         8381         8004          433          323
DOCK10          4040         2295         2024           77           39
CD58            3145         2201         2219           56           49
SMAP2          12461         5928         4385          317          288
NEAT1          57519        21160        26498         1056          896
EPHB4            149           64           47         7104         7266
BCL2            4073         3012         2634          121          111
```

- Most significant changes have high counts in GM128, low counts in H1.hESC, or vice-versa (EPHB4).
- Go back and look at the data. Does it make sense?

Differential Gene Expression with edgeR

- look at differential expression 1:

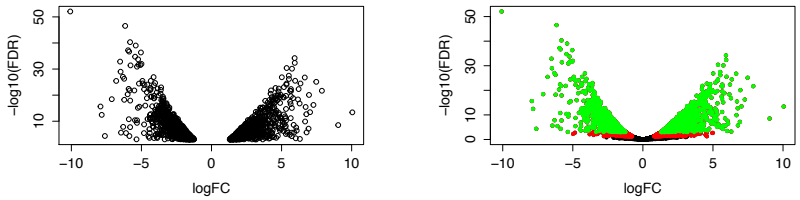
```
> GSE_deg2<-GSE_rn[GSE_tt2$table$FDR < 0.05]   # 3879 genes
> GSE_deg2_001<-GSE_rn[GSE_tt2$table$FDR < 0.001]  # 1840 genes
> plotSmear(GSE_d2,de.tags=GSE_deg2,main="FDR < 0.05")
> plotSmear(GSE_d2,de.tags=GSE_deg2_001,main="FDR < 0.001")
```



Differential Gene Expression with edgeR

- look at differential expression 2: volcano plots

```
> plot(GSE_tt2$table$logFC, -log10(GSE_tt2$table$FDR),
+   xlab="logFC",ylab="-log10(FDR)")
```



look at differential expression 2: volcano plots

```
> plot(GSE_tt2$table[,1], -log10(GSE_tt2$table[,4]),
+   xlab="logFC",ylab="-log10(FDR)",pch=20)
> points(GSE_tt2$table[GSE_deg2,1], -log10(GSE_tt2$table[GSE_deg2,4]),
+   pch=20,col='red')
> points(GSE_tt2$table[GSE_deg2_001,1], -log10(GSE_tt2$table[GSE_deg2_001,4]),
+   pch=20,col='green')
```


Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data.

Rapaport *et al. Genome Biology* 2013, **14**:R95
http://genomebiology.com/2013/14/9/R95

Genome Biology

METHOD Open Access

Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data

Franck Rapaport[1], Raya Khanin[1], Yupu Liang[1], Mono Pirun[1], Azra Krek[1], Paul Zumbo[2,3], Christopher E Mason[2,3], Nicholas D Socci[1] and Doron Betel[3,4*]

Abstract

A large number of computational methods have been developed for analyzing differential gene expression in RNA-seq data. We describe a comprehensive evaluation of common methods using the SEQC benchmark dataset and ENCODE data. We consider a number of key features, including normalization, accuracy of differential expression detection and differential expression analysis when one condition has no detectable expression. We find significant differences among the methods, but note that array-based methods adapted to RNA-seq data perform comparably to methods designed for RNA-seq. Our results demonstrate that increasing the number of replicate samples significantly improves detection power over increased sequencing depth.

Rapaport, F. *et al.*. *Genome Biol* **14,** R95 (2013).

Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data.

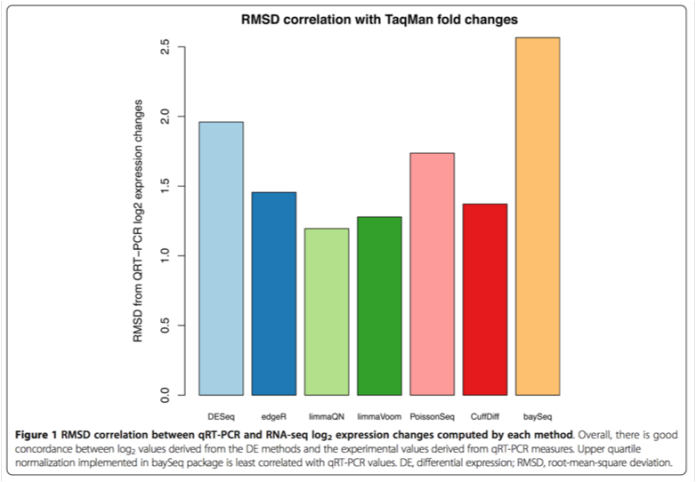


Figure 1 RMSD correlation between qRT-PCR and RNA-seq log_2 expression changes computed by each method. Overall, there is good concordance between log_2 values derived from the DE methods and the experimental values derived from qRT-PCR measures. Upper quartile normalization implemented in baySeq package is least correlated with qRT-PCR values. DE, differential expression; RMSD, root-mean-square deviation.

Rapaport, F. *et al.*. *Genome Biol* **14,** R95 (2013).

Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data.

Number of genes with log2=0.5 change by qPCR vs RNA-seq

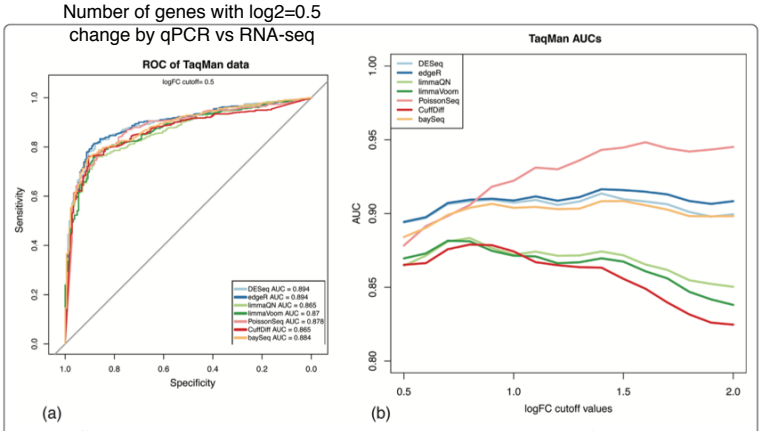


Figure 2 Differential expression analysis using qRT-PCR validated gene set. (a) ROC analysis was performed using a qRT-PCR log_2 expression change threshold of 0.5. The results show a slight advantage for DESeq and edgeR in detection accuracy. **(b)** At increasing log_2 expression ratios (incremented by 0.1), representing a more stringent cutoff for differential expression, the performances of the Cuffdiff and limma methods gradually reduce whereas PoissonSeq performance increases. AUC, area under the curve.

Rapaport, F. *et al.*. *Genome Biol* **14,** R95 (2013).

Identifying differentially expressed genes

1. convert to FPKM (probably not done properly in my example) (cpm)
2. With RNA-seq data, make sure counts > 1
3. Normalize, adjust medians, quantile normalization
4. Look at bulk properties:
   - PCA analysis should group replicates
   - variance should be relatively linear
5. Calculate pair-wise differential expression with t-tests
6. Use topTags to do FDR correction, identify largest changes
   - go back and compare topTags results to actual counts
7. Log2(FC) vs Log10(abundance)
8. Volcano plots show fold-change, q-value tradeoff

Bioconductor: summary

- Bioconductor: a comprehensive 'R' package for expression and genome analysis
  - Obtaining/installing
  - Datasets
  - Vignettes
  - Major packages (affy, edgeR2)
- Using Bioconductor/EdgeR for RNAseq
  - reading in data (what to look for)
  - removing genes with low/no signal
  - normalization
  - finding differentially expressed genes

Always look at the RAW data
that produced the list of genes