edgeR: differential expression analysis of digital gene expression data

User's Guide

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Chapter 1

Introduction

1.1 Scope

This guide provides an overview of the Bioconductor package edgeR for differential expression analyses of read counts arising from RNA-Seq, SAGE or similar technologies [25]. The package can be applied to any technology that produces read counts for genomic features. Of particular interest are summaries of short reads from massively parallel sequencing technologies such as Illumina$^{\text{TM}}$, 454 or ABI SOLiD applied to RNA-Seq, SAGE-Seq or ChIP-Seq experiments and pooled shRNA-seq or CRISPR-Cas9 genetic screens. edgeR provides statistical routines for assessing differential expression in RNA-Seq experiments or differential marking in ChIP-Seq experiments.

The package implements exact statistical methods for multigroup experiments developed by Robinson and Smyth [27, 28]. It also implements statistical methods based on generalized linear models (glms), suitable for multifactor experiments of any complexity, developed by McCarthy et al. [17], Lund et al. [15], Chen et al. [5] and Lun et al. [14]. Sometimes we refer to the former exact methods as *classic* edgeR, and the latter as *glm* edgeR. However the two sets of methods are complementary and can often be combined in the course of a data analysis. Most of the glm functions can be identified by the letters "`glm`" as part of the function name. The glm functions can test for differential expression using either likelihood ratio tests[17, 5] or quasi-likelihood F-tests [15, 14].

A particular feature of edgeR functionality, both classic and glm, are empirical Bayes methods that permit the estimation of gene-specific biological variation, even for experiments with minimal levels of biological replication.

edgeR can be applied to differential expression at the gene, exon, transcript or tag level. In fact, read counts can be summarized by any genomic feature. edgeR analyses at the exon level are easily extended to detect differential splicing or isoform-specific differential expression.

This guide begins with brief overview of some of the key capabilities of package, and then gives a number of fully worked case studies, from counts to lists of genes.

1.2 Citation

The edgeR package implements statistical methods from the following publications. Please try to cite the appropriate articles when you publish results obtained using the software, as such citation is the main means by which the authors receive credit for their work.

Robinson, MD, and Smyth, GK (2008). Small sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* 9, 321–332.

> Proposed the idea of sharing information between genes by estimating the negative binomial variance parameter globally across all genes. This made the use of negative binomial models practical for RNA-Seq and SAGE experiments with small to moderate numbers of replicates. Introduced the terminology *dispersion* for the variance parameter. Proposed conditional maximum likelihood for estimating the dispersion, assuming common dispersion across all genes. Developed an exact test for differential expression appropriate for the negative binomially distributed counts. Despite the official publication date, this was the first of the papers to be submitted and accepted for publication.

Robinson, MD, and Smyth, GK (2007). Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics* 23, 2881–2887.

> Introduced empirical Bayes moderated dispersion parameter estimation. This is a crucial improvement on the previous idea of estimating the dispersions from a global model, because it permits gene-specific dispersion estimation to be reliable even for small samples. Gene-specific dispersion estimation is necessary so that genes that behave consistently across replicates should rank more highly than genes that do not.

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

> Announcement of the edgeR software package. Introduced the terminology *coefficient of biological variation.*

Robinson, MD, and Oshlack, A (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology* 11, R25.

> Introduced the idea of model-based scale normalization of RNA-Seq data. Proposed TMM normalization.

McCarthy, DJ, Chen, Y, Smyth, GK (2012). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research* 40, 4288-4297.

> Extended negative binomial differential expression methods to glms, making the methods applicable to general experiments. Introduced the use of Cox-Reid approximate

conditional maximum likelihood for estimating the dispersion parameters, and used this for empirical Bayes moderation. Developed fast algorithms for fitting glms to thousands of genes in parallel. Gives a more complete explanation of the concept of *biological coefficient of variation*.

Lun, ATL, Chen, Y, and Smyth, GK (2016). It's DE-licious: a recipe for differential expression analyses of RNA-seq experiments using quasi-likelihood methods in edgeR. *Methods in Molecular Biology* 1418, 391–416.

> This book chapter explains the `glmQLFit` and `glmQLFTest` functions, which are alternatives to `glmFit` and `glmLRT`. They replace the chisquare approximation to the likelihood ratio statistic with a quasi-likelihood F-test, resulting in more conservative and rigorous type I error rate control.

Chen, Y, Lun, ATL, and Smyth, GK (2014). Differential expression analysis of complex RNA-seq experiments using edgeR. In: *Statistical Analysis of Next Generation Sequence Data*, Somnath Datta and Daniel S Nettleton (eds), Springer, New York.

> This book chapter explains the `estimateDisp` function and the weighted likelihood empirical Bayes method.

Zhou X, Lindsay H, and Robinson MD (2014). Robustly detecting differential expression in RNA sequencing data using observation weights. *Nucleic Acids Research*, 42, e91.

> Explains `estimateGLMRobustDisp`, which is designed to make the downstream tests done by `glmLRT` robust to outlier observations.

Dai, Z, Sheridan, JM, Gearing, LJ, Moore, DL, Su, S, Wormald, S, Wilcox, S, O'Connor, L, Dickins, RA, Blewitt, ME and Ritchie, ME (2014). edgeR: a versatile tool for the analysis of shRNA-seq and CRISPR-Cas9 genetic screens. *F1000Research* 3, 95.

> This paper explains the `processAmplicons` function for obtaining counts from the fastq files of shRNA-seq and CRISPR-Cas9 genetic screens and outlines a general workflow for analyzing data from such screens.

1.3 How to get help

Most questions about edgeR will hopefully be answered by the documentation or references. If you've run into a question that isn't addressed by the documentation, or you've found a conflict between the documentation and what the software does, then there is an active support community that can offer help.

The edgeR authors always appreciate receiving reports of bugs in the package functions or in the documentation. The same goes for well-considered suggestions for improvements. All other questions or problems concerning edgeR should be posted to the Bioconductor support site `https://support.bioconductor.org`. Please send requests for general assistance and advice to the support site rather than to the individual authors. Posting questions to the Bioconductor support site has a number of advantages. First, the support site includes a community of experienced edgeR users who can answer most common questions. Second, the edgeR authors try hard to ensure that any user posting to Bioconductor receives assistance. Third, the support site allows others with the same sort of questions to gain from the answers. Users posting to the support site for the first time will find it helpful to read the posting guide at `http://www.bioconductor.org/help/support/posting-guide`.

Note that each function in edgeR has its own online help page. For example, a detailed description of the arguments and output of the `exactTest` function can be read by typing `?exactTest` or `help(exactTest)` at the R prompt. If you have a question about any particular function, reading the function's help page will often answer the question very quickly. In any case, it is good etiquette to check the relevant help page first before posting a question to the support site.

The authors do occasionally answer questions posted to other forums, such as SEQAnswers or Biostar, but it is not possible to do this on a regular basis.

1.4 Quick start

edgeR offers many variants on analyses. In general, the glm pipeline is recommended as it offers great flexibilities. There are two testing methods under the glm framework: likelihood ratio test and quasi-likelihood F-test. The details of these two methods are described in Chapter 2.

A typical edgeR analysis might look like the following. Here we assume there are four RNA-Seq libraries in two groups, and the counts are stored in a tab-delimited text file, with gene symbols in a column called `Symbol`.

```
> x <- read.delim("TableOfCounts.txt",row.names="Symbol")
> group <- factor(c(1,1,2,2))
> y <- DGEList(counts=x,group=group)
> y <- calcNormFactors(y)
> design <- model.matrix(~group)
> y <- estimateDisp(y,design)
```

To perform quasi-likelihood F-tests:

```
> fit <- glmQLFit(y,design)
> qlf <- glmQLFTest(fit,coef=2)
> topTags(qlf)
```

To perform likelihood ratio tests:

```
> fit <- glmFit(y,design)
> lrt <- glmLRT(fit,coef=2)
> topTags(lrt)
```

Chapter 2

Overview of capabilities

2.1 Terminology

edgeR performs differential abundance analysis for pre-defined genomic features. Although not strictly necessary, it usually desirable that these genomic features are non-overlapping. For simplicity, we will hence-forth refer to the genomic features as “genes”, although they could in principle be transcripts, exons, general genomic intervals or some other type of feature. For ChIP-seq experiments, abundance might relate to transcription factor binding or to histone mark occupancy, but we will henceforth refer to abundance as in terms of gene expression. In other words, the remainder of this guide will use terminology as for a gene-level analysis of an RNA-seq experiment, although the methodology is more widely applicable than that.

2.2 Aligning reads to a genome

The first step in an RNA-seq analysis is usually to align the raw sequence reads to a reference genome, although there are many variations on this process. Alignment needs to allow for the fact that reads may span multiple exons which may align to well separated locations on the genome. We find the subread-featureCounts pipeline [11, 12] to be very fast and effective for this purpose, but the Bowtie-TopHat-htseq pipeline is also very popular [1].

2.3 Producing a table of read counts

edgeR works on a table of integer read counts, with rows corresponding to genes and columns to independent libraries. The counts represent the total number of reads aligning to each gene (or other genomic locus).

Such counts can be produced from aligned reads by a variety of short read software tools. We find the `featureCounts` function of the Rsubread package [12] to be particularly effective and convenient, but other tools are available such as `findOverlaps` in the GenomicRanges package or the Python software `htseq-counts`.

Reads can be counted in a number of ways. When conducting gene-level analyses, the counts could be for reads mapping anywhere in the genomic span of the gene or the counts could be for exons only. We usually count reads that overlap any exon for the given gene, including the UTR as part of the first exon [12].

For data from pooled shRNA-seq or CRISPR-Cas9 genetic screens, the `processAmplicons` function [7] can be used to obtain counts directly from fastq files.

Note that edgeR is designed to work with actual read counts. We not recommend that predicted transcript abundances are input the edgeR in place of actual counts.

2.4 Reading the counts from a file

If the table of counts has been written to a file, then the first step in any analysis will usually be to read these counts into an R session.

If the count data is contained in a single tab-delimited or comma-separated text file with multiple columns, one for each sample, then the simplest method is usually to read the file into R using one of the standard R read functions such as `read.delim`. See the quick start above, or the case study on LNCaP Cells, or the case study on oral carcinomas later in this guide for examples.

If the counts for different samples are stored in separate files, then the files have to be read separately and collated together. The edgeR function `readDGE` is provided to do this. Files need to contain two columns, one for the counts and one for a gene identifier.

2.5 The DGEList data class

edgeR stores data in a simple list-based data object called a `DGEList`. This type of object is easy to use because it can be manipulated like any list in R. The function `readDGE` makes a `DGEList` object directly. If the table of counts is already available as a matrix or a data.frame, `x` say, then a `DGEList` object can be made by

```
> y <- DGEList(counts=x)
```

A grouping factor can be added at the same time:

```
> group <- c(1,1,2,2)
> y <- DGEList(counts=x, group=group)
```

The main components of an `DGEList` object are a matrix `counts` containing the integer counts, a data.frame `samples` containing information about the samples or libraries, and a optional data.frame `genes` containing annotation for the genes or genomic features. The data.frame `samples` contains a column `lib.size` for the library size or sequencing depth for each sample. If not specified by the user, the library sizes will be computed from the column sums of the counts. For classic edgeR the data.frame `samples` must also contain a column `group`, identifying the group membership of each sample.

2.6 Filtering

Genes with very low counts across all libraries provide little evidence for differential expression. In the biological point of view, a gene must be expressed at some minimal level before it is likely to be translated into a protein or to be biologically important. In addition, the pronounced discreteness of these counts interferes with some of the statistical approximations that are used later in the pipeline. These genes should be filtered out prior to further analysis.

As a rule of thumb, genes are dropped if they can't possibly be expressed in all the samples for any of the conditions. Users can set their own definition of genes being expressed. Usually a gene is required to have a count of 5-10 in a library to be considered expressed in that library. Users should also filter with count-per-million (CPM) rather than filtering on the counts directly, as the latter does not account for differences in library sizes between samples.

Here is a simple example. Suppose the sample information of a `DGEList` object `y` is shown as follows:

```
> y$samples
```

```
        group lib.size norm.factors
Sample1     1 10880519            1
Sample2     1  9314747            1
Sample3     1 11959792            1
Sample4     2  7460595            1
Sample5     2  6714958            1
```

We filter out lowly expressed genes using the following commands:

```
> keep <- rowSums(cpm(y)>1) >= 2
> y <- y[keep, , keep.lib.sizes=FALSE]
```

Here, a CPM of 1 corresponds to a count of 6-7 in the smallest sample. A requirement for expression in two or more libraries is used as the minimum number of samples in each group is two. This ensures that a gene will be retained if it is only expressed in both samples in group 2. It is also recommended to recalculate the library sizes of the `DGEList` object after the filtering though the difference is usually negligible.

2.7 Normalization

2.7.1 Normalization is only necessary for sample-specific effects

edgeR is concerned with differential expression analysis rather than with the quantification of expression levels. It is concerned with relative changes in expression levels between conditions, but not directly with estimating absolute expression levels. This greatly simplifies the technical influences that need to be taken into account, because any technical factor that is unrelated to the experimental conditions should cancel out of any differential expression analysis. For example, read counts can generally be expected to be proportional to length as well as to expression for any transcript, but edgeR does not generally need to adjust for gene length because gene length has the same relative influence on the read counts for each RNA sample. For this reason, normalization issues arise only to the extent that technical factors have sample-specific effects.

2.7.2 Sequencing depth

The most obvious technical factor that affects the read counts, other than gene expression levels, is the sequencing depth of each RNA sample. edgeR adjusts any differential expression analysis for varying sequencing depths as represented by differing library sizes. This is part of the basic modeling procedure and flows automatically into fold-change or p-value calculations. It is always present, and doesn't require any user intervention.

2.7.3 RNA composition

The second most important technical influence on differential expression is one that is less obvious. RNA-seq provides a measure of the relative abundance of each gene in each RNA sample, but does not provide any measure of the total RNA output on a per-cell basis. This commonly becomes important when a small number of genes are very highly expressed in one sample, but not in another. The highly expressed genes can consume a substantial proportion of the total library size, causing the remaining genes to be under-sampled in that sample. Unless this *RNA composition* effect is adjusted for, the remaining genes may falsely appear to be down-regulated in that sample [26].

The `calcNormFactors` function normalizes for RNA composition by finding a set of scaling factors for the library sizes that minimize the log-fold changes between the samples for most genes. The default method for computing these scale factors uses a trimmed mean of M-values (TMM) between each pair of samples [26]. We call the product of the original library size and the scaling factor the *effective library size*. The effective library size replaces the original library size in all downsteam analyses.

TMM is the recommended for most RNA-Seq data where the majority (more than half) of the genes are believed not differentially expressed between any pair of the samples. The following commands perform the TMM normalization and display the normalization factors.

```
> y <- calcNormFactors(y)
> y$samples

        group lib.size norm.factors
Sample1     1 10880519         1.17
Sample2     1  9314747         0.86
Sample3     1 11959792         1.32
Sample4     2  7460595         0.91
Sample5     2  6714958         0.83
```

The normalization factors of all the libraries multiply to unity. A normalization factor below one indicates that a small number of high count genes are monopolizing the sequencing, causing the counts for other genes to be lower than would be usual given the library size. As a result, the library size will be scaled down, analogous to scaling the counts upwards in that library. Conversely, a factor above one scales up the library size, analogous to downscaling the counts.

2.7.4 GC content

The GC-content of each gene does not change from sample to sample, so it can be expected to have little effect on differential expression analyses to a first approximation. Recent publications, however, have demonstrated that sample-specific effects for GC-content can be detected [24, 9]. The EDASeq [24] and cqn [9] packages estimate correction factors that adjust for sample-specific GC-content effects in a way that is compatible with edgeR. In each case, the observation-specific correction factors can be input into the glm functions of edgeR as an *offset* matrix.

2.7.5 Gene length

Like GC-content, gene length does not change from sample to sample, so it can be expected to have little effect on differential expression analyses. Nevertheless, sample-specific effects for gene length have been detected [9], although the evidence is not as strong as for GC-content.

2.7.6 Model-based normalization, not transformation

In edgeR, normalization takes the form of correction factors that enter into the statistical model. Such correction factors are usually computed internally by edgeR functions, but it is also possible for a user to supply them. The correction factors may take the form of scaling factors for the library sizes, such as computed by `calcNormFactors`, which are then used to compute the effective library sizes. Alternatively, gene-specific correction factors can be entered into the glm functions of edgeR as offsets. In the latter case, the offset matrix will be assumed to account for all normalization issues, including sequencing depth and RNA composition.

Note that normalization in edgeR is model-based, and the original read counts are not themselves transformed. This means that users should not transform the read counts in any way before inputing them to edgeR. For example, users should not enter RPKM or FPKM values to edgeR in place of read counts. Such quantities will prevent edgeR from correctly estimating the mean-variance relationship in the data, which is a crucial to the statistical strategies underlying edgeR. Similarly, users should not add artificial values to the counts before inputing them to edgeR.

edgeR is not designed to work with estimated expression levels, for example as might be output by Cufflinks. edgeR can work with expected counts as output by RSEM, but raw counts are still preferred.

2.7.7 Pseudo-counts

The classic edgeR functions `estimateCommonDisp` and `exactTest` produce a matrix of *pseudo-counts* as part of the output object. The pseudo-counts are used internally to speed up computation of the conditional likelihood used for dispersion estimation and exact tests in the classic edgeR pipeline. The pseudo-counts represent the equivalent counts would have been observed had the library sizes all been equal, assuming the fitted model. The pseudo-counts are computed for a specific purpose, and their computation depends on the experimental design as well as the library sizes, so users are advised not to interpret the psuedo-counts as general-purpose normalized counts. They are intended mainly for internal use in the edgeR pipeline.

Disambiguation. Note that some other software packages use the term *pseudo-count* to mean something analogous to *prior counts* in edgeR, i.e., a starting value that is added to a zero count to avoid missing values when computing logarithms. In edgeR, a pseudo-count is a type of normalized count and a prior count is a starting value used to offset small counts.

2.8 Negative binomial models

2.8.1 Introduction

The starting point for an RNA-Seq experiment is a set of n RNA samples, typically associated with a variety of treatment conditions. Each sample is sequenced, short reads are mapped to the appropriate genome, and the number of reads mapped to each genomic feature of interest is recorded. The number of reads from sample i mapped to gene g will be denoted y_{gi}. The set of genewise counts for sample i makes up the expression profile or *library* for that sample. The expected size of each count is the product of the library size and the relative abundance of that gene in that sample.

2.8.2 Biological coefficient of variation (BCV)

RNA-Seq profiles are formed from n RNA samples. Let π_{gi} be the fraction of all cDNA fragments in the ith sample that originate from gene g. Let G denote the total number of genes, so $\sum_{g=1}^{G} \pi_{gi} = 1$ for each sample. Let $\sqrt{\phi_g}$ denote the coefficient of variation (CV) (standard deviation divided by mean) of π_{gi} between the replicates i. We denote the total number of mapped reads in library i by N_i and the number that map to the gth gene by y_{gi}. Then

$$E(y_{gi}) = \mu_{gi} = N_i \pi_{gi}.$$

Assuming that the count y_{gi} follows a Poisson distribution for repeated sequencing runs of the same RNA sample, a well known formula for the variance of a mixture distribution implies:

$$\mathrm{var}(y_{gi}) = E_\pi \left[\mathrm{var}(y|\pi)\right] + \mathrm{var}_\pi \left[E(y|\pi)\right] = \mu_{gi} + \phi_g \mu_{gi}^2.$$

Dividing both sides by μ_{gi}^2 gives

$$\mathrm{CV}^2(y_{gi}) = 1/\mu_{gi} + \phi_g.$$

The first term $1/\mu_{gi}$ is the squared CV for the Poisson distribution and the second is the squared CV of the unobserved expression values. The total CV^2 therefore is the technical CV^2 with which π_{gi} is measured plus the biological CV^2 of the true π_{gi}. In this article, we call ϕ_g the dispersion and $\sqrt{\phi_g}$ the biological CV although, strictly speaking, it captures all sources of the inter-library variation between replicates, including perhaps contributions from technical causes such as library preparation as well as true biological variation between samples.

Two levels of variation can be distinguished in any RNA-Seq experiment. First, the relative abundance of each gene will vary between RNA samples, due mainly to biological causes. Second, there is measurement error, the uncertainty with which the abundance of each gene in each sample is estimated by the sequencing technology. If aliquots of the same RNA sample are sequenced, then the read counts for a particular gene should vary according to a Poisson law [16]. If sequencing variation is Poisson, then it can be shown that the squared coefficient of variation (CV) of each count between biological replicate libraries is the sum of the squared CVs for technical and biological variation respectively,

$$\text{Total CV}^2 = \text{Technical CV}^2 + \text{Biological CV}^2.$$

Biological CV (BCV) is the coefficient of variation with which the (unknown) true abundance of the gene varies between replicate RNA samples. It represents the CV that would remain between biological replicates if sequencing depth could be increased indefinitely. The technical CV decreases as the size of the counts increases. BCV on the other hand does not. BCV is therefore likely to be the dominant source of uncertainty for high-count genes, so reliable estimation of BCV is crucial for realistic assessment of differential expression in

RNA-Seq experiments. If the abundance of each gene varies between replicate RNA samples in such a way that the genewise standard deviations are proportional to the genewise means, a commonly occurring property of measurements on physical quantities, then it is reasonable to suppose that BCV is approximately constant across genes. We allow however for the possibility that BCV might vary between genes and might also show a systematic trend with respect to gene expression or expected count.

The magnitude of BCV is more important than the exact probabilistic law followed by the true gene abundances. For mathematical convenience, we assume that the true gene abundances follow a gamma distributional law between replicate RNA samples. This implies that the read counts follow a negative binomial probability law.

2.8.3 Estimating BCVs

When a negative binomial model is fitted, we need to estimate the BCV(s) before we carry out the analysis. The BCV, as shown in the previous section, is the square root of the dispersion parameter under the negative binomial model. Hence, it is equivalent to estimating the dispersion(s) of the negative binomial model.

The parallel nature of sequencing data allows some possibilities for borrowing information from the ensemble of genes which can assist in inference about each gene individually. The easiest way to share information between genes is to assume that all genes have the same mean-variance relationship, in other words, the dispersion is the same for all the genes [28]. An extension to this "common dispersion" approach is to put a mean-dependent trend on a parameter in the variance function, so that all genes with the same expected count have the same variance.

However, the truth is that the gene expression levels have non-identical and dependent distribution between genes, which makes the above assumptions too naive. A more general approach that allows genewise variance functions with empirical Bayes shrinkage was introduced several years ago [27] and has recently been extended to generalized linear models and thus more complex experimental designs [17]. Only when using tagwise dispersion will genes that are consistent between replicates be ranked more highly than genes that are not. It has been seen in many RNA-Seq datasets that allowing gene-specific dispersion is necessary in order that differential expression is not driven by outliers. Therefore, the tagwise dispersions are strongly recommended in model fitting and testing for differential expression.

In edgeR, we first estimate a common dispersion for all the tags and then apply an empirical Bayes strategy for squeezing the tagwise dispersions towards the common dispersion. The amount of shrinkage is determined by the prior weight given to the common dispersion (or the dispersion trend) and the precision of the tagwise estimates, and can be considered as the prior degrees of freedom. This prior degrees of freedom is estimated by examining the heteroskedasticity of the data [5].

2.8.4 Quasi negative binomial

The NB model can be extended with quasi-likelihood (QL) methods to account for gene-specific variability from both biological and technical sources [15, 14]. Under the QL framework, the variance of the count y_{gi} is a quadratic function of the mean,

$$\mathrm{var}(y_{gi}) = \sigma_g^2(\mu_{gi} + \phi\mu_{gi}^2),$$

where ϕ is the NB dispersion parameter and σ_g^2 is the QL dispersion parameter.

Any increase in the observed variance of y_{gi} will be modelled by an increase in the estimates for ϕ and/or σ_g^2. In this model, the NB dispersion ϕ is a global parameter whereas the QL is gene-specific, so the two dispersion parameters have different roles. The NB dispersion describes the overall biological variability across all genes. It represents the observed variation that is attributable to inherent variability in the biological system, in contrast to the Poisson variation from sequencing. The QL dispersion picks up any gene-specific variability above and below the overall level.

The common NB dispersion for the entire data set can be used for the global parameter. In practice, we use the trended dispersions to account for the empirical mean-variance relationships. Since the NB dispersion under the QL framework reflects the overall biological variability, it does not make sense to use the tagwise dispersions.

Estimation of the gene-specific QL dispersion is difficult as most RNA-seq data sets have limited numbers of replicates. This means that there is often little information to stably estimate the dispersion for each gene. To overcome this, an empirical Bayes (EB) approach is used whereby information is shared between genes [30, 15, 21]. Briefly, a mean-dependent trend is fitted to the raw QL dispersion estimates. The raw estimates are then squeezed towards this trend to obtain moderated EB estimates, which can be used in place of the raw values for downstream hypothesis testing. This EB strategy reduces the uncertainty of the estimates and improves testing power.

2.9 Pairwise comparisons between two or more groups (classic)

2.9.1 Estimating dispersions

edgeR uses the quantile-adjusted conditional maximum likelihood (qCML) method for experiments with single factor.

Compared against several other estimators (e.g. maximum likelihood estimator, Quasi-likelihood estimator etc.) using an extensive simulation study, qCML is the most reliable in terms of bias on a wide range of conditions and specifically performs best in the situation of many small samples with a common dispersion, the model which is applicable to Next-Gen sequencing data. We have deliberately focused on very small samples due to the fact that DNA sequencing costs prevent large numbers of replicates for SAGE and RNA-seq experiments.

The qCML method calculates the likelihood by conditioning on the total counts for each tag, and uses pseudo counts after adjusting for library sizes. Given a table of counts or a `DGEList` object, the qCML common dispersion and tagwise dispersions can be estimated using the `estimateDisp()` function. Alternatively, one can estimate the qCML common dispersion using the `estimateCommonDisp()` function, and then the qCML tagwise dispersions using the `estimateTagwiseDisp()` function.

However, the qCML method is only applicable on datasets with a single factor design since it fails to take into account the effects from multiple factors in a more complicated experiment. When an experiment has more than one factor involved, we need to seek a new way of estimating dispersions.

Here is a simple example of estimating dispersions using the qCML method. Given a `DGEList` object `y`, we estimate the dispersions using the following commands.

To estimate common dispersion and tagwise dispersions in one run (recommended):

```
y <- estimateDisp(y)
```

Alternatively, to estimate common dispersion:

```
y <- estimateCommonDisp(y)
```

Then to estimate tagwise dispersions:

```
y <- estimateTagwiseDisp(y)
```

Note that common dispersion needs to be estimated before estimating tagwise dispersions if they are estimated separately.

2.9.2 Testing for DE genes

For all the Next-Gen squencing data analyses we consider here, people are most interested in finding differentially expressed genes/tags between two (or more) groups. Once negative binomial models are fitted and dispersion estimates are obtained, we can proceed with testing procedures for determining differential expression using the exact test.

The exact test is based on the qCML methods. Knowing the conditional distribution for the sum of counts in a group, we can compute exact p-values by summing over all sums of counts that have a probability less than the probability under the null hypothesis of the observed sum of counts. The exact test for the negative binomial distribution has strong parallels with Fisher's exact test.

As we dicussed in the previous section, the exact test is only applicable to experiments with a single factor. The testing can be done by using the function `exactTest()`, and the function allows both common dispersion and tagwise dispersion approaches. For example:

```
> et <- exactTest(y)
> topTags(et)
```

2.10 More complex experiments (glm functionality)

2.10.1 Generalized linear models

Generalized linear models (GLMs) are an extension of classical linear models to nonnormally distributed response data [20, 19]. GLMs specify probability distributions according to their mean-variance relationship, for example the quadratic mean-variance relationship specified above for read counts. Assuming that an estimate is available for ϕ_g, so the variance can be evaluated for any value of μ_{gi}, GLM theory can be used to fit a log-linear model

$$\log \mu_{gi} = \mathbf{x}_i^T \boldsymbol{\beta}_g + \log N_i$$

for each gene [13, 4]. Here $\mathbf{x}_i$ is a vector of covariates that specifies the treatment conditions applied to RNA sample i, and $\boldsymbol{\beta}_g$ is a vector of regression coefficients by which the covariate effects are mediated for gene g. The quadratic variance function specifies the negative binomial GLM distributional family. The use of the negative binomial distribution is equivalent to treating the π_{gi} as gamma distributed.

2.10.2 Estimating dispersions

For general experiments (with multiple factors), edgeR uses the Cox-Reid profile-adjusted likelihood (CR) method in estimating dispersions. The CR method is derived to overcome the limitations of the qCML method as mentioned above. It takes care of multiple factors by fitting generalized linear models (GLM) with a design matrix.

The CR method is based on the idea of approximate conditional likelihood which reduces to residual maximum likelihood. Given a table counts or a `DGEList` object and the design matrix of the experiment, generalized linear models are fitted. This allows valid estimation of the dispersion, since all systematic sources of variation are accounted for.

The CR method can be used to calculate a common dispersion for all the tags, trended dispersion depending on the tag abundance, or separate dispersions for individual tags. These can be done by calling the function `estimateDisp()` with a specified design. Alternatively, one can estimate the common, trended and tagwise dispersions separately using `estimateGLMCommonDisp()`, `estimateGLMTrendedDisp()` and `estimateGLMTagwiseDisp()`, respectively. The tagwise dispersion approach is strongly recommended in multi-factor experiment cases.

Here is a simple example of estimating dispersions using the GLM method. Given a `DGEList` object `y` and a design matrix, we estimate the dispersions using the following commands.

To estimate common dispersion, trended dispersions and tagwise dispersions in one run (recommended):

```
y <- estimateDisp(y, design)
```

Alternatively, one can use the following calling sequence to estimate them one by one. To estimate common dispersion:

```
y <- estimateGLMCommonDisp(y, design)
```

To estimate trended dispersions:

```
y <- estimateGLMTrendedDisp(y, design)
```

To estimate tagwise dispersions:

```
y <- estimateGLMTagwiseDisp(y, design)
```

Note that we need to estimate either common dispersion or trended dispersions prior to the estimation of tagwise dispersions. When estimating tagwise dispersions, the empirical Bayes method is applied to squeeze tagwise dispersions towards common dispersion or trended dispersions, whichever exists. If both exist, the default is to use the trended dispersions.

For more detailed examples, see the case study in section 4.1 (Tuch's data), section 4.2 (arabidopsis data), section 4.3 (Nigerian data) and section 4.4 (Fu's data).

2.10.3 Testing for DE genes

For general experiments, once dispersion estimates are obtained and negative binomial models are fitted, we can proceed with testing procedures for determining differential expression using either the generalized linear model (GLM) likelihood ratio test or the quasi-likelihood (QL) F-test.

The GLM likelihood ratio test is based on the idea of fitting negative binomial GLMs with the Cox-Reid dispersion estimates. The testing can be done by using the functions `glmFit()` and `glmLRT()`. Given raw counts, dispersion(s) and a design matrix, `glmFit()` fits the negative binomial GLM for each tag and produces an object of class `DGEGLM` with some new components. This `DGEGLM` object can then be passed to `glmLRT()` to carry out the likelihood ratio test. User can select one or more coefficients to drop from the full design matrix. This gives the null model against which the full model is compared using the likelihood ratio test. Tags can then be ranked in order of evidence for differential expression, based on the *p*-value computed for each tag.

As a brief example, consider a situation in which are three treatment groups, each with two replicates, and the researcher wants to make pairwise comparisons between them. A generalized linear model representing the study design can be fitted to the data with commands such as:

```
> group <- factor(c(1,1,2,2,3,3))
> design <- model.matrix(~group)
> fit <- glmFit(y, design)
```

The fit has three parameters. The first is the baseline level of group 1. The second and third are the 2 vs 1 and 3 vs 1 differences.

To compare 2 vs 1:

```
> lrt.2vs1 <- glmLRT(fit, coef=2)
> topTags(lrt.2vs1)
```

To compare 3 vs 1:

```
> lrt.3vs1 <- glmLRT(fit, coef=3)
```

To compare 3 vs 2:

```
> lrt.3vs2 <- glmLRT(fit, contrast=c(0,-1,1))
```

The contrast argument in this case requests a statistical test of the null hypothesis that coefficient3−coefficient2 is equal to zero.

To find genes different between any of the three groups:

```
> lrt <- glmLRT(fit, coef=2:3)
> topTags(lrt)
```

For more detailed examples, see the case study in section 4.1 (Tuch's data)

Alternatively, one can perform QL F-test to test for differential expression. While the likelihood ratio test is a more obvious choice for inferences with GLMs, the QL F-test is preferred as it reflects the uncertainty in estimating the dispersion for each gene. It provides more robust and reliable error rate control when the number of replicates is small. The QL dispersion estimation and hypothesis testing can be done by using the functions `glmQLFit()` and `glmQLFTest()`.

To apply the QL method to the above example and compare 2 vs 1:

```
> fit <- glmQLFit(y, design)
> qlf.2vs1 <- glmQLFTest(fit, coef=2)
> topTags(qlf.2vs1)
```

Similarly for the other comparisons.

For more detailed examples, see the case study in section 4.2 (arabidopsis data), section 4.3 (Nigerian data) and section 4.4 (Fu's data).

2.11 What to do if you have no replicates

edgeR is primarily intended for use with data including biological replication. Nevertheless, RNA-Seq and ChIP-Seq are still expensive technologies, so it sometimes happens that only one library can be created for each treatment condition. In these cases there are no replicate libraries from which to estimate biological variability. In this situation, the data analyst is faced with the following choices, none of which are ideal. We do not recommend any of these choices as a satisfactory alternative for biological replication. Rather, they are the best that can be done at the analysis stage, and options 2–4 may be better than assuming that biological variability is absent.

1. Be satisfied with a descriptive analysis, that might include an MDS plot and an analysis of fold changes. Do not attempt a significance analysis. This may be the best advice.

2. Simply pick a reasonable dispersion value, based on your experience with similar data, and use that for `exactTest` or `glmFit`. Typical values for the common BCV (square-root-dispersion) for datasets arising from well-controlled experiments are 0.4 for human data, 0.1 for data on genetically identical model organisms or 0.01 for technical replicates. Here is a toy example with simulated data:

   ```
   > bcv <- 0.2
   > counts <- matrix( rnbinom(40,size=1/bcv^2,mu=10), 20,2)
   > y <- DGEList(counts=counts, group=1:2)
   > et <- exactTest(y, dispersion=bcv^2)
   ```

   Note that the p-values obtained and the number of significant genes will be very sensitive to the dispersion value chosen, and be aware that less well controlled datasets, with unaccounted-for batch effects and so on, could have in reality much larger dispersions than are suggested here. Nevertheless, choosing a nominal dispersion value may be more realistic than ignoring biological variation entirely.

3. Remove one or more explanatory factors from the linear model in order to create some residual degrees of freedom. Ideally, this means removing the factors that are least important but, if there is only one factor and only two groups, this may mean removing the entire design matrix or reducing it to a single column for the intercept. If your experiment has several explanatory factors, you could remove the factor with smallest fold changes. If your experiment has several treatment conditions, you could try treating the two most similar conditions as replicates. Estimate the dispersion from this reduced model, then insert these dispersions into the data object containing the full design matrix, then proceed to model fitting and testing with `glmFit` and `glmLRT`. This approach will only be successful if the number of DE genes is relatively small.

   In conjunction with this reduced design matrix, you could try `estimateGLMCommonDisp` with `method="deviance"`, `robust=TRUE` and `subset=NULL`. This is our current best attempt at an automatic method to estimate dispersion without replicates, although it will only give good results when the counts are not too small and the DE genes are a small proportion of the whole. Please understand that this is only our best attempt to return something useable. Reliable estimation of dispersion generally requires replicates.

4. If there exist a sizeable number of control transcripts that should not be DE, then the dispersion could be estimated from them. For example, suppose that `housekeeping` is

an index variable identifying housekeeping genes that do not respond to the treatment used in the experiment. First create a copy of the data object with only one treatment group:

```
> y1 <- y
> y1$samples$group <- 1
```

Then estimate the dispersion from the housekeeping genes and all the libraries as one group:

```
> y0 <- estimateCommonDisp(y1[housekeeping,])
```

Then insert this into the full data object and proceed:

```
> y$common.dispersion <- y0$common.dispersion
> et <- exactTest(y)
```

and so on. A reasonably large number of control transcripts is required, at least a few dozen and ideally hundreds.

2.12 Differential expression above a fold-change threshold

All the above testing methods identify differential expression based on statistical significance regardless of how small the difference might be. On the other hand, one might be more interested in studying genes of which the expression levels change by a certain amount. A commonly used approach is to conduct DE tests, apply a fold-change cut-off and then rank all the genes above that fold-change threshold by *p*-value. In some other cases genes are first chosen according to a *p*-value cut-off and then sorted by their fold-changes. These combinations of *p*-value and fold-change threshold criteria seem to give more biological meaningful sets of genes than using either of them alone. However, they are both *ad hoc* and do not give meaningful *p*-values for testing differential expressions relative to a fold-change threshold. They favour lowly expressed but highly variable genes and destroy the control of FDR in general.

edgeR offers a rigorous statistical test for thresholded hypotheses under the GLM framework. It is analogous to TREAT [18] but much more powerful than the original TREAT method. Given a fold-change (or log-fold-change) threshold, the thresholded testing can be done by calling the function `glmTreat()` on a `DGEGLM` object produced by either `glmFit()` or `glmQLFit()`.

In the example shown in section 2.10.3, suppose we are detecting genes of which the log2-fold-changes for 1 vs 2 are significantly greater than 1, i.e., fold-changes significantly greater than 2, we use the following commands:

```
> fit <- glmFit(y, design)
> tr <- glmTreat(fit, coef=2, lfc=1)
> topTags(tr)
```

Note that the fold-change threshold in `glmTreat()` is not the minimum value of the fold-change expected to see from the testing results. Genes will need to exceed this threshold by some way before being declared statistically significant. It is better to interpret the threshold as "the fold-change below which we are definitely not interested in the gene" rather than "the fold-change above which we are interested in the gene". In the presence of a huge number of DE genes, a relatively large fold-change threshold may be appropriate to narrow down the search to genes of interest. In the lack of DE genes, on the other hand, a small or even no fold-change threshold shall be used.

For more detailed examples, see the case study in section 4.4 (Fu's data).

2.13 Gene ontology (GO) and pathway analysis

The gene ontology (GO) enrichment analysis and the KEGG pathway enrichment analysis are the common downstream procedures to interpret the differential expression results in a biological context. Given a set of genes that are up- or down-regulated under a certain contrast of interest, a GO (or pathway) enrichment analysis will find which GO terms (or pathways) are over- or under-represented using annotations for the genes in that set.

The GO analysis can be performed using the `goana()` function in edgeR. The KEGG pathway analysis can be performed using the `kegga()` function in edgeR. Both `goana()` and `kegga()` take a `DGELRT` or `DGEExact` object. They both use the NCBI RefSeq annotation. Therefore, the Entrez Gene identifier (ID) should be supplied for each gene as the row names of the input object. Also users should set `species` according to the organism being studied. The top set of most enriched GO terms can be viewed with the `topGO()` function, and the top set of most enriched KEGG pathways can be viewed with the `topKEGG()` function.

Suppose we want to identify GO terms and KEGG pathways that are over-represented in group 1 compared to group 2 from the previous example in section 2.10.3 assuming the samples are collected from mice. We use the following commands:

```
> qlf <- glmQLFTest(fit, coef=2)
> go <- goana(qlf, species="Mm")
> topGO(go)
> keg <- kegga(qlf, species="Mm")
> topKEGG(keg)
```

For more detailed examples, see the case study in section 4.1 (Tuch's data) and section 4.4 (Fu's data).

2.14 Gene set testing

In addition to the GO and pathway analysis, edgeR offers different types of gene set tests for RNA-Seq data. These gene set tests are the extensions of the original gene set tests in limma in order to handle `DGEList` objects.

The `roast()` function performs ROAST gene set tests [33]. It is a self-contained gene set test. Given a gene set, it tests whether the majority of the genes in the set are DE across the comparison of interest.

The `mroast()` function does ROAST tests for multiple sets, including adjustment for multiple testing.

The `fry()` function is a fast version of `mroast()`. It assumes all the genes in a set have equal variances. Since edgeR uses the z-score equivalents of NB random deviates for the gene set tests, the above assumption is always met. Hence, `fry()` is recommended over `roast()` and `mroast()` in edgeR. It gives the same result as `mroast()` with an infinite number of rotations.

The `camera()` function performs a competitive gene set test accounting for inter-gene correlation. It tests whether a set of genes is highly ranked relative to other genes in terms of differential expression [34].

The `romer()` function performs a gene set enrichment analysis. It implements a GSEA approach [31] based on rotation instead of permutation.

Unlike `goana()` and `kegga()`, the gene set tests are not limited to GO terms or KEGG pathways. Any pre-defined gene set can be used, for example MSigDB gene sets. A common application is to use a set of DE genes that was defined from an analysis of an independent data set.

For more detailed examples, see the case study in section 4.3 (Nigerian's data) and section 4.4 (Fu's data).

2.15 Clustering, heatmaps etc

The function `plotMDS` draws a multi-dimensional scaling plot of the RNA samples in which distances correspond to leading log-fold-changes between each pair of RNA samples. The leading log-fold-change is the average (root-mean-square) of the largest absolute log-fold-changes between each pair of samples. This plot can be viewed as a type of unsupervised clustering. The function also provides the option of computing distances in terms of BCV between each pair of samples instead of leading logFC.

Inputing RNA-seq counts to clustering or heatmap routines designed for microarray data is not straight-forward, and the best way to do this is still a matter of research. To draw a heatmap of individual RNA-seq samples, we suggest using moderated log-counts-per-million. This can be calculated by `cpm` with positive values for `prior.count`, for example

```
> logcpm <- cpm(y, prior.count=2, log=TRUE)
```

where `y` is the normalized `DGEList` object. This produces a matrix of $\log_2$ counts-per-million (logCPM), with undefined values avoided and the poorly defined log-fold-changes for low counts shrunk towards zero. Larger values for `prior.count` produce more shrinkage. The logCPM values can optionally be converted to RPKM or FPKM by subtracting $\log_2$ of gene length, see `rpkm()`.

2.16 Alternative splicing

edgeR can also be used to analyze RNA-Seq data at the exon level to detect differential splicing or isoform-specific differential expression. Alternative splicing events are detected by testing for differential exon usage for each gene, that is testing whether the log-fold-changes differ between exons for the same gene.

Both exon-level and gene-level tests can be performed simultaneously using the `diffSpliceDGE()` function in edgeR. The exon-level test tests for the significant difference between the exon's logFC and the overall logFC for the gene. Two testing methods at the gene-level are provided. The first is to conduct a gene-level statistical test using the exon-level test statistics. Whether it is a likelihood ratio test or a QL F-test depends on the pipeline chosen. The second is to convert the exon-level p-values into a genewise p-value by the Simes' method. The first method is likely to be powerful for genes in which several exons are differentially spliced. The Simes' method is likely to be more powerful when only a minority of the exons for a gene are differentially spliced.

The top set of most significant spliced genes can be viewed by the `topSpliceDGE()` function. The exon-level testing results for a gene of interest can be visualized by the `plotSpliceDGE()` function.

For more detailed examples, see the case study in section 4.5 (Pasilla's data).

2.17 CRISPR-Cas9 and shRNA-seq screen analysis

edgeR can also be used to analyze data from CRISPR-Cas9 and shRNA-seq genetic screens as described in Dai *et al.* (2014) [7]. Screens of this kind typically involve the comparison of two or more cell populations either in the presence or absence of a selective pressure, or as a time-course before and after a selective pressure is applied. The goal is to identify sgRNAs (or shRNAs) whose representation changes (either increases or decreases) suggesting that disrupting the target gene's function has an effect on the cell.

To begin, the `processAmplicons` function can be used to obtain counts for each sgRNA (or shRNA) in the screen in each sample and organise them in a `DGEList` for down-stream analysis using either the classic edgeR or GLM pipeline mentioned above. Next, gene set testing methods such as `camera` and `roast` can be used to summarize results from multiple sgRNAs or shRNAs targeting the same gene to obtain gene-level results.

For a detailed example, see the case study in section 4.6 (CRISPR-Cas9 knockout screen analysis).

Chapter 3

Specific experimental designs

3.1 Introduction

In this chapter, we outline the principles for setting up the design matrix and forming contrasts for some typical experimental designs.

3.2 Two or more groups

3.2.1 Introduction

The simplest and most common type of experimental design is that in which a number of experimental conditions are compared on the basis of independent biological replicates of each condition. Suppose that there are three experimental conditions to be compared, treatments A, B and C, say. The `samples` component of the `DGEList` data object might look like:

```
> y$samples
         group lib.size norm.factors
sample.1     A   100001            1
sample.2     A   100002            1
sample.3     B   100003            1
sample.4     B   100004            1
sample.5     C   100005            1
```

Note that it is not necessary to have multiple replicates for all the conditions, although it is usually desirable to do so. By default, the conditions will be listed in alphabetical order, regardless of the order that the data were read:

```
> levels(y$samples$group)
[1] "A" "B" "C"
```

3.2.2 Classic approach

The classic edgeR approach is to make pairwise comparisons between the groups. For example,

```
> et <- exactTest(y, pair=c("A","B"))
> topTags(et)
```

will find genes differentially expressed (DE) in B vs A. Similarly

```
> et <- exactTest(y, pair=c("A","C"))
```

for C vs A, or

```
> et <- exactTest(y, pair=c("C","B"))
```

for B vs C.

Alternatively, the conditions to be compared can be specified by number, so that

```
> et <- exactTest(y, pair=c(3,2))
```

is equivalent to `pair=c("C","B")`, given that the second and third levels of `group` are `B` and `C` respectively.

Note that the levels of `group` are in alphabetical order by default, but can be easily changed. Suppose for example that C is a control or reference level to which conditions A and B are to be compared. Then one might redefine the group levels, in a new data object, so that C is the first level:

```
> y2 <- y
> y2$samples$group <- relevel(y2$samples$group, ref="C")
> levels(y2$samples$group)
[1] "C" "A" "B"
```

Now

```
> et <- exactTest(y2, pair=c("A","B"))
```

would still compare B to A, but

```
> et <- exactTest(y2, pair=c(1,2))
```

would now compare A to C.

When `pair` is not specified, the default is to compare the first two group levels, so

```
> et <- exactTest(y)
```

compares B to A, whereas

```
> et <- exactTest(y2)
```

compares A to C.

3.2.3 GLM approach

The glm approach to multiple groups is similar to the classic approach, but permits more general comparisons to be made. The glm approach requires a design matrix to describe the treatment conditions. We will usually use the `model.matrix` function to construct the design matrix, although it could be constructed manually. There are always many equivalent ways to define this matrix. Perhaps the simplest way is to define a coefficient for the expression level of each group:

```
> design <- model.matrix(~0+group, data=y$samples)
> colnames(design) <- levels(y$samples$group)
> design
         A B C
sample.1 1 0 0
sample.2 1 0 0
sample.3 0 1 0
sample.4 0 1 0
sample.5 0 0 1
```

Here, the `0+` in the model formula is an instruction not to include an intercept column and instead to include a column for each group.

One can compare any of the treatment groups using the `contrast` argument of the `glmLRT` function. For example,

```
> fit <- glmFit(y, design)
> lrt <- glmLRT(fit, contrast=c(-1,1,0))
> topTags(lrt)
```

will compare B to A. The meaning of the contrast is to make the comparison `-1*A + 1*B + 0*C`, which is of course is simply `B-A`.

The contrast vector can be constructed using `makeContrasts` if that is convenient. The above comparison could have been made by

```
> BvsA <- makeContrasts(B-A, levels=design)
> lrt <- glmLRT(fit, contrast=BvsA)
```

One could make three pairwise comparisons between the groups by

```
> my.contrasts <- makeContrasts(BvsA=B-A, CvsB=C-B, CvsA=A-C, levels=design)
> lrt.BvsA <- glmLRT(fit, contrast=my.contrasts[,"BvsA"])
> topTags(lrt.BvsA)
> lrt.CvsB <- glmLRT(fit, contrast=my.contrasts[,"CvsB"])
> topTags(lrt.CvsB)
> lrt.CvsA <- glmLRT(fit, contrast=my.contrasts[,"CvsA"])
> topTags(lrt.CvsA)
```

which would compare B to A, C to B and C to A respectively.

Any comparison can be made. For example,

```
> lrt <- glmLRT(fit, contrast=c(-0.5,-0.5,1))
```

would compare C to the average of A and B. Alternatively, this same contrast could have been specified by

```
> my.contrast <- makeContrasts(C-(A+B)/2, levels=design)
> lrt <- glmLRT(fit, contrast=my.contrast)
```

with the same results.

3.2.4 Questions and contrasts

The glm approach allows an infinite variety of contrasts to be tested between the groups. This embarassment of riches leads to the question, which specific contrasts should we test? This answer is that we should form and test those contrasts that correspond to the scientific questions that we want to answer. Each statistical test is an answer to a particular question, and we should make sure that our questions and answers match up.

To clarify this a little, we will consider a hypothetical experiment with four groups. The groups correspond to four different types of cells: white and smooth, white and furry, red and smooth and red furry. We will think of white and red as being the major group, and smooth and furry as being a sub-grouping. Suppose the RNA samples look like this:

Sample	Color	Type	Group
1	White	Smooth	A
2	White	Smooth	A
3	White	Furry	B
4	White	Furry	B
5	Red	Smooth	C
6	Red	Smooth	C
7	Red	Furry	D
8	Red	Furry	D

To decide which contrasts should be made between the four groups, we need to be clear what are our scientific hypotheses. In other words, what are we seeking to show?

First, suppose that we wish to find genes that are always higher in red cells than in white cells. Then we will need to form the four contrasts `C-A`, `C-B`, `D-A` and `D-B`, and select genes that are significantly up for all four contrasts.

Or suppose we wish to establish that the difference between Red and White is large compared to the differences between Furry and Smooth. An efficient way to establish this would be to form the three contrasts `B-A`, `D-C` and `(C+D)/2-(A+B)/2`. We could confidently make this assertion for genes for which the third contrast is far more significant than the first two. Even if `B-A` and `D-C` are statistically significant, we could still look for genes for which the fold changes for `(C+D)/2-(A+B)/2` are much larger than those for `B-A` or `D-C`.

We might want to find genes that are more highly expressed in Furry cells regardless of color. Then we would test the contrasts `B-A` and `D-C`, and look for genes that are significantly up for both contrasts.

Or we want to assert that the difference between Furry over Smooth is much the same regardless of color. In that case you need to show that the contrast `(B+D)/2-(A+C)/2` (the average Furry effect) is significant for many genes but that `(D-C)-(B-A)` (the interaction) is not.

3.2.5 A more traditional glm approach

A more traditional way to create a design matrix in R is to include an intercept term that represents the first level of the factor. We included `0+` in our model formula above. Had we omitted it, the design matrix would have had the same number of columns as above, but the first column would be the intercept term and the meanings of the second and third columns would change:

```
> design <- model.matrix(~group, data=y$samples)
> design
         (Intercept) groupB groupC
sample.1           1      0      0
sample.2           1      0      0
sample.3           1      1      0
sample.4           1      1      0
sample.5           1      0      1
```

Now the first coefficient will measure the baseline logCPM expression level in the first treatment condition (here group A), and the second and third columns are relative to the baseline. Here the second and third coefficients represent B vs A and C vs A respectively. In other words, `coef=2` now means `B-A` and `coef=3` means `C-A`, so

```
> fit <- glmFit(y, design)
> lrt <- glmLRT(fit, coef=2)
```

would test for differential expression in B vs A. and

```
> lrt <- glmLRT(fit, coef=3)
```

would test for differential expression in C vs A.

This parametrization makes good sense when the first group represents a reference or control group, as all comparison are made with respect to this condition. If we releveled the factor to make level C the first level (see Section 3.2.2), then the design matrix becomes:

```
> design2 <- model.matrix(~group, data=y2$samples)
> design2
         (Intercept) groupA groupB
sample.1           1      1      0
sample.2           1      1      0
sample.3           1      0      1
sample.4           1      0      1
sample.5           1      0      0
```

Now

```
> fit2 <- glmFit(y2, design2)
> lrt <- glmLRT(fit2, coef=2)
```

compares A to C, and

```
> lrt <- glmLRT(fit2, coef=3)
```

compares B to C. With this parametrization, one could still compare B to A using

```
> lrt <- glmLRT(fit2, contrast=c(0,-1,1))
```

Note that

```
> lrt <- glmLRT(fit2, coef=1)
```

should not be used. It would test whether the first coefficient is zero, but it is not meaningful to compare the logCPM in group A to zero.

3.2.6 An ANOVA-like test for any differences

It might be of interest to find genes that are DE between any of the groups, without specifying before-hand which groups might be different. This is analogous to a one-way ANOVA test. In edgeR, this is done by specifying multiple coefficients to `glmLRT`, when the design matrix includes an intercept term. For example, with `fit` as defined in the previous section,

```
> lrt <- glmLRT(fit, coef=2:3)
> topTags(lrt)
```

will find any genes that differ between any of the treatment conditions A, B or C. Technically, this procedure tests whether either of the contrasts `B-A` or `C-A` are non-zero. Since at least one of these must be non-zero when differences exist, the test will detect any differences. To have this effect, the `coef` argument should specify all the coefficients except the intercept.

Note that this approach does not depend on how the group factor was defined, or how the design matrix was formed, as long as there is an intercept column. For example

```
> lrt <- glmLRT(fit2, coef=2:3)
```

gives exactly the results, even though `fit2` and `fit` were computed using different design matrices. Here `fit2` is as defined in the previous section.

3.3 Experiments with all combinations of multiple factors

3.3.1 Defining each treatment combination as a group

We now consider experiments with more than one experimental factor, but in which every combination of experiment conditions can potentially have a unique effect. For example, suppose that an experiment has been conducted with an active drug and a placebo, at three times from 0 hours to 2 hours, with all samples obtained from independent subjects. The data frame `targets` describes the treatment conditions applied to each sample:

```
> targets
           Treat Time
Sample1  Placebo   0h
Sample2  Placebo   0h
Sample3  Placebo   1h
Sample4  Placebo   1h
Sample5  Placebo   2h
Sample6  Placebo   2h
Sample7     Drug   0h
Sample8     Drug   0h
Sample9     Drug   1h
Sample10    Drug   1h
Sample11    Drug   2h
Sample12    Drug   2h
```

As always, there are many ways to setup a design matrix. A simple, multi-purpose approach is to combine all the experimental factors into one combined factor:

```
> Group <- factor(paste(targets$Treat,targets$Time,sep="."))
> cbind(targets,Group=Group)
           Treat Time      Group
Sample1  Placebo   0h Placebo.0h
Sample2  Placebo   0h Placebo.0h
Sample3  Placebo   1h Placebo.1h
Sample4  Placebo   1h Placebo.1h
Sample5  Placebo   2h Placebo.2h
Sample6  Placebo   2h Placebo.2h
Sample7     Drug   0h    Drug.0h
Sample8     Drug   0h    Drug.0h
Sample9     Drug   1h    Drug.1h
Sample10    Drug   1h    Drug.1h
Sample11    Drug   2h    Drug.2h
Sample12    Drug   2h    Drug.2h
```

Then we can take the same approach as in the previous section on two or more groups. Each treatment time for each treatment drug is a group:

```
> design <- model.matrix(~0+Group)
> colnames(design) <- levels(Group)
> fit <- glmFit(y, design)
```

Then we can make any comparisons we wish. For example, we might wish to make the following contrasts:

```
> my.contrasts <- makeContrasts(
+         Drug.1vs0 = Drug.1h-Drug.0h,
+         Drug.2vs0 = Drug.2h-Drug.0h,
+         Placebo.1vs0 = Placebo.1h-Placebo.0h,
+         Placebo.2vs0 = Placebo.2h-Placebo.0h,
+         DrugvsPlacebo.0h = Drug.0h-Placebo.0h,
+         DrugvsPlacebo.1h = (Drug.1h-Drug.0h)-(Placebo.1h-Placebo.0h),
+         DrugvsPlacebo.2h = (Drug.2h-Drug.0h)-(Placebo.2h-Placebo.0h),
+ levels=design)
```

To find genes responding to the drug at 1 hour:

```
> lrt <- glmLRT(fit, contrast=my.contrasts[,"Drug.1vs0"])
```

or at 2 hours:

```
> lrt <- glmLRT(fit, contrast=my.contrasts[,"Drug.2vs0"])
```

To find genes with baseline differences between the drug and the placebo at 0 hours:

```
> lrt <- glmLRT(fit, contrast=my.contrasts[,"DrugvsPlacebo.0h"])
```

To find genes that have responded *differently* to the drug and the placebo at 2 hours:

```
> lrt <- glmLRT(fit, contrast=my.contrasts[,"DrugvsPlacebo.2h"])
```

Of course, it is not compulsory to use `makeContrasts` to form the contrasts. The coefficients are the following:

```
> colnames(fit)
[1] "Drug.0h"    "Drug.1h"    "Drug.2h"    "Placebo.0h" "Placebo.1h" "Placebo.2h"
```

so

```
> lrt <- glmLRT(fit, contrast=c(-1,0,1,0,0,0))
```

would find the `Drug.2vs0` contrast, and

```
> lrt <- glmLRT(fit, contrast=c(-1,0,1,1,0,-1))
```

is another way of specifying the `DrugvsPlacebo.2h` contrast.

3.3.2 Nested interaction formulas

We generally recommend the approach of the previous section, because it is so explicit and easy to understand. However it may be useful to be aware of more short-hand approach to form the same contrasts in the previous section using a model formula. First, make sure that the placebo is the reference level:

```
> targets$Treat <- relevel(targets$Treat, ref="Placebo")
```

Then form the design matrix:

```
> design <- model.matrix(~Treat + Treat:Time, data=targets)
> fit <- glmFit(y, design)
```

The meaning of this formula is to consider all the levels of time for each treatment drug separately. The second term is a nested interaction, the interaction of Time within Treat. The coefficient names are:

```
> colnames(fit)
[1] "(Intercept)"         "TreatDrug"
[3] "TreatPlacebo:Time1h" "TreatDrug:Time1h"
[5] "TreatPlacebo:Time2h" "TreatDrug:Time2h"
```

Now most of the above contrasts are directly available as coefficients:

```
> lrt <- glmLRT(fit, coef=2)
```

is the baseline drug vs placebo comparison,

```
> lrt <- glmLRT(fit, coef=4)
```

is the drug effect at 1 hour,

```
> lrt <- glmLRT(fit, coef=6)
```

is the drug effect at 2 hours, and finally

```
> lrt <- glmLRT(fit, contrast=c(0,0,0,0-1,1))
```

is the `DrugvsPlacebo.2h` contrast.

3.3.3 Treatment effects over all times

The nested interaction model makes it easy to find genes that respond to the treatment at any time, in a single test. Continuing the above example,

```
> lrt <- glmLRT(fit, coef=c(4,6))
```

finds genes that respond to the treatment at either 1 hour or 2 hours versus the 0 hour baseline. This is analogous to an ANOVA F-test for a normal linear model.

3.3.4 Interaction at any time

The full interaction formula is

```
> design <- model.matrix(~Treat * Time, data=targets)
```

which is equivalent to

```
> design <- model.matrix(~Treat + Time + Treat:Time, data=targets)
> fit <- glmFit(y, design)
```

This formula is primarily useful as a way to conduct an overall test for interaction. The coefficient names are:

```
> colnames(design)
[1] "(Intercept)"      "TreatDrug"
[3] "Time1h"           "Time2h"
[5] "TreatDrug:Time1h" "TreatDrug:Time2h"
```

Now

```
> lrt <- glmLRT(fit, coef=2)
```

is again the baseline drug vs placebo comparison, but

```
> lrt <- glmLRT(fit, coef=3)
```

and

```
> lrt <- glmLRT(fit, coef=4)
```

are the effects of the reference drug, i.e., the effects of the placebo at 1 hour and 2 hours. The last two coefficients give the `DrugvsPlacebo.1h` and `DrugvsPlacebo.2h` contrasts, so that

```
> lrt <- glmLRT(fit, coef=5:6)
```

is useful because it detects genes that respond *differently* to the drug, relative to the placebo, at either of the times.

3.4 Additive models and blocking

3.4.1 Paired samples

Paired samples occur whenever we compare two treatments and each independent subject in the experiment receives both treatments. Suppose for example that an experiment is conducted to compare a new treatment (T) with a control (C). Suppose that both the control and the treatment are administered to each of three patients. This produces the sample data:

FileName	Subject	Treatment
File1	1	C
File2	1	T
File3	2	C
File4	2	T
File5	3	C
File6	3	T

This is a paired design in which each subject receives both the control and the active treatment. We can therefore compare the treatment to the control for each patient separately, so that baseline differences between the patients are subtracted out.

The design matrix is formed from an additive model formula without an interaction term:

```
> Subject <- factor(targets$Subject)
> Treat <- factor(targets$Treatment, levels=c("C","T"))
> design <- model.matrix(~Subject+Treat)
```

The omission of an interaction term is characteristic of paired designs. We are not interested in the effect of the treatment on an individual patient (which is what an interaction term would examine). Rather we are interested in the average effect of the treatment over a population of patients.

As always, the dispersion has to be estimated:

```
> y <- estimateGLMCommonDisp(y,design)
> y <- estimateGLMTrendedDisp(y,design)
> y <- estimateGLMTagwiseDisp(y,design)
```

We proceed to fit a linear model and test for the treatment effect. Note that we can omit the `coef` argument to `glmLRT` because the treatment effect is the last coefficient in the model.

```
> fit <- glmFit(y, design)
> lrt <- glmLRT(fit)
> topTags(lrt)
```

This test detects genes that are differentially expressed in response to the active treatment compared to the control, adjusting for baseline differences between the patients. This test can be viewed as a generalization of a paired *t*-test.

See the oral carcinomas case study of Section 4.1 for a fully worked analysis with paired samples.

3.4.2 Blocking

Paired samples are a simple example of what is called "blocking" in experimental design. The idea of blocking is to compare treatments using experimental subjects that are as similar as possible, so that the treatment difference stands out as clearly as possible.

Suppose for example that we wish to compare three treatments A, B and C using experimental animals. Suppose that animals from the same litter are appreciably more similar than animals from different litters. This might lead to an experimental setup like:

FileName	Litter	Treatment
File1	1	A
File2	1	B
File3	1	C
File4	2	B
File5	2	A
File6	2	C
File7	3	C
File8	3	B
File9	3	A

Here it is the differences between the treatments that are of interest. The differences between the litters are not of primary interest, nor are we interested in a treatment effect that occurs for in only one litter, because that would not be reproducible.

We can compare the three treatments adjusting for any baseline differences between the litters by fitting an additive model:

```
> Litter <- factor(targets$Litter)
> Treatment <- factor(targets$Treatment)
> design <- model.matrix(~Litter+Treatment)
```

This creates a design matrix with five columns: three for the litters and two more for the differences between the treatments.

If `fit` is the fitted model with this design matrix, then we may proceed as follows. To detect genes that are differentially expressed between any of the three treatments, adjusting for litter differences:

```
> lrt <- glmLRT(fit, coef=4:5)
> topTags(lrt)
```

To detect genes that are differentially expressed in treatment B vs treatment A:

```
> lrt <- glmLRT(fit, coef=4)
> topTags(lrt)
```

To detect genes that are differentially expressed in treatment C vs treatment A:

```
> lrt <- glmLRT(fit, coef=5)
> topTags(lrt)
```

To detect genes that are differentially expressed in treatment C vs treatment B:

```
> lrt <- glmLRT(fit, contrast=c(0,0,0,-1,1))
> topTags(lrt)
```

The advantage of using litter as a blocking variable in the analysis is that this will make the comparison between the treatments more precise, if litter-mates are more alike that animals from different litters. On the other hand, if litter-mates are no more alike than animals from different litters, which might be so for genetically identical inbred laboratory animals, then the above analysis is somewhat inefficient because the litter effects are being estimated unnecessarily. In that case, it would be better to omit litter from the model formula.

3.4.3 Batch effects

Another situation in which additive model formulas are used is when correcting for batch effects in an experiment. The situation here is analogous to blocking, the only difference being that the batch effects were probably unintended rather than a deliberate aspect of the experimental design. The analysis is the same as for blocking. The treatments can be adjusted for differences between the batches by using an additive model formula of the form:

```
> design <- model.matrix(~Batch+Treatment)
```

In this type of analysis, the treatments are compared only within each batch. The analysis is corrected for baseline differences between the batches.

The Arabidopsis case study in Section 4.2 gives a fully worked example with batch effects.

3.5 Comparisons both between and within subjects

Here is a more complex scenario, posed by a poster to the Bioconductor mailing list. The experiment has 18 RNA samples collected from 9 subjects. The samples correspond to cells from 3 healthy patients, either treated or not with a hormone; cells from 3 patients with disease 1, either treated or not with the hormone; and cells from 3 patients with disease 2, either treated or not with the hormone. The targets frame looks like this:

```
> targets
    Disease Patient Treatment
1   Healthy       1      None
2   Healthy       1   Hormone
3   Healthy       2      None
4   Healthy       2   Hormone
5   Healthy       3      None
6   Healthy       3   Hormone
7  Disease1       4      None
8  Disease1       4   Hormone
9  Disease1       5      None
10 Disease1       5   Hormone
11 Disease1       6      None
12 Disease1       6   Hormone
13 Disease2       7      None
```

```
14 Disease2       7   Hormone
15 Disease2       8      None
16 Disease2       8   Hormone
17 Disease2       9      None
18 Disease2       9   Hormone
```

If all the RNA samples were collected from independent subjects, then this would be nested factorial experiment, from which we would want to estimate the treatment effect for each disease group. As it is, however, we have a paired comparison experiment for each disease group. The feature that makes this experiment complex is that some comparisons (between the diseases) are made *between* patients while other comparisons (hormone treatment vs no treatment) are made *within* patients.

The design matrix will be easier to construct in R if we re-number the patients within each disease group:

```
> Patient <- gl(3,2,length=18)
```

We also define Disease and Treatment to be factors, with the control state as the first level in each case:

```
> Disease <- factor(targets$Disease, levels=c("Healthy","Disease1","Disease2"))
> Treatment <- factor(targets$Treatment, levels=c("None","Hormone"))
```

This gives us a revised targets frame:

```
> data.frame(Disease,Patient,Treatment)
    Disease Patient Treatment
1   Healthy       1      None
2   Healthy       1   Hormone
3   Healthy       2      None
4   Healthy       2   Hormone
5   Healthy       3      None
6   Healthy       3   Hormone
7  Disease1       1      None
8  Disease1       1   Hormone
9  Disease1       2      None
10 Disease1       2   Hormone
11 Disease1       3      None
12 Disease1       3   Hormone
13 Disease2       1      None
14 Disease2       1   Hormone
15 Disease2       2      None
16 Disease2       2   Hormone
17 Disease2       3      None
18 Disease2       3   Hormone
```

Now we can construct the design matrix. The critical feature to appreciate is that Patient and Treatment are of interest within each disease group, so we use the nested factorial formula discussed in a previous section. The patients are nested with the disease groups, because we have different patients in each group. The treatment is nested within disease groups, because we are interested in the disease-specific treatment effects. The model formula has the main effect for disease plus nested interactions with Patient and Treatment:

```
> design <- model.matrix(~Disease+Disease:Patient+Disease:Treatment)
> colnames(design)
 [1] "(Intercept)"                      "DiseaseDisease1"
 [3] "DiseaseDisease2"                  "DiseaseHealthy:Patient2"
 [5] "DiseaseDisease1:Patient2"         "DiseaseDisease2:Patient2"
 [7] "DiseaseHealthy:Patient3"          "DiseaseDisease1:Patient3"
 [9] "DiseaseDisease2:Patient3"         "DiseaseHealthy:TreatmentHormone"
[11] "DiseaseDisease1:TreatmentHormone" "DiseaseDisease2:TreatmentHormone"
```

After estimating the dispersions (code not shown), we can fit a linear model:

```
> fit <- glmFit(y, design)
```

To find genes responding to the hormone in healthy patients:

```
> lrt <- glmLRT(fit, coef="DiseaseHealthy:TreatmentHormone")
> topTags(lrt)
```

To find genes responding to the hormone in disease1 patients:

```
> lrt <- glmLRT(fit, coef="DiseaseDisease1:TreatmentHormone")
> topTags(lrt)
```

To find genes responding to the hormone in disease2 patients:

```
> lrt <- glmLRT(fit, coef="DiseaseDisease2:TreatmentHormone")
> topTags(lrt)
```

To find genes that respond to the hormone in *any* disease group:

```
> lrt <- glmLRT(fit, coef=10:12)
> topTags(lrt)
```

To find genes that respond differently to the hormone in disease1 vs healthy patients:

```
> lrt <- glmLRT(fit, contrast=c(0,0,0,0,0,0,0,0,0,-1,1,0))
> topTags(lrt)
```

To find genes that respond differently to the hormone in disease2 vs healthy patients:

```
> lrt <- glmLRT(fit, contrast=c(0,0,0,0,0,0,0,0,0,-1,0,1))
> topTags(lrt)
```

To find genes that respond differently to the hormone in disease2 vs disease1 patients:

```
> lrt <- glmLRT(fit, contrast=c(0,0,0,0,0,0,0,0,0,0,-1,1))
> topTags(lrt)
```

Chapter 4

Case studies

4.1 RNA-Seq of oral carcinomas vs matched normal tissue

4.1.1 Introduction

This section provides a detailed analysis of data from a paired design RNA-seq experiment, featuring oral squamous cell carcinomas and matched normal tissue from three patients [32]. The aim of the analysis is to detect genes differentially expressed between tumor and normal tissue, adjusting for any differences between the patients. This provides an example of the GLM capabilities of edgeR.

RNA was sequenced on an Applied Biosystems SOLiD System 3.0 and reads mapped to the UCSC hg18 reference genome [32]. Read counts, summarised at the level of refSeq transcripts, are available in Table S1 of Tuch et al. [32].

4.1.2 Reading in the data

The read counts for the six individual libraries are stored in one tab-delimited file. To make this file, we downloaded Table S1 from Tuch et al. [32], deleted some unnecessary columns and edited the column headings slightly:

```
> rawdata <- read.delim("TableS1.txt", check.names=FALSE, stringsAsFactors=FALSE)
> head(rawdata)
     RefSeqID    Symbol NbrOfExons   8N 8T   33N 33T   51N  51T
1   NM_182502 TMPRSS11B         10 2592  3  7805 321  3372    9
2   NM_003280     TNNC1          6 1684  0  1787   7  4894  559
3   NM_152381     XIRP2         10 9915 15 10396  48 23309 7181
4   NM_022438       MAL          3 2496  2  3585 239  1596    7
5 NM_001100112      MYH2         40 4389  7  7944  16  9262 1818
6   NM_017534      MYH2         40 4402  7  7943  16  9244 1815
```

For easy manipulation, we put the data into a `DGEList` object:

```
> library(edgeR)
> y <- DGEList(counts=rawdata[,4:9], genes=rawdata[,1:3])
```

4.1.3 Annotation

The study by Tuch et al. [32] was undertaken a few years ago, so not all of the RefSeq IDs provided by match RefSeq IDs currently in use. We retain only those transcripts with IDs in the current NCBI annotation, which is provided by the `org.HS.eg.db` package:

```
> library(org.Hs.eg.db)
> idfound <- y$genes$RefSeqID %in% mappedRkeys(org.Hs.egREFSEQ)
> y <- y[idfound,]
> dim(y)
[1] 15559     6
```

We add Entrez Gene IDs to the annotation:

```
> egREFSEQ <- toTable(org.Hs.egREFSEQ)
> head(egREFSEQ)
  gene_id    accession
1       1    NM_130786
2       1    NP_570602
3       2    NM_000014
4       2    NP_000005
5       2 XM_006719056
6       2 XP_006719119
> m <- match(y$genes$RefSeqID, egREFSEQ$accession)
> y$genes$EntrezGene <- egREFSEQ$gene_id[m]
```

Now use the Entrez Gene IDs to update the gene symbols:

```
> egSYMBOL <- toTable(org.Hs.egSYMBOL)
> head(egSYMBOL)
  gene_id symbol
1       1   A1BG
2       2    A2M
3       3  A2MP1
4       9   NAT1
5      10   NAT2
6      11   NATP
> m <- match(y$genes$EntrezGene, egSYMBOL$gene_id)
> y$genes$Symbol <- egSYMBOL$symbol[m]
> head(y$genes)
```

```
      RefSeqID    Symbol NbrOfExons EntrezGene
1    NM_182502 TMPRSS11B         10     132724
2    NM_003280     TNNC1          6       7134
3    NM_152381     XIRP2         10     129446
4    NM_022438       MAL          3       4118
5 NM_001100112      MYH2         40       4620
6    NM_017534      MYH2         40       4620
```

4.1.4 Filtering and normalization

Different RefSeq transcripts for the same gene symbol count predominantly the same reads. So we keep one transcript for each gene symbol. We choose the transcript with highest overall count:

```
> o <- order(rowSums(y$counts), decreasing=TRUE)
> y <- y[o,]
> d <- duplicated(y$genes$Symbol)
> y <- y[!d,]
> nrow(y)
[1] 10523
```

Normally we would also filter lowly expressed genes. For this data, all transcripts already have at least 50 reads for all samples of at least one of the tissues types.

Recompute the library sizes:

```
> y$samples$lib.size <- colSums(y$counts)
```

Use Entrez Gene IDs as row names:

```
> rownames(y$counts) <- rownames(y$genes) <- y$genes$EntrezGene
> y$genes$EntrezGene <- NULL
```

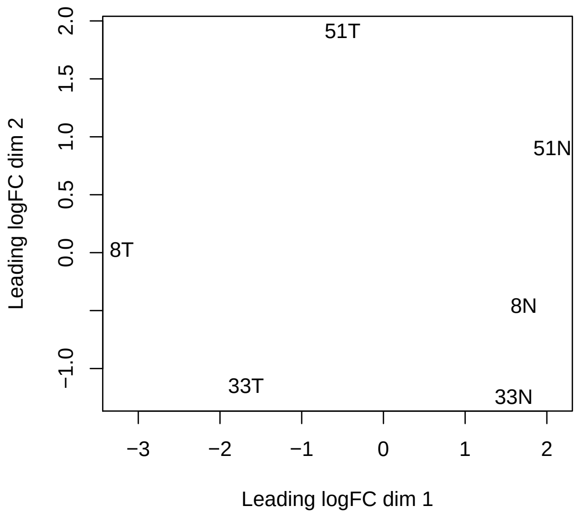
TMM normalization is applied to this dataset to account for compositional difference between the libraries.

```
> y <- calcNormFactors(y)
> y$samples
    group lib.size norm.factors
8N      1  7992800        1.146
8T      1  7374090        1.086
33N     1 15759922        0.673
33T     1 14050463        0.974
51N     1 21547308        1.032
51T     1 15199698        1.190
```

4.1.5 Data exploration

The first step of an analysis should be to examine the samples for outliers and for other relationships. The function `plotMDS` produces a plot in which distances between samples correspond to leading biological coefficient of variation (BCV) between those samples:

```
> plotMDS(y)
```



In the plot, dimension 1 separates the tumor from the normal samples, while dimension 2 roughly corresponds to patient number. This confirms the paired nature of the samples. The tumor samples appear more heterogeneous than the normal samples.

4.1.6 The design matrix

Before we fit negative binomial GLMs, we need to define our design matrix based on the experimental design. Here we want to test for differential expression between tumour and normal tissues within patients, i.e. adjusting for differences between patients. In statistical terms, this is an additive linear model with patient as the blocking factor:

```
> Patient <- factor(c(8,8,33,33,51,51))
> Tissue <- factor(c("N","T","N","T","N","T"))
> data.frame(Sample=colnames(y),Patient,Tissue)
  Sample Patient Tissue
1     8N       8      N
2     8T       8      T
3    33N      33      N
```

```
4    33T      33      T
5    51N      51      N
6    51T      51      T
```

```
> design <- model.matrix(~Patient+Tissue)
> rownames(design) <- colnames(y)
> design
```

```
    (Intercept) Patient33 Patient51 TissueT
8N            1         0         0       0
8T            1         0         0       1
33N           1         1         0       0
33T           1         1         0       1
51N           1         0         1       0
51T           1         0         1       1
attr(,"assign")
[1] 0 1 1 2
attr(,"contrasts")
attr(,"contrasts")$Patient
[1] "contr.treatment"

attr(,"contrasts")$Tissue
[1] "contr.treatment"
```

This sort of additive model is appropriate for paired designs, or experiments with batch effects.

4.1.7 Estimating the dispersion

We estimate the NB dispersion for the dataset.

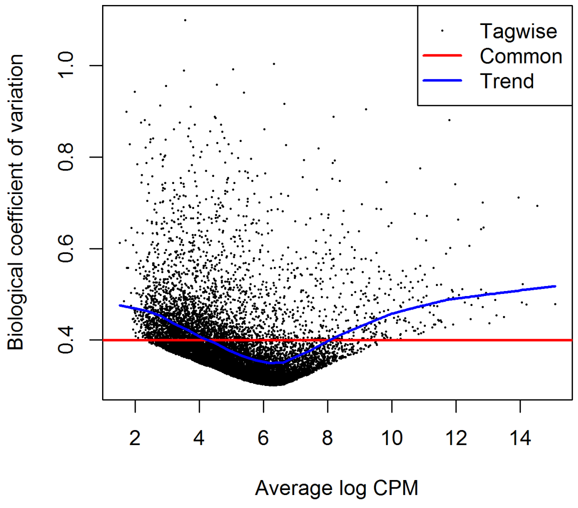
```
> y <- estimateDisp(y, design, robust=TRUE)
> y$common.dispersion
```

```
[1] 0.159
```

The square root of the common dispersion gives the coefficient of variation of biological variation. Here the common dispersion is found to be 0.159, so the coefficient of biological variation is around 0.4.

The dispersion estimates can be viewed in a BCV plot:

```
> plotBCV(y)
```



4.1.8 Differential expression

Now proceed to determine differentially expressed genes. Fit genewise glms:

```
> fit <- glmFit(y, design)
```

Conduct likelihood ratio tests for tumour vs normal tissue differences and show the top genes:

```
> lrt <- glmLRT(fit)
> topTags(lrt)
Coefficient:  TissueT
           RefSeqID   Symbol NbrOfExons logFC logCPM   LR   PValue      FDR
5737   NM_001039585    PTGFR          4 -5.18   4.74 98.7 2.98e-23 3.14e-19
5744      NM_002820    PTHLH          4  3.97   6.21 92.1 8.10e-22 4.26e-18
3479   NM_001111283     IGF1          5 -3.99   5.71 86.5 1.38e-20 4.83e-17
1288      NM_033641   COL4A6         45  3.66   5.71 77.5 1.32e-18 3.47e-15
10351     NM_007168    ABCA8         38 -3.98   4.94 75.9 2.97e-18 6.24e-15
5837      NM_005609     PYGM         20 -5.48   5.99 75.4 3.82e-18 6.70e-15
487       NM_004320   ATP2A1         23 -4.62   5.96 74.8 5.16e-18 7.75e-15
27179     NM_014440    IL36A          4 -6.17   5.40 72.2 1.96e-17 2.58e-14
196374    NM_173352    KRT78          9 -4.25   7.61 70.8 3.98e-17 4.66e-14
83699     NM_031469 SH3BGRL2          4 -3.93   5.53 67.8 1.83e-16 1.93e-13
```

Note that `glmLRT` has conducted a test for the last coefficient in the linear model, which we can see is the tumor vs normal tissue effect:

```
> colnames(design)
```

```
[1] "(Intercept)" "Patient33"   "Patient51"   "TissueT"
```

The genewise tests are for tumor vs normal differential expression, adjusting for baseline differences between the three patients. The tests can be viewed as analogous to paired t-tests. The top DE tags have tiny p-values and FDR values, as well as large fold changes.

Here's a closer look at the counts-per-million in individual samples for the top genes:

```
> o <- order(lrt$table$PValue)
> cpm(y)[o[1:10],]
           8N      8T    33N     33T    51N      51T
5737    49.69   0.874  27.08   0.877  78.10   2.5425
5744     7.32  95.799  11.79 204.034   6.88 116.2917
3479    50.24   3.123  32.36   1.901 211.59  14.2048
1288    12.12 140.138   6.32  94.378   4.86  56.8193
10351   52.64   3.123  39.43   2.120  79.18   6.0799
5837   152.80   2.748 119.53   1.170  97.67   5.6930
487    107.91   3.123 146.98   3.801 102.80   8.8987
27179   40.08   1.249 172.08   3.290  36.08   0.0553
196374 372.22  20.733 580.96  47.737 145.05   4.5323
83699   96.22   5.121 117.08   5.410  48.18   5.4166
```

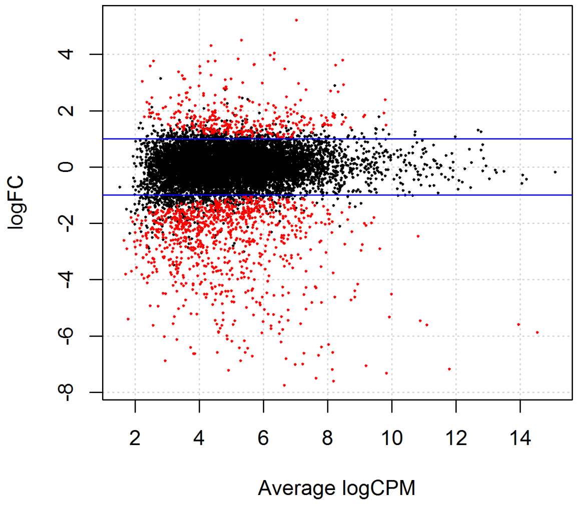
We see that all the top genes have consistent tumour vs normal changes for the three patients.

The total number of differentially expressed genes at 5% FDR is given by:

```
> summary(de <- decideTestsDGE(lrt))
   [,1]
-1  938
0  9255
1   330
```

Plot log-fold change against log-counts per million, with DE genes highlighted:

```
> detags <- rownames(y)[as.logical(de)]
> plotSmear(lrt, de.tags=detags)
> abline(h=c(-1, 1), col="blue")
```



The blue lines indicate 2-fold changes.

4.1.9 Gene ontology analysis

We perform a gene ontology analysis focusing on the ontology of biological process (BP). The genes up-regulated in the tumors tend to be associated with cell differentiation, cell migration and tissue morphogenesis:

```
> go <- goana(lrt)
> topGO(go, ont="BP", sort="Up", n=30)
                                               Term Ont    N  Up Down     P.Up   P.Down
GO:0040011                                   locomotion  BP 1142  87  163 3.13e-15 2.39e-10
GO:0006928 movement of cell or subcellular component  BP 1235  85  182 2.38e-12 8.40e-13
GO:0016477                               cell migration  BP  791  61  128 7.08e-11 7.34e-12
GO:0048870                                cell motility  BP  832  62  130 1.99e-10 5.96e-11
GO:0051674                         localization of cell  BP  832  62  130 1.99e-10 5.96e-11
GO:0030154                         cell differentiation  BP 2249 121  285 3.12e-10 9.69e-12
GO:0009888                           tissue development  BP 1130  74  179 9.87e-10 1.33e-15
GO:0030198             extracellular matrix organization  BP  278  31   52 1.23e-09 2.04e-07
GO:0043062          extracellular structure organization  BP  278  31   52 1.23e-09 2.04e-07
GO:0007155                                cell adhesion  BP  876  62  149 1.60e-09 1.26e-15
GO:0044707         single-multicellular organism process  BP 3532 165  439 1.65e-09 9.00e-19
GO:0022610                          biological adhesion  BP  880  62  150 1.91e-09 8.15e-16
GO:0048699                        generation of neurons  BP  987  66  116 4.39e-09 1.06e-03
GO:0060429                        epithelium development  BP  712  53   96 5.48e-09 2.22e-05
GO:0022008                                 neurogenesis  BP 1042  68  122 6.61e-09 9.04e-04
GO:0007275            multicellular organism development  BP 2921 141  353 9.92e-09 4.67e-12
GO:0048869               cellular developmental process  BP 2418 122  296 1.59e-08 2.06e-10
```

```
GO:0048731                       system development  BP 2624 129  334 2.35e-08 1.77e-14
GO:0032501         multicellular organismal process  BP 3756 168  487 3.54e-08 7.79e-27
GO:0048729                    tissue morphogenesis  BP  411  36   56 3.73e-08 9.23e-04
GO:0022617         extracellular matrix disassembly  BP   94  16   12 4.23e-08 1.34e-01
GO:0009887                     organ morphogenesis  BP  570  44   81 4.54e-08 1.54e-05
GO:0006775     fat-soluble vitamin metabolic process  BP   33  10    1 4.91e-08 9.55e-01
GO:0048513                 animal organ development  BP 1891 100  259 6.53e-08 1.70e-14
GO:0009653        anatomical structure morphogenesis  BP 1745  94  238 8.11e-08 5.39e-13
GO:0048856         anatomical structure development  BP 3091 143  384 1.34e-07 1.76e-15
GO:0030182                   neuron differentiation  BP  915  58  109 2.98e-07 9.44e-04
GO:0002009           morphogenesis of an epithelium  BP  339  30   39 4.42e-07 6.18e-02
GO:0009605            response to external stimulus  BP 1483  81  186 5.21e-07 3.27e-07
GO:0032502                    developmental process  BP 3453 153  405 6.15e-07 2.91e-12
```

4.1.10 Setup

This analysis was conducted on:

```
> sessionInfo()
R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] org.Hs.eg.db_3.3.0    RSQLite_1.0.0         DBI_0.3.1             AnnotationDbi_1.33.11
 [5] IRanges_2.5.46        S4Vectors_0.9.51      Biobase_2.31.3        BiocGenerics_0.17.5
 [9] edgeR_3.13.9          limma_3.27.19

loaded via a namespace (and not attached):
[1] locfit_1.5-9.1  lattice_0.20-33 GO.db_3.3.0     grid_3.3.0      splines_3.3.0   statmod_1.4.24
[7] tools_3.3.0
```

4.2 RNA-Seq of pathogen inoculated arabidopsis with batch effects

4.2.1 Introduction

This case study re-analyses Arabidopsis thaliana RNA-Seq data described by Cumbie et al. [6]. Summarized count data is available as a data object in the CRAN package `NBPSeq` comparing ΔhrcC challenged and mock-inoculated samples [6]. Samples were collected in three batches, and adjustment for batch effects proves to be important. The aim of the analysis therefore is to detect genes differentially expressed in response to ΔhrcC challenge, while correcting for any differences between the batches.

4.2.2 RNA samples

Pseudomonas syringae is a bacterium often used to study plant reactions to pathogens. In this experiment, six-week old Arabidopsis plants were inoculated with the ΔhrcC mutant of *P. syringae*, after which total RNA was extracted from leaves. Control plants were inoculated with a mock pathogen.

Three biological replicates of the experiment were conducted at separate times and using independently grown plants and bacteria.

The six RNA samples were sequenced one per lane on an Illumina Genome Analyzer. Reads were aligned and summarized per gene using GENE-counter. The reference genome was derived from the TAIR9 genome release (`www.arabidopsis.org`).

4.2.3 Loading the data

Load the data from the NBPSeq package:

```
> library(NBPSeq)
> library(edgeR)
> data(arab)
> head(arab)
          mock1 mock2 mock3 hrcc1 hrcc2 hrcc3
AT1G01010    35    77    40    46    64    60
AT1G01020    43    45    32    43    39    49
AT1G01030    16    24    26    27    35    20
AT1G01040    72    43    64    66    25    90
AT1G01050    49    78    90    67    45    60
AT1G01060     0    15     2     0    21     8
```

There are two experimental factors, treatment (hrcc vs mock) and the time that each replicate was conducted:

```
> Treat <- factor(substring(colnames(arab),1,4))
> Treat <- relevel(Treat, ref="mock")
> Time <- factor(substring(colnames(arab),5,5))
```

We then create a `DGEList` object:

```
> y <- DGEList(counts=arab, group=Treat)
```

4.2.4 Filtering and normalization

There is no purpose in analysing genes that are not expressed in either experimental condition. We consider a gene to be expressed at a reasonable level in a sample if it has at least two counts for each million mapped reads in that sample. This cutoff is ad hoc, but serves to require at least 4–6 reads in this case. Since this experiment has three replicates for each condition, a gene should be expressed in at least three samples if it responds to at least one condition. Hence we keep genes with at least two counts per million (CPM) in at least three samples:

```
> keep <- rowSums(cpm(y)>2) >= 3
> table(keep)
keep
FALSE  TRUE
 9696 16526
> y <- y[keep, , keep.lib.sizes=FALSE]
```

Note that the filtering does not use knowledge of what treatment corresponds to each sample, so the filtering does not bias the subsequent differential expression analysis.

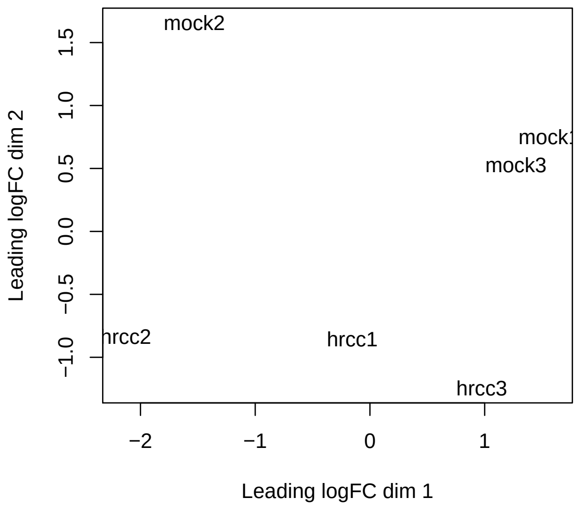
The TMM normalization is applied to account for the compositional biases:

```
> y <- calcNormFactors(y)
> y$samples
      group lib.size norm.factors
mock1  mock  1896802        0.979
mock2  mock  1898690        1.054
mock3  mock  3249396        0.903
hrcc1  hrcc  2119367        1.051
hrcc2  hrcc  1264927        1.096
hrcc3  hrcc  3516253        0.932
```

4.2.5 Data exploration

An MDS plot shows the relative similarities of the six samples.

```
> plotMDS(y)
```



Distances on an MDS plot of a DGEList object correspond to *leading log-fold-change* between each pair of samples. Leading log-fold-change is the root-mean-square average of the largest $\log_2$-fold-changes between each pair of samples. Each pair of samples extracted at each time tend to cluster together, suggesting a batch effect. The hrcc treated samples tend to be below the mock samples for each time, suggesting a treatment effect within each time. The two samples at time 1 are less consistent than at times 2 and 3.

To examine further consistency of the three replicates, we compute predictive log2-fold-changes (logFC) for the treatment separately for the three times.

```
> design <- model.matrix(~Time+Time:Treat)
> logFC <- predFC(y,design,prior.count=1,dispersion=0.05)
```

The logFC at the three times are positively correlated with one another, as we would hope:

```
> cor(logFC[,4:6])
                Time1:Treathrcc Time2:Treathrcc Time3:Treathrcc
Time1:Treathrcc           1.000           0.315           0.400
Time2:Treathrcc           0.315           1.000           0.437
Time3:Treathrcc           0.400           0.437           1.000
```

The correlation is highest between times 2 and 3.

4.2.6 The design matrix

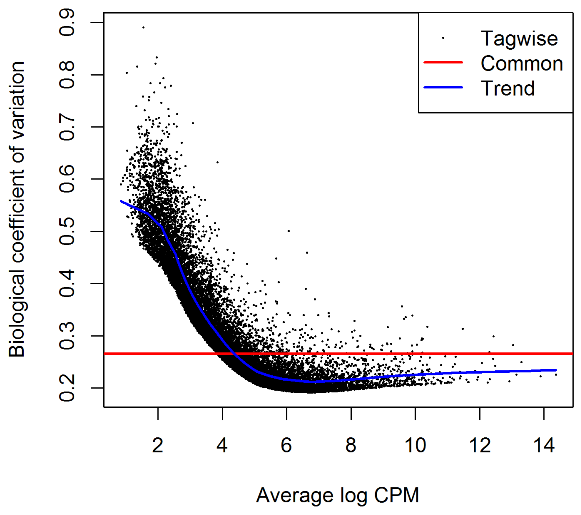
Before we fit GLMs, we need to define our design matrix based on the experimental design. We want to test for differential expressions between ΔhrcC challenged and mock-inoculated samples within batches, i.e. adjusting for differences between batches. In statistical terms, this is an additive linear model. So the design matrix is created as:

```
> design <- model.matrix(~Time+Treat)
> rownames(design) <- colnames(y)
> design
      (Intercept) Time2 Time3 Treathrcc
mock1           1     0     0         0
mock2           1     1     0         0
mock3           1     0     1         0
hrcc1           1     0     0         1
hrcc2           1     1     0         1
hrcc3           1     0     1         1
attr(,"assign")
[1] 0 1 1 2
attr(,"contrasts")
attr(,"contrasts")$Time
[1] "contr.treatment"

attr(,"contrasts")$Treat
[1] "contr.treatment"
```

4.2.7 Estimating the dispersion

Estimate the genewise dispersion estimates over all genes, allowing for a possible abundance trend. The estimation is also robustified against potential outlier genes.

```
> y <- estimateDisp(y, design, robust=TRUE)
> y$common.dispersion
[1] 0.0705
> plotBCV(y)
```

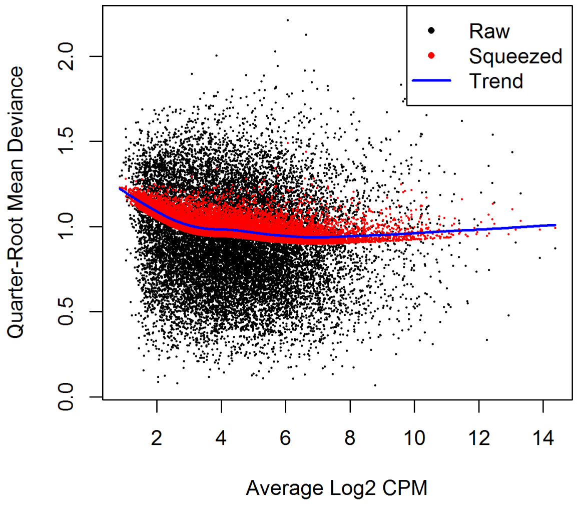


The square root of dispersion is the coefficient of biological variation (BCV). The common BCV is on the high side, considering that this is a designed experiment using genetically identical plants. The trended dispersion shows a decreasing trend with expression level. At low logCPM, the dispersions are very large indeed.

Note that only the trended dispersion is used under the quasi-likelihood (QL) pipeline. The tagwise and common estimates are shown here but will not be used further.

The QL dispersions can be estimated using the `glmQLFit` function, and then be visualized with the `plotQLDisp` function.

```
> fit <- glmQLFit(y, design, robust=TRUE)
> plotQLDisp(fit)
```



4.2.8 Differential expression

Now we test for significant differential expression in each gene using the QL F-test.

First we check whether there was a genuine need to adjust for the experimental times. We do this by testing for differential expression between the three times. There is considerable differential expression, justifying our decision to adjust for the batch effect:

```
> qlf <- glmQLFTest(fit, coef=2:3)
> topTags(qlf)
Coefficient:  Time2 Time3
          logFC.Time2 logFC.Time3 logCPM     F   PValue      FDR
AT5G66800        5.58      -1.065   5.48 150.1 8.93e-10 1.48e-05
AT5G23000        5.58      -0.292   5.71 127.5 2.48e-09 2.05e-05
```

```
AT5G31702        5.83      -2.568   5.95 113.9 5.03e-09 2.77e-05
AT2G45830        5.42      -0.589   4.71 108.4 6.84e-09 2.83e-05
AT3G33004        4.81      -1.763   5.63 102.8 9.47e-09 3.13e-05
AT2G11230        3.50      -1.532   5.60  98.7 1.22e-08 3.36e-05
AT2G07782        3.48      -1.616   5.28  93.5 1.70e-08 4.01e-05
AT2G18193        3.05      -2.396   5.08  84.8 3.09e-08 6.04e-05
AT2G23910        3.59      -0.384   5.13  83.9 3.29e-08 6.04e-05
AT5G54830        3.07      -0.367   6.07  79.7 4.51e-08 7.31e-05
> FDR <- p.adjust(qlf$table$PValue, method="BH")
> sum(FDR < 0.05)
[1] 1628
```

Now conduct QL F-tests for the pathogen effect and show the top genes. By default, the test is for the last coefficient in the design matrix, which in this case is the treatment effect:

```
> qlf <- glmQLFTest(fit)
> topTags(qlf)
Coefficient:  Treathrcc
          logFC logCPM   F   PValue      FDR
AT2G19190  4.50   7.37 304 1.83e-10 2.62e-06
AT2G39530  4.34   6.71 278 3.17e-10 2.62e-06
AT3G46280  4.78   8.10 247 6.70e-10 2.78e-06
AT2G39380  4.94   5.77 247 6.72e-10 2.78e-06
AT1G51800  3.97   7.71 232 9.92e-10 3.28e-06
AT1G51850  5.32   5.42 209 1.89e-09 4.30e-06
AT5G48430  6.32   6.73 203 2.30e-09 4.30e-06
AT2G44370  5.41   5.20 200 2.50e-09 4.30e-06
AT1G51820  4.34   6.37 198 2.64e-09 4.30e-06
AT3G55150  5.78   4.90 196 2.80e-09 4.30e-06
```

Here's a closer look at the individual counts-per-million for the top genes. The top genes are very consistent across the three replicates:

```
> top <- rownames(topTags(qlf))
> cpm(y)[top,]
           mock1 mock2 mock3 hrcc1 hrcc2 hrcc3
AT2G19190 16.696  12.0 13.29 341.3 254.7 351.1
AT2G39530  7.001   9.0 13.29 158.1 191.9 243.1
AT3G46280 18.850  17.0 18.40 384.8 374.5 820.9
AT2G39380  2.154   3.0  4.77  91.6  84.4 135.1
AT1G51800 29.083  16.5 30.66 362.4 347.8 464.0
AT1G51850  1.077   1.0  3.75  78.1  56.3 108.9
AT5G48430  4.309   4.5  0.00 189.1 314.6 125.1
AT2G44370  2.154   1.0  1.70  57.0  67.1  86.0
AT1G51820  9.694   7.5  6.13 121.2 156.6 191.3
AT3G55150  0.539   1.0  1.36  43.1  64.9  64.4
```

The total number of genes significantly up-regulated or down-regulated at 5% FDR is summarized as follows:

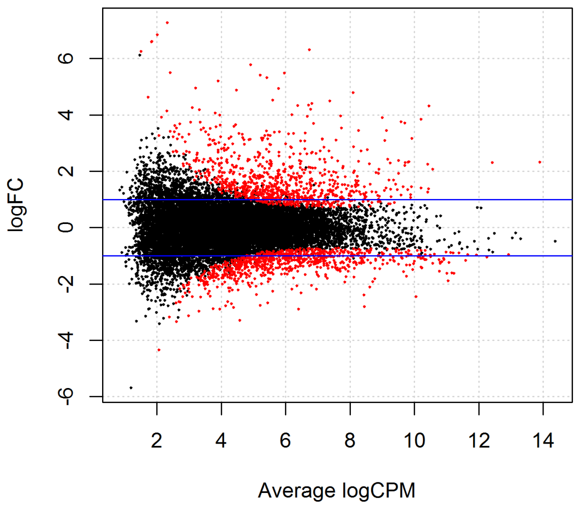
```
> summary(dt <- decideTestsDGE(qlf))
   [,1]
-1   837
0  14797
1    892
```

We can pick out which genes are DE:

```
> isDE <- as.logical(dt)
> DEnames <- rownames(y)[isDE]
```

Then we can plot all the logFCs against average count size, highlighting the DE genes:

```
> plotSmear(qlf, de.tags=DEnames)
> abline(h=c(-1,1), col="blue")
```



The blue lines indicate 2-fold up or down.

4.2.9 Setup

This analysis was conducted on:

```
> sessionInfo()
```

```
R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] NBPSeq_0.3.0          org.Hs.eg.db_3.3.0    RSQLite_1.0.0         DBI_0.3.1
 [5] AnnotationDbi_1.33.11 IRanges_2.5.46        S4Vectors_0.9.51      Biobase_2.31.3
 [9] BiocGenerics_0.17.5   edgeR_3.13.9          limma_3.27.19

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.4.5    magrittr_1.5     splines_3.3.0    munsell_0.4.3    statmod_1.4.24
 [6] colorspace_1.2-6 lattice_0.20-33  stringr_1.0.0    plyr_1.8.3       tools_3.3.0
[11] grid_3.3.0       gtable_0.2.0     reshape2_1.4.1   ggplot2_2.1.0    qvalue_2.3.2
[16] stringi_1.0-1    GO.db_3.3.0      scales_0.4.0     locfit_1.5-9.1
```

4.3 Profiles of Yoruba HapMap individuals

4.3.1 Background

RNA-Seq profiles were made of cell lines derived from lymphoblastoid cells from 69 different Yoruba individuals from Ibadan, Nigeria [22] [23]. The profiles were generated as part of the International HapMap project [10]. RNA from each individual was sequenced on at least two lanes of an Illumina Genome Analyser 2, and mapped reads to the human genome using MAQ v0.6.8.

The study group here is essentially an opportunity sample and the individuals are likely to be genetically diverse. In this analysis we look at genes that are differentially expressed between males and female.

4.3.2 Loading the data

Read counts summarized by Ensembl gene identifiers are available in the tweeDEseqCountData package:

```
> library(tweeDEseqCountData)
> data(pickrell1)
> Counts <- exprs(pickrell1.eset)
> dim(Counts)
```

```
[1] 38415    69
```

```
> Counts[1:5,1:5]
```

```
                NA18486 NA18498 NA18499 NA18501 NA18502
ENSG00000127720       6      32      14      35      14
ENSG00000242018      20      21      24      22      16
ENSG00000224440       0       0       0       0       0
ENSG00000214453       0       0       0       0       0
ENSG00000237787       0       0       1       0       0
```

In this analysis we will compare female with male individuals.

```
> Gender <- pickrell1.eset$gender
> table(Gender)
```

```
Gender
female   male
    40     29
```

```
> rm(pickrell1.eset)
```

Annotation for each Ensemble gene is also available from the tweeDEseqCountData package:

```
> data(annotEnsembl63)
> annot <- annotEnsembl63[,c("Symbol","Chr")]
> annot[1:5,]
```

```
                Symbol Chr
ENSG00000252775     U7   5
ENSG00000207459     U6   5
ENSG00000252899     U7   5
ENSG00000201298     U6   5
ENSG00000222266     U6   5
```

```
> rm(annotEnsembl63)
```

Form a DGEList object combining the counts and associated annotation:

```
> library(edgeR)
> y <- DGEList(counts=Counts, genes=annot[rownames(Counts),])
```

4.3.3 Filtering and normalization

Keep genes with least 1 count-per-million reads (cpm) in at least 20 samples:

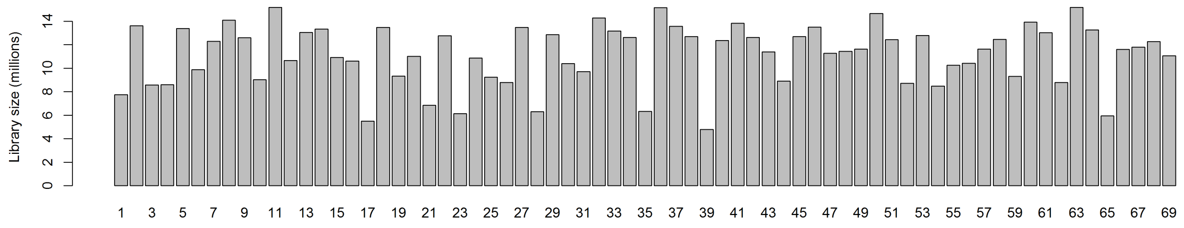
```
> isexpr <- rowSums(cpm(y)>1) >= 20
```

Keep only genes with defined annotation, and recompute library sizes:

```
> hasannot <- rowSums(is.na(y$genes))==0
> y <- y[isexpr & hasannot, , keep.lib.sizes=FALSE]
> dim(y)

[1] 17310    69
```

The library sizes vary from about 5 million to over 15 million:

```
> barplot(y$samples$lib.size*1e-6, names=1:69, ylab="Library size (millions)")
```



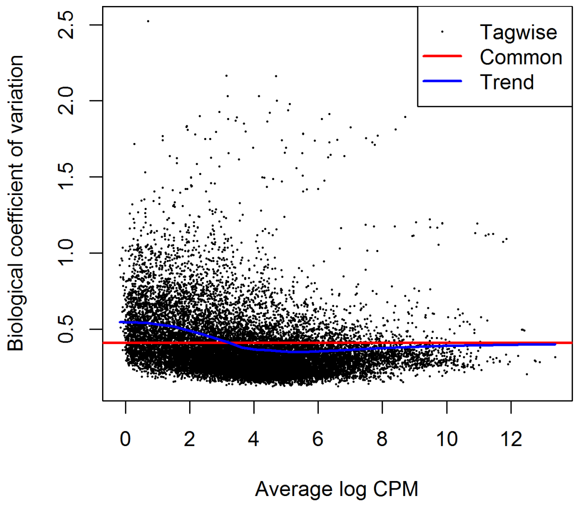
Apply TMM normalization to account for the composition biases:

```
> y <- calcNormFactors(y)
> head(y$samples)

        group lib.size norm.factors
NA18486     1  7749527        0.939
NA18498     1 13612983        1.110
NA18499     1  8569631        0.963
NA18501     1  8595024        1.201
NA18502     1 13375275        0.938
NA18504     1  9881732        0.979
```

4.3.4 Estimating the dispersion

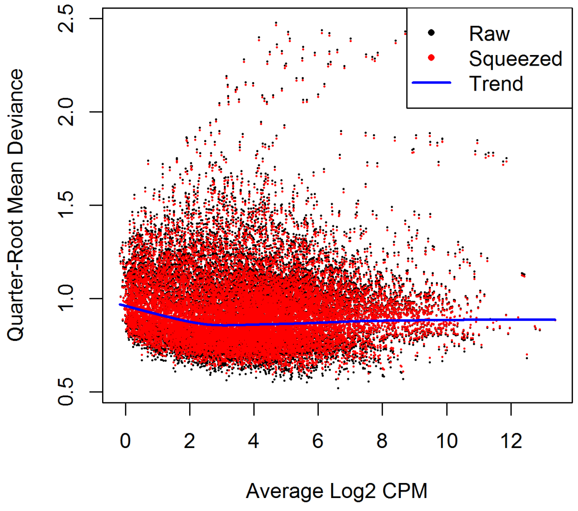
We are interested in the differences between male and female. Hence, we create a design matrix using the gender factor. We estimate the NB dispersion using `estimateDisp`. The estimation is robustified against potential outlier genes.

```
> design <- model.matrix(~Gender)
> y <- estimateDisp(y, design, robust=TRUE)
> plotBCV(y)
```



We then estimate the QL dispersions around the dispersion trend using `glmQLFit`. The large number of cases and the high variability means that the QL dispersions are not squeezed very heavily from the raw values:

```
> fit <- glmQLFit(y, design, robust=TRUE)
> plotQLDisp(fit)
```



4.3.5 Differential expression

Now find genes differentially expressed between male and females. Positive log-fold-changes mean higher expression in males. The highly ranked genes are mostly on the X or Y chromosomes. Top ranked is the famous XIST gene, which is known to be expressed only in females.

```
> qlf <- glmQLFTest(fit)
> topTags(qlf,n=15)
```

```
Coefficient:  Gendermale 
                      Symbol Chr logFC logCPM    F   PValue      FDR
ENSG00000229807         XIST   X -9.49  7.249 1213 1.03e-46 1.78e-42
ENSG00000099749     CYorf15A   Y  4.28  1.757  857 1.19e-41 1.03e-37
ENSG00000131002     CYorf15B   Y  5.63  2.055  587 2.66e-36 1.31e-32
ENSG00000157828       RPS4Y2   Y  3.18  4.207  585 3.02e-36 1.31e-32
ENSG00000233864       TTTY15   Y  4.84  1.254  538 4.42e-35 1.53e-31
ENSG00000198692       EIF1AY   Y  2.36  3.247  376 3.04e-30 8.78e-27
ENSG00000165246       NLGN4Y   Y  5.09  1.676  303 1.70e-27 4.21e-24
ENSG00000183878          UTY   Y  1.86  3.137  253 3.24e-25 7.01e-22
ENSG00000243209   AC010889.1   Y  2.66  0.797  232 3.63e-24 6.29e-21
ENSG00000129824       RPS4Y1   Y  2.53  5.401  232 3.63e-24 6.29e-21
ENSG00000012817        KDM5D   Y  1.47  4.950  222 1.16e-23 1.82e-20
ENSG00000213318  RP11-331F4.1  16  3.67  3.688  217 2.87e-23 4.13e-20
ENSG00000067048        DDX3Y   Y  1.62  5.621  181 2.48e-21 3.30e-18
ENSG00000146938       NLGN4X   X  3.94  1.048  139 1.81e-18 2.24e-15
ENSG00000232928 RP13-204A15.4   X  1.44  3.558  111 3.19e-16 3.68e-13
```

```
> summary(decideTestsDGE(qlf))

   [,1]
-1    46
0  17243
1     21
```

4.3.6 Gene set testing

The tweeDEseqCountData package includes a list of genes belonging to the male-specific region of chromosome Y, and a list of genes located in the X chromosome that have been reported to escape X-inactivation. We expect genes in the first list to be up-regulated in males, whereas genes in the second list should be up-regulated in females.

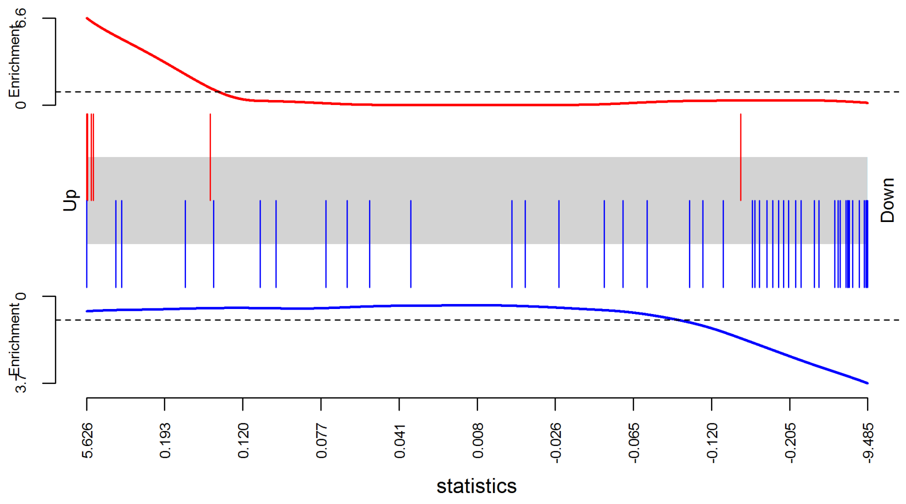
```
> data(genderGenes)
> Ymale <- rownames(y) %in% msYgenes
> Xescape <- rownames(y) %in% XiEgenes
```

Roast gene set tests by `fry()` confirm that the male-specific genes are significantly up as a group in our comparison of males with females, whereas the X genes are significantly down as a group [33].

```
> index <- list(Y=Ymale, X=Xescape)
> fry(y, index=index, design=design)

  NGenes Direction   PValue      FDR PValue.Mixed FDR.Mixed
Y     12        Up 7.04e-46 1.41e-45     5.86e-11  5.86e-11
X     46      Down 7.25e-17 7.25e-17     2.28e-66  4.55e-66
```

A barcode plot can be produced to visualize the results. Genes are ranked from left to right by decreasing log-fold-change in the background of the barcode plot. Genes in the set of `msYgenes` are represented by red bars whereas genes in the set of `XiEgenes` are represented by blue bars. The line above the barcode shows the relative local enrichment of the vertical bars in each part of the plot. This particular plot suggests that the male-specific genes tend to have large positive log-fold-changes, whereas the X genes tend to have large negative log-fold-changes.

```
> barcodeplot(qlf$table$logFC, index[[1]], index[[2]])
```

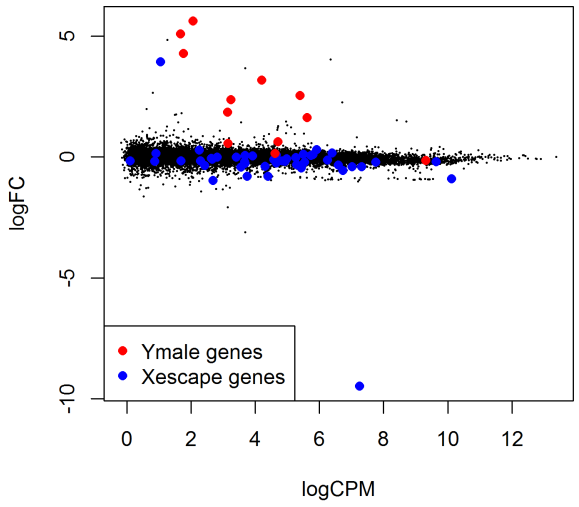


The results from competitive camera gene sets tests are even more convincing [34]. The positive intergene correlations here show that the genes in each set tend to be biologically correlated:

```
> camera(y, index, design)

  NGenes Correlation Direction   PValue      FDR
Y     12      0.0846        Up 4.18e-39 8.36e-39
X     46      0.0239      Down 1.34e-12 1.34e-12
```

See where the X and Y genes fall on the MA plot:

```
> with(qlf$table, plot(logCPM,logFC,pch=16,cex=0.2))
> with(qlf$table, points(logCPM[Xescape],logFC[Xescape],pch=16,col="blue"))
> with(qlf$table, points(logCPM[Ymale],logFC[Ymale],pch=16,col="red"))
> legend("bottomleft",legend=c("Ymale genes","Xescape genes"),pch=16,,col=c("red","blue"))
```



4.3.7 Setup

This analysis was conducted on:

```
> sessionInfo()

R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] tweeDEseqCountData_1.9.0 NBPSeq_0.3.0             org.Hs.eg.db_3.3.0
 [4] RSQLite_1.0.0            DBI_0.3.1                AnnotationDbi_1.33.11
 [7] IRanges_2.5.46           S4Vectors_0.9.51         Biobase_2.31.3
[10] BiocGenerics_0.17.5      edgeR_3.13.9             limma_3.27.19

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.4.5    magrittr_1.5     splines_3.3.0    munsell_0.4.3    statmod_1.4.24
 [6] colorspace_1.2-6 lattice_0.20-33  stringr_1.0.0    plyr_1.8.3       tools_3.3.0
```

```
[11] grid_3.3.0       gtable_0.2.0     reshape2_1.4.1   ggplot2_2.1.0    qvalue_2.3.2
[16] stringi_1.0-1    GO.db_3.3.0      scales_0.4.0     locfit_1.5-9.1
```

4.4 RNA-Seq profiles of mouse mammary gland

4.4.1 Introduction

The RNA-Seq data of this case study is described in Fu *et al.* [8]. The sequence and count data are publicly available from the Gene Expression Omnibus (GEO) at the series accession number GSE60450. This study examines the expression profiles of basal stem-cell enriched cells (B) and committed luminal cells (L) in the mammary gland of virgin, pregnant and lactating mice. Six groups are present, with one for each combination of cell type and mouse status. Each group contains two biological replicates. This is summarized in the table below, where the basal and luminal cell types are abbreviated with `B` and `L` respectively.

```
> targets <- read.delim("targets.txt", header=TRUE)
> targets

          FileName GEOAccession CellType   Status
1  SRR1552450.fastq   GSM1480297        B   virgin
2  SRR1552451.fastq   GSM1480298        B   virgin
3  SRR1552452.fastq   GSM1480299        B pregnant
4  SRR1552453.fastq   GSM1480300        B pregnant
5  SRR1552454.fastq   GSM1480301        B  lactate
6  SRR1552455.fastq   GSM1480302        B  lactate
7  SRR1552444.fastq   GSM1480291        L   virgin
8  SRR1552445.fastq   GSM1480292        L   virgin
9  SRR1552446.fastq   GSM1480293        L pregnant
10 SRR1552447.fastq   GSM1480294        L pregnant
11 SRR1552448.fastq   GSM1480295        L  lactate
12 SRR1552449.fastq   GSM1480296        L  lactate
```

The name of the file containing the read sequences for each library is also shown. Each file is downloaded from the Sequence Read Archive and has an accession number starting with SRR, e.g., SRR1552450 for the first library in `targets`.

4.4.2 Read alignment and processing

Prior to read alignment, these files are converted into the FASTQ format using the `fastq-dump` utility from the SRA Toolkit. See `http://www.ncbi.nlm.nih.gov/books/NBK158900` for how to download and use the SRA Toolkit.

Before the differential expression analysis can proceed, these reads must be aligned to the mouse genome and counted into annotated genes. This can be achieved with functions in the Rsubread package [11]. We assume that an index of the mouse genome is already available - if not, this can be constructed from a FASTA file of the genome sequence with the `buildindex` command. In this example, we assume that the prefix for the index files is `mm10`. The reads in each FASTQ file are then aligned to the mouse genome, as shown below.

```
> library(Rsubread)
> output.files <- sub(".fastq", ".bam", targets$FileName)
> align("mm10", readfile1=targets$FileName, phredOffset=33,
+     input_format="FASTQ", output_file=output.files)
```

This produces a set of BAM files, where each file contains the read alignments for each library. The mapped reads can be counted into mouse genes by using the `featureCounts` function. It uses the exon intervals defined in the NCBI annotation of the mm10 genome.

```
> fc <- featureCounts(output.files, annot.inbuilt="mm10")
> colnames(fc$counts) <- 1:12
> head(fc$counts)

            1   2   3   4   5   6   7   8   9  10  11  12
497097    438 300  65 237 354 287   0   0   0   0   0   0
100503874   1   0   1   1   0   4   0   0   0   0   0   0
100038431   0   0   0   0   0   0   0   0   0   0   0   0
19888       1   1   0   0   0   0  10   3  10   2   0   0
20671     106 182  82 105  43  82  16  25  18   8   3  10
27395     309 234 337 300 290 270 560 464 489 328 307 342
```

The row names of the matrix represent the Entrez gene identifiers for each gene. In the output from `featureCounts`, the column names of `fc$counts` are the output file names from `align`. Here, we simplify them for brevity.

4.4.3 Count loading and annotation

We create a `DGEList` object as follows

```
> group <- factor(paste0(targets$CellType, ".", targets$Status))
> y <- DGEList(fc$counts, group=group)
> colnames(y) <- targets$GEO
```

Human-readable gene symbols can also be added to complement the Entrez identifiers for each gene, using the annotation in the `org.Mm.eg.db` package.

```
> require(org.Mm.eg.db)
> y$genes <- select(org.Mm.eg.db,keys=rownames(y),columns="SYMBOL")
> head(y$genes)

   ENTREZID  SYMBOL
1    497097    Xkr4
2 100503874 Gm19938
3 100038431 Gm10568
4     19888     Rp1
5     20671   Sox17
6     27395  Mrpl15
```

4.4.4 Filtering and normalization

Here, a gene is only retained if it is expressed at a count-per-million (CPM) above 0.5 in at least two samples.

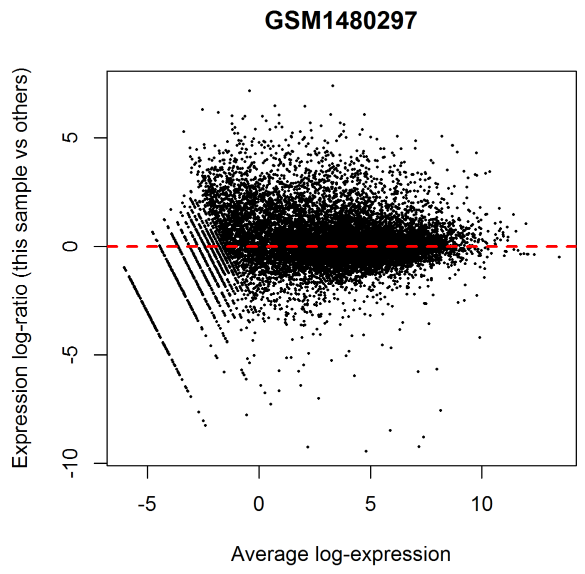
```
> keep <- rowSums(cpm(y) > 0.5) >= 2
> summary(keep)

   Mode   FALSE    TRUE    NA's
logical   11375   15804       0

> y <- y[keep, , keep.lib.sizes=FALSE]
```

TMM normalization is performed to eliminate composition biases between libraries.

```
> y <- calcNormFactors(y)
> y$samples

                group lib.size norm.factors
GSM1480297   B.virgin 23218026        1.237
GSM1480298   B.virgin 21768136        1.214
GSM1480299 B.pregnant 24091588        1.126
GSM1480300 B.pregnant 22656713        1.070
GSM1480301  B.lactate 21522033        1.036
GSM1480302  B.lactate 20008326        1.087
GSM1480291   L.virgin 20384562        1.368
GSM1480292   L.virgin 21698793        1.365
GSM1480293 L.pregnant 22235847        1.005
GSM1480294 L.pregnant 21982745        0.923
GSM1480295  L.lactate 24719697        0.529
GSM1480296  L.lactate 24652963        0.535
```

The performance of the TMM normalization procedure can be examined using mean-difference (MD) plots. This visualizes the library size-adjusted log-fold change between two libraries (the difference) against the average log-expression across those libraries (the mean). The following MD plot is generated by comparing sample 1 against an artificial library constructed from the average of all other samples.

```
> plotMD(cpm(y, log=TRUE), column=1)
> abline(h=0, col="red", lty=2, lwd=2)
```

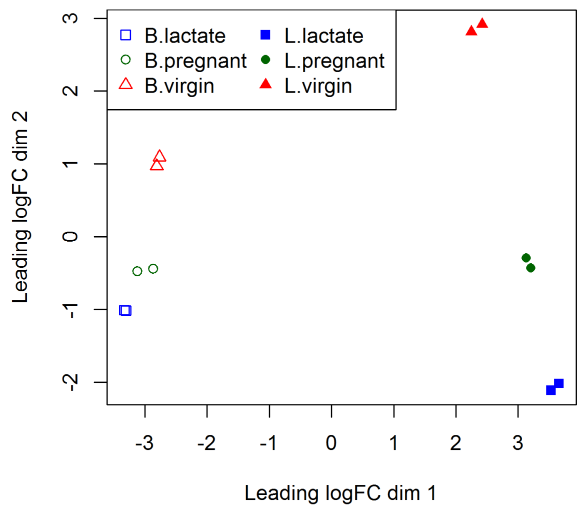


Ideally, the bulk of genes should be centred at a log-fold change of zero. This indicates that any composition bias between libraries has been successfully removed. This quality check should be repeated by constructing a MD plot for each sample.

4.4.5 Data exploration

The data can be explored by generating multi-dimensional scaling (MDS) plots. This visualizes the differences between the expression profiles of different samples in two dimensions.

```
> points <- c(0,1,2,15,16,17)
> colors <- rep(c("blue", "darkgreen", "red"), 2)
> plotMDS(y, col=colors[group], pch=points[group])
> legend("topleft", legend=levels(group), pch=points, col=colors, ncol=2)
```



Replicate samples from the same group cluster together in the plot, while samples from different groups form separate clusters. This indicates that the differences between groups are larger than those within groups, i.e., differential expression is greater than the variance and can be detected. The distance between basal samples on the left and luminal cells on the right is about 6 units, corresponding to a leading fold change of about 64-fold ($2^6 = 64$) between basal and luminal. The expression differences between virgin, pregnant and lactating are greater for luminal cells than for basal.

4.4.6 The design matrix

The experimental design for this study can be parametrized with a one-way layout, whereby one coefficient is assigned to each group. The design matrix contains the predictors for each sample and and is constructed using the code below.

```
> design <- model.matrix(~ 0 + group)
> colnames(design) <- levels(group)
> design
```

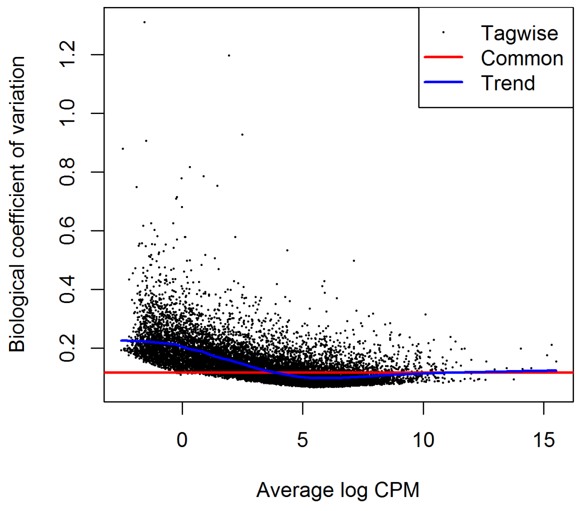
```
  B.lactate B.pregnant B.virgin L.lactate L.pregnant L.virgin
1         0          0        1         0          0        0
2         0          0        1         0          0        0
3         0          1        0         0          0        0
4         0          1        0         0          0        0
5         1          0        0         0          0        0
6         1          0        0         0          0        0
7         0          0        0         0          0        1
```

```
8           0           0         0          0           0         1
9           0           0         0          0           1         0
10          0           0         0          0           1         0
11          0           0         0          1           0         0
12          0           0         0          1           0         0
attr(,"assign")
[1] 1 1 1 1 1 1
attr(,"contrasts")
attr(,"contrasts")$group
[1] "contr.treatment"
```

4.4.7 Estimating the dispersion

The NB dispersion is estimated using the `estimateDisp` function. This returns the `DGEList` object with additional entries for the estimated NB dispersions for all gene. These estimates can be visualized with `plotBCV`, which shows the root-estimate, i.e., the biological coefficient of variation for each gene.

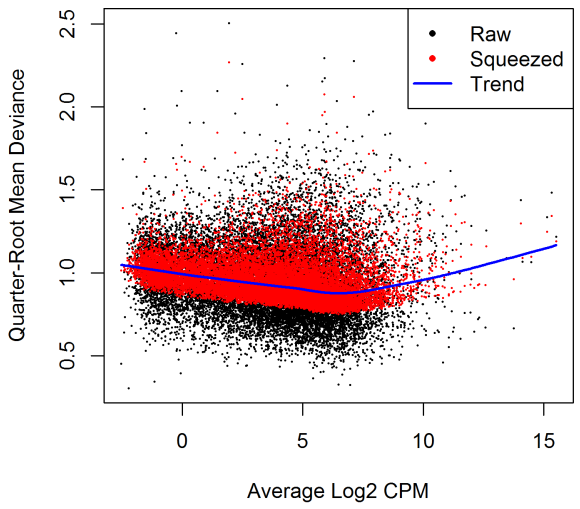
```
> y <- estimateDisp(y, design, robust=TRUE)
> y$common.dispersion

[1] 0.0134

> plotBCV(y)
```



Note that only the trended dispersion is used under the quasi-likelihood (QL) pipeline. The tagwise and common estimates are shown here but will not be used further.

For the QL dispersions, estimation can be performed using the `glmQLFit` function. This returns a `DGEGLM` object containing the estimated values of the GLM coefficients for each gene, as well as the fitted mean-QL dispersion trend, the squeezed QL estimates and the prior degrees of freedom (df). These can be visualized with the `plotQLDisp` function.

```
> fit <- glmQLFit(y, design, robust=TRUE)
> head(fit$coefficients)

       B.lactate B.pregnant B.virgin L.lactate L.pregnant L.virgin
497097    -11.14     -12.02   -11.23     -19.0     -19.03    -19.0
20671     -12.77     -12.51   -12.15     -14.5     -14.31    -14.1
27395     -11.27     -11.30   -11.53     -10.6     -10.87    -10.9
18777     -10.15     -10.21   -10.77     -10.1     -10.39    -10.4
21399      -9.89      -9.74    -9.79     -10.2      -9.97    -10.0
58175     -16.16     -14.85   -15.99     -13.3     -12.29    -12.1

> plotQLDisp(fit)
```



Setting `robust=TRUE` in `glmQLFit` is strongly recommended [21]. Setting `robust=TRUE` in `estimateDisp` has no effect on the downstream analysis, but is nevertheless very useful as it identifies genes that are outliers from the mean-NB dispersion trend.

4.4.8 Differential expression

We test for significant differential expression in each gene, using the QL F-test. The contrast of interest can be specified using the `makeContrasts` function. Here, genes are tested for DE between the basal pregnant and lactating groups. This is done by defining the null hypothesis as `B.pregnant - B.lactate = 0`.

```
> con <- makeContrasts(B.pregnant - B.lactate, levels=design)
> qlf <- glmQLFTest(fit, contrast=con)
```

The top set of most significant genes can be examined with `topTags`. Here, a positive log-fold change represents genes that are up in `B.pregnant` over `B.lactate`. Multiplicity correction is performed by applying the Benjamini-Hochberg method on the *p*-values, to control the false discovery rate (FDR).

```
> topTags(qlf)

Coefficient:  -1*B.lactate 1*B.pregnant
      ENTREZID  SYMBOL logFC logCPM   F   PValue      FDR
18071    12992 Csn1s2b -6.09  10.18 421 4.86e-11 7.67e-07
22881   211577  Mrgprf -5.15   2.74 345 1.30e-10 8.05e-07
12177   226101    Myof -2.32   6.44 322 1.97e-10 8.05e-07
851     381290  Atp2b4 -2.14   6.14 320 2.04e-10 8.05e-07
9279    140474    Muc4  7.17   6.05 308 2.64e-10 8.34e-07
18829   231830 Micall2  2.25   5.18 282 4.47e-10 1.18e-06
2491     24117    Wif1  1.82   6.76 260 7.30e-10 1.65e-06
18684    12740   Cldn4  5.32   9.87 298 8.88e-10 1.71e-06
22829    21953   Tnni2 -5.75   3.86 313 9.76e-10 1.71e-06
19483   231991   Creb5 -2.57   4.87 241 1.16e-09 1.83e-06
```

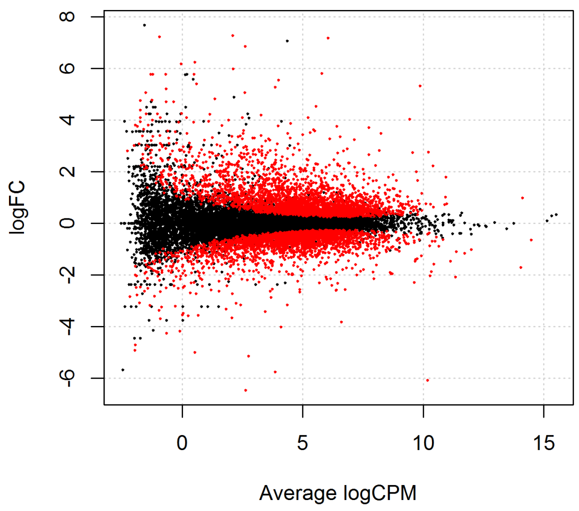
The top gene Csn1s2b has a large negative log2-fold-change, showing that it is far more highly expressed in lactating than pregnant mice. This gene is known to be a major source of protein in milk.

The total number of DE genes in each direction at a FDR of 5% can be examined with `decideTestsDGE`. There are in fact nearly 4500 DE genes an FDR cut-off of 5% in this comparison:

```
> is.de <- decideTestsDGE(qlf, p.value=0.05)
> summary(is.de)

   [,1]
-1  2505
0  10529
1   2770
```

The differential expression test results can be visualized using a smear plot. The log-fold change for each gene is plotted against the average abundance, i.e., `logCPM` in the result table above. Significantly DE genes at a FDR of 5% are highlighted in red.

```
> plotSmear(qlf, de.tags=rownames(qlf)[is.de!=0])
```



We use `glmTreat` to narrow down the list of DE genes and focus on genes that are more biologically meaningful. We test whether the differential expression is significantly above a log2-fold-change of $\log_2 1.2$, i.e., a fold-change of 1.2.

```
> tr <- glmTreat(fit, contrast=con, lfc=log2(1.2))
> topTags(tr)

Coefficient:  -1*B.lactate 1*B.pregnant
      ENTREZID  SYMBOL logFC unshrunk.logFC logCPM   PValue      FDR
18071    12992 Csn1s2b -6.09          -6.09  10.18 5.08e-11 8.02e-07
22881   211577  Mrgprf -5.15          -5.15   2.74 1.38e-10 9.13e-07
12177   226101    Myof -2.32          -2.32   6.44 2.66e-10 9.13e-07
851     381290  Atp2b4 -2.14          -2.14   6.14 2.89e-10 9.13e-07
9279    140474    Muc4  7.17           7.34   6.05 2.89e-10 9.13e-07
18829   231830 Micall2  2.25           2.25   5.18 6.15e-10 1.62e-06
18684    12740   Cldn4  5.32           5.32   9.87 9.30e-10 2.00e-06
22829    21953   Tnni2 -5.75          -5.76   3.86 1.01e-09 2.00e-06
2491     24117    Wif1  1.82           1.82   6.76 1.18e-09 2.08e-06
19483   231991   Creb5 -2.57          -2.57   4.87 1.47e-09 2.32e-06
```

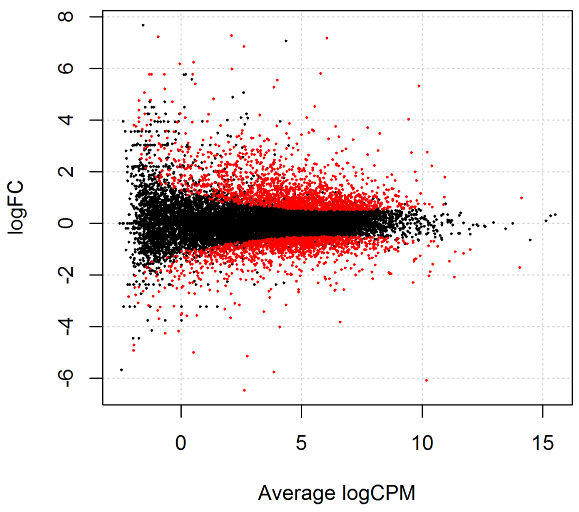
Around 3000 genes are detected as DE with fold-change significantly above 1.2 at an FDR cut-off of 5%.

```
> is.de <- decideTestsDGE(tr, p.value=0.05)
> summary(is.de)
```

```
   [,1]
-1  1291
0  12827
1   1686
```

The test results are visualized in the following smear plot. Genes that are significantly DE above a fold-change of 1.2 at an FDR of 5% are highlighted in red.

```
> plotSmear(tr, de.tags=rownames(tr)[is.de!=0])
```



4.4.9 ANOVA-like testing

The differential expression analysis of two-group comparison can be easily extended to comparisons between three or more groups. This is done by creating a matrix of contrasts, where which each column represents a contrast between two groups of interest. In this manner, users can perform a one-way analysis of variance (ANOVA) for each gene.

As an example, suppose we want to compare the three groups in the luminal population, i.e., virgin, pregnant and lactating. An appropriate contrast matrix can be created as shown below, to make pairwise comparisons between all three groups.

```
> con <- makeContrasts(
+      L.PvsL = L.pregnant - L.lactate,
+      L.VvsL = L.virgin - L.lactate,
+      L.VvsP = L.virgin - L.pregnant, levels=design)
```

The QL F-test is then applied to identify genes that are DE among the three groups. This combines the three pairwise comparisons into a single F-statistic and *p*-value. The top set of significant genes can be displayed with `topTags`.

```
> anov <- glmQLFTest(fit, contrast=con)
> topTags(anov)

Coefficient:  LR test of 2 contrasts
      ENTREZID  SYMBOL logFC.L.PvsL logFC.L.VvsL logCPM    F   PValue      FDR
19230    19242     Ptn        -1.54         7.26   7.97 2387 3.77e-17 5.96e-13
15679    13645     Egf        -5.36        -7.22   3.67 1126 4.46e-15 3.22e-11
21207    52150   Kcnk6        -2.42        -7.00   5.91 1020 8.40e-15 3.22e-11
23907    15439      Hp         1.08         5.42   4.93  992 1.00e-14 3.22e-11
18071    12992 Csn1s2b        -8.55       -11.36  10.18 1050 1.03e-14 3.22e-11
22626    14183   Fgfr2        -1.15         3.95   7.38  953 1.29e-14 3.22e-11
2901     20856    Stc2        -1.81         3.19   6.10  917 1.64e-14 3.22e-11
20062    11941  Atp2b2        -7.37       -10.56   6.60 1133 1.79e-14 3.22e-11
9083     13358 Slc25a1        -4.13        -4.91   7.49  888 2.01e-14 3.22e-11
8278     17068    Ly6d         3.42         9.24   4.68  886 2.04e-14 3.22e-11
```

Note that the three contrasts of pairwise comparisons are linearly dependent. Constructing the contrast matrix with any two of the contrasts would be sufficient to specify an ANOVA test. For instance, the contrast matrix shown below produces the same test results but with a different column of log-fold changes.

```
> con <- makeContrasts(
+      L.PvsL = L.pregnant - L.lactate,
+      L.VvsP = L.virgin - L.pregnant, levels=design)
```

If all three contrasts are present in the contrast matrix, then only the log-fold changes of the first two contrasts are shown in the output of `topTags`.

4.4.10 Gene ontology analysis

Further analyses are required to interpret the differential expression results in a biological context. One common downstream procedure is a gene ontology (GO) enrichment analysis.

Suppose we want to identify GO terms that are over-represented in the basal lactating group compared to the basal pregnancy group. This can be achieved by applying the `goana` function to the differential expression results of that comparison. The top set of most enriched GO terms can be viewed with the `topGO` function.

```
> con <- makeContrasts(B.lactate - B.pregnant, levels=design)
> qlf <- glmQLFTest(fit, contrast=con)
> go <- goana(qlf, species = "Mm")
> topGO(go, n=30)
```

```
                                              Term Ont    N  Up Down  P.Up   P.Down
GO:0044822                      poly(A) RNA binding  MF 1092 107  394 1.000 3.44e-53
GO:0003723                              RNA binding  MF 1429 155  469 1.000 4.29e-50
GO:0042254                      ribosome biogenesis  BP  223   7  127 1.000 1.13e-40
GO:0022613     ribonucleoprotein complex biogenesis  BP  336  24  161 1.000 1.58e-38
GO:0022626                       cytosolic ribosome  CC   97   1   75 1.000 4.19e-38
GO:0030529 intracellular ribonucleoprotein complex  CC  646  43  246 1.000 3.87e-37
GO:1990904                ribonucleoprotein complex  CC  646  43  246 1.000 3.87e-37
GO:0005730                                nucleolus  CC  663  79  239 1.000 9.61e-32
GO:0003676                    nucleic acid binding  MF 2834 423  712 0.998 1.42e-30
GO:0006364                          rRNA processing  BP  146   3   85 1.000 1.57e-28
GO:0016072                  rRNA metabolic process  BP  149   5   85 1.000 1.17e-27
GO:0031974                 membrane-enclosed lumen  CC 2850 409  698 1.000 2.62e-26
GO:0070013           intracellular organelle lumen  CC 2793 401  684 1.000 1.11e-25
GO:0005840                                 ribosome  CC  203   4  100 1.000 1.71e-25
GO:0043233                          organelle lumen  CC 2797 402  684 1.000 1.72e-25
GO:0044391                        ribosomal subunit  CC  157   1   84 1.000 9.64e-25
GO:0022625        cytosolic large ribosomal subunit  CC   52   0   43 1.000 1.49e-24
GO:0031981                            nuclear lumen  CC 2558 384  631 0.996 2.88e-24
GO:1901363            heterocyclic compound binding  MF 4346 668  975 0.998 8.95e-24
GO:0044445                           cytosolic part  CC  189  17   93 0.999 9.45e-24
GO:0032991                    macromolecular complex  CC 3900 584  890 1.000 1.26e-23
GO:0097159          organic cyclic compound binding  MF 4393 676  980 0.998 5.15e-23
GO:0044446             intracellular organelle part  CC 5362 874 1158 0.871 7.88e-23
GO:0044428                             nuclear part  CC 2955 452  700 0.992 4.74e-22
GO:0003735       structural constituent of ribosome  MF  190   8   90 1.000 1.43e-21
GO:0044422                           organelle part  CC 5508 909 1176 0.740 3.05e-21
GO:0006396                           RNA processing  BP  672  57  215 1.000 5.97e-21
GO:0005634                                  nucleus  CC 5357 881 1145 0.780 1.35e-20
GO:0034660                   ncRNA metabolic process  BP  353  24  133 1.000 6.45e-20
GO:0034470                         ncRNA processing  BP  269  14  110 1.000 8.47e-20
```

The row names of the output are the universal identifiers of the GO terms, with one term per row. The `Term` column gives the names of the GO terms. These terms cover three domains - biological process (BP), cellular component (CC) and molecular function (MF), as shown in the `Ont` column. The `N` column represents the total number of genes that are annotated with each GO term. The `Up` and `Down` columns represent the number of genes with the GO term that are significantly up- and down-regulated in this differential expression comparison, respectively. The `P.Up` and `P.Down` columns contain the *p*-values for over-representation of the GO term across the set of up- and down-regulated genes, respectively. The output table is sorted by the minimum of `P.Up` and `P.Down` by default.

The `goana` function uses the NCBI RefSeq annotation. Therefore, the Entrez Gene identifier (ID) should be supplied for each gene as the row names of `qlf`.

4.4.11 Gene set testing

Another downstream step uses the rotation gene set test (ROAST) [33]. Given a set of genes, we can test whether the majority of the genes in the set are DE across the contrast of interest. It is useful when the specified set contains all genes involved in some pathway or process.

In our case study, suppose we are interested in three GO terms related to cytokinesis. Each term will be used to define a set containing all genes that are annotated with that term. The names of these terms can be viewed as shown below.

```
> library(GO.db)
> cyt.go <- c("GO:0032465", "GO:0000281", "GO:0000920")
> term <- select(GO.db, keys=cyt.go, columns="TERM")
> term

        GOID                            TERM
1 GO:0032465         regulation of cytokinesis
2 GO:0000281                 mitotic cytokinesis
3 GO:0000920 cell separation after cytokinesis
```

We construct a list of three components, each of which is a vector of Entrez Gene IDs for all genes annotated with one of the GO terms. We then convert the Gene IDs into row indices of the `fit` object using the function `ids2indices`.

```
> Rkeys(org.Mm.egGO2ALLEGS) <- cyt.go
> ind <- ids2indices(as.list(org.Mm.egGO2ALLEGS), fit$genes$ENTREZID)
```

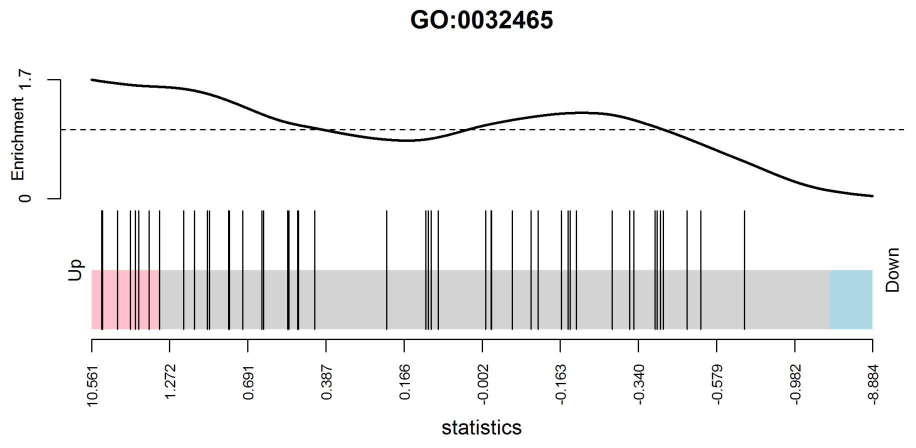
We proceed to run ROAST on the defined gene sets for the contrast of interest. Suppose the comparison of interest is between the virgin and lactating groups in the basal population. We use `fry` to test for multiple gene sets.

```
> con <- makeContrasts(B.virgin-B.lactate, levels=design)
> fr <- fry(y, index=ind, design=design, contrast=con)
> fr

           NGenes Direction   PValue     FDR PValue.Mixed FDR.Mixed
GO:0032465     48        Up 0.000471 0.00141     3.72e-06  6.96e-06
GO:0000920     16      Down 0.001839 0.00276     4.64e-06  6.96e-06
GO:0000281     30        Up 0.007394 0.00739     2.50e-05  2.50e-05
```

Each row corresponds to a single gene set, i.e., GO term. The `NGenes` column gives the number of genes in each set. The net direction of change is determined from the significance of changes in each direction, and is shown in the `Direction` column. The `PValue` provides evidence for whether the majority of genes in the set are DE in the specified direction, whereas the `PValue.Mixed` tests for differential expression in any direction. FDRs are computed from the corresponding p-values across all sets.

A barcode plot can be produced with the `barcodeplot` function to visualize the results for any particular set. In this case, visualization is performed for the gene set defined by GO:0032465. Here, genes are represented by bars and are ranked from left to right by decreasing log-fold change. This forms the barcode-like pattern. The line above the barcode shows the relative local enrichment of the vertical bars in each part of the plot. This particular plot suggests that most genes in this set are up-regulated in the virgin group compared to the lactating group.

```
> res <- glmQLFTest(fit, contrast=con)
> barcodeplot(res$table$logFC, ind[[1]], main=names(ind)[1])
```



4.4.12 Setup

This analysis was conducted on:

```
> sessionInfo()

R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
```

```
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] GO.db_3.3.0             org.Mm.eg.db_3.3.0      tweeDEseqCountData_1.9.0
 [4] NBPSeq_0.3.0            org.Hs.eg.db_3.3.0      RSQLite_1.0.0
 [7] DBI_0.3.1               AnnotationDbi_1.33.11   IRanges_2.5.46
[10] S4Vectors_0.9.51        Biobase_2.31.3          BiocGenerics_0.17.5
[13] edgeR_3.13.9            limma_3.27.19

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.4.5    magrittr_1.5     splines_3.3.0    munsell_0.4.3    statmod_1.4.24
 [6] colorspace_1.2-6 lattice_0.20-33  stringr_1.0.0    plyr_1.8.3       tools_3.3.0
[11] grid_3.3.0       gtable_0.2.0     reshape2_1.4.1   ggplot2_2.1.0    qvalue_2.3.2
[16] stringi_1.0-1    scales_0.4.0     locfit_1.5-9.1
```

4.5 Differential splicing after Pasilla knockdown

4.5.1 Introduction

The RNA-Seq data of this case study was produced by Brooks *et al* [3]. Drosophila melanogaster was used as a model system to study the proteins NOVA1 and NOVA2 which are known to regulate splicing in mammals. In particular, an RNA interference system (RNAi) was used to knock down the expression of the D. melanogaster ortholog of NOVA1 and NOVA2, which is Pasilla.

The experiment compared treated and untreated cells from the S2-DRSC cell line. In this case study we are interested in exons and genes that are differentially expressed after Pasilla knockdown, as well as genes that are differentially spliced in the knockdown samples as compared to wildtype.

4.5.2 RNA-Seq samples

The RNA-Seq data of the six samples were deposited on GEO `http://www.ncbi.nlm.nih.gov/geo`. The GEO accession numbers and titles were prepared in a csv file:

```
> library(edgeR)
> GEO <- readTargets("GEO-samples.csv", sep=",")
> GEO

        GEO                Title Pasilla
1 GSM461176   S2_DRSC_Untreated-1  Normal
```

```
2 GSM461177   S2_DRSC_Untreated-3  Normal
3 GSM461178   S2_DRSC_Untreated-4  Normal
4 GSM461179 S2_DRSC_CG8144_RNAi-1    Down
5 GSM461180 S2_DRSC_CG8144_RNAi-3    Down
6 GSM461181 S2_DRSC_CG8144_RNAi-4    Down
```

There are three untreated biological samples, in which Pasilla should be expressed at normal levels, and three treated biological samples, in which Pasilla should be expressed at reduced levels.

While GEO records the sample information, the sequencing data file are actually held on the NCBI Short Read Archive (SRA). The RNA samples were sequenced on an Illumina Genome Analyzer II. Multiple sequencing runs were used for several of the samples, resulting in a total of 20 SRA files:

```
> SRA <- readTargets("SRA-Files.csv", sep=",")
> SRA
```

```
         SRA       GEO                 Title  RunDate FlowCellID Type ReadLength
1  SRR031708 GSM461176   S2_DRSC_Untreated-1  7/15/08  308T2AAXX   SE         45
2  SRR031709 GSM461176   S2_DRSC_Untreated-1  7/15/08  308T2AAXX   SE         45
3  SRR031710 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
4  SRR031711 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
5  SRR031712 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
6  SRR031713 GSM461176   S2_DRSC_Untreated-1  8/15/08  30AYWAAXX   SE         45
7  SRR031714 GSM461177   S2_DRSC_Untreated-3 11/14/08  30MNEAAXX   PE         37
8  SRR031715 GSM461177   S2_DRSC_Untreated-3 12/23/08  30M2BAAXX   PE         37
9  SRR031716 GSM461178   S2_DRSC_Untreated-4 12/23/08  30M2BAAXX   PE         37
10 SRR031717 GSM461178   S2_DRSC_Untreated-4 12/23/08  30M2BAAXX   PE         37
11 SRR031718 GSM461179 S2_DRSC_CG8144_RNAi-1  7/15/08  308T2AAXX   SE         45
12 SRR031719 GSM461179 S2_DRSC_CG8144_RNAi-1  7/18/08  308UEAAXX   SE         45
13 SRR031720 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
14 SRR031721 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
15 SRR031722 GSM461179 S2_DRSC_CG8144_RNAi-1  8/15/08  30AYWAAXX   SE         45
16 SRR031723 GSM461179 S2_DRSC_CG8144_RNAi-1  8/21/08  308A0AAXX   SE         45
17 SRR031724 GSM461180 S2_DRSC_CG8144_RNAi-3 12/23/08  30M2BAAXX   PE         37
18 SRR031725 GSM461180 S2_DRSC_CG8144_RNAi-3 12/23/08  30M2BAAXX   PE         37
19 SRR031726 GSM461181 S2_DRSC_CG8144_RNAi-4 12/23/08  30M2BAAXX   PE         37
20 SRR031727 GSM461181 S2_DRSC_CG8144_RNAi-4 12/23/08  30M2BAAXX   PE         37
```

The last two columns of the above target file indicate whether the samples are single end (SE) sequencing with 45 base-pair reads or paired end (PE) sequencing with 37 bp reads.

4.5.3 Read alignment and processing

The SRA format files were first converted to FASTQ format using the SRA Toolkit. Then an index file of the D. melanogaster reference genome was built in Rsubread[11] using the FASTA files downloaded from `ftp://ftp.ncbi.nlm.nih.gov/genomes/Drosophila_melanogaster/RELEASE_5_48`. Finally, reads were aligned to the reference D. melanogaster genome using Rsubread.

Next we counted the number of reads or fragments overlapping each annotated exon of each gene. GFF files containing gene and exon annotation were downloaded from `ftp://ftp.ncbi.nlm.nih.gov/genomes/Drosophila_melanogaster/RELEASE_5_48`. The five *.gff files, one for each chromosome, were concatenated into one file, and repeated exons instances of the same exon (same start and stop position) were removed to create a data frame of start/stop positions called `unique.gff`. The single end (SE) reads were counted by:

```
> fc_SE <- featureCounts(SE_bam_files, annot.ext="unique.gff",
+    isGTFAnnotationFile=TRUE, GTF.featureType="exon", GTF.attrType="ID",
+    useMetaFeatures=FALSE, allowMultiOverlap=TRUE)
```

where `SE_bam_files` is a vector of BAM file names for the SE reads. The paired end (PE) reads were counted by:

```
> fc_PE <- featureCounts(PE_bam_files, annot.ext="unique.gff",
+    isGTFAnnotationFile=TRUE, GTF.featureType="exon", GTF.attrType="ID",
+    useMetaFeatures=FALSE, allowMultiOverlap=TRUE, isPairedEnd=TRUE)
```

where `PE_bam_files` is a vector of BAM file names for the PE reads.

4.5.4 Count loading and annotation

We create a `DGEList` object as follows

```
> y.all <- DGEList(counts=cbind(fc_SE$counts, fc_PE$counts), genes=fc_SE$annotation)
> dim(y.all)

[1] 74184    20

> head(y.all$genes)

       GeneID         Chr  Start    End Strand Length
138088  30970 NC_004354.3 138094 139379      -   1286
138087  30970 NC_004354.3 139445 139611      -    167
138089  30970 NC_004354.3 139445 139889      -    445
138086  30970 NC_004354.3 139713 139889      -    177
138091  30971 NC_004354.3 140011 141629      +   1619
138092  30971 NC_004354.3 142415 144271      +   1857
```

The annotation includes Entrez ID and the length, chromosome and start and stop position of each exon. We resort the samples back to original SRA order and collapse the data so that there is a single column for each GEO sample by summing the counts over the technical replicates:

```
> y.all <- y.all[, SRA$SRA]
> y <- sumTechReps(y.all, ID=SRA$GEO)
> y$samples

          group lib.size norm.factors
GSM461176     1 31007529            1
GSM461177     1 13040952            1
GSM461178     1 15030819            1
GSM461179     1 28143539            1
GSM461180     1 14901292            1
GSM461181     1 16264066            1

> colnames(y) <- c("N1","N3","N4","D1","D3","D4")
```

Annotation for D. melanogaster genes was downloaded from `ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/GENE_INFO/Invertebrates`. We now add selected annotation columns to the DGEList object:

```
> ncbi.L1 <- readLines("Drosophila_melanogaster.gene_info", n = 1)
> ncbi.colname <- unlist(strsplit(substring(ncbi.L1, 10, 234), ' '))
> ncbi <- read.delim("Drosophila_melanogaster.gene_info", skip=1,
+     header=FALSE, stringsAsFactors=FALSE)
> colnames(ncbi) <- ncbi.colname
> m <- match(y$genes$GeneID, ncbi$GeneID)
> y$genes$Chr <- ncbi$chromosome[m]
> y$genes$Symbol <- ncbi$Symbol[m]
> y$genes$Strand <- NULL
> head(y$genes)

       GeneID Chr  Start    End Length Symbol
138088  30970   X 138094 139379   1286 CG3038
138087  30970   X 139445 139611    167 CG3038
138089  30970   X 139445 139889    445 CG3038
138086  30970   X 139713 139889    177 CG3038
138091  30971   X 140011 141629   1619    G9a
138092  30971   X 142415 144271   1857    G9a
```

4.5.5 Filtering and normalization

Here, an exon is only retained if it is expressed at a count-per-million (CPM) above 1 in at least 3 samples.

```
> keep <- rowSums(cpm(y) > 1) >=3
> summary(keep)
```

```
   Mode   FALSE    TRUE    NA's
logical   36926   37258       0
```

```
> y <- y[keep, , keep.lib.sizes=FALSE]
```

TMM normalization is performed to eliminate composition biases between libraries.

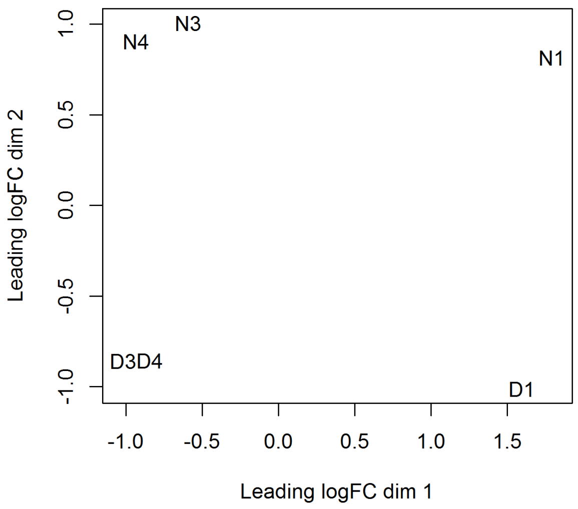
```
> y <- calcNormFactors(y)
> y$samples
```

```
   group lib.size norm.factors
N1     1 30872843        0.955
N3     1 12962245        1.031
N4     1 14908555        0.976
D1     1 27989806        1.005
D3     1 14760887        1.022
D4     1 16172265        1.014
```

4.5.6 Data exploration

The data can be explored by generating multi-dimensional scaling (MDS) plots. This visualizes the differences between the expression profiles of different samples in two dimensions.

```
> plotMDS(y)
```



The MDS plot shows clear separation of the Pasilla down vs normal samples, but also a batch effect associated with sequencing type and date.

4.5.7 The design matrix

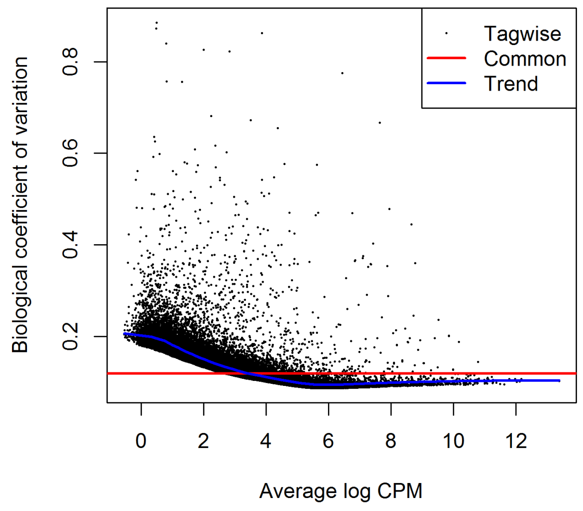
To account for the batch effect observed from the MDS plot, we create a design matrix as follows:

```
> Batch <- factor(c(1,3,4,1,3,4))
> Pasilla <- factor(GEO$Pasilla, levels=c("Normal","Down"))
> design <- model.matrix(~ Batch + Pasilla)
> design

  (Intercept) Batch3 Batch4 PasillaDown
1           1      0      0           0
2           1      1      0           0
3           1      0      1           0
4           1      0      0           1
5           1      1      0           1
6           1      0      1           1
attr(,"assign")
[1] 0 1 1 2
attr(,"contrasts")
attr(,"contrasts")$Batch
[1] "contr.treatment"

attr(,"contrasts")$Pasilla
[1] "contr.treatment"
```

4.5.8 Estimating the dispersion

We estimate NB dispersions using the `estimateDisp` function. The estimated dispersions can be visualized with `plotBCV`.

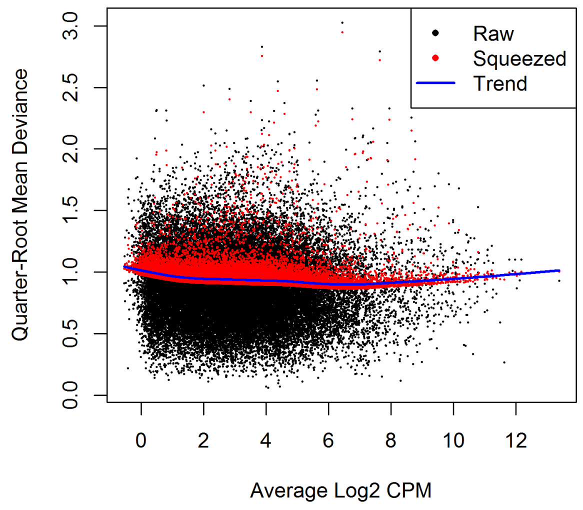
```
> y <- estimateDisp(y, design, robust=TRUE)
> y$common.dispersion

[1] 0.0141

> plotBCV(y)
```



Note that only the trended dispersion is used under the quasi-likelihood (QL) pipeline. The tagwise and common estimates are shown here but will not be used further.

For the QL dispersions, estimation can be performed using the `glmQLFit` function. The results can be visualized with the `plotQLDisp` function.

```
> fit <- glmQLFit(y, design, robust=TRUE)
> plotQLDisp(fit)
```



4.5.9 Differential expression

We test for differentially expressed exons between Pasilla knockdown and normal using the QL F-test.

```
> qlf <- glmQLFTest(fit, coef=4)
```

The top set of most significant exons can be examined with `topTags`. Here, a positive log-fold change represents exons that are up in Pasilla knockdown over normal. Multiplicity correction is performed by applying the Benjamini-Hochberg method on the *p*-values, to control the false discovery rate (FDR).

```
> topTags(qlf)

Coefficient:  PasillaDown
       GeneID Chr    Start      End Length      Symbol logFC logCPM   F   PValue      FDR
150709  32007   X 10674926 10676128   1203        sesB -3.26   7.21 949 7.41e-15 1.39e-10
150713  32007   X 10675026 10676128   1103        sesB -3.26   7.21 948 7.46e-15 1.39e-10
150697  32008   X 10672987 10673728    742        Ant2  2.85   6.14 863 1.48e-14 1.84e-10
91614   42865  3R 19970915 19971592    678        Kal1 -4.43   3.81 756 3.88e-14 3.61e-10
107856  44030  3L  2561932  2562843    912         msn -2.46   5.59 615 1.74e-13 1.30e-09
150702  32008   X 10674230 10674694    465        Ant2  2.97   4.55 575 2.83e-13 1.33e-09
150695  32008   X 10674230 10674559    330        Ant2  2.96   4.55 574 2.86e-13 1.33e-09
70750   44258  3R  5271691  5272628    938          ps -2.27   5.95 572 2.97e-13 1.33e-09
11333   44548  2R  6407125  6408782   1658        lola  2.26   6.15 565 3.21e-13 1.33e-09
96433   43230  3R 22697648 22697717     70 BM-40-SPARC -2.17   6.65 542 4.37e-13 1.63e-09
```

The total number of DE exons in each direction at a FDR of 5% can be examined with `decideTestsDGE`.

```
> is.de <- decideTestsDGE(qlf, p.value=0.05)
> summary(is.de)

   [,1]
-1  2059
0  33385
1   1814
```

4.5.10 Alternative splicing

We detect alternative splicing by testing for differential exon usage between Pasilla knockdown and normal in each gene.

```
> sp <- diffSpliceDGE(fit, coef=4, geneid="GeneID", exonid="Start")

Total number of exons:  37258
Total number of genes:  8192
Number of genes with 1 exon:  1619
Mean number of exons in a gene:  5
Max number of exons in a gene:  56
```

Two testing methods at the gene-level are provided. The Simes' method is likely to be more powerful when only a minority of the exons for a gene are differentially spliced. The F-tests are likely to be powerful for genes in which several exons are differentially spliced.

The top spliced genes under the Simes' method are shown below:

```
> topSpliceDGE(sp, test="Simes", n=20)

        GeneID Chr    Symbol NExons  P.Value      FDR
141235   45320   X      trol     44 1.64e-30 1.08e-26
11214    44548  2R      lola     30 4.28e-30 1.41e-26
95956    44448  3R     scrib     35 1.10e-20 2.40e-17
107810   44030  3L       msn     24 1.15e-18 1.89e-15
19880    36773  2R        Dg     15 2.17e-18 2.86e-15
16060    36542  2R      shot     38 2.03e-17 2.22e-14
82117    42130  3R       osa     17 2.50e-17 2.35e-14
32242    37893  2R      slik     19 2.59e-15 2.13e-12
131170   40205  3L   CG42674     16 3.88e-15 2.84e-12
163416   32817   X CrebB-17A     12 1.00e-14 6.58e-12
150694   32008   X      Ant2      5 1.02e-13 6.07e-11
110493   38491  3L       ens     16 1.88e-13 1.03e-10
41795  3771968  2L   Msp-300     33 2.80e-12 1.41e-09
115767   38879  3L       pbl     12 3.84e-12 1.80e-09
2032   2768716  2R       mim     25 8.98e-12 3.93e-09
526      35494  2R  laccase2      9 2.81e-11 1.11e-08
150710   32007   X      sesB      7 2.88e-11 1.11e-08
166094   33098   X   CG32521      8 4.29e-11 1.57e-08
52823    34652  2L     vir-1      7 8.31e-11 2.88e-08
85970    42428  3R   Stat92E     14 4.41e-10 1.45e-07
```

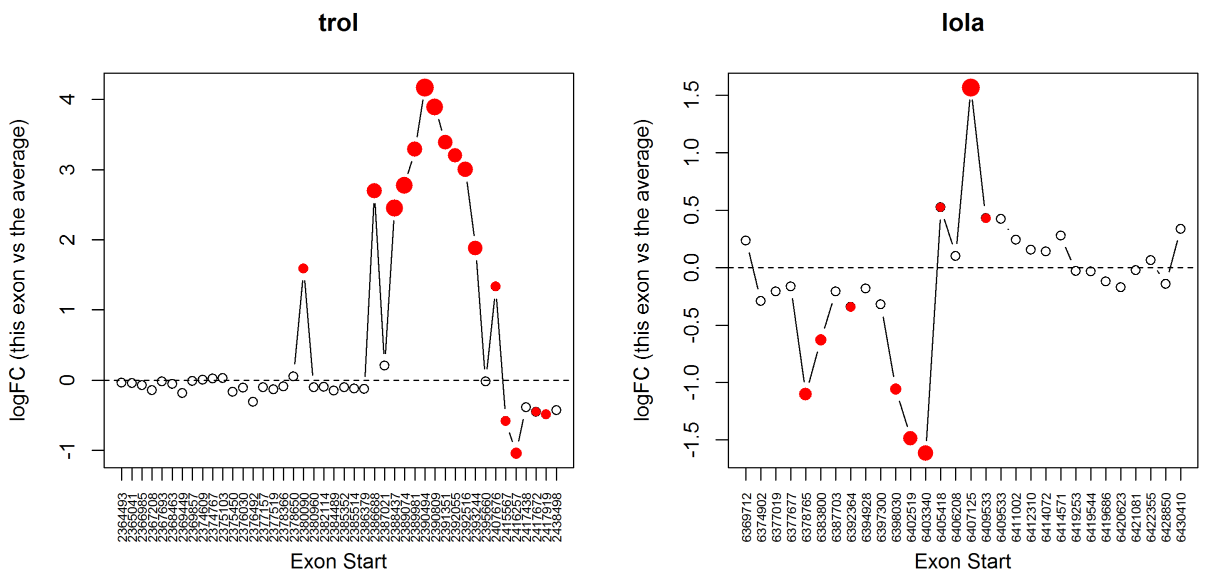
The top spliced genes identified by F-tests are shown below:

```
> topSpliceDGE(sp, test="gene", n=20)

        GeneID Chr      Symbol NExons gene.F  P.Value      FDR
141235   45320   X        trol     44   52.5 2.53e-51 1.66e-47
11214    44548  2R        lola     30   42.7 6.84e-34 2.25e-30
41795  3771968  2L     Msp-300     33   22.7 2.34e-27 5.13e-24
95956    44448  3R       scrib     35   17.2 1.08e-24 1.78e-21
16060    36542  2R        shot     38   11.1 6.90e-20 9.07e-17
```

```
32242     37893  2R         slik      19    25.8 1.22e-18 1.34e-15
166094    33098   X     CG32521        8    64.1 1.14e-14 1.07e-11
19880     36773  2R           Dg      15    21.8 2.57e-14 2.11e-11
2032    2768716  2R          mim      25    10.7 8.49e-14 6.20e-11
107810    44030  3L          msn      24    10.6 3.12e-13 2.05e-10
150694    32008   X         Ant2       5   108.1 4.05e-13 2.36e-10
82117     42130  3R          osa      17    15.6 4.30e-13 2.36e-10
163416    32817   X   CrebB-17A       12    22.3 2.49e-12 1.26e-09
150710    32007   X         sesB       7    51.5 5.26e-12 2.47e-09
131170    40205  3L     CG42674       16    12.7 4.91e-11 2.15e-08
115767    38879  3L          pbl      12    15.8 2.81e-10 1.16e-07
134207    40464  3L        Ten-m      12    15.6 3.49e-10 1.35e-07
11103     36104  2R G-oalpha47A       13    14.1 3.87e-10 1.41e-07
108973    38376  3L       BtbVII      10    18.7 5.84e-10 2.02e-07
110493    38491  3L          ens      16    10.6 7.63e-10 2.51e-07
```

We plot all the exons for the top two most differentially spliced genes. Exons that are individually significant are highlighted.

```
> par(mfrow=c(1,2))
> plotSpliceDGE(sp, geneid="trol", genecol="Symbol")
> plotSpliceDGE(sp, geneid="lola", genecol="Symbol")
```



We can see that a block of five or six exons at the right end of the trol gene are relatively lost when Pasilla is down. Most exons in the first half of the gene behave similarly to each other. This gene is on the negative strand, so transcription is from right to left. The gene trol was identified by Brooks et al [3] to have a novel set of coordinately regulated exons.

4.5.11 Setup

This analysis was conducted on:

```
> sessionInfo()

R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] GO.db_3.3.0            org.Mm.eg.db_3.3.0      tweeDEseqCountData_1.9.0
 [4] NBPSeq_0.3.0           org.Hs.eg.db_3.3.0      RSQLite_1.0.0
 [7] DBI_0.3.1              AnnotationDbi_1.33.11   IRanges_2.5.46
[10] S4Vectors_0.9.51       Biobase_2.31.3          BiocGenerics_0.17.5
[13] edgeR_3.13.9           limma_3.27.19

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.4.5    magrittr_1.5     splines_3.3.0    munsell_0.4.3    statmod_1.4.24
 [6] colorspace_1.2-6 lattice_0.20-33  stringr_1.0.0    plyr_1.8.3       tools_3.3.0
[11] grid_3.3.0       gtable_0.2.0     reshape2_1.4.1   ggplot2_2.1.0    qvalue_2.3.2
[16] stringi_1.0-1    scales_0.4.0     locfit_1.5-9.1
```

4.5.12 Acknowledgements

Thanks to Yang Liao for mapping the reads and running featureCounts and Yifang Hu for the initial analysis of the data.

4.6 CRISPR-Cas9 knockout screen analysis

4.6.1 Introduction

Dai *et al.* (2014) [7] describe the use of edgeR to analyze data from pooled genetic screens utilizing either shRNAs or CRISPR-Cas9 to disrupt gene expression in a population of cells.

In this case study we analyze data from a pooled screen that uses CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-associated nuclease Cas9) knockout technology. In this example, a library of around 64,000 sgRNAs (as used in Shalem *et al.* 2014 [29]) were screened to look for genes that may lead to resistance from a particular drug. This unpublished data set has been anonymised.

4.6.2 Sequence processing

Multiple single guide RNAs (sgRNAs) per gene (generally between 3-6) were included in the screen. Below we read in the raw sequences from the paired end fastq files *screen4_R1.fastq* and *screen4_R2.fastq* using the `processAmplicons` function in edgeRThis screen employed a dual indexing strategy where the first 8 bases from each pair of reads contained an index sequence that uniquely identifies which sample a particular sgRNA sequence originated from. Matches between sample indexes and sgRNAs listed in the files *Samples4.txt* and *sgRNAs4.txt* are identified by `processAmplicons` to produce a `DGEList` of counts.

```
> # Read in sample & sgRNA information
> sampleanno <- read.table("Samples4.txt", header=TRUE, sep="\t")
> sampleanno[1:5,]
> sgseqs <- read.table("sgRNAs4.txt", header=TRUE, sep="\t")
> sgseqs[1:5,]
> # Process raw sequences from fastq files
> x <- processAmplicons("screen4_R1.fastq", readfile2="screen4_R2.fastq",
+       barcodefile="Samples4.txt", hairpinfile="sgRNAs4.txt",
+       barcodeStart=1, barcodeEnd=8, hairpinStart=33, hairpinEnd=52,
+       barcodeStartRev=1, barcodeEndRev=8, verbose=TRUE)
```

Note that this dual indexing strategy requires an additional column named *'Sequences-Rev'* in the file that contains the sample annotation information. Also, `readFile2` must be specified, along with position information (`barcodeStartRev`, `barcodeEndRev`) for the second index in the second read from each pair (in this case the index can be found in the first 8 bases).

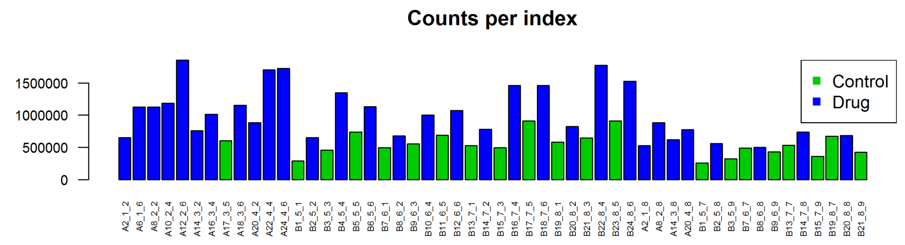
4.6.3 Filtering and data exploration

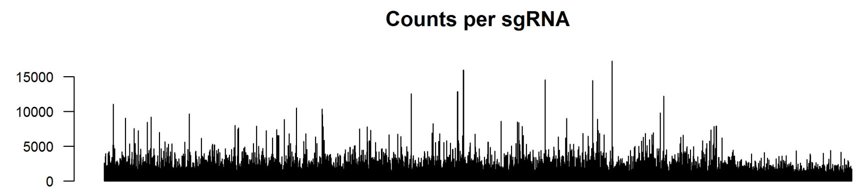
We next filter out sgRNAs and samples with low numbers of reads.

```
> x

An object of class "DGEList"
$counts
       A1_1_1 A2_1_2 A3_1_3 A4_1_4 A5_1_5 A6_1_6 A7_2_1 A8_2_2 A9_2_3 A10_2_4 A11_2_5 A12_2_6
sgRNA1      0     14      0      0      3     36      1     55      0      23       0      62
sgRNA2      0     18      0      0      1     22      0     26      0      29       0      43
sgRNA3      0     50      0      0      4     52      1     98      0      60       0     111
```

```
sgRNA4      0     32      0      0      3     55      2     54      0      51       0      57
sgRNA5      0      7      0      0      1      3      0      3      0       5       1       5
       A13_3_1 A14_3_2 A15_3_3 A16_3_4 A17_3_5 A18_3_6 A19_4_1 A20_4_2 A21_4_3 A22_4_4 A23_4_5
sgRNA1       0      21       0      30      21      36       0      37       0      39       1
sgRNA2       0      27       0      27      26      30       1      23       0      44       0
sgRNA3       0      59       0      62      24      63       0      44       0     106       0
sgRNA4       0      43       0      50      19      26       0      44       0      94       0
sgRNA5       0       3       0       3       1       7       0       1       0       8       0
       A24_4_6 B1_5_1 B2_5_2 B3_5_3 B4_5_4 B5_5_5 B6_5_6 B7_6_1 B8_6_2 B9_6_3 B10_6_4 B11_6_5
sgRNA1      63      3      5      5     26     11      4      3      4     14       3       8
sgRNA2      34     14     27     16     77     22     42      9     24     12      27      15
sgRNA3      70     16     39     27     53     41     43     31     24     14      35      43
sgRNA4     107     15     13     12     24     12     22     10     10     11      20      15
sgRNA5       3      0      5     12     15      6     25      6      9      6       3       9
       B12_6_6 B13_7_1 B14_7_2 B15_7_3 B16_7_4 B17_7_5 B18_7_6 B19_8_1 B20_8_2 B21_8_3 B22_8_4
sgRNA1       6       9      10       6       8      11      31       6      13       9      25
sgRNA2      41      11      23      14      64      46      66      18      31      12      94
sgRNA3      45      38      51      14      59      38      73      31      29      31      63
sgRNA4      18      13      12       7      28      15      23      14      18      19      27
sgRNA5      16       1       5       0       3       7      11       1       7       3      18
       B23_8_5 B24_8_6 A1_1_7 A2_1_8 A3_1_9 A7_2_7 A8_2_8 A9_2_9 A13_3_7 A14_3_8 A15_3_9 A19_4_7
sgRNA1      13      19      0     18      0      0     38      0       0      16       0       0
sgRNA2      36      64      0     12      0      0     18      0       0      15       0       0
sgRNA3      58      74      0     30      0      4     71      0       0      32       0       1
sgRNA4      19      36      0     27      0      1     39      0       0      34       0       1
sgRNA5      24      12      0      2      0      0      2      0       0       8       0       0
       A20_4_8 A21_4_9 B1_5_7 B2_5_8 B3_5_9 B7_6_7 B8_6_8 B9_6_9 B13_7_7 B14_7_8 B15_7_9 B19_8_7
sgRNA1      32       0      5      6      5     10      5     15       2       9       3      10
sgRNA2      16       0      9     20      8     11     22      7       7      22      16      20
sgRNA3      39       0     10     19     23     19     14     30      30      35      24      27
sgRNA4      28       0      8      9      9      8     10      5      11       9       8      11
sgRNA5       0       0      0      9      4      4     10      3       2       2       0       3
       B20_8_8 B21_8_9
sgRNA1      15       8
sgRNA2      20       9
sgRNA3      18      17
sgRNA4      12      11
sgRNA5      10       8
64746 more rows ...

$samples
      ID lib.size norm.factors SequencesReverse  group Infection Replicate IndexF IndexR
1 A1_1_1      188            1         TAAGGCGA   Drug         1         1      1      1
2 A2_1_2   667530            1         CGTACTAG NoDrug         1         1      1      2
3 A3_1_3     1340            1         AGGCAGAA   Drug         1         1      1      3
4 A4_1_4     2473            1         TCCTGAGC NoDrug         1         1      1      4
5 A5_1_5    69161            1         GGACTCCT   Drug         1         1      1      5
67 more rows ...
```

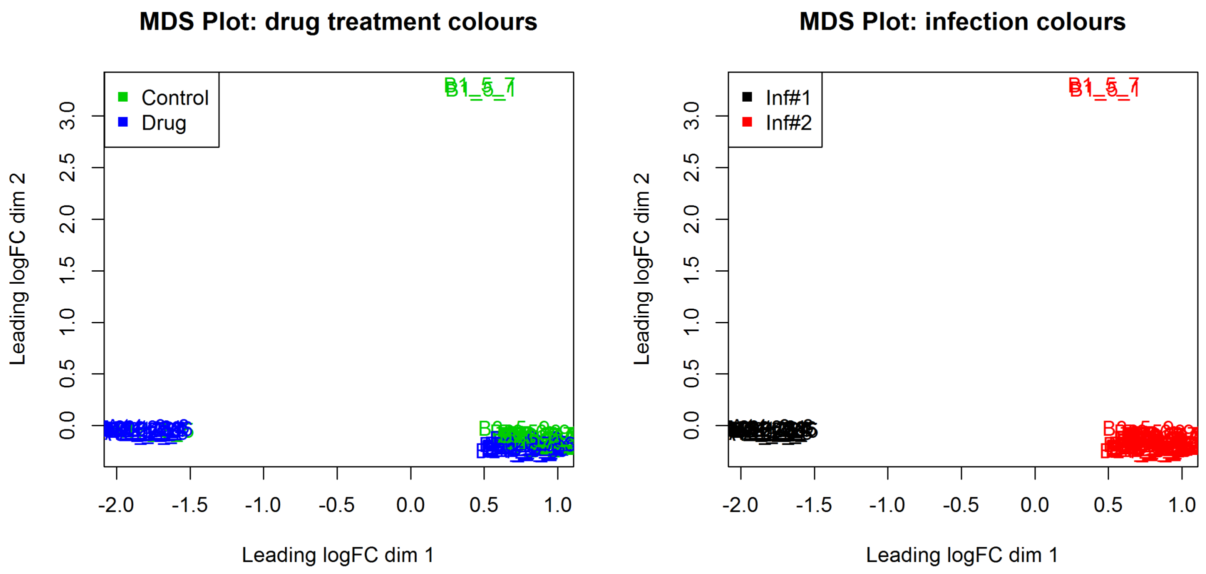
```
$genes
      ID             Sequences Gene
1 sgRNA1 TACCCTGGGACTGTACCCCC   99
2 sgRNA2 ACCCTTGCTGCACGACCTGC   99
3 sgRNA3 TCGCTCGCCCCGCTCTTCCT   99
4 sgRNA4 TGACGCCTCGGACGTGTCTG   19
5 sgRNA5 CGTCATAGCCAATCTTCTTC   19
64746 more rows ...

> table(x$samples$group)

  Drug NoDrug
    40     32

> # Filter sgRNAs and samples with low counts
> # Need a CPM greater than 5 in 15 or more samples to keep sgRNAs
> selr <- rowSums(cpm(x$counts)>5)>=15
> # Need at least 100,000 reads to keep a given sample
> selc <- colSums(x$counts)>=100000
> x <- x[selr,selc]
> # Set up drug treatment colours
> cols <- as.numeric(x$samples$group)+2
> # Plot number of sgRNAs that could be matched per sample
> # and total for each sgRNA across all samples
> par(mfrow=c(2,1))
> barplot(colSums(x$counts), las=2, main="Counts per index", col=cols, cex.names=0.5, cex.axis=0.8)
> legend("topright", legend=c("Control", "Drug"), col=c(3,4), pch=15)
> barplot(rowSums(x$counts), las=2, main="Counts per sgRNA", axisnames=FALSE, cex.axis=0.8)
```





A multidimensional scaling plot was generated to assess the consistency between replicate samples. There is a clear separation between the two infections, indicating the need to incorporate an effect for this in the GLM.

```
> # Make an MDS plot to visualise relationships between replicate samples
> # Set up infection colours
> cols2 <- x$samples$Infection
> par(mfrow=c(1,2))
> plotMDS(x, col=cols, main="MDS Plot: drug treatment colours")
> legend("topleft", legend=c("Control", "Drug"), col=c(3,4), pch=15)
> plotMDS(x, col=cols2, main="MDS Plot: infection colours")
> legend("topleft", legend=c("Inf#1", "Inf#2"), col=c(1,2), pch=15)
```



4.6.4 The design matrix and dispersion estimation

A design matrix is set up for the GLM analysis, and the sgRNA-specific variation is estimated and plotted (while taking into account both drug treatment and infection number).

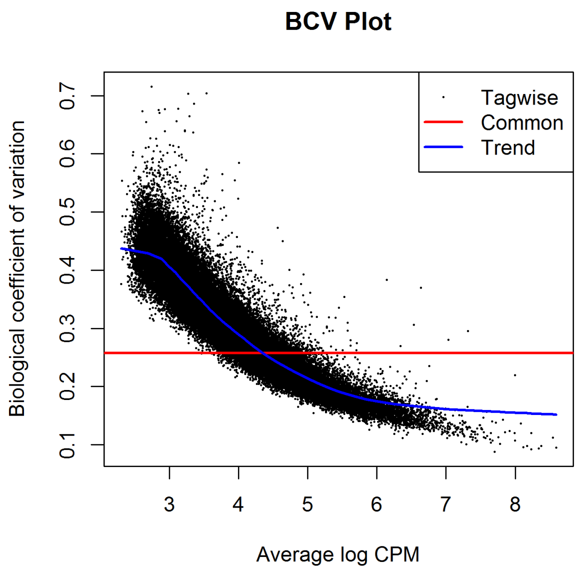
```
> # Set up design matrix for GLM
> treatment <- relevel(as.factor(x$samples$group), "NoDrug")
> infection <- as.factor(x$samples$Infection)
> des <- model.matrix(~treatment+infection)
> des[1:5,]
```

```
  (Intercept) treatmentDrug infection2
1           1             0          0
2           1             0          0
3           1             0          0
4           1             0          0
5           1             0          0
```

```
> colnames(des)[2:3] <- c("Drug", "Infection2")
> # Estimate dispersions
> xglm <- estimateDisp(x, des)
> sqrt(xglm$common.disp)
```

```
[1] 0.258
```

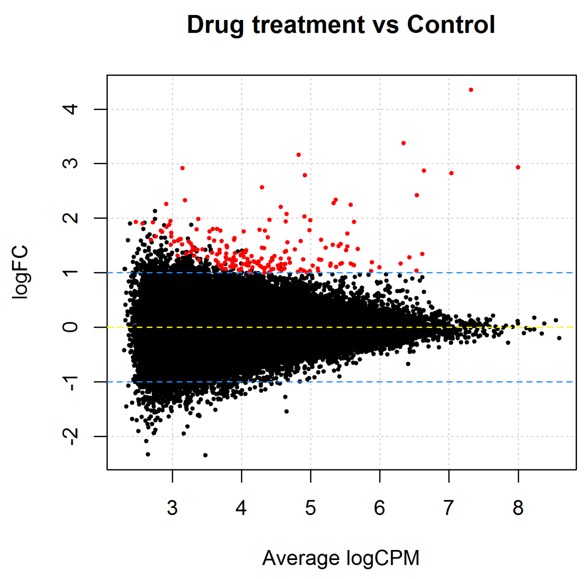
```
> # Plot BCVs versus abundance
> plotBCV(xglm, main="BCV Plot")
```



4.6.5 Differential representation analysis

We use the function `glmFit` to fit the sgRNA-specific models and `glmLRT` to do the testing between the drug treated and control samples. The top ranked sgRNAs are listed using the `topTags` function and sgRNAs with FDR < 0.0001 [2] and log-fold-change ≥ 1 are highlighted on a plot of log-fold-change versus log-counts-per-millions by the `plotSmear` function. Since this is a positive screen, we highlight over-represented sgRNAs (i.e. those with positive log-fold-changes) since the model is parameterized to compare drug treatment versus control (coefficient 2 in the design matrix).

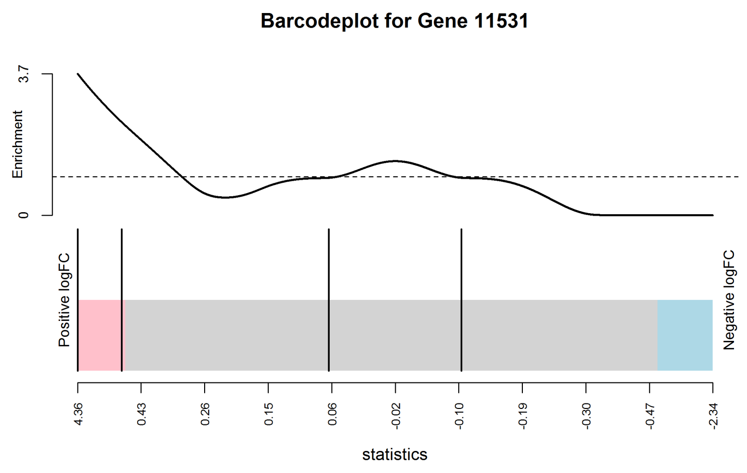
```
> # Fit negative bionomial GLM
> fit <- glmFit(xglm, des)
> # Carry out Likelihood ratio test
> lrt <- glmLRT(fit, coef=2)
> # Show top ranked sgRNAs
> topTags(lrt)

Coefficient:  Drug
              ID            Sequences  Gene logFC logCPM  LR    PValue       FDR
816     sgRNA816 TCCGAACTCCCCCTTCCCGA   269  4.36   7.32 699 4.41e-154 2.49e-149
4070   sgRNA4070 GTTGTGCTCAGTACTGACTT  1252  2.94   8.00 659 2.11e-145 5.94e-141
6351   sgRNA6351 AAAAACGTATCTATTTTTAC  1957  3.37   6.34 422  8.43e-94  1.58e-89
12880 sgRNA12880 CTGCACCGAAGAGAGCTGCT  3979  2.83   7.04 322  5.38e-72  7.58e-68
23015 sgRNA23015 CAATTTGATCTCTTCTACTG  6714  3.16   4.83 233  1.34e-52  1.51e-48
62532 sgRNA62532 AAACACGTCCAGTGCAGCCC 19612  2.79   4.91 216  6.15e-49  5.78e-45
38819 sgRNA38819 TACGTTGTCGGGCGCCGCCA 11531  2.42   6.54 204  2.93e-46  2.36e-42
3887   sgRNA3887 AACGCTGGACTCGAATGGCC  1194  2.28   5.33 203  4.03e-46  2.84e-42
19299 sgRNA19299 GGGGTCTTACCCGAGGCTCC  5732  1.94   5.63 202  7.64e-46  4.78e-42
52924 sgRNA52924 CCACCGCGTTCCACTTCTTG 16395  2.87   6.64 193  5.49e-44  3.09e-40

> # Select sgRNAs with FDR < 0.0001 and logFC <= -1 to highlight on plot
> thresh <- 0.0001
> lfc <- 1
> top4 <- topTags(lrt, n=Inf)
> top4ids <- top4$table[top4$table$FDR<thresh & top4$table$logFC>=lfc,1]
> # Plot logFC versus logCPM
> plotSmear(lrt, de.tags=top4ids, pch=20, cex=0.6, main="Drug treatment vs Control")
> abline(h = c(-1, 0, 1), col = c("dodgerblue", "yellow", "dodgerblue"), lty=2)
```



4.6.6 Gene set tests to summarize over multiple sgRNAs targeting the same gene

We finish this analysis by summarising data across multiple sgRNAs that target the same gene in order to get a gene-by-gene ranking, rather than a sgRNA-specific one. The *camera* gene-set test [34] is used for this purpose. For this analysis, the collection of sgRNAs that target a specific gene can be regarded as a 'set'. In the code below, we restrict our analysis to genes with more than 3 sgRNAs. A barcode plot, highlighting the rank of sgRNAs for a given gene relative to the entire data set is generated for the top-ranked gene (11531). Abundance of sgRNAs targeting this gene tend to increase with drug treatment (FDR=0.0003).

```
> # Carry out camera gene-set analysis
> genesymbols <- x$genes[,3]
> genesymbollist <- list()
> unq <- unique(genesymbols)
> unq <- unq[!is.na(unq)]
> for(i in unq) {
+   sel <- genesymbols==i & !is.na(genesymbols)
+   if(sum(sel)>3)
+     genesymbollist[[i]] <- which(sel)
+ }
> # Run camera for all genes
> camera.res <- camera(xglm, index=genesymbollist, des, contrast=2)
> # Display results for top ranked genes
> camera.res[1:10,]

      NGenes Correlation Direction   PValue     FDR
11531      4     -0.1744        Up 1.08e-07 0.00032
19612      5     -0.0324        Up 1.16e-07 0.00032
10784      4     -0.1885        Up 7.31e-07 0.00134
8808       4     -0.1059        Up 1.65e-06 0.00228
10386      4     -0.1050        Up 2.36e-05 0.02606
3979       4     -0.0121        Up 4.16e-05 0.03823
8493       4     -0.1267        Up 8.30e-05 0.06537
4635       5     -0.0959        Up 1.05e-04 0.07214
2005       4     -0.0240        Up 2.11e-04 0.12951
9860       5     -0.0996        Up 2.55e-04 0.14031

> # Make a barcode plot for an example that ranks highly
> # Gene 11531
> barcodeplot(lrt$table$logFC,index=genesymbollist[[11531]],
+             main="Barcodeplot for Gene 11531",
+             labels=c("Positive logFC", "Negative logFC"),
+             quantile=c(-0.5,0.5))
```



The raw data from this example and several other case studies for this technology can be found at `http://bioinf.wehi.edu.au/shRNAseq/`.

4.6.7 Setup

This analysis was conducted on:

```
> sessionInfo()

R version 3.3.0 beta (2016-04-14 r70486)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 7 x64 (build 7601) Service Pack 1

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] parallel  stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] GO.db_3.3.0              org.Mm.eg.db_3.3.0       tweeDEseqCountData_1.9.0
 [4] NBPSeq_0.3.0             org.Hs.eg.db_3.3.0       RSQLite_1.0.0
 [7] DBI_0.3.1                AnnotationDbi_1.33.11    IRanges_2.5.46
[10] S4Vectors_0.9.51         Biobase_2.31.3           BiocGenerics_0.17.5
[13] edgeR_3.13.9             limma_3.27.19

loaded via a namespace (and not attached):
 [1] Rcpp_0.12.4.5    magrittr_1.5     splines_3.3.0    munsell_0.4.3    statmod_1.4.24
 [6] colorspace_1.2-6 lattice_0.20-33  stringr_1.0.0    plyr_1.8.3       tools_3.3.0
```

```
[11] grid_3.3.0       gtable_0.2.0     reshape2_1.4.1   ggplot2_2.1.0    qvalue_2.3.2
[16] stringi_1.0-1    scales_0.4.0     locfit_1.5-9.1
```

4.6.8 Acknowledgements

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