

# CQN (Conditional Quantile Normalization)

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## Introduction

This package contains the CQN (conditional quantile normalization) method for normalizing RNA-seq datasets. This method is described in [1].

```
> library(cqn)
> library(scales)
```

## Data

As an example we use ten samples from Montgomery [2]. The data has been processed as described in [1]. First we have the region by sample count matrix

```
> data(montgomery.subset)
> dim(montgomery.subset)
```

```
[1] 23552    10
```

```
> montgomery.subset[1:4,1:4]
```

	NA06985	NA06994	NA07037	NA10847
ENSG00000000419	69	54	67	70
ENSG00000000457	53	37	27	41
ENSG00000000460	12	25	33	22
ENSG00000000938	168	270	140	103

```
> colnames(montgomery.subset)
```

```
[1] "NA06985" "NA06994" "NA07037" "NA10847" "NA11920" "NA11918"
[7] "NA11931" "NA12003" "NA12006" "NA12287"
```

Because of (disc) space issues, We have removed all genes that have zero counts in all 10 samples. Next we have the *sizeFactors* which simply tells us how deep each sample was sequenced:

```
> data(sizeFactors.subset)
> sizeFactors.subset[1:4]

NA06985 NA06994 NA07037 NA10847
3107420 2388948 3087234 2852972
```

Finally, we have a matrix containing length and GC-content for each gene.

```
> data(uCovar)
> head(uCovar)

                length gccontent
ENSG00000000419    1207 0.3976802
ENSG00000000457    2861 0.4606781
ENSG00000000460    4912 0.4338355
ENSG00000000938    3524 0.5749149
ENSG00000000971    8214 0.3613343
ENSG00000001036    2590 0.4312741
```

Note that the row ordering of the count matrix is the same as the row ordering of the matrix containing length and GC-content and that the sizeFactor vector has the same column order as the count matrix. We can formally check this

```
> stopifnot(all(rownames(montgomery.subset) == rownames(uCovar)))
> stopifnot(colnames(montgomery.subset) == names(sizeFactors.subset))
```

## Normalization

The methodology is described in [1]. The main workhorse is the function `cqn` which fits the following model

$$\log_2(\text{RPM}) = s(x) + s(\log_2(\text{length}))$$

where  $x$  is some covariate,  $s$  are smooth functions (specifically natural cubic splines with 5 knots), and RPM are “reads per millions”. We also have the function `cqn.fixedlength` which fits the model

$$\log_2(\text{RPKM}) = s(x)$$

In this model gene length is included as a known offset.

The basic call to `cqn` is relatively easy, we need the count matrix, a vector of lengths, a vector of GC content and a vector of sizeFactors. Make sure that they all have the same ordering.

```
> cqn.subset <- cqn(montgomery.subset, lengths = uCovar$length,  
+                 x = uCovar$gccontent, sizeFactors = sizeFactors.subset,  
+                 verbose = TRUE)
```

```
RQ fit .....
```

```
SQN .
```

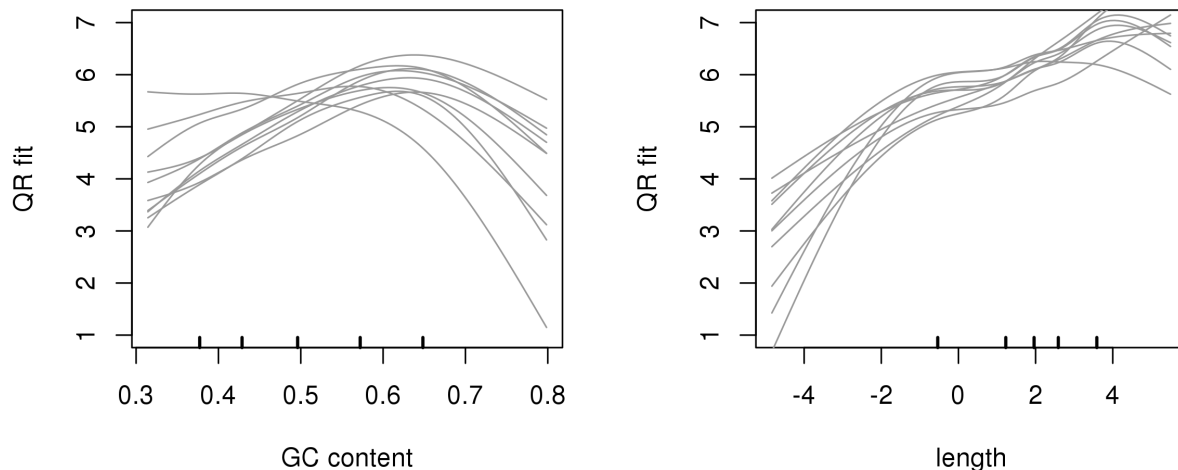
```
> cqn.subset
```

```
object of class 'cqn' with 23552 regions  
 10 samples  
originating from a fit with smooth length
```

This normalized matrix is similar, but not equivalent, to the data examined in [1]. The main differences are (1) in [1] we normalize 60 samples together, not 10 and (2) we have removed all genes with zero counts in all 10 samples.

We can examine plots of systematic effects by using `cqnplot`. The `n` argument refers to the systematic effect, `n=1` is always the covariate specified by the `x` argument above, while `n=2` is lengths.

```
> par(mfrow=c(1,2))  
> cqnplot(cqn.subset, n = 1, xlab = "GC content", lty = 1, ylim = c(1,7))  
> cqnplot(cqn.subset, n = 2, xlab = "length", lty = 1, ylim = c(1,7))
```



The normalized expression values are

```
> RPKM.cqn <- cqn.subset$y + cqn.subset$offset
> RPKM.cqn[1:4,1:4]

           NA06985  NA06994  NA07037  NA10847
ENSG00000000419  5.761813  5.568754  5.547280  5.975746
ENSG00000000457  4.436652  4.110397  3.394524  4.139537
ENSG00000000460  2.603203  3.444776  3.777542  3.068756
ENSG00000000938  5.152035  6.084140  4.698142  4.281873
```

These values are on the  $\log_2$ -scale.

We can do a MA plot of these fold changes, and compare it to fold changes based on standard RPKM. First we compute the standard RPKM (on a  $\log_2$  scale):

```
> RPM <- sweep(log2(montgomery.subset + 1), 2, log2(sizeFactors.subset/10^6))
> RPKM.std <- sweep(RPM, 1, log2(uCovar$length / 10^3))
```

We now look at differential expression between two groups of samples. We use the same grouping as in [1], namely

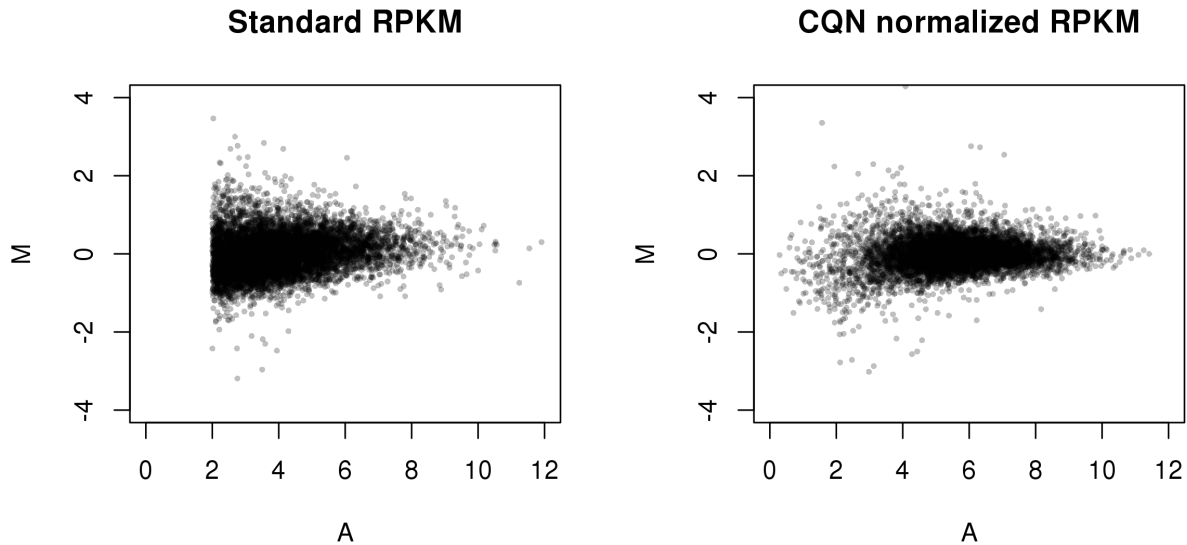
```
> grp1 <- c("NA06985", "NA06994", "NA07037", "NA10847", "NA11920")
> grp2 <- c("NA11918", "NA11931", "NA12003", "NA12006", "NA12287")
```

We now do an MA-plot, but we only choose to plot genes with average standard  $\log_2$ -RPKM of  $\log_2(5)$  or greater, and we also form the M and A values:

```
> whGenes <- which(rowMeans(RPKM.std) >= 2 & uCovar$length >= 100)
> M.std <- rowMeans(RPKM.std[whGenes, grp1]) - rowMeans(RPKM.std[whGenes, grp2])
> A.std <- rowMeans(RPKM.std[whGenes,])
> M.cqn <- rowMeans(RPKM.cqn[whGenes, grp1]) - rowMeans(RPKM.cqn[whGenes, grp2])
> A.cqn <- rowMeans(RPKM.cqn[whGenes,])
```

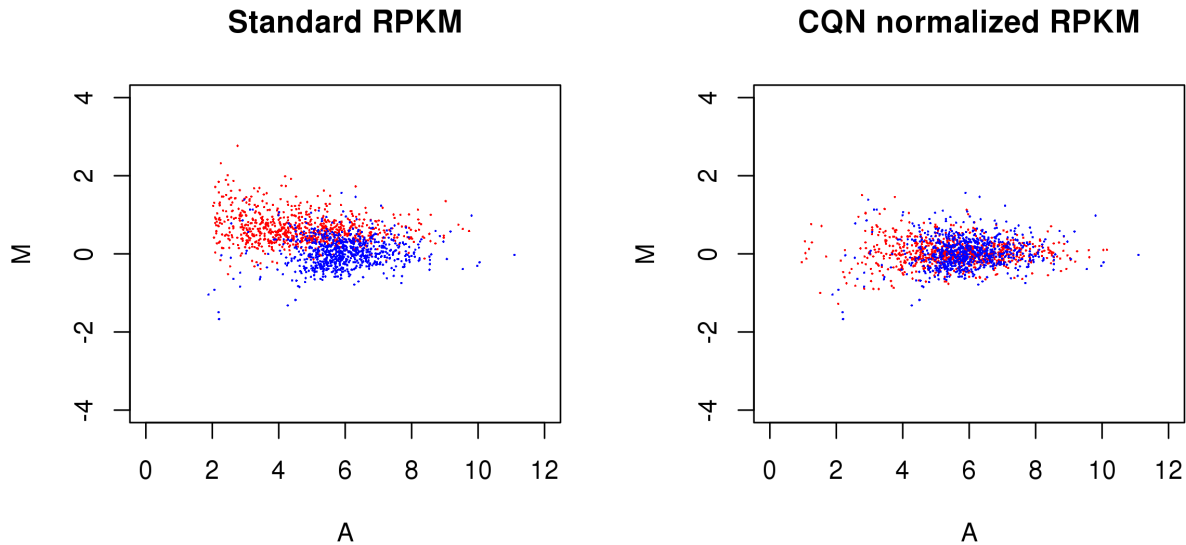
Now we do the MA plots, with alpha-blending

```
> par(mfrow = c(1,2))
> plot(A.std, M.std, cex = 0.5, pch = 16, xlab = "A", ylab = "M",
+      main = "Standard RPKM", ylim = c(-4,4), xlim = c(0,12),
+      col = alpha("black", 0.25))
> plot(A.cqn, M.cqn, cex = 0.5, pch = 16, xlab = "A", ylab = "M",
+      main = "CQN normalized RPKM", ylim = c(-4,4), xlim = c(0,12),
+      col = alpha("black", 0.25))
```



We can also color the genes according to whether they have high/low GC-content. Here one needs to be careful, because of overplotting. One solution is to leave out all genes with intermediate GC content. We define high/low GC content as the 10% most extreme genes:

```
> par(mfrow = c(1,2))
> gcccontent <- uCovar$gcccontent[whGenes]
> whHigh <- which(gcccontent > quantile(gcccontent, 0.9))
> whLow <- which(gcccontent < quantile(gcccontent, 0.1))
> plot(A.std[whHigh], M.std[whHigh], cex = 0.2, pch = 16, xlab = "A", ylab = "M",
+      main = "Standard RPKM", ylim = c(-4,4), xlim = c(0,12),
+      col = "red")
> points(A.cqn[whLow], M.cqn[whLow], cex = 0.2, pch = 16, col = "blue")
> plot(A.cqn[whHigh], M.cqn[whHigh], cex = 0.2, pch = 16, xlab = "A", ylab = "M",
+      main = "CQN normalized RPKM", ylim = c(-4,4), xlim = c(0,12),
+      col = "red")
> points(A.cqn[whLow], M.cqn[whLow], cex = 0.2, pch = 16, col = "blue")
```



Note that genes/regions with very small counts should not be relied upon, even if the CQN normalized fold change are big. They should be filtered out using some kind of statistical test, good packages for this are *DESeq*[3] and *edgeR*[4].

## Import into edgeR

First we construct a `DGEList`. In the `groups` argument we use that the first 5 samples (columns) in `montgomery.subset` is what we earlier called `grp1` and the last 5 samples (columns) are `grp2`.

```
> library(edgeR)
> d.mont <- DGEList(counts = montgomery.subset, lib.size = sizeFactors.subset,
+                   group = rep(c("grp1", "grp2"), each = 5), genes = uCovar)
```

In this object we cannot (unfortunately, yet) also store the computed offsets. Since we will use the offsets computed by *cqn*, there is no need to normalize using the normalization tools from *edgeR*, such as `calcNormFactors`. Also, as is clearly described in the *edgeR* user's guide, the `lib.size` is unnecessary, since we plan to use the offsets computed from *cqn*.

Using *edgeR* is well described in the user's guide, and we refer to that document for further information. The analysis presented below should be thought of as an example, and not necessarily the best analysis of this data.

The first step is estimating the dispersion parameter(s). Several methods exists, such as `estimateGLMCommonDisp` or `estimateTagwiseDisp`. We also need to setup a design matrix, which is particular simple for this two group comparison. Further information about con-

structuring design matrices may be found in both the *edgeR* user's guide and the *limma* user's guide.

```
> design <- model.matrix(~ d.mont$sample$group)
> d.mont.cqn <- estimateGLMCommonDisp(d.mont, design = design, offset = cqn.subset$offset)
```

After fitting the dispersion parameter(s), we need to fit the model, and do a test for significance of the parameter of interest. With this design matrix, there are two coefficients. The first coefficient is just an intercept (overall level of expression for the gene) and it is (usually) not meaningful to test for this effect. Instead, the interesting coefficient is the second one that encodes a group difference.

```
> efit.cqn <- glmFit(d.mont.cqn, design = design, offset = cqn.subset$offset)
> elrt.cqn <- glmLRT(d.mont.cqn, efit.cqn, coef = 2)
> topTags(elrt.cqn, n = 2)
```

```
Coefficient: d.mont$sample$groupgrp2
              length gccontent  logConc    logFC      LR
ENSG00000211890  1071 0.6358543 10.313924 -10.90978 66.02393
ENSG00000211660   411 0.5888078  5.580851 -12.92733 65.53574
              P.Value      FDR
ENSG00000211890 4.454808e-16 6.720512e-12
ENSG00000211660 5.706956e-16 6.720512e-12
```

`topTags` shows (per default) the "top 10" genes. In this case, since we have biological replicates and just a random group structure, we would expect no differentially expression genes. Instead we get

```
> summary(decideTestsDGE(elrt.cqn))
```

```
 [,1]
-1  195
 0 23189
 1   168
```

significantly differentially expressed at an FDR (false discovery rate) of 5%. We may contrast this with the result of not using *cqn*:

```
> d.mont.std <- estimateGLMCommonDisp(d.mont, design = design)
> efit.std <- glmFit(d.mont.std, design = design)
> elrt.std <- glmLRT(d.mont.std, efit.std, coef = 2)
> summary(decideTestsDGE(elrt.std))
```

```
[,1]
-1  211
0  23086
1   255
```

What is arguably as important is that we achieve a much better estimation of the fold change using *cqn*.

## SessionInfo

- R version 2.14.1 (2011-12-22), x86\_64-unknown-linux-gnu
- Locale: LC\_CTYPE=en\_US.UTF-8, LC\_NUMERIC=C, LC\_TIME=en\_US.UTF-8, LC\_COLLATE=C, LC\_MONETARY=en\_US.UTF-8, LC\_MESSAGES=en\_US.UTF-8, LC\_PAPER=C, LC\_NAME=C, LC\_ADDRESS=C, LC\_TELEPHONE=C, LC\_MEASUREMENT=en\_US.UTF-8, LC\_IDENTIFICATION=C
- Base packages: base, datasets, grDevices, graphics, methods, splines, stats, utils
- Other packages: SparseM 0.91, cqn 1.0.1, edgeR 2.4.3, limma 3.10.2, mclust 3.4.11, nor1mix 1.1-3, preprocessCore 1.16.0, quantreg 4.76, scales 0.1.0
- Loaded via a namespace (and not attached): RColorBrewer 1.0-5, colorspace 1.1-1, dichromat 1.2-4, munsell 0.3, plyr 1.7.1, stringr 0.6, tools 2.14.1

## References

- [1] Hansen, K.D., Irizarry, R.A., and Wu, W. Removing technical variability in RNA-seq data using conditional quantile normalization. *Johns Hopkins, Dept of Biostatistics Working Papers*, Working Paper 227 (2011). url: <http://www.bepress.com/jhubiostat/paper227>
- [2] Montgomery, S.B. *et al.* Transcriptome genetics using second generation sequencing in a Caucasian population. *Nature* **464**, 773–777. doi: [10.1038/nature08903](https://doi.org/10.1038/nature08903)
- [3] Anders, S. and Huber, W. Differential expression analysis for sequence count data. *Genome Biology* **11**(10), R106 (2010). doi: [10.1186/gb-2010-11-10-r106](https://doi.org/10.1186/gb-2010-11-10-r106)
- [4] Robinson, M.D., McCarthy, D.J., Smyth, G.K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**(1), 139–140 (2010). doi: [10.1093/bioinformatics/btp616](https://doi.org/10.1093/bioinformatics/btp616)