

NormqPCR: Functions for normalisation of RT-qPCR data

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1 Introduction

The package "NormqPCR" provides methods for the normalization of real-time quantitative RT-PCR data. In this vignette we describe and demonstrate the available functions. Firstly we show how the user may combine technical replicates, deal with undetermined values and deal with values above a user-chosen threshold. The rest of the vignette is split into two distinct sections, the first giving details of different methods to select the best housekeeping gene/genes for normalisation, and the second showing how to use the selected housekeeping gene(s) to produce $2^{-\Delta Cq}$ normalised estimators and $2^{-\Delta\Delta Cq}$ estimators of differential expression.

2 Combining technical replicates

When a raw data file read in using `read.qPCR` contains technical replicates, they are dealt with by concatenating the suffix `_TechRep.n` to the detector name, where `n` in `1, 2...N` is the number of the replication in the total number of replicates, `N`, based on order of appearance in the `qPCR` data file.

So if we read in a file with technical replicates, we can see that the detector/feature names are thus suffixed:

```
> library(ReadqPCR) # load the ReadqPCR library
> library(NormqPCR)
> path <- system.file("exData", package = "NormqPCR")
> qPCR.example.techReps <- file.path(path, "qPCR.techReps.txt")
> qPCRBatch.qPCR.techReps <- read.qPCR(qPCR.example.techReps)
> rownames(exprs(qPCRBatch.qPCR.techReps))[1:8]

[1] "gene_aj_TechReps.1" "gene_aj_TechReps.2" "gene_al_TechReps.1"
[4] "gene_al_TechReps.2" "gene_ax_TechReps.1" "gene_ax_TechReps.2"
[7] "gene_bo_TechReps.1" "gene_bo_TechReps.2"
```

It is likely that before continuing with the analysis, the user would wish to average the technical replicates by using the arithmetic mean of the raw `Cq` values. This can be achieved using the `combineTechReps` function, which will produce a new `qPCRBatch` object, with all tech reps reduced to one reading:

```
> combinedTechReps <- combineTechReps(qPCRBatch.qPCR.techReps)
> combinedTechReps
```

```
qPCRBatch (storageMode: lockedEnvironment)
assayData: 8 features, 3 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: one three two
  varLabels: sample
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
Annotation:
```

3 Dealing with undetermined values

When an RT-qPCR experiment does not produce a reading after a certain number of cycles (the cycle threshold), the reading is given as undetermined. These are represented in `qPCRBatch` objects as `NA`. Different users may have different ideas about how many cycles they wish to allow before declaring a detector as not present in the sample. There are two methods for the user to decide what to do with numbers above a given cycle threshold:

First the user might decide that anything above 38 cycles means there is nothing present in their sample, instead of the standard 40 used by the `taqman` software. They can replace the value of all readings above 38 as `NA` using the following:

Firstly read in the `taqman` example file which has 96 detectors, with 4 replicates for `mia` (case) and 4 non-`mia` (control):

```
> path <- system.file("exData", package = "NormqPCR")
> taqman.example <- file.path(path, "/example.txt")
> qPCRBatch.taqman <- read.taqman(taqman.example)
```

We can see that for the detector: `Cc120.Rn00570287_m1` we have these readings for the different samples:

```
> exprs(qPCRBatch.taqman)["Cc120.Rn00570287_m1",]

      fp1.day3.v  fp2.day3.v  fp5.day3.mia  fp6.day3.mia  fp.3.day.3.v
              NA           NA          35.74190       34.05922       35.02052
fp.4.day.3.v fp.7.day.3.mia fp.8.day.3.mia
              NA           35.93689       36.57921
```

We can now use the `replaceAboveCutOff` method in order to replace anything above 35 with `NA`:

```
> qPCRBatch.taqman.replaced <- replaceAboveCutOff(qPCRBatch.taqman,
+                                                  newVal = NA, cutOff = 35)
> exprs(qPCRBatch.taqman.replaced)["Cc120.Rn00570287_m1",]

      fp1.day3.v  fp2.day3.v  fp5.day3.mia  fp6.day3.mia  fp.3.day.3.v
              NA           NA           NA          34.05922       NA
fp.4.day.3.v fp.7.day.3.mia fp.8.day.3.mia
              NA           NA           NA
```

It may also be the case that the user wants to get rid of all `NA` values, and replace them with an arbitrary number. This can be done using the `replaceNAs` method. So if the user wanted to replace all `NAs` with 40, it can be done as follows:

```

> qPCRBatch.taqman.replaced <- replaceNAs(qPCRBatch.taqman, newNA = 40)
> exprs(qPCRBatch.taqman.replaced)[ "Cc120.Rn00570287_m1", ]

      fp1.day3.v    fp2.day3.v    fp5.day3.mia    fp6.day3.mia    fp.3.day.3.v
      40.00000      40.00000      35.74190      34.05922      35.02052
fp.4.day.3.v fp.7.day.3.mia fp.8.day.3.mia
      40.00000      35.93689      36.57921

```

In addition, the situation sometimes arises where some readings for a given detector are above a given cycle threshold, but some others are not. The user may decide for example that if a given number of readings are NAs, then all of the readings for this detector should be NAs. This is important because otherwise an unusual reading for one detector might lead to an inaccurate estimate for the expression of a given gene.

This process will necessarily be separate for the different sample types, since you might expect a given gene to show expression in one sample type compared to another. Therefore it is necessary to designate the replicates per sample type using a contrast matrix. It is also necessary to make a sampleMaxMatrix which gives a maximum number of NAs allowed for each sample type.

So in the example file above we two sample types, with 4 biological replicates for each, the contrastMatrix and sampleMaxMatrix might be constructed like this:

```

> sampleNames(qPCRBatch.taqman)

[1] "fp1.day3.v"      "fp2.day3.v"      "fp5.day3.mia"    "fp6.day3.mia"
[5] "fp.3.day.3.v"    "fp.4.day.3.v"    "fp.7.day.3.mia"  "fp.8.day.3.mia"

> a <- c(0,0,1,1,0,0,1,1) # one for each sample type, with 1 representing
> b <- c(1,1,0,0,1,1,0,0) # position of sample type in sampleNames vector
> contM <- cbind(a,b)
> colnames(contM) <- c("case","control") # set the names of each sample type
> rownames(contM) <- sampleNames(qPCRBatch.taqman) # set row names
> contM

      case control
fp1.day3.v      0      1
fp2.day3.v      0      1
fp5.day3.mia    1      0
fp6.day3.mia    1      0
fp.3.day.3.v    0      1
fp.4.day.3.v    0      1
fp.7.day.3.mia  1      0
fp.8.day.3.mia  1      0

```

```
> sMaxM <- t(as.matrix(c(3,3))) # now make the contrast matrix
> colnames(sMaxM) <- c("case","control") # make sure these line up with samples
> sMaxM
```

```
      case control
[1,]    3      3
```

More details on contrast matrices can be found in the limma manual, which requires a similar matrix when testing for differential expression between samples.

For example, if the user decides that if at least 3 out of 4 readings are NAs for a given detector, then all readings should be NA, they can do the following, using the `makeAllNewVal` method:

```
> qPCRBatch.taqman.replaced <- makeAllNewVal(qPCRBatch.taqman, contM,
+                                           sMaxM, newVal=NA)
```

Here you can see for the `Ccl20.Rn00570287_m1` detector, the control values have been made all NA, whereas before 3 were NA and one was 35. However the case values have been kept, since they were all below the NA threshold. It is important to filter the data in this way to ensure the correct calculations are made downstream when calculating variation and other parameters.

```
> exprs(qPCRBatch.taqman.replaced)["Ccl20.Rn00570287_m1",]

      fp1.day3.v    fp2.day3.v    fp5.day3.mia    fp6.day3.mia    fp.3.day.3.v
              NA              NA             35.74190             34.05922              NA
fp.4.day.3.v fp.7.day.3.mia fp.8.day.3.mia
              NA             35.93689             36.57921
```

4 Selection of most stable reference/housekeeping genes

This section contains two subsections containing different methods for the selection of appropriate housekeeping genes.

4.1 `geNorm`

We describe the selection of the best (most stable) reference/housekeeping genes using the method of Vandesompele et al (2002) [3] (in the sequel: Vand02) which is called *geNorm*. We first load the package and the data

```
> options(width = 68)
> data(geNorm)
> str(exprs(geNorm.qPCRBatch))
```

```

num [1:10, 1:85] 0.0425 0.0576 0.1547 0.1096 0.118 ...
- attr(*, "dimnames")=List of 2
..$ : chr [1:10] "ACTB" "B2M" "GAPD" "HMBS" ...
..$ : chr [1:85] "BM1" "BM2" "BM3" "BM4" ...

```

We start by ranking the selected reference/housekeeping genes. The `geNorm` algorithm implemented in function `selectHKs` proceeds stepwise; confer Section “Materials and methods” in Vand02. That is, the gene stability measure `M` of all candidate genes is computed and the gene with the highest `M` value is excluded. Then, the gene stability measure `M` for the remaining gene is calculated and so on. This procedure is repeated until two respectively, `minNrHK` genes remain.

```

> tissue <- as.factor(c(rep("BM", 9), rep("FIB", 20), rep("LEU", 13),
+                       rep("NB", 34), rep("POOL", 9)))
> res.BM <- selectHKs(geNorm.qPCRBatch[,tissue == "BM"], method = "geNorm",
+                     Symbols = featureNames(geNorm.qPCRBatch),
+                     minNrHK = 2, log = FALSE)

```

| | | | | | |
|-----------|-----------|-----------|-----------|-----------|-----------|
| HPRT1 | YWHAZ | RPL13A | UBC | GAPD | SDHA |
| 0.5160313 | 0.5314564 | 0.5335963 | 0.5700961 | 0.6064919 | 0.6201470 |
| TBP | HMBS | B2M | ACTB | | |
| 0.6397969 | 0.7206013 | 0.7747634 | 0.8498739 | | |
| HPRT1 | RPL13A | YWHAZ | UBC | GAPD | SDHA |
| 0.4705664 | 0.5141375 | 0.5271169 | 0.5554718 | 0.5575295 | 0.5738460 |
| TBP | HMBS | B2M | | | |
| 0.6042110 | 0.6759176 | 0.7671985 | | | |
| HPRT1 | RPL13A | SDHA | YWHAZ | UBC | GAPD |
| 0.4391222 | 0.4733732 | 0.5243665 | 0.5253471 | 0.5403137 | 0.5560120 |
| TBP | HMBS | | | | |
| 0.5622094 | 0.6210820 | | | | |
| HPRT1 | RPL13A | YWHAZ | UBC | SDHA | GAPD |
| 0.4389069 | 0.4696398 | 0.4879728 | 0.5043292 | 0.5178634 | 0.5245346 |
| TBP | | | | | |
| 0.5563591 | | | | | |
| HPRT1 | RPL13A | UBC | YWHAZ | GAPD | SDHA |
| 0.4292808 | 0.4447874 | 0.4594181 | 0.4728920 | 0.5012107 | 0.5566762 |
| UBC | RPL13A | HPRT1 | YWHAZ | GAPD | |
| 0.4195958 | 0.4204997 | 0.4219179 | 0.4424631 | 0.4841646 | |
| RPL13A | UBC | YWHAZ | HPRT1 | | |
| 0.3699163 | 0.3978736 | 0.4173706 | 0.4419220 | | |
| UBC | RPL13A | YWHAZ | | | |
| 0.3559286 | 0.3761358 | 0.3827933 | | | |

```
RPL13A      UBC
0.3492712  0.3492712
```

```
> res.POOL <- selectHKs(geNorm.qPCRBatch[,tissue == "POOL"],
+                       method = "geNorm",
+                       Symbols = featureNames(geNorm.qPCRBatch),
+                       minNrHK = 2, trace = FALSE, log = FALSE)
> res.FIB <- selectHKs(geNorm.qPCRBatch[,tissue == "FIB"],
+                      method = "geNorm",
+                      Symbols = featureNames(geNorm.qPCRBatch),
+                      minNrHK = 2, trace = FALSE, log = FALSE)
> res.LEU <- selectHKs(geNorm.qPCRBatch[,tissue == "LEU"],
+                      method = "geNorm",
+                      Symbols = featureNames(geNorm.qPCRBatch),
+                      minNrHK = 2, trace = FALSE, log = FALSE)
> res.NB <- selectHKs(geNorm.qPCRBatch[,tissue == "NB"],
+                     method = "geNorm",
+                     Symbols = featureNames(geNorm.qPCRBatch),
+                     minNrHK = 2, trace = FALSE, log = FALSE)
```

We obtain the following ranking of genes (see Table 3 in Vand02)

```
> ranks <- data.frame(c(1, 1:9), res.BM$ranking, res.POOL$ranking,
+                     res.FIB$ranking, res.LEU$ranking,
+                     res.NB$ranking)
> names(ranks) <- c("rank", "BM", "POOL", "FIB", "LEU", "NB")
> ranks
```

| | rank | BM | POOL | FIB | LEU | NB |
|----|------|--------|--------|--------|--------|--------|
| 1 | 1 | RPL13A | GAPD | GAPD | UBC | GAPD |
| 2 | 1 | UBC | SDHA | HPRT1 | YWHAZ | HPRT1 |
| 3 | 2 | YWHAZ | HMBS | YWHAZ | B2M | SDHA |
| 4 | 3 | HPRT1 | HPRT1 | UBC | GAPD | UBC |
| 5 | 4 | GAPD | TBP | ACTB | RPL13A | HMBS |
| 6 | 5 | SDHA | UBC | TBP | TBP | YWHAZ |
| 7 | 6 | TBP | RPL13A | SDHA | SDHA | TBP |
| 8 | 7 | HMBS | YWHAZ | RPL13A | HPRT1 | ACTB |
| 9 | 8 | B2M | ACTB | B2M | HMBS | RPL13A |
| 10 | 9 | ACTB | B2M | HMBS | ACTB | B2M |

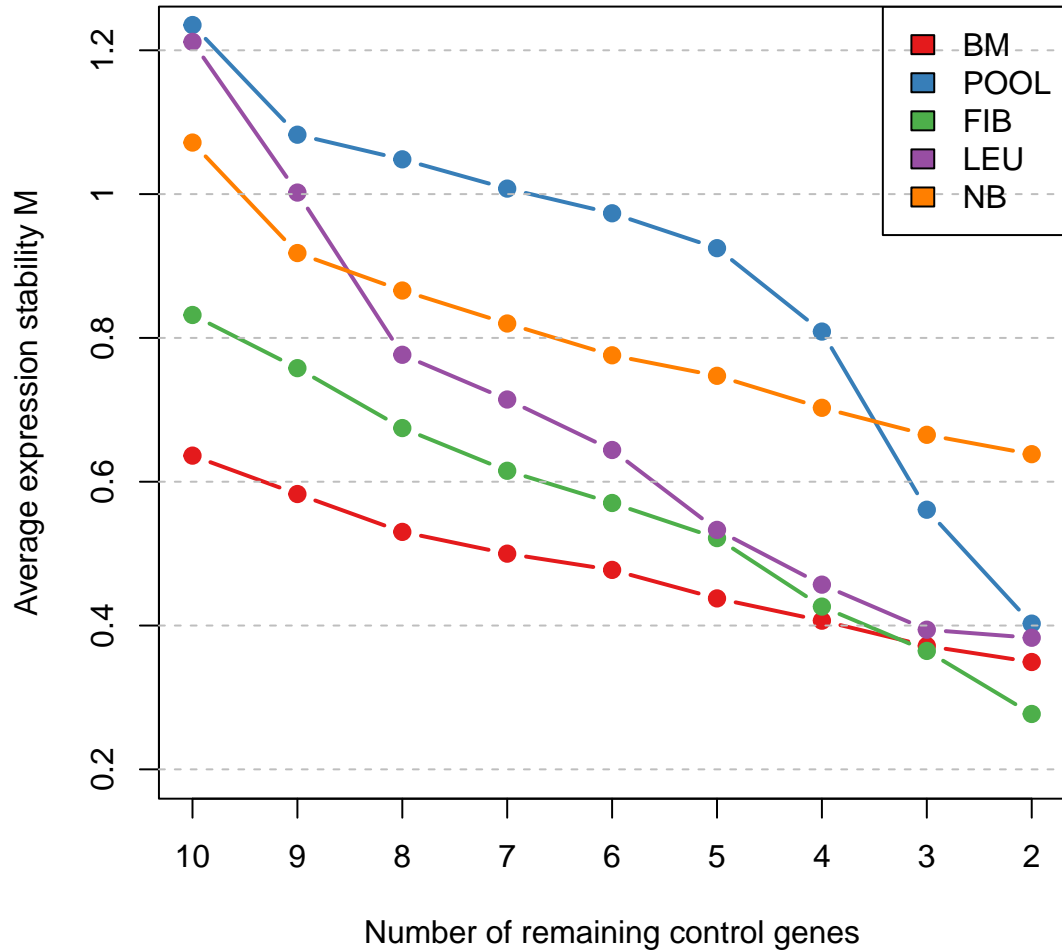
Remark 1:

Since the computation is based on gene ratios, the two most stable control genes in each cell type cannot be ranked.

We plot the average expression stability M for each cell type (see Figure 2 in Vand02).

```
> library(RColorBrewer)
> mypalette <- brewer.pal(5, "Set1")
> matplot(cbind(res.BM$meanM, res.POOL$meanM, res.FIB$meanM,
+             res.LEU$meanM, res.NB$meanM), type = "b",
+       ylab = "Average expression stability M",
+       xlab = "Number of remaining control genes",
+       axes = FALSE, pch = 19, col = mypalette,
+       ylim = c(0.2, 1.22), lty = 1, lwd = 2,
+       main = "Figure 2 in Vandesompele et al. (2002)")
> axis(1, at = 1:9, labels = as.character(10:2))
> axis(2, at = seq(0.2, 1.2, by = 0.2), labels = seq(0.2, 1.2, by = 0.2))
> box()
> abline(h = seq(0.2, 1.2, by = 0.2), lty = 2, lwd = 1, col = "grey")
> legend("topright", legend = c("BM", "POOL", "FIB", "LEU", "NB"),
+       fill = mypalette)
```


Figure 2 in Vandesompele et al. (2002)



Second, we plot the pairwise variation for each cell type (see Figure 3 (a) in Vand02)

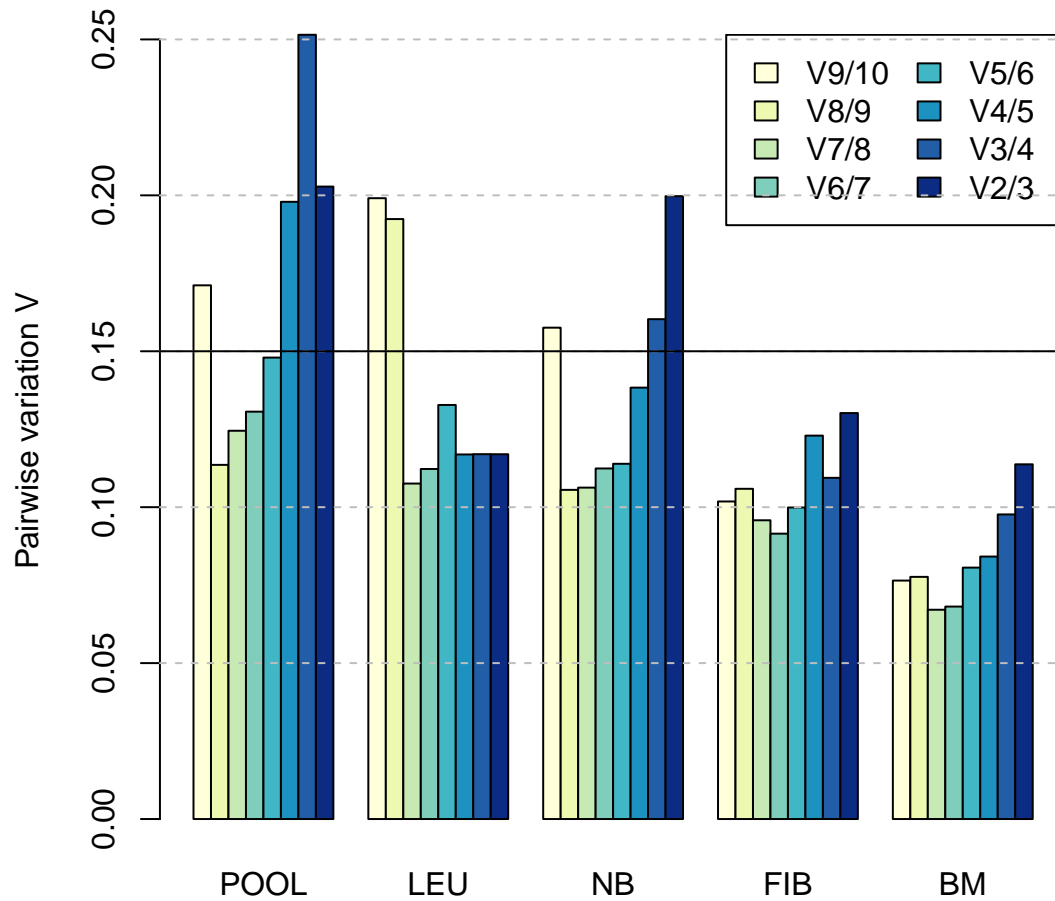
```
> mypalette <- brewer.pal(8, "YlGnBu")
> barplot(cbind(res.POOL$variation, res.LEU$variation, res.NB$variation,
+             res.FIB$variation, res.BM$variation), beside = TRUE,
+         col = mypalette, space = c(0, 2),
+         names.arg = c("POOL", "LEU", "NB", "FIB", "BM"),
+         ylab = "Pairwise variation V",
+         main = "Figure 3(a) in Vandesompele et al. (2002)")
```

```

> legend("topright", legend = c("V9/10", "V8/9", "V7/8", "V6/7",
+                               "V5/6", "V4/5", "V3/4", "V2/3"),
+       fill = mypalette, ncol = 2)
> abline(h = seq(0.05, 0.25, by = 0.05), lty = 2, col = "grey")
> abline(h = 0.15, lty = 1, col = "black")

```

Figure 3(a) in Vandesompele et al. (2002)



Remark 2:

Vand02 recommend a cut-off value of 0.15 for the pairwise variation. Below this bound the inclusion of an additional housekeeping gene is not required.

4.2 NormFinder

The second method for selection reference/housekeeping genes implemented in package is the method derived by [1] (in the sequel: And04) called *NormFinder*.

The ranking contained in Table 3 of And04 can be obtained via

```
> data(Colon)
> Colon

qPCRBatch (storageMode: lockedEnvironment)
assayData: 13 features, 40 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: I459N 90 ... I-C1056T (40 total)
  varLabels: Sample.no. Classification
  varMetadata: labelDescription
featureData
  featureNames: UBC UBB ... TUBA6 (13 total)
  fvarLabels: Symbol Gene.name
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Table 1 in Andersen et al. (2004)

> Class <- pData(Colon)[,"Classification"]
> res.Colon <- stabMeasureRho(Colon, group = Class, log = FALSE)
> sort(res.Colon) # see Table 3 in Andersen et al (2004)
```

| | UBC | GAPD | TPT1 | UBB | TUBA6 | RPS13 |
|--|-----------|-----------|-----------|-----------|-----------|-----------|
| | 0.1821707 | 0.2146061 | 0.2202956 | 0.2471573 | 0.2700641 | 0.2813039 |
| | NACA | CFL1 | SUI1 | ACTB | CLTC | RPS23 |
| | 0.2862397 | 0.2870467 | 0.3139404 | 0.3235918 | 0.3692880 | 0.3784909 |
| | FLJ20030 | | | | | |
| | 0.3935173 | | | | | |

```
> data(Bladder)
> Bladder

qPCRBatch (storageMode: lockedEnvironment)
assayData: 14 features, 28 samples
  element names: exprs
protocolData: none
phenoData
```

```

sampleNames: 335-6 1131-1 ... 1356-1 (28 total)
varLabels: Sample.no. Grade
varMetadata: labelDescription
featureData
  featureNames: ATP5B HSPCB ... FLJ20030 (14 total)
  fvarLabels: Symbol Gene.name
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation: Table 1 in Andersen et al. (2004)

> grade <- pData(Bladder)[,"Grade"]
> res.Bladder <- stabMeasureRho(Bladder, group = grade,
+                               log = FALSE)
> sort(res.Bladder)

      HSPCB      TEGT      ATP5B      UBC      RPS23      RPS13
0.1539598 0.1966556 0.1987227 0.2033477 0.2139626 0.2147852
      CFL1  FLJ20030      TPT1      UBB      FLOT2      GAPD
0.2666129 0.2672918 0.2691553 0.2826051 0.2960429 0.3408742
      S100A6      ACTB
0.3453435 0.3497295

```

Of course, we can also reproduce the geNorm ranking also included in Table 3 of And04.

```

> selectHKs(Colon, log = FALSE, trace = FALSE,
+           Symbols = featureNames(Colon))$ranking

      1      1      3      4      5      6
"RPS23"  "TPT1"  "RPS13"  "SUI1"  "UBC"  "GAPD"
      7      8      9     10     11     12
"UBA6"   "UBB"   "NACA"  "CFL1"  "CLTC" "ACTB"
      13
"FLJ20030"

> selectHKs(Bladder, log = FALSE, trace = FALSE,
+           Symbols = featureNames(Bladder))$ranking

      1      1      3      4      5      6
"CFL1"   "UBC"  "ATP5B"  "HSPCB"  "GAPD"  "TEGT"
      7      8      9     10     11     12
"RPS23"  "RPS13"  "TPT1"  "FLJ20030"  "FLOT2"  "UBB"
      13      14
"ACTB"   "S100A6"

```

As we are often interested in more than one reference/housekeeping gene we also implemented a step-wise procedure of the NormFinder algorithm explained in Section “Average control gene” in the supplementary information of And04. This procedure is available via function `selectHKs`.

```
> Class <- pData(Colon)[,"Classification"]
> selectHKs(Colon, group = Class, log = FALSE, trace = TRUE,
+           Symbols = featureNames(Colon), minNrHKs = 12,
+           method = "NormFinder")$ranking
```

| | | | | | |
|------------|------------|------------|------------|------------|------------|
| UBC | GAPD | TPT1 | UBB | TUBA6 | RPS13 |
| 0.1821707 | 0.2146061 | 0.2202956 | 0.2471573 | 0.2700641 | 0.2813039 |
| NACA | CFL1 | SUI1 | ACTB | CLTC | RPS23 |
| 0.2862397 | 0.2870467 | 0.3139404 | 0.3235918 | 0.3692880 | 0.3784909 |
| FLJ20030 | | | | | |
| 0.3935173 | | | | | |
| GAPD | TPT1 | UBB | NACA | CFL1 | RPS13 |
| 0.1375298 | 0.1424519 | 0.1578360 | 0.1657364 | 0.1729069 | 0.1837057 |
| TUBA6 | SUI1 | ACTB | RPS23 | FLJ20030 | CLTC |
| 0.1849021 | 0.2065531 | 0.2131651 | 0.2188277 | 0.2359623 | 0.2447588 |
| TPT1 | NACA | UBB | RPS13 | CFL1 | TUBA6 |
| 0.1108474 | 0.1299802 | 0.1356690 | 0.1411173 | 0.1474242 | 0.1532953 |
| FLJ20030 | SUI1 | ACTB | RPS23 | CLTC | |
| 0.1583031 | 0.1586250 | 0.1682972 | 0.1686139 | 0.1926907 | |
| UBB | TUBA6 | ACTB | CFL1 | RPS13 | SUI1 |
| 0.09656546 | 0.09674897 | 0.10753445 | 0.10830099 | 0.11801680 | 0.12612399 |
| CLTC | NACA | FLJ20030 | RPS23 | | |
| 0.12773131 | 0.13422958 | 0.14609897 | 0.16530522 | | |
| RPS13 | SUI1 | TUBA6 | NACA | FLJ20030 | CFL1 |
| 0.09085973 | 0.09647829 | 0.09943424 | 0.10288912 | 0.11097074 | 0.11428399 |
| ACTB | RPS23 | CLTC | | | |
| 0.11495336 | 0.12635109 | 0.13286210 | | | |
| ACTB | TUBA6 | CFL1 | FLJ20030 | NACA | CLTC |
| 0.09215478 | 0.09499893 | 0.09674032 | 0.10528784 | 0.10718604 | 0.10879846 |
| SUI1 | RPS23 | | | | |
| 0.11368091 | 0.13134766 | | | | |
| SUI1 | NACA | FLJ20030 | RPS23 | TUBA6 | CFL1 |
| 0.08281504 | 0.08444905 | 0.08922236 | 0.09072667 | 0.10559279 | 0.10993755 |
| CLTC | | | | | |
| 0.13142181 | | | | | |
| NACA | CFL1 | TUBA6 | FLJ20030 | CLTC | RPS23 |
| 0.08336046 | 0.08410148 | 0.09315528 | 0.09775742 | 0.10499056 | 0.10554332 |

| | | | | | |
|------------|------------|------------|------------|------------|--------|
| CFL1 | TUBA6 | CLTC | FLJ20030 | RPS23 | |
| 0.07222968 | 0.07722737 | 0.08440691 | 0.09831958 | 0.12735605 | |
| FLJ20030 | TUBA6 | CLTC | RPS23 | | |
| 0.08162006 | 0.08189011 | 0.10705192 | 0.11430674 | | |
| TUBA6 | CLTC | RPS23 | | | |
| 0.06978897 | 0.08069582 | 0.13702726 | | | |
| CLTC | RPS23 | | | | |
| 0.1199009 | 0.1245241 | | | | |
| 1 | 2 | 3 | 4 | 5 | 6 |
| "UBC" | "GAPD" | "TPT1" | "UBB" | "RPS13" | "ACTB" |
| 7 | 8 | 9 | 10 | 11 | 12 |
| "SUI1" | "NACA" | "CFL1" | "FLJ20030" | "TUBA6" | "CLTC" |

In case of the Bladder dataset the two top ranked genes are HSPCB and RPS13; see Figure 1 in And04.

```
> grade <- pData(Bladder)[,"Grade"]
> selectHKs(Bladder, group = grade, log = FALSE, trace = FALSE,
+           Symbols = featureNames(Bladder), minNrHKs = 13,
+           method = "NormFinder")$ranking
```

| | | | | | |
|---------|------------|--------|----------|---------|--------|
| 1 | 2 | 3 | 4 | 5 | 6 |
| "HSPCB" | "RPS13" | "UBC" | "RPS23" | "ATP5B" | "TEGT" |
| 7 | 8 | 9 | 10 | 11 | 12 |
| "UBB" | "FLJ20030" | "CFL1" | "S100A6" | "FLOT2" | "ACTB" |
| 13 | | | | | |
| "TPT1" | | | | | |

5 Normalization by means of reference/housekeeping genes

5.1 ΔCq method using a single housekeeper

The ΔCq method normalises detectors within a sample by subtracting the cycle time value of the housekeeper gene from the other genes. This can be done in NormqPCR as follows:

for the example dataset from "ReadqPCR" we must first read in the data:

```
> path <- system.file("exData", package = "NormqPCR")
> taqman.example <- file.path(path, "example.txt")
> qPCR.example <- file.path(path, "qPCR.example.txt")
> qPCRBatch.taqman <- read.taqman(taqman.example)
```

We then need to supply a housekeeper gene to be subtracted:

```
> hkgs<-"Actb-Rn00667869_m1"
> qPCRBatch.norm <- deltaCq(qPCRBatch = qPCRBatch.taqman, hkgs = hkgs, calc="arith")
> head(exprs(qPCRBatch.norm))
```

| | fp1.day3.v | fp2.day3.v | fp5.day3.mia |
|----------------------|------------|------------|--------------|
| Actb.Rn00667869_m1 | 0.000000 | 0.000000 | 0.000000 |
| Adipoq.Rn00595250_m1 | 0.016052 | -0.116520 | 2.933523 |
| Adrbk1.Rn00562822_m1 | NA | NA | 6.566628 |
| Agtrl1.Rn00580252_s1 | 4.899380 | 5.035841 | 6.397364 |
| Alpl.Rn00564931_m1 | 12.531942 | 11.808657 | 13.035166 |
| B2m.Rn00560865_m1 | 0.741558 | 0.890717 | 2.040470 |

| | fp6.day3.mia | fp.3.day.3.v | fp.4.day.3.v |
|----------------------|--------------|--------------|--------------|
| Actb.Rn00667869_m1 | 0.000000 | 0.000000 | 0.000000 |
| Adipoq.Rn00595250_m1 | 2.540987 | -0.178971 | -0.563263 |
| Adrbk1.Rn00562822_m1 | 6.642561 | NA | NA |
| Agtrl1.Rn00580252_s1 | 5.680837 | 5.220796 | 4.425364 |
| Alpl.Rn00564931_m1 | 12.239549 | 12.394802 | 11.772896 |
| B2m.Rn00560865_m1 | 2.234605 | 0.505516 | 0.877598 |

| | fp.7.day.3.mia | fp.8.day.3.mia |
|----------------------|----------------|----------------|
| Actb.Rn00667869_m1 | 0.000000 | 0.000000 |
| Adipoq.Rn00595250_m1 | 2.458509 | 2.736475 |
| Adrbk1.Rn00562822_m1 | 3.737100 | 6.873568 |
| Agtrl1.Rn00580252_s1 | 4.794776 | 5.345202 |
| Alpl.Rn00564931_m1 | 12.110000 | 12.255186 |
| B2m.Rn00560865_m1 | 1.927563 | 1.903269 |

This returns a new `qPCRBatch`, with new values in the `exprs` slot. This will be compatible with many other bioconductor and R packages, such as `heatmap`.

Note these numbers might be negative. For further analysis requiring positive values only, $2^{\Delta CT}$ can be used to transform the data into $2^{\Delta CT}$ values.

5.2 ΔCq method using a combination of housekeeping genes

If the user wishes to normalise by more than one housekeeping gene, for example if they have found a more than one housekeeping gene using the `NormFinder/geNorm` algorithms described above, they can. This is implemented by calculating the average of these values to form a "pseudo-housekeeper" which is subtracted from the other values. So using the same dataset as above, using housekeeping genes `GAPDH`, `Beta-2-microglobulin` and `Beta-actin`, the following steps would be taken:

```
> hkgs<-c("Actb-Rn00667869_m1", "B2m-Rn00560865_m1", "Gapdh-Rn99999916_s1")
> qPCRBatch.norm <- deltaCq(qPCRBatch = qPCRBatch.taqman, hkgs = hkgs, calc="arith")
> head(exprs(qPCRBatch.norm))
```

| | fp1.day3.v | fp2.day3.v | fp5.day3.mia |
|----------------------|----------------|----------------|--------------|
| Actb.Rn00667869_m1 | -1.2998917 | -1.2816963 | -1.380296 |
| Adipoq.Rn00595250_m1 | -1.2838397 | -1.3982163 | 1.553227 |
| Adrbk1.Rn00562822_m1 | NA | NA | 5.186332 |
| Agtrl1.Rn00580252_s1 | 3.5994883 | 3.7541447 | 5.017068 |
| Alpl.Rn00564931_m1 | 11.2320503 | 10.5269607 | 11.654870 |
| B2m.Rn00560865_m1 | -0.5583337 | -0.3909793 | 0.660174 |
| | fp6.day3.mia | fp.3.day.3.v | fp.4.day.3.v |
| Actb.Rn00667869_m1 | -1.5106197 | -1.1644617 | -1.1714227 |
| Adipoq.Rn00595250_m1 | 1.0303673 | -1.3434327 | -1.7346857 |
| Adrbk1.Rn00562822_m1 | 5.1319413 | NA | NA |
| Agtrl1.Rn00580252_s1 | 4.1702173 | 4.0563343 | 3.2539413 |
| Alpl.Rn00564931_m1 | 10.7289293 | 11.2303403 | 10.6014733 |
| B2m.Rn00560865_m1 | 0.7239853 | -0.6589457 | -0.2938247 |
| | fp.7.day.3.mia | fp.8.day.3.mia | |
| Actb.Rn00667869_m1 | -1.323712 | -1.286277 | |
| Adipoq.Rn00595250_m1 | 1.134797 | 1.450198 | |
| Adrbk1.Rn00562822_m1 | 2.413388 | 5.587291 | |
| Agtrl1.Rn00580252_s1 | 3.471064 | 4.058925 | |
| Alpl.Rn00564931_m1 | 10.786288 | 10.968909 | |
| B2m.Rn00560865_m1 | 0.603851 | 0.616992 | |

5.3 $2^{-\Delta\Delta Cq}$ method using a single housekeeper

It is possible to use the $2^{-\Delta\Delta Cq}$ method for calculating relative gene expression between two sample types. Both the same well and the separate well methods as detailed in [2] can be used for this purpose, and will produce the same answers, but with different levels of variation. By default detectors in the same sample will be paired with the housekeeper, and the standard deviation used will be that of the differences between detectors and the housekeepers. However, if the argument `paired=FALSE` is added, standard deviation between case and control will be calculated as $s = \sqrt{s_1^2 + s_2^2}$, where s_1 is the standard deviation for the detector readings and s_2 is the standard deviation the housekeeper gene readings. The latter approach is not recommended when the housekeeper and genes to be compared are from the same sample, as is the case when using the taqman cards, but is included for completeness and for situations where readings for the housekeeper might be taken from a separate biological replicate (for example in a *post hoc* manner due to the originally designated housekeeping genes not performing well), or for when NormqPCR is used for more traditional qPCR where the products undergo amplifications from separate wells.

for the example dataset from "ReadqPCR" we must first read in the data:

```
> path <- system.file("exData", package = "NormqPCR")
```



```

> taqman.example <- file.path(path, "example.txt")
> qPCR.example <- file.path(path, "qPCR.example.txt")
> qPCRBatch.taqman <- read.taqman(taqman.example)

```

deltaDeltaCq also requires a contrast matrix. This is to contain columns which will be used to specify the samples representing `case` and `control` which are to be compared, in a similar way to the "limma" package. these columns should contain 1s or 0s which refer to the samples in either category:

```

> contM <- cbind(c(0,0,1,1,0,0,1,1),c(1,1,0,0,1,1,0,0))
> colnames(contM) <- c("interestingPhenotype","wildTypePhenotype")
> rownames(contM) <- sampleNames(qPCRBatch.taqman)
> contM

```

| | interestingPhenotype | wildTypePhenotype |
|----------------|----------------------|-------------------|
| fp1.day3.v | 0 | 1 |
| fp2.day3.v | 0 | 1 |
| fp5.day3.mia | 1 | 0 |
| fp6.day3.mia | 1 | 0 |
| fp.3.day.3.v | 0 | 1 |
| fp.4.day.3.v | 0 | 1 |
| fp.7.day.3.mia | 1 | 0 |
| fp.8.day.3.mia | 1 | 0 |

We can now normalise each sample by a given housekeeping gene and then look at the ratio of expression between the case and control samples. Results show (by column): 1) Name of gene represented by detector. 2) Case ΔCq for the detector: the average cycle time for this detector in the samples denoted as "case" - the housekeeper cycle time. 3) the standard deviation for the cycle times used to calculate the value in column 2). 4) Control ΔCq for the detector: the average cycle time for this detector in the samples denoted as "controller", or the "callibrator" samples - the housekeeper cycle time. 5) The standard deviation for the cycle times used to calculate the value in column 4). 6) $2^{-\Delta\Delta Cq}$ - The difference between the ΔCq values for case and control. We then find 2^{-} of this value. 7) and 8) correspond to 1 s.d. either side of the mean value, as detailed in [2].

```

> hkg <- "Actb-Rn00667869_m1"
> ddCq.taqman <- deltaDeltaCq(qPCRBatch = qPCRBatch.taqman, maxNAcase=1, maxNAcontrol=1,
+                             hkg=hkg, contrastM=contM, case="interestingPhenotype",
+                             control="wildTypePhenotype", statCalc="geom", hkgCalc="arith")
> head(ddCq.taqman)

```

| | ID | 2^{-dCt} .interestingPhenotype |
|---|--------------------|----------------------------------|
| 1 | Actb.Rn00667869_m1 | 1.000e+00 |

```

2 Adipoq.Rn00595250_m1          1.587e-01
3 Adrbk1.Rn00562822_m1          2.602e-02
4 Agtrl1.Rn00580252_s1          2.300e-02
5 Alpl.Rn00564931_m1            1.892e-04
6 B2m.Rn00560865_m1            2.464e-01
interestingPhenotype.sd 2^-dCt.wildTypePhenotype
1          0.000e+00          1.000e+00
2          2.280e-02          1.171e+00
3          3.266e-02          NA
4          1.014e-02          3.434e-02
5          4.770e-05          2.298e-04
6          2.498e-02          5.965e-01
wildTypePhenotype.sd          2^-ddCt 2^-ddCt.min 2^-ddCt.max
1          0.000e+00 1          NA          NA
2          2.131e-01 0.135541545192243          NA          NA
3          NA +          NA          NA
4          8.584e-03 0.669721905042939          NA          NA
5          6.107e-05 0.823327272466571          NA          NA
6          7.668e-02 0.413128242070071          NA          NA

```

We can also average the taqman data using the separate samples/wells method . Here standard deviation is calculated separately and then combined, as described above. Therefore the pairing of housekeeper with the detector value within the same sample is lost. This can potentially increase variance.

```

> hkg <- "Actb-Rn00667869_m1"
> ddCqAvg.taqman <- deltaDeltaCq(qPCRBatch = qPCRBatch.taqman, maxNACase=1, maxNAControl=1,
+                               hkg=hkg, contrastM=contM, case="interestingPhenotype",
+                               control="wildTypePhenotype", paired=FALSE, statCalc="geom",
+                               hkgCalc="arith")
> head(ddCqAvg.taqman)
          ID 2^-dCt.interestingPhenotype
1 Actb.Rn00667869_m1          1.000e+00
2 Adipoq.Rn00595250_m1          1.587e-01
3 Adrbk1.Rn00562822_m1          2.602e-02
4 Agtrl1.Rn00580252_s1          2.300e-02
5 Alpl.Rn00564931_m1            1.892e-04
6 B2m.Rn00560865_m1            2.464e-01
interestingPhenotype.sd 2^-dCt.wildTypePhenotype
1          0.000e+00          1.000e+00
2          2.280e-02          1.171e+00

```

| | | | |
|---|----------------------|-------------------|--|
| 3 | 3.266e-02 | | NA |
| 4 | 1.014e-02 | | 3.434e-02 |
| 5 | 4.770e-05 | | 2.298e-04 |
| 6 | 2.498e-02 | | 5.965e-01 |
| | wildTypePhenotype.sd | | 2 ⁻ -ddCt 2 ⁻ -ddCt.min 2 ⁻ -ddCt.max |
| 1 | 0.000e+00 | 1 | NA NA |
| 2 | 2.131e-01 | 0.135541545192243 | NA NA |
| 3 | NA | + | NA NA |
| 4 | 8.584e-03 | 0.669721905042939 | NA NA |
| 5 | 6.107e-05 | 0.823327272466571 | NA NA |
| 6 | 7.668e-02 | 0.413128242070071 | NA NA |

5.4 $2^{\Delta\Delta Cq}$ method using a combination of housekeeping genes

If the user wishes to normalise by more than one housekeeping gene, for example if they have found a more than one housekeeping gene using the NormFinder/geNorm algorithms described above, they can. This is implemented by calculating the average of these values using the geometric mean to form a "pseudo-housekeeper" which is subtracted from the other values. For the dataset above, using housekeeping genes GAPDH, Beta-2-microglobulin and Beta-actin:

```
> qPCRBatch.taqman <- read.taqman(taqman.example)
> contM <- cbind(c(0,0,1,1,0,0,1,1),c(1,1,0,0,1,1,0,0))
> colnames(contM) <- c("interestingPhenotype","wildTypePhenotype")
> rownames(contM) <- sampleNames(qPCRBatch.taqman)
> hkgs<-c("Actb-Rn00667869_m1", "B2m-Rn00560865_m1", "Gapdh-Rn99999916_s1")
> ddCq.gM.taqman <- deltaDeltaCq(qPCRBatch = qPCRBatch.taqman, maxNACase=1, maxNAControl=1,
+                               hkgs=hkgs, contrastM=contM, case="interestingPhenotype",
+                               control="wildTypePhenotype", statCalc="arith", hkgCalc="ari
> head(ddCq.gM.taqman)
```

| | ID | 2 ⁻ -dCt.interestingPhenotype |
|---|-------------------------|--|
| 1 | Actb.Rn00667869_m1 | 2.594e+00 |
| 2 | Adipoq.Rn00595250_m1 | 4.083e-01 |
| 3 | Adrbk1.Rn00562822_m1 | 4.182e-02 |
| 4 | Agtrl1.Rn00580252_s1 | 5.520e-02 |
| 5 | Alpl.Rn00564931_m1 | 4.767e-04 |
| 6 | B2m.Rn00560865_m1 | 6.367e-01 |
| | interestingPhenotype.sd | 2 ⁻ -dCt.wildTypePhenotype |
| 1 | 0.09819 | 2.345e+00 |
| 2 | 0.24929 | 2.713e+00 |
| 3 | 1.45844 | NA |

| | | | |
|---|----------------------|-------------------|---|
| 4 | 0.63719 | | 7.878e-02 |
| 5 | 0.42589 | | 5.242e-04 |
| 6 | 0.05413 | | 1.390e+00 |
| | wildTypePhenotype.sd | | 2 ⁻ ddCt 2 ⁻ ddCt.min 2 ⁻ ddCt.max |
| 1 | 0.071373 | 1.10638851325547 | 1.034e+00 1.184310 |
| 2 | 0.201905 | 0.150497255530234 | 1.266e-01 0.178884 |
| 3 | NA | + | NA NA |
| 4 | 0.333840 | 0.700597907024805 | 4.505e-01 1.089636 |
| 5 | 0.386280 | 0.909381199520663 | 6.769e-01 1.221662 |
| 6 | 0.163975 | 0.457939394245865 | 4.411e-01 0.475448 |

There is also the option of using the mean housekeeper method using shared variance between the samples being compared, similar to the second `deltaDeltaCq` method shown above.

```

> qPCRBatch.taqman <- read.taqman(taqman.example)
> contM <- cbind(c(0,0,1,1,0,0,1,1),c(1,1,0,0,1,1,0,0))
> colnames(contM) <- c("interestingPhenotype","wildTypePhenotype")
> rownames(contM) <- sampleNames(qPCRBatch.taqman)
> hkg<-c("Actb-Rn00667869_m1", "B2m-Rn00560865_m1", "Gapdh-Rn99999916_s1")
> ddAvgCq.gM.taqman <-deltaDeltaCq(qPCRBatch = qPCRBatch.taqman, maxNACase=1, maxNAControl=1
+                               hkg=hkgs, contrastM=contM, case="interestingPhenotype",
+                               control="wildTypePhenotype", paired=FALSE, statCalc="arith
+                               hkgCalc="arith")
> head(ddAvgCq.gM.taqman)

```

| | ID | 2 ⁻ dCt.interestingPhenotype |
|---|-------------------------|---|
| 1 | Actb.Rn00667869_m1 | 2.594e+00 |
| 2 | Adipoq.Rn00595250_m1 | 4.083e-01 |
| 3 | Adrbk1.Rn00562822_m1 | 4.182e-02 |
| 4 | Agtrl1.Rn00580252_s1 | 5.520e-02 |
| 5 | Alpl.Rn00564931_m1 | 4.767e-04 |
| 6 | B2m.Rn00560865_m1 | 6.367e-01 |
| | interestingPhenotype.sd | 2 ⁻ dCt.wildTypePhenotype |
| 1 | 0.3849 | 2.345e+00 |
| 2 | 0.4822 | 2.713e+00 |
| 3 | 1.4545 | NA |
| 4 | 0.6905 | 7.878e-02 |
| 5 | 0.5846 | 5.242e-04 |
| 6 | 0.2777 | 1.390e+00 |
| | wildTypePhenotype.sd | 2 ⁻ ddCt 2 ⁻ ddCt.min 2 ⁻ ddCt.max |
| 1 | 0.3574 | 1.10638851325547 8.473e-01 1.444684 |

| | | | | |
|---|--------|-------------------|-----------|----------|
| 2 | 0.2495 | 0.150497255530234 | 1.077e-01 | 0.210221 |
| 3 | NA | + | NA | NA |
| 4 | 0.2813 | 0.700597907024805 | 4.341e-01 | 1.130625 |
| 5 | 0.3689 | 0.909381199520663 | 6.064e-01 | 1.363762 |
| 6 | 0.4576 | 0.457939394245865 | 3.778e-01 | 0.555126 |

TO SHOW EXAMPLE USING GENORM/NORMFINDER DATA

5.5 Compute NRQs

THIS FUNCTION IS STILL EXPERIMENTAL!

We load a dataset including technical replicates.

```
> path <- system.file("exData", package = "ReadqPCR")
> qPCR.example <- file.path(path, "qPCR.example.txt")
> Cq.data <- read.qPCR(qPCR.example)
```

We combine the technical replicates and in addition compute standard deviations.

```
> Cq.data1 <- combineTechRepsWithSD(Cq.data)
```

We load efficiencies for the dataset and add them to the dataset.

```
> Effs <- file.path(path, "Efficiencies.txt")
> Cq.effs <- read.table(file = Effs, row.names = 1, header = TRUE)
> rownames(Cq.effs) <- featureNames(Cq.data1)
> effs(Cq.data1) <- as.matrix(Cq.effs[, "efficiency", drop = FALSE])
> se.effs(Cq.data1) <- as.matrix(Cq.effs[, "SD.efficiency", drop = FALSE])
```

Now we can compute normalized relative quantities for the dataset where we consider two of the included features as reference/housekeeping genes.

```
> res <- ComputeNRQs(Cq.data1, hkgs = c("gene_az", "gene_gx"))
> ## NRQs
> exprs(res)
```

| | caseA | caseB | controlA | controlB |
|---------|-----------|-----------|-----------|-----------|
| gene_ai | 1.9253072 | 1.3586729 | 0.6479659 | 0.8749479 |
| gene_az | 1.0567118 | 1.1438982 | 1.0331980 | 0.9134997 |
| gene_bc | 1.1024935 | 0.7193500 | 0.7030487 | 1.2140836 |
| gene_by | 1.5102316 | 0.9573047 | 0.7527082 | 1.6008850 |
| gene_dh | 1.2982037 | 1.0722522 | 0.9623335 | 0.9392871 |
| gene_dm | 0.6590246 | 1.1690720 | 1.2475372 | 0.9366210 |
| gene_dq | 0.7541955 | 0.7036408 | 0.8327917 | 1.6165326 |

```

gene_dr 2.2192305 1.0581211 0.7026411 0.6900584
gene_eg 0.9366671 0.5800339 0.8313720 1.1848856
gene_er 0.5269062 0.9375427 0.6953326 2.3195978
gene_ev 1.4622280 2.3457021 0.9038912 1.1454535
gene_fr 1.4954763 1.6200792 0.9641192 0.7295680
gene_fw 0.6944248 0.8051075 1.5698382 0.7978611
gene_gx 0.9463318 0.8742037 0.9678687 1.0946911
gene_hl 1.0009372 1.4015267 0.7683665 0.7713712
gene_il 1.4632019 1.2595559 0.7216891 0.9318860
gene_iv 1.7263335 1.2275001 1.5464212 0.8881605
gene_jr 0.8984351 0.9834026 0.8754813 0.6637941
gene_jw 1.4655948 0.9340184 1.0505200 1.5504136
gene_qs 0.6730225 0.7610418 1.0665938 3.5329891
gene_qy 0.5287127 1.5722670 1.0615326 3.3252907
gene_rz 0.8690600 1.5588299 0.7287288 1.4812753
gene_sw 0.5975288 1.2406438 0.6982954 1.6007333
gene_vx 0.6942254 0.7168408 2.0253177 1.3190943
gene_xz 0.7668030 1.0218209 0.6136038 1.6729352

```

```

> ## SD of NRQs
> se.exprs(res)

```

```

           caseA      caseB  controlA  controlB
gene_ai 1.3996554 0.8787290 0.4855882 1.0034912
gene_az 0.6832730 0.7601966 0.8971054 0.6031927
gene_bc 0.7225348 0.4746146 0.9626570 1.0478385
gene_by 1.1522746 0.6116269 0.6088836 2.0409211
gene_dh 1.2483072 0.7889984 0.6165041 0.9767947
gene_dm 0.4711409 0.7780238 0.8476053 0.7294405
gene_dq 0.7023561 0.4849899 0.5813310 1.4067670
gene_dr 1.4407662 1.0804211 0.4543153 0.5149367
gene_eg 0.7355269 0.5497433 0.5588801 1.0938601
gene_er 0.4301195 0.6119514 0.4471454 1.5115897
gene_ev 1.0094209 2.4267114 0.6337126 0.7782519
gene_fr 1.6760391 1.1119157 0.6226081 0.5040967
gene_fw 0.5041070 0.9131565 1.1153268 0.7234551
gene_gx 0.6046042 0.9027816 0.6713914 1.7394961
gene_hl 0.7633174 0.9123997 1.0000329 0.5005813
gene_il 1.4621406 0.9540445 0.5678634 0.6067147
gene_iv 1.2668346 0.8039841 0.9995225 0.8171996
gene_jr 0.5749672 0.6786989 0.5595295 0.4405919

```

gene_jw 0.9626606 0.7890401 0.7194378 1.1512512
gene_qs 0.5830335 0.5309990 0.6828952 2.8253799
gene_qy 0.5947918 1.1294199 0.6794829 2.1713340
gene_rz 0.5846751 1.7926435 0.4911506 2.1424580
gene_sw 0.6284440 0.8062083 0.9638307 1.8398593
gene_vx 0.5285361 1.0126959 1.3861226 0.8683886
gene_xz 0.5231477 0.9270275 0.3972901 1.3643840

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