

Visualization and assessment of RNA-Seq data normalization - the Normalization Visualization Tool

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Abstract

Measuring differential gene expression is a common task in the analysis of RNA-Seq data. To identify differentially expressed genes between two samples, it is crucial to normalize the data sets. While multiple normalization methods are available, all of them are based on certain assumptions that may or may not be suitable for the type of data they are applied on. Researchers therefore need to select an adequate normalization strategy for each RNA-Seq experiment. This selection includes exploration of different normalization methods as well as their comparison. Methods that agree with each other most likely represent realistic assumptions under the particular experimental conditions. We developed the *NVT* package, which provides a fast and simple way to analyze and evaluate multiple normalization methods via visualization and representation of correlation values, based on a user-defined set of uniformly expressed genes. This vignette explains the use of the package and demonstrates a typical work flow.

NVT version: 1.0

If you use *NVT* in published research, please cite:

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1 Installation

The latest NVT package can be downloaded from: <https://github.com/Edert/NVT/releases>. The installation of the package can be done directly in R.

```
install.packages(file.path("/home/user/Downloads/", "NVT_1.0.tar.gz"),
repos=NULL, type="source")
```

2 Standard work flow

Initially the library has to be loaded, which provides access to the data examples 2.1.1 and the *NVT*functions.

```
library("NVT")
```

2.1 Load data

For the demonstration of the *NVT*functions we need gene expression data, so we load the example data provided with the package. We use the two human expression data sets *GSM1275862* and *GSM1275863* from the *airway*[1] package.

2.1.1 Load expression data

We simply load the provided data sets *mylen* from *GSM1275862*, *myexp2* from *GSM1275863* and the length data per gene as *mylen*.

```
data(mylen)
data(myexp1)
data(myexp2)

#show just the first four elements of the loaded data
head(mylen,4)

##                Length
## ENSG000000000003    3688
## ENSG000000000005    1881
## ENSG00000000419     4032
## ENSG00000000457    10784

head(myexp1,4)

##                 GSM1275862
## ENSG000000000003      679
## ENSG000000000005       0
## ENSG00000000419      467
## ENSG00000000457     260
```

In order to evaluate the expression between the two RNA-seq expression samples, we have to define a list of human housekeeping genes *GAPDH*, *ALB*, *ACTB*, *HPRT1*, *ADA*, *POLR2L*, *VCP*, *GPI*, *HBS1L* and *SDHA*. For the evaluation with the XY-Scatter or MA-plots a linear model is calculated in order to achieve reasonable results it is recommended to use at least 10 [2] data points.

```
mylist1<-c("ENSG00000111640", "ENSG00000163631", "ENSG00000075624",
          "ENSG00000165704", "ENSG00000196839", "ENSG00000177700",
          "ENSG00000165280", "ENSG00000105220", "ENSG00000112339",
          "ENSG00000073578")
```

But there is also a list of human housekeeping genes supplied by the *NVT* tool, which can be retrieved the following way:

```
data(mylist_hs)

mylist_hs
## [1] "ENSG00000111640" "ENSG00000163631" "ENSG00000075624" "ENSG00000165704"
## [5] "ENSG00000196839" "ENSG00000177700" "ENSG00000165280" "ENSG00000105220"
## [9] "ENSG00000112339" "ENSG00000073578"
```

2.1.2 Load gene length data

Instead of using the length data generated directly in R or using a simple flat file, it is also possible to load the gene or exon length data directly from an annotation file in gtf or gff format. This function utilizes the [GenomicRanges](#)[3] and [rtracklayer](#)[4] packages.

- gff-version: version of the provided gff file [*gff1*, *gff2*, *gff3*, *gtf*]
- gff-feature: feature to use [*default* : *exon*]
- gff-name: name to use [*default* : *gene_id*]

```
#this line gets the path to the gff file provided in the NVT package
#(annotation from Chlamydia trachomatis, ACCESSION: NC_000117 )
mygffpath <- system.file("extdata", "Ctr-D-UW3CX.gff", package = "NVT")

#this function loads the gff file from the gffpath
mylen1 <- NVTloadgff(mygffpath,"gff3","gene","locus_tag")

head(mylen1)

##      Length
## CT001     273
## CT002     303
## CT003    1476
## CT004    1467
## CT005    1092
## CT006     570
```

2.2 Generate NVTdata objects

In the first step you need to generate an *NVTdata* object with the *NVTinit* function. Here you have to provide the list of housekeeping genes, the two gene expression samples and the normalization method. Optionally you can also add the gene or exon length data.

The normalization methods are:

- N = No normalization
- TC = Total count normalization
- Med = Median normalization
- TMM = Trimmed Mean of M-values normalization
- UQ = Upper Quartile normalization
- UQ2 = Upper Quartile normalization (from [NOISEq\[5\]](#))
- Q = Quantile normalization implemented in [limma\[6\]](#)
- RPKM = Reads Per Kilobase per Million mapped reads normalization
- RPM = Reads Per Million mapped reads normalization
- TPM = Transcripts Per Million normalization
- DEQ = Relative log expression method included in the [DESeq\[7\]](#) package
- G = Use the provided genes to normalize

For the most methods no length information is required.

```
mynvt <- NVTinit(mylist1,myexp1,myexp2, "TMM")
```

But for RPKM and TPM normalization the length data has to be provided.

```
mynvt <- NVTinit(mylist1,myexp1,myexp2, "RPKM",mylen)
```

2.2.1 Normalize the NVTdata

The now initialized *NVTdata* object can be normalized in the next step.

```
mynorm <- NVTnormalize(mynvt)
## [1] "RPKM normalization!"
```

The text output can be suppressed via `verbose=FALSE`.

```
mynvt <- NVTnormalize(mynvt,verbose=F)
```

If required the now normalized data can be retrieved easily.

```
mynvalues <- show(mynorm)

head(mynvalues)

##                      GSM1275862  GSM1275863
## ENSG000000000003  8.9209656  5.8859979
## ENSG000000000005  0.0000000  0.0000000
## ENSG00000000419   5.6121512  6.1889890
## ENSG00000000457   1.1682249  0.9480595
## ENSG00000000460   0.2395964  0.2196300
```

```
## ENSG000000000938 0.0000000 0.0000000
```

2.3 Visualize expression data

One of the key features of *NVT* is the plotting of the XY-Scatter-Plots and MA-Plots. This can be done with the functions: *NVTplot*, *NVTadvancedplot*, *NVTmaplot* and *NVTadvancedmaplot*.

2.3.1 Simple plot functions

The normalized *NVTdata* object can be visualized with the `plot` function. With the second parameter you can modify the size-ratio of the text and the data points. All expression values are shown as grey dots and the linear model as red line, it is calculated with the data from the housekeeping genes only. The dashed grey line highlights the diagonal, so the perfect correlation between the two data sets.

```
NVTplot(mynorm, 0.4)
```

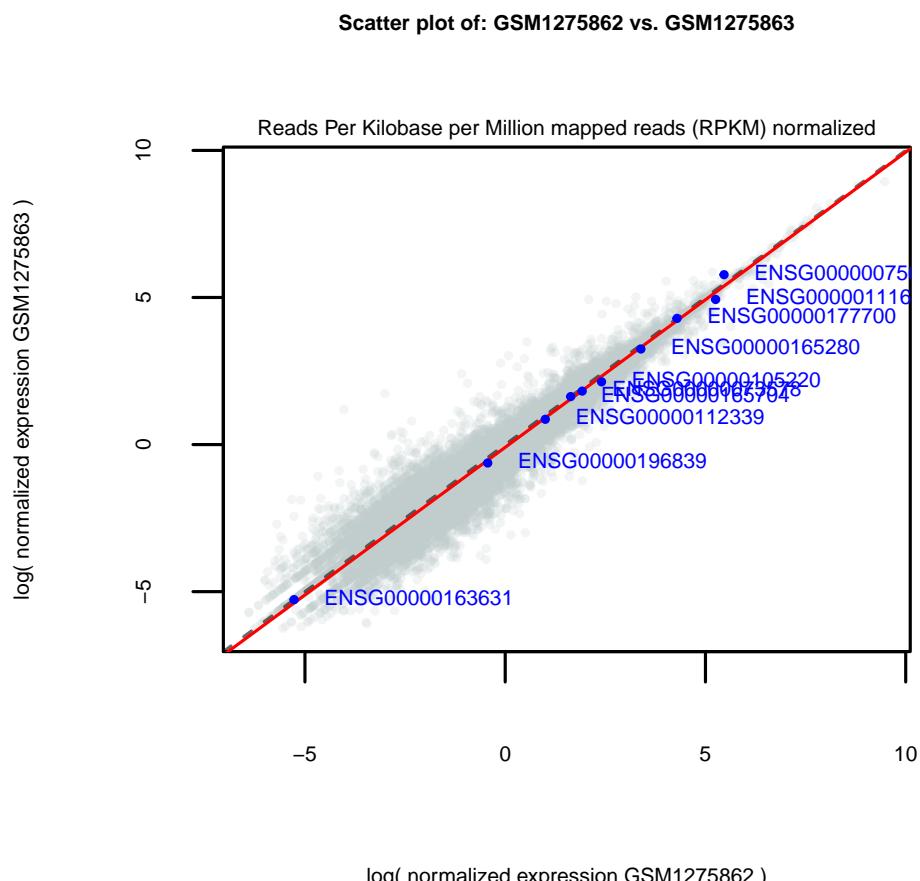


Figure 1: **XY-Scatter-Plot.** The resulting plot from the simple `NVTplot` function

The *NVTplotma* function works similar but produces an MA-Plot instead of a Scatter-Plot.

```
NVTmaplot(mynorm, 0.4)
```

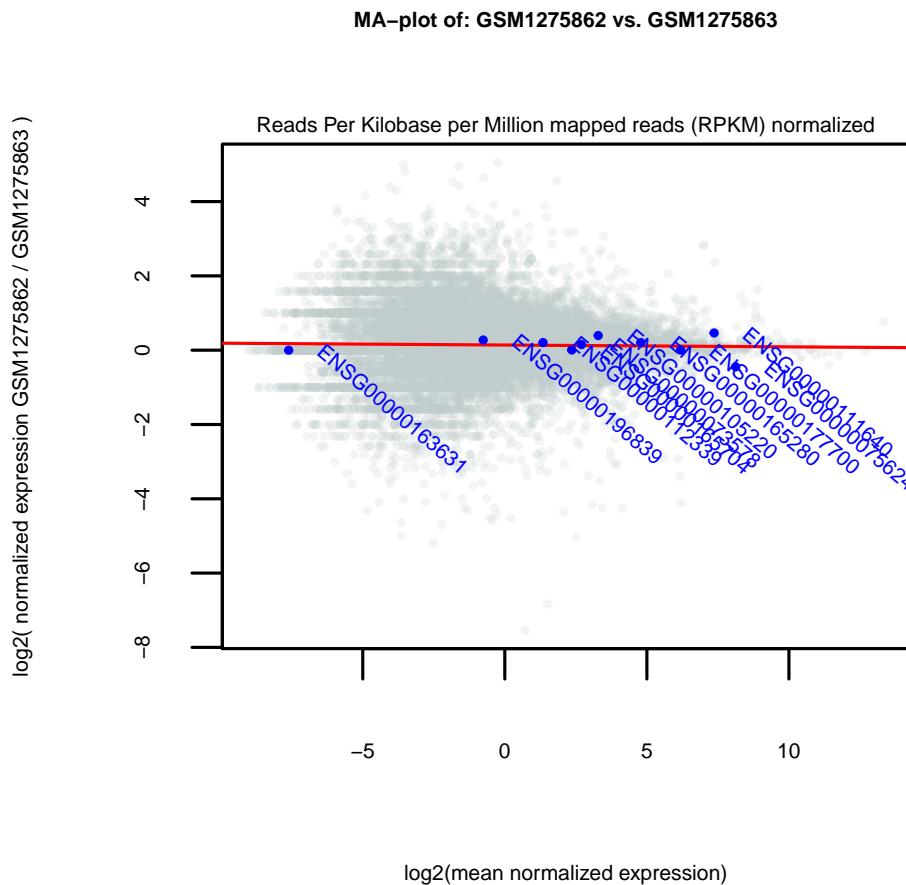


Figure 2: **MA-Plot.** The resulting plot from the simple NVTmaplot function

2.3.2 Advanced plot functions

The *NVTadvancedplot* plots via [ggplot2\[8\]](#) and the size parameters control data points, text and labels separately. Here we use the default values of 1 for the data points and the text and increase the labels of the housekeeping genes to 2. Again the grey dots represent the expression data and their density is visualized by the rug in dark red for x- and y-axis. The linear model of the housekeeping gene data is shown in red and the diagonal is highlighted as grey dashed line. As we want to compare different methods we now normalize with the TMM method.

```
mynvt <- NVTinit(mylist1,myexp1,myexp2,"TMM",mylen)
mynorm <- NVTnormalize(mynvt)
## [1] "Trimmed Mean of M-values normalization!"
NVTadvancedplot(mynorm,1,1,1)
```

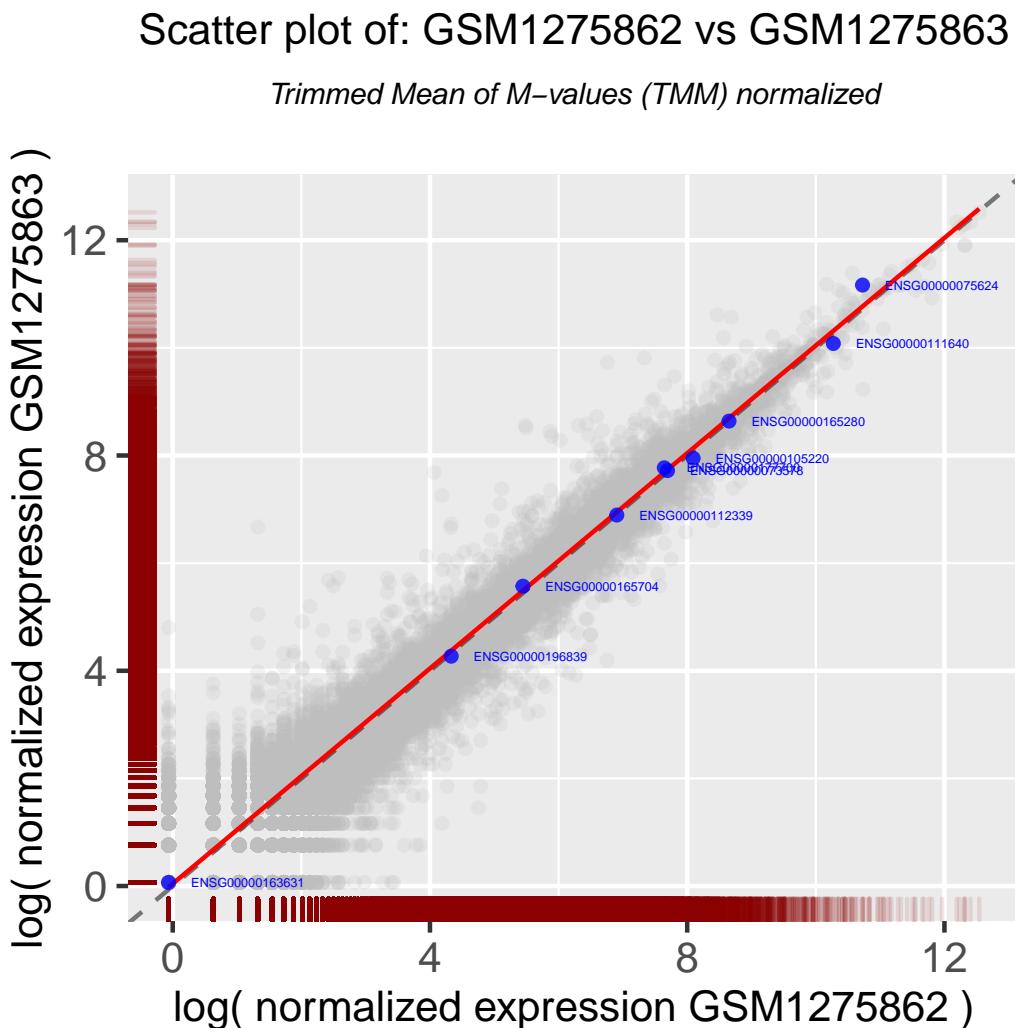


Figure 3: **Advanced XY-Scatter-Plot.** The *NVTadvancedplot* function results in a plot produced by [ggplot2](#) with a rug on both axis, indicating the density of the data-points

As alternative there is also the possibility to create an MA-Plot with the *NVTadvancedmaplot* function.

```
NVTadvancedmaplot(mynorm, 1, 1, 1)
```

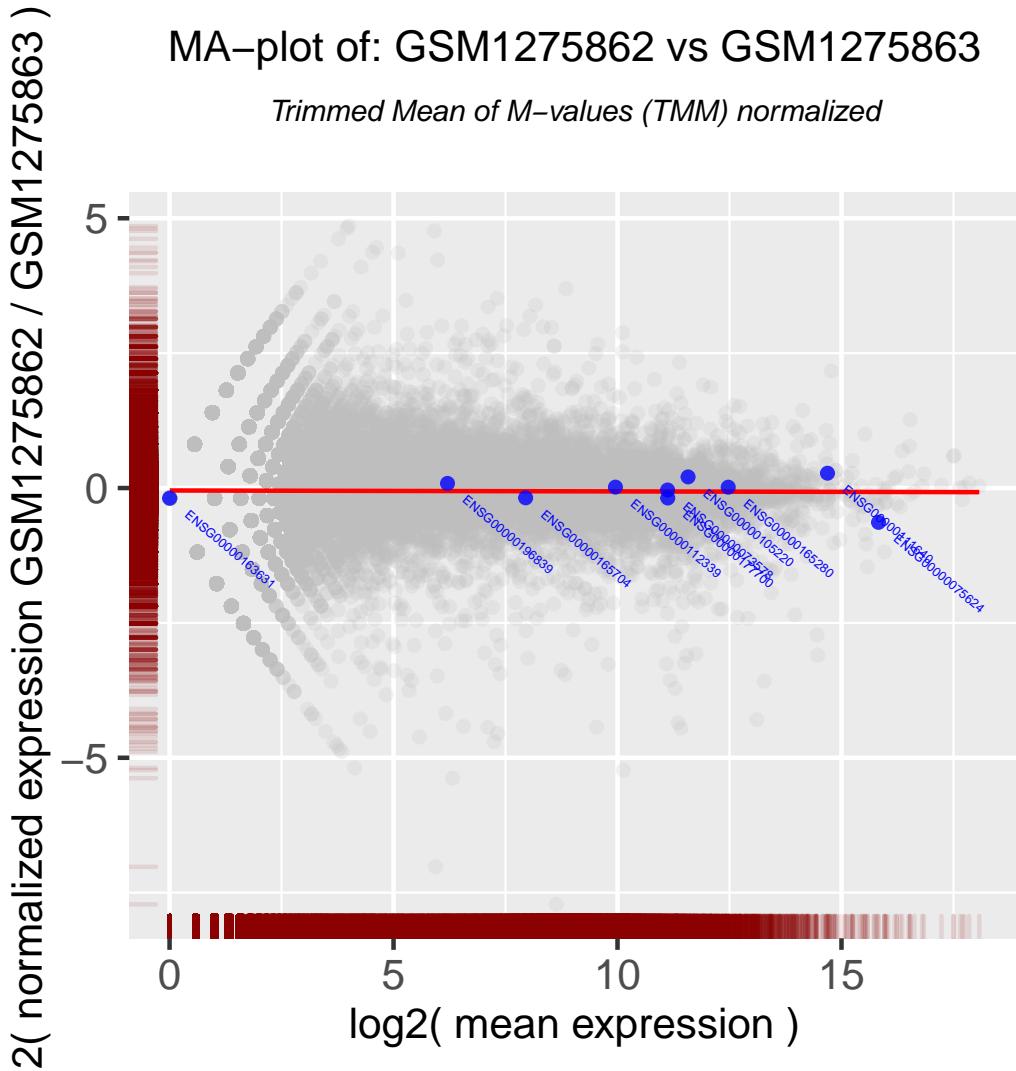


Figure 4: **Advanced MA-Plot.** The *NVTadvancedmaplot* function results in a plot produced by ggplot2 with a rug on both axis, indicating the density of the data-points

2.3.3 Linear model

If required, the linear model plotted as red line can be retrieved with the *NVTlm* function.

```
mylm <- NVTlm(mynorm)

summary(mylm)

##
## Call:
## lm(formula = sample2 ~ sample1)
##
## Residuals:
##       Min        1Q    Median        3Q       Max
## -0.23427 -0.09105 -0.03633  0.08405  0.39746
##
## Coefficients:
##             Estimate Std. Error t value Pr(>|t|)    
## (Intercept) 0.04327   0.15208   0.284   0.783    
## sample1     1.00002   0.02006  49.846 2.91e-11 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.1891 on 8 degrees of freedom
## Multiple R-squared:  0.9968, Adjusted R-squared:  0.9964 
## F-statistic: 2485 on 1 and 8 DF,  p-value: 2.905e-11
```

2.4 Correlation values

In addition to the graphical representation of the gene expression data, the correlation coefficients of the housekeeping genes of the two samples can be calculated with the functions *NVTpearson*, *NVTrmsd* and *NVTmae*.

2.4.1 Pearson correlation

The Pearson correlation coefficient of the normalized expression of the housekeeping genes is calculated with the following command, using an already normalized *NVTdata* object.

```
NVTpearson(mynorm)

##      pearson      p-value
## 9.645647e-01 6.608992e-06
```

2.4.2 RMSD and MEA correlation

The root mean square error (RMSE) also called the root mean square deviation (RMSD) is calculated with the *NVTrmsd* function.

```
NVTrmsd(mynorm)
##      RMSD
## 8137.41
```

And the mean absolute error (MAE) can be calculated and retrieved with the *NVTmae* function.

```
NVTmae(mynorm)
##      MAE
## 3114.019
```

2.5 Test all methods

To test more normalization methods on the provided data sets in one single step, the correlation coefficients of all implemented normalization methods can be calculated with the *NVTtestall* function. It requires a normalized *NVTdata* object and the correlation coefficient you are interested in. The method can be defined with:

- p = Pearson correlation
- rmsd = root mean square deviation
- mae = mean absolute error

```
NVTtestall(mynorm, "p")
## [1] "No normalization!"
## [1] "Total count normalization!"
## [1] "Median normalization!"
## [1] "Trimmed Mean of M-values normalization!"
## [1] "Upper Quartile normalization!"
## [1] "Upper Quartile normalization (from NOISeq)!"
## [1] "Quantile normalization!"
## [1] "RPKM normalization!"
## [1] "RPM normalization!"
## [1] "DESeq normalization!"
## [1] "Using DESeq"
## [1] "Input counts are not integer, converting them!"
## [1] "TPM normalization!"
## [1] "Normalization by given gene-set!"
## [1] "ENSG00000111640" "ENSG00000163631" "ENSG00000075624" "ENSG00000165704"
## [5] "ENSG00000196839" "ENSG00000177700" "ENSG00000165280" "ENSG00000105220"
## [9] "ENSG00000112339" "ENSG00000073578"
##      pearson      p-value
## Q    0.9724102 2.452009e-06
## TC   0.9645647 6.608992e-06
## N    0.9645647 6.608992e-06
## G    0.9645647 6.608992e-06
## Med  0.9645647 6.608992e-06
## TMM  0.9645647 6.608992e-06
## UQ   0.9645647 6.608992e-06
```

```
## UQ2 0.9645647 6.608992e-06
## RPM 0.9645647 6.608992e-06
## DEQ 0.9645647 6.608992e-06
## TPM 0.9515575 2.272016e-05
## RPKM 0.9515575 2.272016e-05
```

It is also possible to oppress text output via `verbose=FALSE` (default: `verbose=TRUE`)

```
NVTtestall(mynorm, "p", verbose=F)

##          pearson      p-value
## Q      0.9724102 2.452009e-06
## TC     0.9645647 6.608992e-06
## N      0.9645647 6.608992e-06
## G      0.9645647 6.608992e-06
## Med    0.9645647 6.608992e-06
## TMM    0.9645647 6.608992e-06
## UQ     0.9645647 6.608992e-06
## UQ2    0.9645647 6.608992e-06
## RPM    0.9645647 6.608992e-06
## DEQ    0.9645647 6.608992e-06
## TPM    0.9515575 2.272016e-05
## RPKM   0.9515575 2.272016e-05
```

2.6 Example use case

To show a typical use case, the RNA-Seq data of GEO accession: [GSE60052](#) was used. The following data sets of healthy control lung cells and small cell lung carcinoma cells were used: healthy *GSM1464282*, *GSM1464283* *GSM1464284* and small cell lung cancer *GSM1464289*, *GSM1464290*, *GSM1464291*. We expect that the overall expression patterns will be quite diverse if we compare healthy with cancer cell expression and it is strongly recommended to use genes which are stably expressed.

First we load the *NVTlibrary*, the gene length data, the housekeeping gene list and the expression data.

```
library("NVT")
data(myusecaseexp)
data(myusecaselen)
data(mylist_hs)

head(myusecaseexp)

##          GSM1464282  GSM1464283  GSM1464284  GSM1464289  GSM1464290  GSM1464291
## ENSG00000000003     186        54       28         8       22       534
## ENSG00000000005     100        4       52         0         0         0
## ENSG00000000419      66       96       22         2       22      143
## ENSG00000000457      93      234       50      112         0      234
## ENSG00000000460      68      130       38       98         0      426
## ENSG00000000938     203      794      347       24         0       28
```

Now we create an *NVTobject* with the expression of the first healthy and the first small cell lung cancer

samples, add the gene length data and set the first normalization method to test to RPKM. We also apply the normalization and have a look at the resulting expression values.

```
mynvt <- NVTinit(mylist_hs,myusecaseexp[,1,drop=FALSE],myusecaseexp[,4,drop=FALSE],  
"RPKM",myusecaselen)  
mynorm <- NVTnormalize(mynvt)  
## [1] "RPKM normalization!"  
  
mynvalues <- show(mynorm)  
head(mynvalues)  
  
##          GSM1464282  GSM1464289  
## ENSG000000000003  3.4284335  0.1474595  
## ENSG000000000005  5.1919942  0.0000000  
## ENSG000000000419  4.5708476  0.1385105  
## ENSG000000000457  1.1294454  1.3601924  
## ENSG000000000460  0.9526052  1.3728722  
## ENSG000000000938  4.8845696  0.5774861
```

Then we create the first plot.

```
NVTplot(mynorm, 0.4)
```

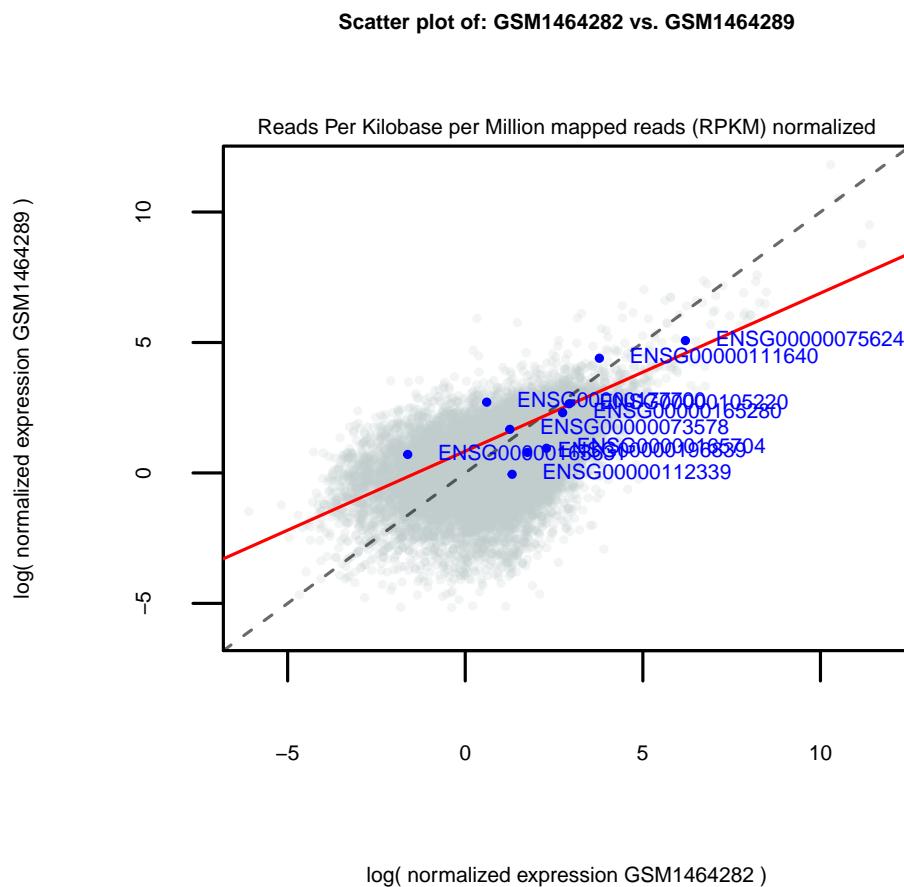


Figure 5: **XY-Scatter-Plot.** The resulting plot from the simple NVTplot function shows a broad cloud of expression values and the linear model is not very close to the diagonal

In addition we also create the MA-plot.

```
NVTadvancedmaplot(mynorm,1,1,1)
```

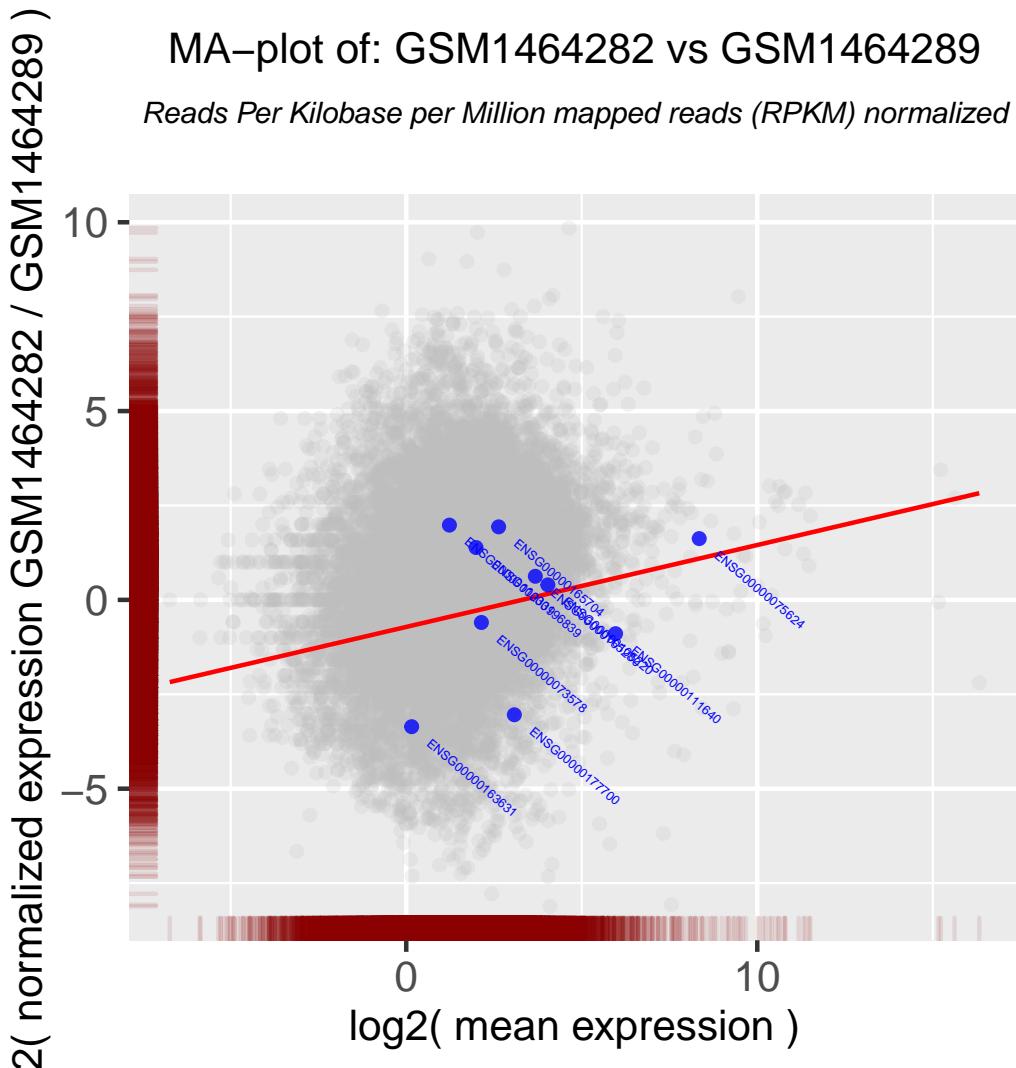


Figure 6: **Advanced MA-Plot.** The NVTAdvancedmaplot function shows also that the expression of the genes is quite diverse resulting in this broad cloud and also the chosen genes are widely distributed and some are very distant to the horizontal 0-line indicating that their expression is strongly varying.

Despite we have seen that the chosen gene set is not optimal, we test all implemented normalization methods. We apply the `NVTtestall` function, which indicates that Q is the best normalization method based on the selected genes.

```
NVTtestall(mynorm, "p")
```

```
## [1] "No normalization!"  
## [1] "Total count normalization!"  
## [1] "Median normalization!"  
## [1] "Trimmed Mean of M-values normalization!"  
## [1] "Upper Quartile normalization!"
```

```

## [1] "Upper Quartile normalization (from NOISeq)!"
## [1] "Quantile normalization!"
## [1] "RPKM normalization!"
## [1] "RPM normalization!"
## [1] "DESeq normalization!"
## [1] "Using DESeq"
## [1] "Input counts are not integer, converting them!"
## [1] "TPM normalization!"
## [1] "Normalization by given gene-set!"
## [1] "ENSG00000111640" "ENSG00000163631" "ENSG00000075624" "ENSG00000165704"
## [5] "ENSG00000196839" "ENSG00000177700" "ENSG00000165280" "ENSG00000105220"
## [9] "ENSG00000112339" "ENSG00000073578"
##          pearson      p-value
## Q      0.9670602 4.949840e-06
## DEQ    0.9265992 1.161464e-04
## G      0.9265992 1.161464e-04
## TC     0.9265992 1.161464e-04
## TMM    0.9265992 1.161464e-04
## UQ     0.9265992 1.161464e-04
## RPM    0.9265992 1.161464e-04
## N      0.9265992 1.161464e-04
## Med    0.9265992 1.161464e-04
## UQ2    0.9265992 1.161464e-04
## RPKM   0.9183081 1.763889e-04
## TPM    0.9183081 1.763889e-04

```

In order to apply this test to all data sets we can use a simple loop to go through all healthy versus cancer comparisons.

```

for (dataset1 in 1:3){
  dataset2 <- dataset1+3
  mynvt <- NVTinit(mylist_hs,myusecaseexp[,dataset1,drop=FALSE],
  myusecaseexp[,dataset2,drop=FALSE], "N",myusecaselen)
  mynorm <- NVTnormalize(mynvt)

  print(NVTtestall(mynorm, "p",verbose=F))
}

## [1] "No normalization!"
##          pearson      p-value
## Q      0.9670602 4.949840e-06
## DEQ    0.9265992 1.161464e-04
## G      0.9265992 1.161464e-04
## TC     0.9265992 1.161464e-04
## TMM    0.9265992 1.161464e-04
## UQ     0.9265992 1.161464e-04
## RPM    0.9265992 1.161464e-04
## N      0.9265992 1.161464e-04
## Med    0.9265992 1.161464e-04

```

```
## UQ2 0.9265992 1.161464e-04
## RPKM 0.9183081 1.763889e-04
## TPM 0.9183081 1.763889e-04
## [1] "No normalization!"
##      pearson      p-value
## Q 0.9903064 3.818231e-08
## TPM 0.9869293 1.257041e-07
## RPKM 0.9869293 1.257041e-07
## Med 0.9772349 1.143249e-06
## RPM 0.9772349 1.143249e-06
## N 0.9772349 1.143249e-06
## TC 0.9772349 1.143249e-06
## UQ 0.9772349 1.143249e-06
## UQ2 0.9772349 1.143249e-06
## G 0.9772349 1.143249e-06
## TMM 0.9772349 1.143249e-06
## DEQ 0.9772349 1.143249e-06
## [1] "No normalization!"
##      pearson      p-value
## RPKM 0.9995837 1.313739e-13
## TPM 0.9995837 1.313739e-13
## RPM 0.9984395 2.589449e-11
## DEQ 0.9984395 2.589449e-11
## G 0.9984395 2.589449e-11
## TC 0.9984395 2.589449e-11
## TMM 0.9984395 2.589449e-11
## UQ 0.9984395 2.589449e-11
## UQ2 0.9984395 2.589449e-11
## N 0.9984395 2.589449e-11
## Med 0.9984395 2.589449e-11
## Q 0.9967627 4.786437e-10
```

3 Session Info

- R version 3.2.5 (2016-04-14), x86_64-pc-linux-gnu
- Locale: LC_CTYPE=en_US.UTF-8, LC_NUMERIC=C, LC_TIME=de_AT.UTF-8, LC_COLLATE=en_US.UTF-8, LC_MONETARY=de_AT.UTF-8, LC_MESSAGES=en_US.UTF-8, LC_PAPER=de_AT.UTF-8, LC_NAME=C, LC_ADDRESS=C, LC_TELEPHONE=C, LC_MEASUREMENT=de_AT.UTF-8, LC_IDENTIFICATION=C
- Base packages: base, datasets, graphics, grDevices, methods, stats, utils
- Other packages: knitr 1.12.3, NVT 1.0
- Loaded via a namespace (and not attached): annotate 1.48.0, AnnotationDbi 1.32.3, Biobase 2.30.0, BiocGenerics 0.16.1, BiocParallel 1.4.3, BiocStyle 1.8.0, Biostrings 2.38.4, bitops 1.0-6, colorspace 1.2-6, DBI 0.3.1, DESeq 1.22.1, evaluate 0.8.3, formatR 1.3, futile.logger 1.4.1, futile.options 1.0.0, genefilter 1.52.1, geneplotter 1.48.0, GenomeInfoDb 1.6.3, GenomicAlignments 1.6.3, GenomicRanges 1.22.4, ggplot2 2.1.0, grid 3.2.5, gtable 0.2.0, highr 0.5.1, IRanges 2.4.8, labeling 0.3, lambda.r 1.1.7, lattice 0.20-33, limma 3.26.9, magrittr 1.5, Matrix 1.2-5, munsell 0.4.3, NOISeq 2.14.1, parallel 3.2.5, plyr 1.8.3, RColorBrewer 1.1-2, Rcpp 0.12.4, RCurl 1.95-4.8, Rsamtools 1.22.0, RSQLite 1.0.0, rtracklayer 1.30.4, S4Vectors 0.8.11, scales 0.4.0, splines 3.2.5, stats4 3.2.5, stringi 1.0-1, stringr 1.0.0, SummarizedExperiment 1.0.2, survival 2.39-2, tools 3.2.5, XML 3.98-1.4, xtable 1.8-2, XVector 0.10.0, zlibbioc 1.16.0

References

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