

— Exploring cDNA Data—

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The following exercise will guide you through the first steps of a spotted cDNA microarray analysis. These steps comprise loading data into R/Bioconductor, quality control of the measurements, and preprocessing of the raw data via normalization. Make extensive use of the `help(<object>)` command to find information about particular objects. Use the `vignette(<package>)` command to get introductory material for a certain package.

- 1.) Preliminaries.** To go through this exercise, you need to have installed R \geq 2.0.1, the release 1.5 versions of the Bioconductor libraries Biobase, marray, multtest, limma, vsn, and arrayMagic.

```
> library("vsn")
> library("marray")
> library("limma")
> library("arrayMagic")
> library("cluster")
> library("RColorBrewer")
```

- 2.) Reading data files.** For a first time quick start, we suggest to skip the data acquisition part and to continue with step 3 (the data you need is already present in your current working directory). When working through the exercises a second time, we strongly encourage you to execute the data acquisition steps. Experience tells that this is one of the most error prone steps.

- a.** Your data has to be stored in one folder, with one file corresponding to one sample. Our example folder is located at `<R library path>/lymphoma/extdata/`. Set the working directory path to that location. With a little luck,

```
> setwd(paste(.Library, "/lymphoma/extdata/", sep = ""))
```

does the job. The file names are `1c7b047rex.DAT`, `1c7b048rex.DAT`, ... On the command line, you can use the commands `dir()`, `getwd()` and `setwd()` to navigate around. In the GUI, you can use *File, Change dir* in the menu.

- b.** Open the file `1c7b048rex.DAT` in a text editor. This is the typical file format for the results from the image analysis on a cDNA slide. Different image analysis programs use slightly different conventions and column headings, but we will describe an import method which is suited to the most common software (Genepix, Spot, ...??).

- c.** For a both easy and flexible import of the data, there has to be a description file. The description file contains a table with all the hybridization data file names in one column and possibly additional sample information in further columns. Create a tab-delimited text file of this kind. We have done this for you, the description file is named `phenoData.txt`. You may examine its structure with any text editor. There is a convenient method for converting such a table into a `phenoData` object.

```
> lymphenoData = read.phenoData("phenoData.txt")
```

- d.** The `phenoData` object is used to simultaneously import all data files.

```

> lymphRaw = readIntensities(pData(lymphenoData), fileNameColumn = "fileName",
+   slideNameColumn = "slideNumber", type = "ScanAlyze")
> lymphNormvsn = normalise(lymphRaw, subtractBackground = T, method = "vsn",
+   spotIdentifier = "SPOT")
> lymphoma = as.exprSet(lymphNormvsn)

```

3.) The Bioconductor class `exprSet`.

- a. The object `lymphoma` is of class `exprSet`. This class is the standard representation of a microarray experiment in Bioconductor. It consists of the objects ("slots")

```

exprs      : A spots × samples matrix containing the expression levels
se.exprs   : A spots × samples matrix containing an estimate of the standard error
             of each single spot measurement
phenoData  : An object of class phenoData, essentially a data frame containing
             phenotypical information about the samples that were hybridized
annotation : Textual annotation
description : Object of class MIAME which incorporates those MIAME-entries
             that are not covered by other objects of the exprSet class
notes      : Text containing additional remarks

```

Slots can be accessed directly with "@" (e.g. `lymphoma@phenoData`), but one should use the accessor methods for the class `exprSets`. See `help(exprSets)` for details. The most interesting objects to us are the expression matrix, given by `exprs(lymphoma)` and the data frame with the phenotype data, `pData(lymphoma)`. Have a look at them.

```

> dim(exprs(lymphoma)) # genes × samples
[1] 9216  16
> exprs(lymphoma)[1:3, 1:6]
      [,1] [,2] [,3] [,4] [,5] [,6]
gene #1 4.709008 5.155799 5.324186 5.961231 6.192032 4.735077
gene #2 6.543069 6.557879 7.380401 6.242252 7.223298 6.467218
gene #3 7.118181 7.047634 7.464352 6.625402 7.737590 7.015837
> dim(pData(lymphoma)) # samples × descriptors
[1] 16  5
> pData(lymphoma)
      fileName sampleid tumortype sex slideNumber
1 lc7b047rex.DAT  CLL-13      CLL  m          1
2 lc7b048rex.DAT  CLL-13      CLL  m          2
3 lc7b069rex.DAT  CLL-52      CLL  f          3
:

```

- b. You might want to add a column containing the hybridization colour.

```

> colour = c(rep("red", 8), rep("green", 8))
> pData(lymphoma) = cbind(pData(lymphoma), colour)
      fileName sampleid tumortype sex slideNumber colour
1 lc7b047rex.DAT  CLL-13      CLL  m          1      red
2 lc7b048rex.DAT  CLL-13      CLL  m          2      red
3 lc7b069rex.DAT  CLL-52      CLL  f          3      red
:

```

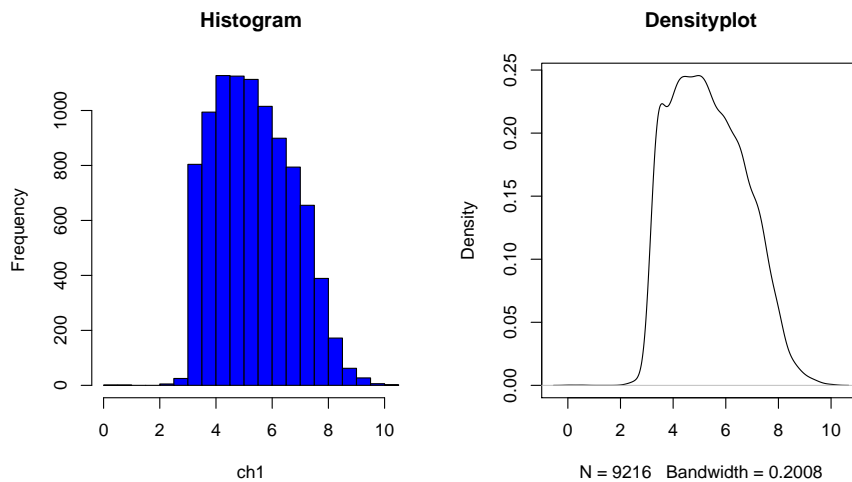
4.) Simple plots.

- a. We will perform some elementary diagnostic plots for quality control. Most analyses are carried out on the log transformed data, so `lymphoma` contains (generalized) log transformed expression values. For convenience, we extract these values into another variable.

```
> logexpr = exprs(lymphoma)
```

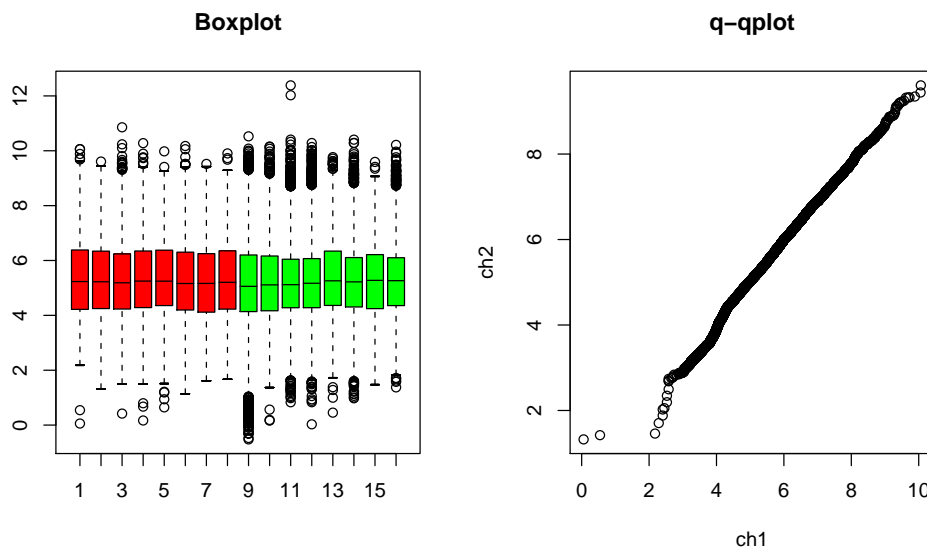
It is possible to examine each single channel. Produce a histogram and a density plot of the log intensities in channel 1 of slide 1. The command `x11()` opens a new graphics window.

```
> ch1=logexpr[,1]; ch2=logexpr[,2] # we will need the second channel later
> x11()
> plot(hist(ch1), main = "Histogram")
> plot(density(ch1), main = "Densityplot")
```



Some plots that help to detect bad hybridizations. Compare the log intensity boxplots of the slides

```
> boxplot(split(t(logexpr), 1:ncol(logexpr)), col = as.vector(pData(lymphoma)[,
+ "colour"]), main = "boxplot")
```



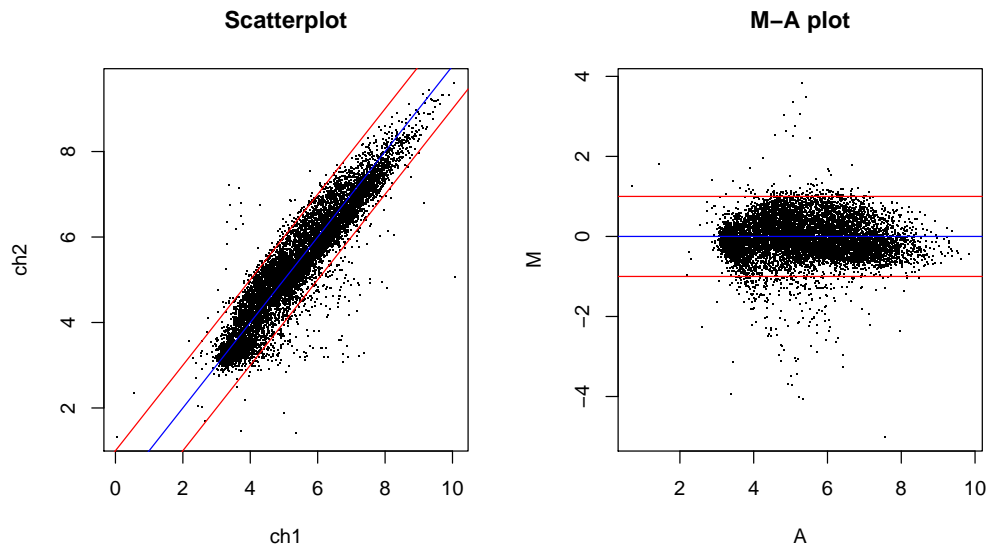
A convenient way to compare the expression distributions between two samples is a quantile-quantile plot

```
> qqplot(ch1, ch2, main = "q-q plot")
```

- b. Save one of the plots as a PDF. Copy and paste it into an MS-Office application.

```
> pdf(file = "savedplot")
> qqplot(ch1, ch2, main = "q-q plot")
> dev.off()
```

- c. A more detailed view is provided by a scatter plot and its corresponding M-A plot
- ```
> plot(ch1, ch2, pch=".", main="Scatterplot")
> abline(a=0, b=1, col="blue"); abline(a=1, b=1, col="red"); abline(a=-1, b=1, col="red")
> plot((ch1+ch2)/2, ch2-ch1, pch=".", xlab="A", ylab="M", main="M-A plot")
> abline(h=0, col="blue"); abline(h=1, col="red"); abline(h=-1, col="red")
```
- d. We hid one "outlier slide" among the original lymphoma data. Find it using diagnostic plots!

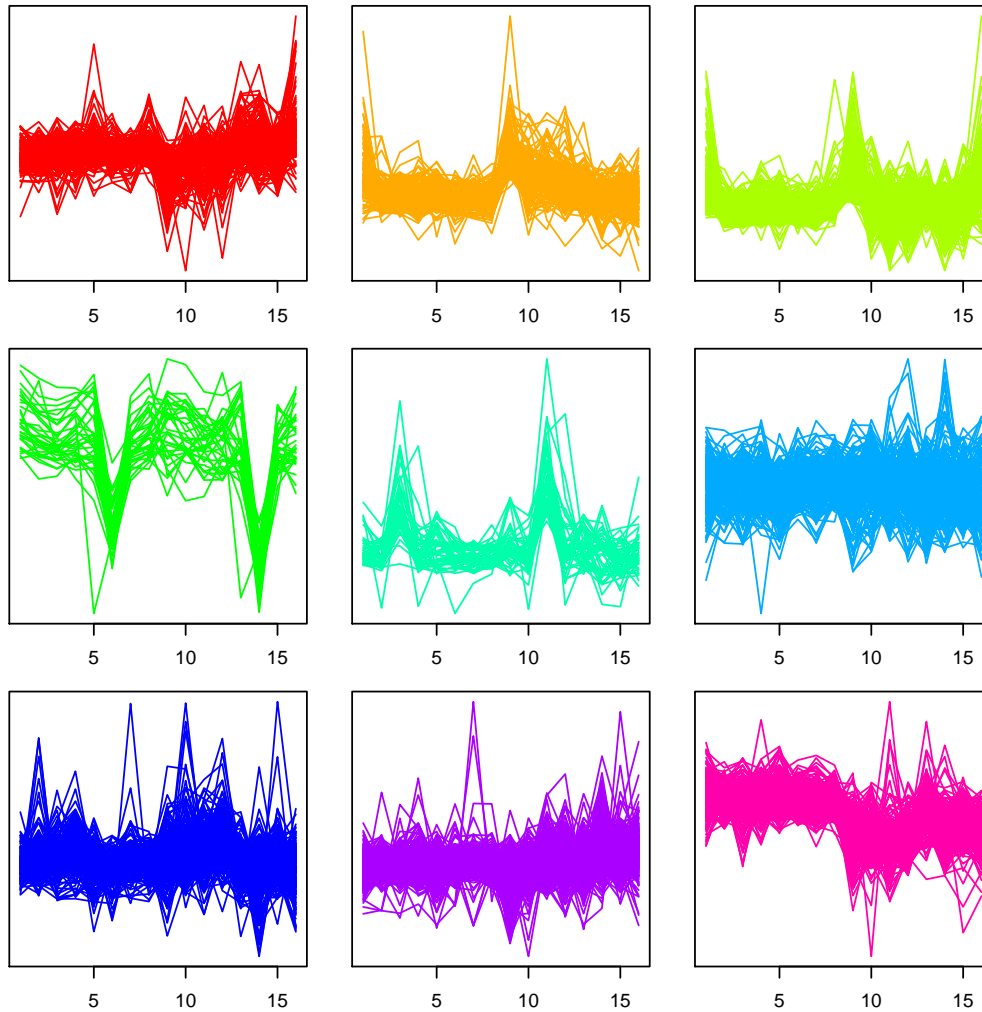


## 5.) Normalization

- a. Before we started analysis, we tacitly normalized our data (cf. `normalise(lymphraw...)`). Try two other commonly used normalization methods:
- ```
> lymphNormloess = normalise(lymphRaw, subtractBackground = T,
+   method = "loess", spotIdentifier = "SPOT")
> logexpr2 = as.exprSet(lymphNormloess)
> lymphNormquantile = normalise(lymphRaw, subtractBackground = T,
+   method = "quantile", spotIdentifier = "SPOT")
> logexpr3 = as.exprSet(lymphNormquantile)
```
- b. These commands take their time! You can save the results into a file with the `save` function, and later restore them with the `load` function. In MS-Windows, you can use the GUI for the latter.
- c. Compare the results of the variance stabilization method to the loess method! Which Plots are appropriate for that?

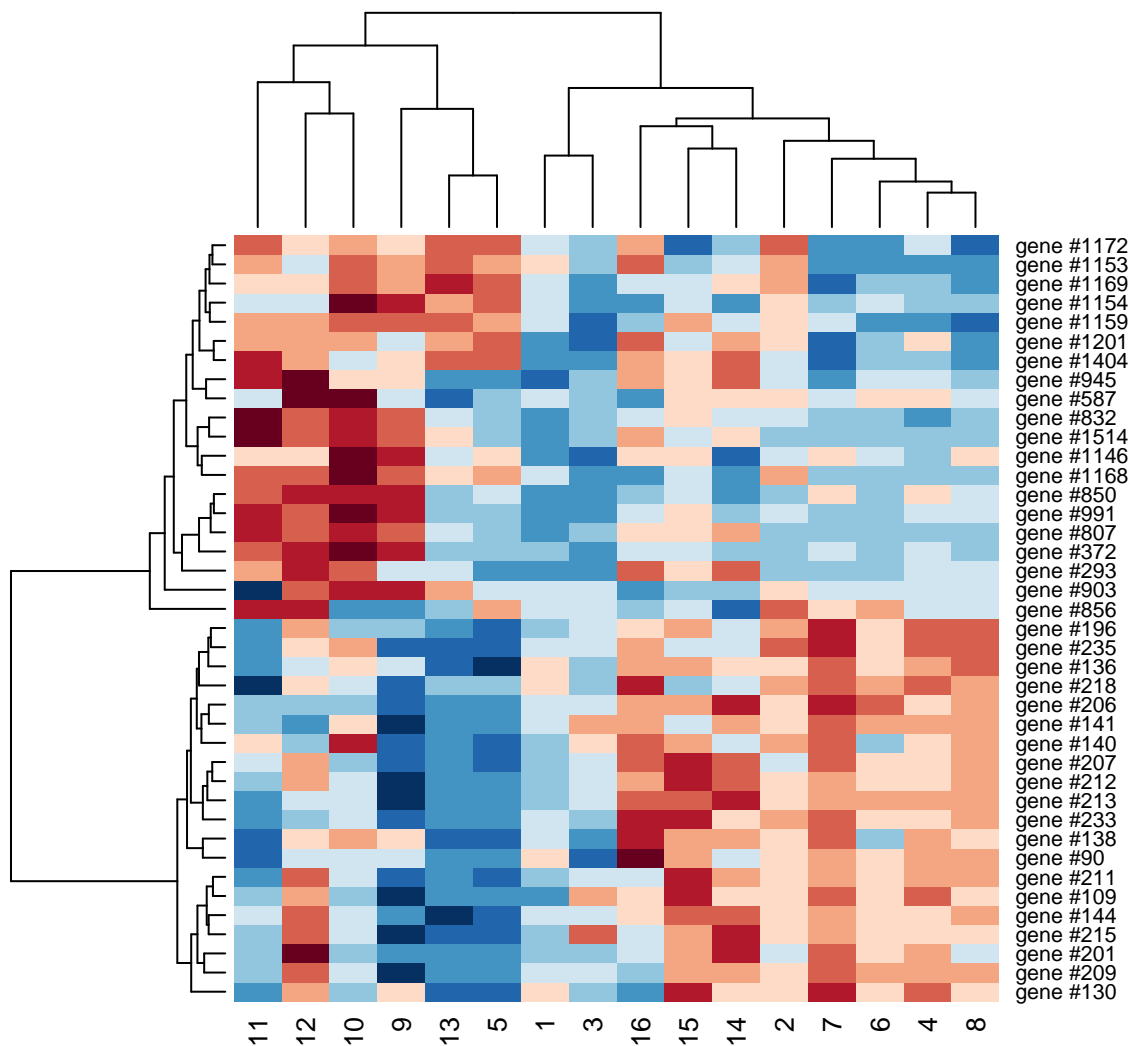
6.) Further data exploration

- a. Another explorative tool is clustering of genes, which can be done in many ways. We use k-means clustering to obtain 50 gene clusters, say.
- ```
> set.seed(0)
> palette(rainbow(9))
> result = kmeans(logexpr, centers = 50, iter.max = 30)
> x11()
> par(mfrow = c(3, 3))
> for (j in 1:9) {
+ selection = (1:nrow(logexpr))[result$cluster %in% j]
+ plot(c(0, 0), xlim = c(1, 16), ylim = c(min(logexpr[selection,
+]), max(logexpr[selection,])), type = "n", xlab = "",
+ ylab = "")
+ apply(logexpr[selection,], 1, points, type = "l", col = j)
+ }
```



- b. Due to their pervasive power, heatmaps enjoy high popularity (although they hardly prove anything). We can produce them in a few lines of R code.

```
> selection1 = (1:nrow(logexpr))[result$cluster %in% 11]
> selection2 = (1:nrow(logexpr))[result$cluster %in% 20]
> heatmap(logexpr[c(selection1[1:20], selection2[1:20]),], col = brewer.pal(10,
+ "RdBu"))
```



## 7.) Further quality assessment

The package `arrayMagic` provides additional measures for quality control. It produces a bunch of graphics which are saved in your current working directory. Take the time to examine some of them. Have a look at the vignette `arrayMagicVignette` for details before proceeding.

```
> vignette("arrayMagicVignette")
> qP <- qualityParameters(lymphRaw, lymphNormvsN, resultFileName = "qP.txt",
+ spotIdentifier = "SPOT", slideNameColumn = "fileName")
> qualityDiagnostics(lymphRaw, lymphNormvsN, qP)
```

```
windows
 2
```

```
> visualiseHybridisations(lymphRaw[, 1], mappingColumns = list(Block = "GRID",
+ Column = "COL", Row = "ROW"))
```

here are some samples of the output:

