Analysis of bead-summary data using beadarray

Mark Dunning and Matt Ritchie

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Introduction

Illumina have created an alternative microarray technology (BeadArray) based on randomly arranged beads. A specific oligonucleotide sequence is assigned to each *bead type*, which is replicated many times (typically ~ 30) on an array. A series of decoding hybridisations is used to identify each bead on the array. The high degree of replication makes robust measurements for each bead type possible.

BeadArrays are used in many applications, including gene expression studies, SNP genotyping and methylation profiling and are processed in parallel as a Sentrix Array Matrix (SAM) or BeadChip. A SAM is a plate of 96 uniquely prepared hexagonal BeadArrays, each of which contains around 1,500 bead types. The BeadChip technology comprises a series of rectangular strips on a slide with each strip containing about 24,000 bead types. For example, there are six pairs of strips on each Human-6 BeadChip. Depending on the particular assay used, the data from a BeadArray may be single channel or two-colour.

The data from Illumina BeadArrays is available in different formats. We refer to the raw TIFF images and text files output by the BeadScan software as *bead-level data*. For details on how to use the beadarray package to read in and process data in this format, refer to the bead-level user's guide which can be launched with the following command

```
> library(beadarray)
> beadarrayUsersGuide(topic = "beadlevel")
```

The second format is produced by Illumina's BeadStudio software. We refer to this output as *bead-summary data* as these files contain summary intensities for each bead type on each array. In this user guide we describe how to process summarised gene expression data from Illumina BeadArrays using the beadarray package. Most of the analysis outlined in this guide can equally be applied to the summary values produced by reading and processing the bead-level data using beadarray.

1 Importing bead-summary data

BeadStudio is Illumina's proprietary software for analysing raw bead-level data from BeadScan. It contains different modules for analysing data from different platforms. For further information on the software and how to export summarised data, refer to the user's manual. In this section we consider how to read in and analyse BeadStudio output from the gene expression module.

The example data used in this guide (`BeadSummaryExample.zip`) can be downloaded from

`http://www.compbio.group.cam.ac.uk/Resources/illumina/BeadSummaryExample.zip`

The example data set consists of 3 Human-6 version 1 BeadChips. These arrays were part of a pilot study into BeadArray technology and 7 different samples (IC, IH, MC, MD, MT, P and Norm) were hybridised to the arrays. MC, MD, MT, P and Norm are all samples from different cell lines and IH and

IC are samples provided by Illumina. The non-normalised data and control information were produced using BeadStudio version 3.2.3.

1.1 Description of files

Each file contained in `BeadSummaryExample.zip` is briefly described below.

- `SampleProbeProfile.txt` (*required*) - This file contains the raw, non-normalised bead-summary values as output by BeadStudio. The file begins with several lines of header information followed by a data matrix with around 48,000 rows. Each row gives expression measures for a different probe, while the columns give various measurements from different arrays. For each array, we record the summarised expression level (AVG_Signal), standard error of the bead replicates (BEAD_STDERR), number of beads used (Avg_NBEADS) and detection scores (Detection Pval - which estimates the probability of a probe being detected above the background level). When exporting data from BeadStudio, the user is able to choose which columns to save. The columns AVG_Signal, BEAD_STDERR, Avg_NBEADS and Detection Pval, along with the ProbeID column, which contains a unique identifier for each probe, should be selected at a minimum.

  It is also possible to export annotation information. We recommend this data not be exported if the file is to be read into beadarray, as some of the special characters used in the annotation fields cause problems for the `readBeadSummaryData` function. This information can be retrieved at a later stage from other Bioconductor packages, such as illuminaHumanv1BeadID.db. These packages are based on the reannotation tables available from `http://www.compbio.group.cam.ac.uk/Resources/Annotation/`.

- `SampleSheet.csv` (*optional*) - This is a file format that Illumina recommend for users of BeadStudio to specify the array IDs and samples hybridised to each array. This file is created by the user.

- `ControlGeneProfile.txt` (*optional*) - Gives the summarised data for each control type on each array as output by BeadStudio, which can be used for quality assessment purposes. The format of the control file differs slightly between BeadStudio version 1 and later versions of the software. The current format is similar to that of `SampleProbeProfile.txt` with columns for the intensities, standard errors, number of beads and detection scores (AVG_Signal, BEAD_STDERR, Avg_NBEADS and Detection Pval respectively) for each array. Each row contains data for a different control type, whose identifier is given in the TargetID column. Refer to the Illumina documentation for information on each control.

The following code can be used to read the example data into `R` (provided that the contents of `BeadSummaryExample.zip` have been extracted to the current working directory).

```
> library(beadarray)
> dataFile = "SampleProbeProfile.txt"
> sampleSheet = "SampleSheet.csv"
> qcFile = "ControlGeneProfile.txt"
> BSData = readBeadSummaryData(dataFile = dataFile,
+     qcFile = qcFile, sampleSheet = sampleSheet,
+     controlID = "TargetID")
```

The arguments of `readBeadSummaryData` can be modified to suit data from versions 1, 2 or 3 of BeadStudio. The current default settings should work for version 3 output. Users may need to change the argument `sep`, which specifies if the `dataFile` is comma or tab delimited and the `skip` argument which specifies the number of lines of header information at the top of the file. Possible `skip` arguments of 0, 7 and 8 have been observed, depending on the version of BeadStudio or way in

which the data was exported. The `columns` argument is used to specify which column headings to read from `dataFile` and store in various matrices. Note that the naming of the columns containing the standard errors changed between versions of BeadStudio (earlier versions used BEAD_STDEV in place of BEAD_STDERR - be sure to check that the `columns` argument is appropriate for your data). Equivalent arguments (`qc.sep`, `qc.skip` and `qc.columns`) are used to read the data from `qcFile`. See the help page (`?readBeadSummaryData`) for a complete description of each argument to the function. Control information from Illumina experiments can also be read into beadarray independently using the `readQC` function.

2 The BSData object

`BSData` is an object of type *ExpressionSetIllumina* which is an extension of the *ExpressionSet* class from the `Biobase` package. Objects of this type use a series of slots to store the data.

```
> BSData

ExpressionSetIllumina (storageMode: list)
assayData: 47312 features, 18 samples
  element names: exprs, se.exprs, NoBeads, Detection
phenoData
  rowNames: IH-1, IH-2, ..., Norm-2  (18 total)
  varLabels and varMetadata description:
    Sample_Name: Sample_Name
    Sample_Well: Sample_Well
    ...: ...
    Sentrix_Position: Sentrix_Position
    (7 total)
featureData
  featureNames: 360450, 1690139, ..., 103060372  (47312 total)
  fvarLabels and fvarMetadata description:
    ProbeID: NA
experimentData: use 'experimentData(object)'
Annotation:
QC Information
 Available Slots:  exprs se.exprs Detection NoBeads
  featureNames: biotin, cy3_hyb, ..., low_stringency_hyb, negative
  sampleNames: IH-1, IH-2, ..., Norm-1, Norm-2

> dim(BSData)

Features  Samples
   47312       18

> slotNames(BSData)

[1] "QC"                "BeadLevelQC"
[3] "assayData"         "phenoData"
[5] "featureData"       "experimentData"
[7] "annotation"        ".__classVersion__"

> names(assayData(BSData))
```

```
[1] "exprs"     "se.exprs"  "NoBeads"
[4] "Detection"
```

```
> exprs(BSData)[1:5, 1:2]
```

```
             IH-1      IH-2
360450    87.8092  231.9214
1690139  161.7640  258.5725
5420594  481.1803  499.3547
3060411  633.6545  581.3866
450341  1535.5670 1302.9530
```

```
> se.exprs(BSData)[1:5, 1:2]
```

```
             IH-1     IH-2
360450   5.071144 15.53351
1690139 12.018600 18.97802
5420594 21.652110 34.37503
3060411 21.579310 25.81822
450341  42.663560 80.21368
```

```
> pData(BSData)[1:2, ]
```

```
     Sample_Name Sample_Well Sample_Plate
IH-1        IH-1          NA           NA
IH-2        IH-2          NA           NA
     Sample_Group Pool_ID Sentrix_ID
IH-1           IH      NA 1475542114
IH-2           IH      NA 1475542114
     Sentrix_Position
IH-1                A
IH-2                C
```

The data from the file `SampleProbeProfile.txt` is stored in the `assayData` slot of the object. This slot contains a number of matrices, each of which has a column for each array in the experiment and a row for each probe. There is a matrix for each column specified by the `columns` parameter in `readBeadSummaryData`. If the character strings specified in `columns` cannot be matched in the file, the matrix will be filled with `NAs`.

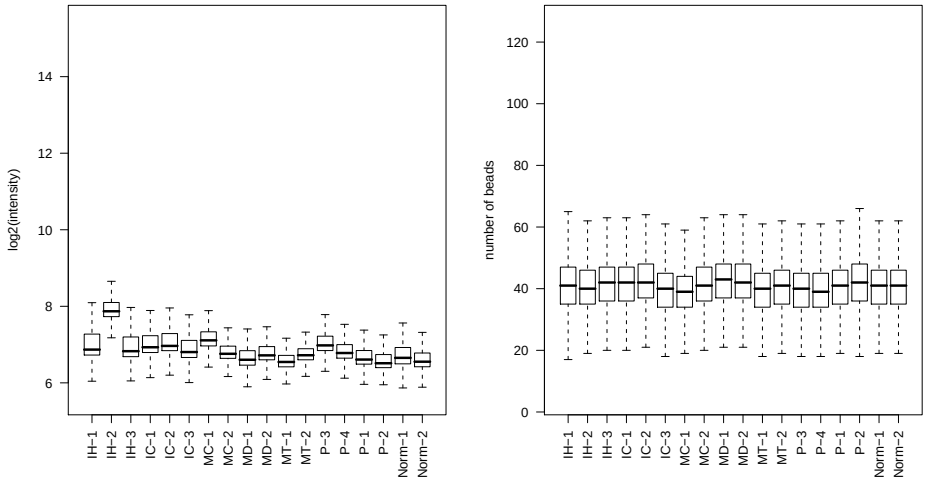
For consistency with the definition of other *ExpressionSet* objects, we now refer to the expression values as the *exprs* matrix which can be accessed using `exprs` and subsetted in the usual manner. Similarly, the standard errors for each bead, which are stored in the *se.exprs* matrix can be accessed using `se.exprs`. The number of beads and detection scores can be accessed using the functions `NoBeads` and `Detection` respectively. The rows names of each of these matrices are from the column in `SampleProbeProfile.txt` that matches the `ProbeID` argument of `readBeadSummaryData`.

Sample information for the experiment can be accessed using `pData` and the `QC` slot contains the control probe intensities.

3 Quality assessment and normalisation

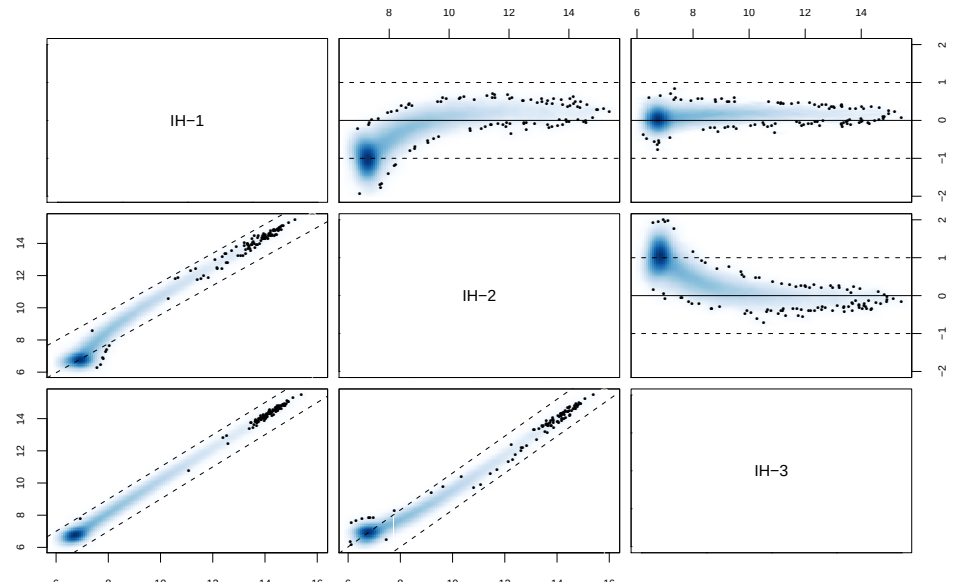
Boxplots of intensity levels and the number of beads are useful for quality assessment purposes. Below is the code to produce boxplots of these quantities for each array in the experiment.

```
> par(mfrow = c(1, 2))
> boxplot(as.data.frame(log2(exprs(BSData))),
+     las = 2, outline = FALSE, ylab = "log2(intensity)")
> boxplot(as.data.frame(NoBeads(BSData)),
+     las = 2, outline = FALSE, ylab = "number of beads")
```



Recall that there are 6 arrays per BeadChip and differences between chips hybridised on different days can be expected (NOTE: in this example, the samples are not ordered by BeadChip. Instead replicate samples appear consecutively). In this experiment, differences in intensity level between arrays are generally small, with the exception of array 2, which has much higher signal than the other arrays. The sample on this array is replicated three times in the experiment and comparing the MA and XY plots for the replicates of this sample using the `plotMAXY` function can be informative.

```
> plotMAXY(exprs(BSData), arrays = 1:3,
+     pch = 16)
```

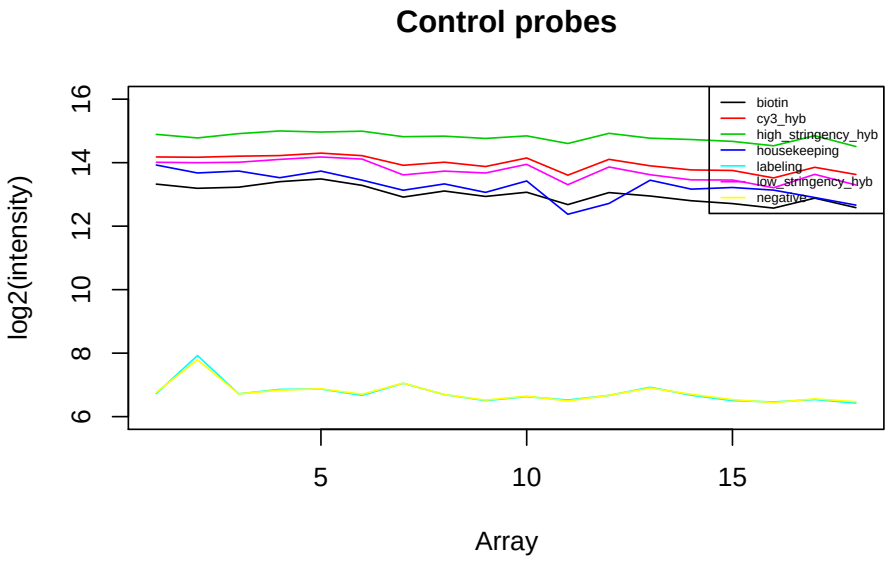


In the top right corner we see the MA plots for all pairwise comparisons involving the 3 arrays. On an MA plot, for each probe we plot the average of the log_2-intensities from the two arrays on the x-axis and the difference in intensities (log_2-ratios) on the y-axis. For replicate arrays we would expect all probes to be unchanged between the two samples and hence most points on the plot should lie along the line y=0. In the lower left corner of the MAXY plot we see the XY plot and for replicate arrays we would expect to see most points along the diagonal $y = x$. From this MAXY plot it is obvious that the second array is systematically different to the other replicates and may benefit from normalisation.

Both XY and MA plots are available separately for a particular comparison of arrays using `plotXY` and `plotMA`.

The control probe intensities can be plotted as a further quality diagnostic. To retrieve this control data, which is stored in the QC slot, use the `QCInfo` function. `QCInfo` returns a list object containing several matrices which can be accessed using the `$` operator. The row names of each matrix indicate the control type (as specified by the column in `qcFile` matching the `controlID` argument of `readBeadSummaryData`). In the following example we plot summary intensities for each control type on each array.

```
> QC = QCInfo(BSData)
> names(QC)

[1] "exprs"     "se.exprs"  "Detection"
[4] "NoBeads"

> rownames(QC$exprs)

[1] "biotin"              "cy3_hyb"
[3] "high_stringency_hyb" "housekeeping"
[5] "labeling"            "low_stringency_hyb"
[7] "negative"

> QC$exprs[, 1:2]

                          IH-1       IH-2
biotin               10255.0200  9358.6680
cy3_hyb              18556.4400 18442.0200
high_stringency_hyb  30390.5100 28120.1700
housekeeping         15551.8900 13086.8300
labeling               105.9794   243.0619
low_stringency_hyb   16526.4700 16378.6700
negative               108.7406   221.2646

> numContr = nrow(QC$exprs)
> plot(log2(QC$exprs[1, ]), type = "l",
+     xlab = "Array", ylab = "log2(intensity)",
+     main = "Control probes", cex = 0.5,
+     ylim = c(6, 16))
> for (i in 2:numContr) {
+     points(log2(QC$exprs[i, ]), type = "l",
+         col = i)
+ }
> legend("topright", legend = rownames(QC$exprs),
+     col = 1:numContr, lty = 1, cex = 0.5)
```



To correct for differences in expression level across a chip and between chips we need to normalise the signal to make the arrays comparable. The normalisation methods available in the affy package, or variance-stabilising transformation from the lumi package may be applied using the `normaliseIllumina` function. Below we quantile normalise the log_2 transformed data.

```
> BSData.quantile = normaliseIllumina(BSData,
+     method = "quantile", transform = "log2")
> plotMAXY(exprs(BSData.quantile), arrays = 1:3,
+     log = FALSE, pch = 16)
```

4 Differential expression

The differential expression methods available in the limma package can be used to identify differentially expressed genes. The functions `lmFit` and `eBayes` can be applied to the normalised data.

In the example below, we set up a design matrix for the example experiment and fit a linear model to summarise the data from the IC, IH, MC, MD, MT, P and Norm replicates to give one value per condition. We then define contrasts comparing the IH sample to the P sample, IH to Norm and P to Norm and calculate moderated t-statistics with empirical Bayes' shrinkage of the sample variances. In this particular experiment, the IH and P samples are very different and we would expect to see many differentially expressed genes.

```
> samples = pData(BSData.quantile)$Sample_Group
> samples

 [1] "IH"   "IH"   "IH"   "IC"   "IC"   "IC"
 [7] "MC"   "MC"   "MD"   "MD"   "MT"   "MT"
[13] "P"    "P"    "P"    "P"    "Norm" "Norm"

> samples = as.factor(samples)
> design = model.matrix(~0 + samples)
> colnames(design) = levels(samples)
```

```
> fit = lmFit(exprs(BSData.quantile), design)
> cont.matrix = makeContrasts(IHvsP = IH -
+     P, IHvsNorm = IH - Norm, PvsNorm = P -
+     Norm, levels = design)
> fit = contrasts.fit(fit, cont.matrix)
> ebFit = eBayes(fit)
> topTable(ebFit, coef = 1, number = 5)

           ID     logFC  AveExpr         t
13746 2030280 -6.400270 8.646376 -88.39647
9262  4570301  7.085818 9.047241  86.78303
21843 3800358  5.497822 8.475639  74.16995
22146 3800435  5.162826 9.459757  72.14053
9261  6900332  6.805046 9.031485  69.66278
          P.Value    adj.P.Val        B
13746 1.351034e-33 5.135877e-29 64.73139
9262  2.171067e-33 5.135877e-29 64.36352
21843 1.235732e-31 1.948832e-27 61.09813
22146 2.522020e-31 2.983045e-27 60.49917
9261  6.193555e-31 5.860590e-27 59.73601
```

For more information about `lmFit` and `eBayes`, refer to the limma documentation.

Annotation

Within Bioconductor, annotation packages are available for most types of Illumina BeadChips. For this experiment, the illuminaHumanv1BeadID.db package will be used to provide further information on each probe. The reannotation tables at `http://www.compbio.group.cam.ac.uk/Resources/Annotation/` have been used to generate the following packages:

illuminaHumanv1.db, illuminaHumanv1BeadID.db, illuminaHumanv2.db, illuminaHumanv2BeadID.db, illuminaHumanv3.db, illuminaHumanv3BeadID.db, illuminaMousev1.db, illuminaMousev1BeadID.db, illuminaMousev1p1.db, illuminaMousev1p1BeadID.db, illuminaMousev2.db, illuminaMousev2BeadID.db, illuminaRatv1.db, illuminaRatv1BeadID.db and illuminaHumanDASLBeadID.db.

The packages with BeadID in the name use the numeric probe identifier as the key in the database. These packages should be used when analysing summarised data obtained from a bead-level analysis, as the numeric identifiers from the raw text files are stored by beadarray. These packages should also be used when analysing BeadStudio output if the entries in the *ProbeID* column have been chosen as the identifiers (this is the default setting in `readBeadSummaryData` when `ProbeID="ProbeID"`). The other packages use the *TargetID* column from the BeadStudio output as the key, and should be used when `ProbeID="TargetID"` is specified in `readBeadSummaryData`.

```
> library(illuminaHumanv1BeadID.db)
> illuminaHumanv1BeadID()

Quality control information for illuminaHumanv1BeadID:


This package has the following mappings:

illuminaHumanv1BeadIDACCNUM has 21435 mapped keys (of 47296 keys)
```

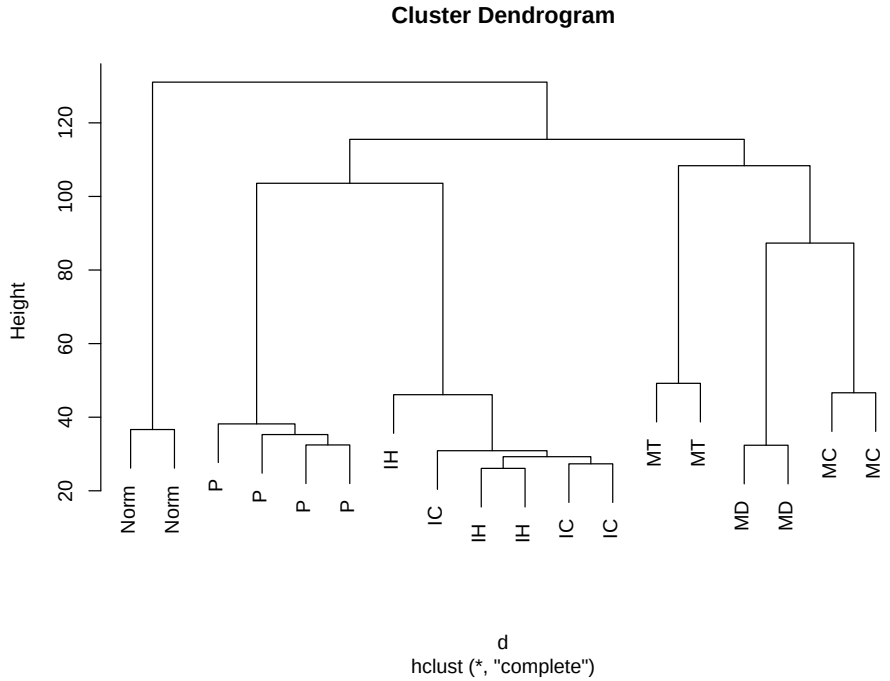
```
illuminaHumanv1BeadIDALIAS2PROBE has 54210 mapped keys (of 54210 keys)
illuminaHumanv1BeadIDCHR has 16926 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDCHRLENGTHS has 25 mapped keys (of 25 keys)
illuminaHumanv1BeadIDCHRLOC has 15536 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDCHRLOCEND has 15536 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDENSEMBL has 15149 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDENSEMBL2PROBE has 13524 mapped keys (of 13524 keys)
illuminaHumanv1BeadIDENTREZID has 16929 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDENZYME has 1676 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDENZYME2PROBE has 718 mapped keys (of 718 keys)
illuminaHumanv1BeadIDGENENAME has 16929 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDGO has 14449 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDGO2ALLPROBES has 8782 mapped keys (of 8782 keys)
illuminaHumanv1BeadIDGO2PROBE has 6235 mapped keys (of 6235 keys)
illuminaHumanv1BeadIDMAP has 16787 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDOMIM has 10484 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDPATH has 4029 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDPATH2PROBE has 212 mapped keys (of 212 keys)
illuminaHumanv1BeadIDPFAM has 16477 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDPMID has 16158 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDPMID2PROBE has 170443 mapped keys (of 170443 keys)
illuminaHumanv1BeadIDPROSITE has 16477 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDREFSEQ has 16750 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDSYMBOL has 16929 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDUNIGENE has 16772 mapped keys (of 47296 keys)
illuminaHumanv1BeadIDUNIPROT has 15936 mapped keys (of 47296 keys)


Additional Information about this package:

DB schema: HUMANCHIP_DB
DB schema version: 1.0
Organism: Homo sapiens
Date for NCBI data: 2008-Sep2
Date for GO data: 200808
Date for KEGG data: 2008-Sep2
Date for Golden Path data: 2006-Apr14
Date for IPI data: 2008-Sep02
Date for Ensembl data: 2008-Jul23

> ids = rownames(exprs(BSData))
> chr = mget(ids, illuminaHumanv1BeadIDCHR,
+     ifnotfound = NA)
> chrloc = mget(ids, illuminaHumanv1BeadIDCHR,
+     ifnotfound = NA)
> refseq = mget(ids, illuminaHumanv1BeadIDREFSEQ,
+     ifnotfound = NA)
> genename = mget(ids, illuminaHumanv1BeadIDGENENAME,
+     ifnotfound = NA)
> anno = cbind(Ill_ID = as.character(ids),
+     Chr = as.character(chr), Loc = as.character(chrloc),
```

```
+     RefSeq = as.character(refseq), Name = as.character(genename))
> ebFit$genes = anno
> write.fit(ebFit, file = "results.txt")
```

5 Further analysis

The clustering functionality available in BeadStudio can be performed in R using the `hclust` function once a distance matrix has been defined. The `heatmap` function could also be used.

```
> d = dist(t(exprs(BSData.quantile)))
> plot(hclust(d), labels = samples)
```



This user guide was built using the following packages:

```
> sessionInfo()

R version 2.8.0 RC (2008-10-12 r46696)
x86_64-unknown-linux-gnu

locale:
LC_CTYPE=en_GB.UTF-8;LC_NUMERIC=C;LC_TIME=en_GB.UTF-8;LC_COLLATE=en_GB.UTF-8;LC_MONETARY=C;LC_MESSAGES

attached base packages:
[1] tools     stats     graphics  grDevices
[5] utils     datasets  methods   base

other attached packages:
 [1] illuminaHumanv1BeadID.db_1.1.2
 [2] beadarray_1.9.11
```

```
 [3] sma_0.5.15
 [4] hwriter_0.93
 [5] affy_1.19.4
 [6] preprocessCore_1.3.4
 [7] affyio_1.9.1
 [8] geneplotter_1.19.6
 [9] annotate_1.19.3
[10] xtable_1.5-4
[11] AnnotationDbi_1.3.12
[12] RSQLite_0.7-0
[13] DBI_0.2-4
[14] lattice_0.17-15
[15] Biobase_2.1.7
[16] limma_2.15.15
[17] weaver_1.7.0
[18] codetools_0.2-1
[19] digest_0.3.1

loaded via a namespace (and not attached):
[1] grid_2.8.0         KernSmooth_2.22-22
[3] RColorBrewer_1.0-2
```

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