

massiR: MicroArray Sample Sex Identifier

Sam Buckberry

April 28, 2026

Contents

1	The Problem	2
2	Importing data and beginning the analysis	2
3	Extracting the Y chromosome probe data	3
4	Predicting the sex of samples	5
5	Visualizing the massiR analysis data	6
6	Check for potential sex bias using the dip test	10
7	Performing massiR analysis with an ExpressionSet object	13
8	Using the included massiR Y chromosome probe lists	14
9	Using biomaRt to obtain y chromosome probe lists	15
10	References	17

1 The Problem

Given that the sex of many species is an easily observable and usually unambiguous classification, it is surprising the number of microarray data sets in public repositories that lack the associated sample sex information. Sex-biased gene expression in normal and pathological tissues is a well recognized for both sex chromosome and autosomal genes. Sex biases also exist in the prevalence and severity of many common human diseases, such as cardiovascular disease and some cancers. As sex is a potential influencing factor of both pathological and non-pathological phenotypes, gene expression analyses that do not account for sex-specific effects could fail to identify a significant proportion of genes that contribute the condition under investigation. Therefore, the absence of sample sex information restricts the reuse of gene expression data sets where the researcher intends to factor the effect of sex in reanalysis or reinterpretation, or when intending to include such data sets in larger gene expression meta-analyses.

This is why we developed *massiR*, a package for predicting the sex of samples in microarray data sets. This package allows researchers to expand their analyses to retrospectively incorporate sex as a variable, generate or confirm sex information associated with publicly available data sets, to accurately predict the sex of samples missing sex information, or as a simple sanity check for your own microarray gene expression data.

2 Importing data and beginning the analysis

The *massiR* analysis begins by importing standard gene expression data of normalized and log transformed probe values. The gene expression data can be in the form of a `data.frame` object and have the sample identifiers as the column names and the probe identifiers as the row names, or as an `ExpressionSet` object. The identifiers for probes corresponding to Y chromosome genes must be as a `data.frame` object with the probe identifiers as `row.names`.

To load the included test *massiR* gene expression data:

```
> library(massiR)
> data(massi.test.dataset)
```

The included gene expression data is composed of 60 samples and 1026 probes as a `data.frame` object.

To load the test Y chromosome probes corresponding to the included data:

```
> data(massi.test.probes)
```

The Included list of Y chromosome probes contains probe identifiers as `row.names` in the `data.frame` class.

3 Extracting the Y chromosome probe data

The first step of the *massiR* analysis involves extracting the expression values for probes that correspond to Y chromosome genes. The user has the option of using their own list of probes corresponding to Y chromosome genes or using the probe lists included with the package. The included lists correspond to popular microarray platforms and contain identifiers for probes that map uniquely to Y chromosome genes. See section 8 for details on using the included probes and section 9 for details on obtaining Y chromosome probes easily from Ensembl Biomart.

When the expression values for Y chromosome probes are extracted, the expression variance for each probe across all samples is calculated. This allows the identification of low variance probes, which are unlikely to be informative in sex classification. The user has the option of selecting a probe variation threshold, so only the most informative probes are used in the classification process. Deciding on a probe variation threshold can be informed by inspecting a probe variation plot (Figure 1) generated by the *massi.y.plot* function. In our experience, using the most variable 25-50% of probes (typically 10-40 probes, depending on platform) produces good results.

To extract data corresponding to Y chromosome probes from the test data set and look at a probe variation plot:

```
> massi.y.out <- massi_y(massi.test.dataset, massi.test.probes)  
  
> massi.y.plot(massi.y.out)
```

This plot (Figure 1) is output to the R graphics device.

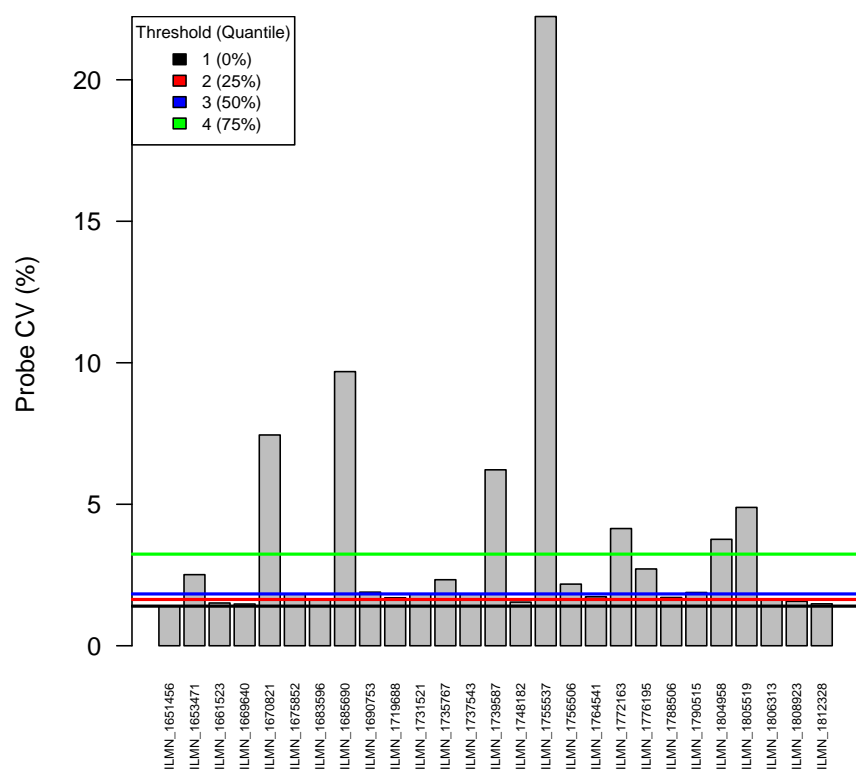


Figure 1: Expression variation (CV) of Y chromosome probes across all samples

After viewing the probe variation plot, a decision can be made regarding which probes to use in the clustering step. The `massiR` package includes methods for selecting probe variation thresholds based on quantiles. The threshold can be determined by quantiles of probe variance (CV): 1=All probes, 2=Upper 75%, 3=Upper 50%, 4=Upper 25%. It is highly recommended that probe CV plot generated using the `massi_y_plot` function be inspected to inform threshold choice (Figure 1). The default threshold value is 3.

Once a probe threshold has been decided upon, run the `massi_select` function. This will return a `data.frame` with the samples as columns and the subset of selected y chromosome probes as row names.

```
> massi.select.out <-  
+   massi_select(massi.test.dataset, massi.test.probes, threshold=4)
```

Check the output for the first 5 samples:

```
> head(massi.select.out)[,1:5]
```

	S1	S2	S3	S4	S5
ILMN_1670821	5.746427	5.686032	6.307110	6.179258	6.594808
ILMN_1685690	5.459125	5.567289	6.919465	6.789817	6.559376
ILMN_1739587	5.883483	5.764190	6.441775	6.438789	6.707278
ILMN_1755537	5.882456	5.831844	8.133164	8.052959	8.298985
ILMN_1772163	5.696833	5.680091	5.907170	6.017871	6.465122
ILMN_1804958	5.815093	5.654395	5.929610	6.104089	5.868732

4 Predicting the sex of samples

To classify samples as either male or female, clustering is performed using the values from the subset of Y chromosome probes by implementing the partitioning around medoids algorithm which performs k-medoids clustering (Hennig 2013), where samples are assigned to one of two clusters. The two clusters are then compared using the probe expression values across all samples in each cluster. Samples within the cluster featuring the highest Y chromosome probe values are classed as male and those within the cluster with the lowest Y probe values classed as female. Results such sample probe mean, standard deviation and z-scores are reported in a table together with the sex predicted for each sample.

To predict the sex of the samples using `massi_cluster`:

```
> results <- massi_cluster(massi.select.out)
```

Extract the results for each sample from the returned list:

```
> sample.results <- data.frame(results[[2]])  
  
> head(sample.results)
```

	ID	mean_y_probes_value	y_probes_sd	z_score	sex
1	S1	5.911089	0.4572756	-0.6453629	female
2	S10	6.749520	0.8418586	0.7773050	male
3	S11	5.689586	0.4750484	-1.1074329	female
4	S12	6.702993	0.7894613	0.7045705	male
5	S13	5.838450	0.6759924	-0.7193198	female
6	S14	5.819845	0.6593184	-0.7524047	female

As you can see, it is a relatively straightforward procedure to produce a table with the predicted sex of each sample with some basic metrics.

5 Visualizing the massiR analysis data

The massiR package includes a function which allows various aspects of the data used in the analysis to be visualized. These plots enable to used to inspect sample and clustering characteristics which could aid in identifying problematic samples and outliers.

To run the `massi.plot` function with the output from the `massi_select` and `massi_cluster` functions:

```
> massi_cluster_plot(massi.select.out, results)
```

This function will generate a heat map with dendrogram of Y chromosome probes as rows and individual samples in columns (Figure 2), a bar plot of mean values and standard deviation from the subset of Y chromosome probes used in K-medoids clustering (Figure 3), with the bars colored with respect to predicted sex and a principal component plot showing clusters (Figure 4). These plots can aid the user in identifying sample outliers or probes that may not be informative in the clustering step.

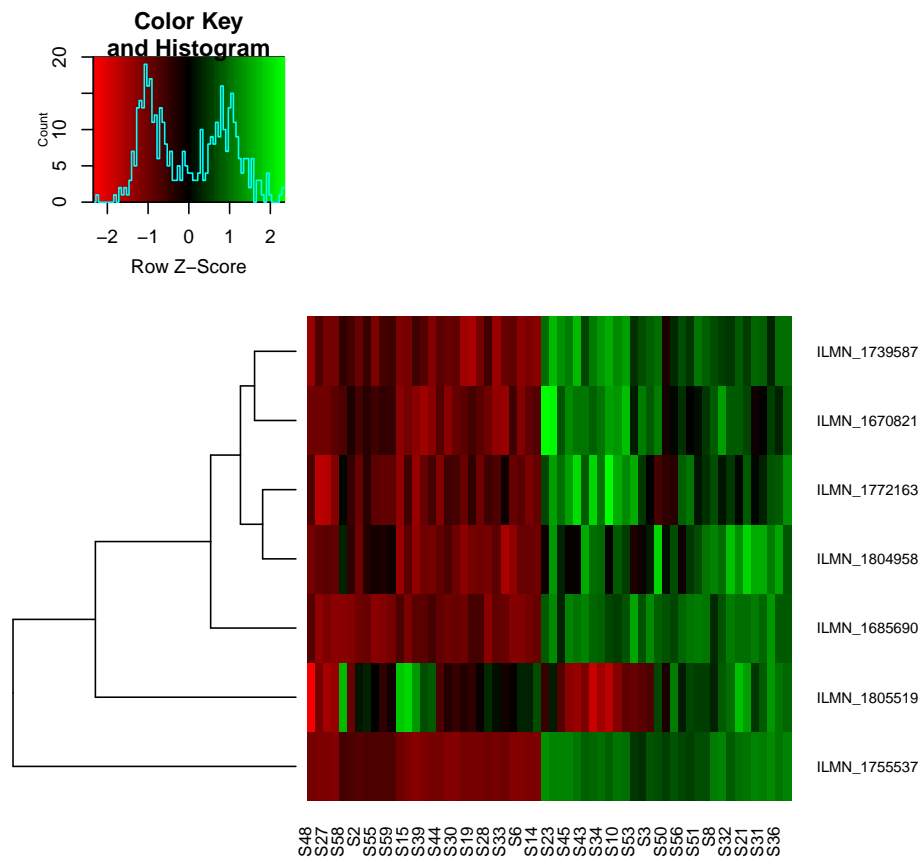


Figure 2: Heat map with dendrogram of Y chromosome probes as rows and individual samples in columns. Notice that the values for the probe in the fifth row are reasonably variable but do not show the same pattern seen with other probes. Therefore viewing the heatmap may help identify problematic probes.

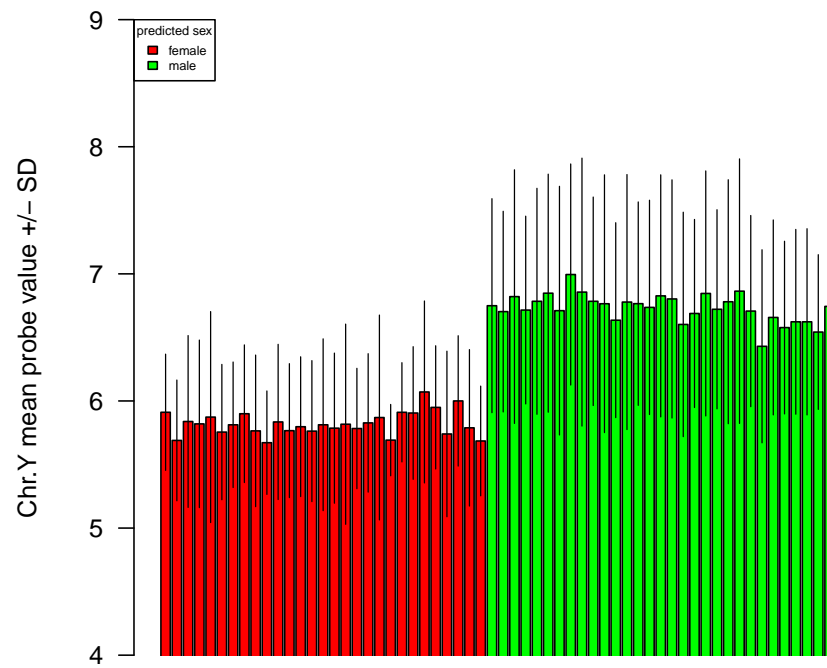


Figure 3: Mean values of the subset of Y chromosome probes used in K-medoids clustering. The bar colors represent clusters, which were assigned as female (red) and male (green)

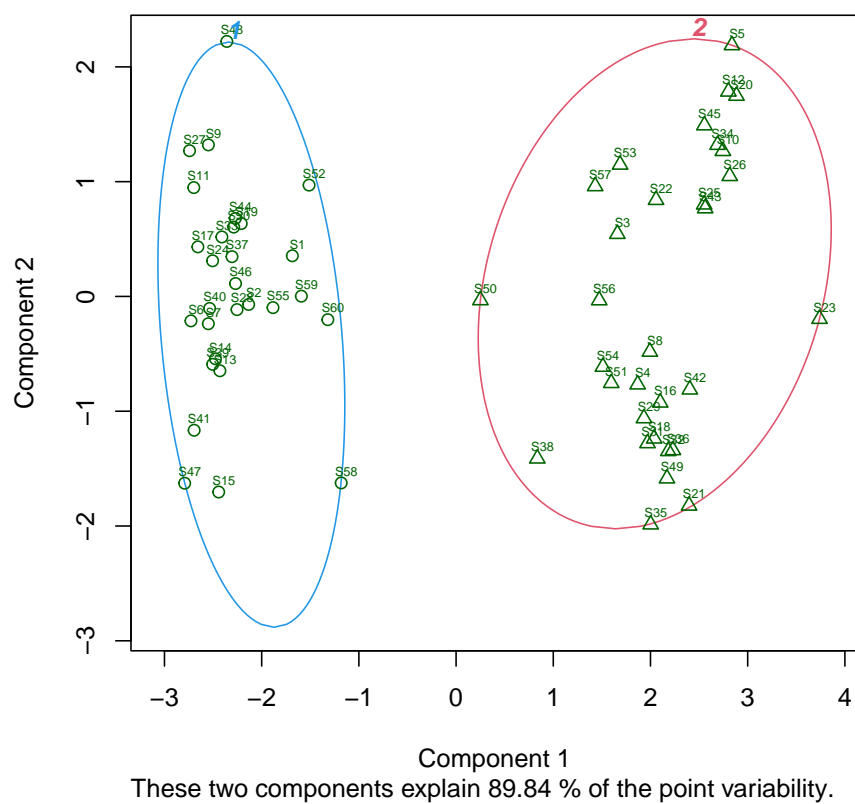


Figure 4: Principal component plot of male and female clusters

6 Check for potential sex bias using the dip test

The massiR method for predicting the sex of samples is >97% accurate for data sets with 6 or more samples and with at least of 15% of either males or females. Outside of this range, this method still performs well in most cases. As there is no guarantee that publicly available data sets will fall within these limits, the function `massi.dip` can be used to test if the data set might have a male/female ratio that might affect performance.

The massiR method was tested using empirical data sets for five different human tissues. Individual data subsets were randomly generated for each tissue data set ranging from 6-50 samples and with a wide-range of Male/Female ratios. The results of this testing suggest for data sets with >10 samples a dip statistic >0.08 is indicative of at least 15% of males or females in the data set.

The `massi_dip` function calculates z-scores for each sample and implements the dip test to test for unimodality (Maechler 2013). As a relatively balanced dataset would typically show a bi-modal distribution of the z-scores, the dip statistic is then used to predict if a dataset shows a unimodal distribution that would be expected if a vast majority of samples were of one sex.

To use `massi_dip` function, which calculates the dip statistic using the data output from the `massi_select` function:

```
> dip.result <- massi_dip(massi.select.out)
```

This returns the message: **dip test statistic is >0.08. This suggests that the proportion of male and female samples in this data set is relatively balanced**

Visually inspecting this distribution as a density plot (figure 5) or a histogram plot (figure 6) enables the user to see if there is the expected bi-modal distribution (as there should be distinct distributions for each sex).

To produce a density plot and histogram of sample z-scores:

```
> dip.result <- massi_dip(massi.select.out)
> plot(dip.result[[3]])
```

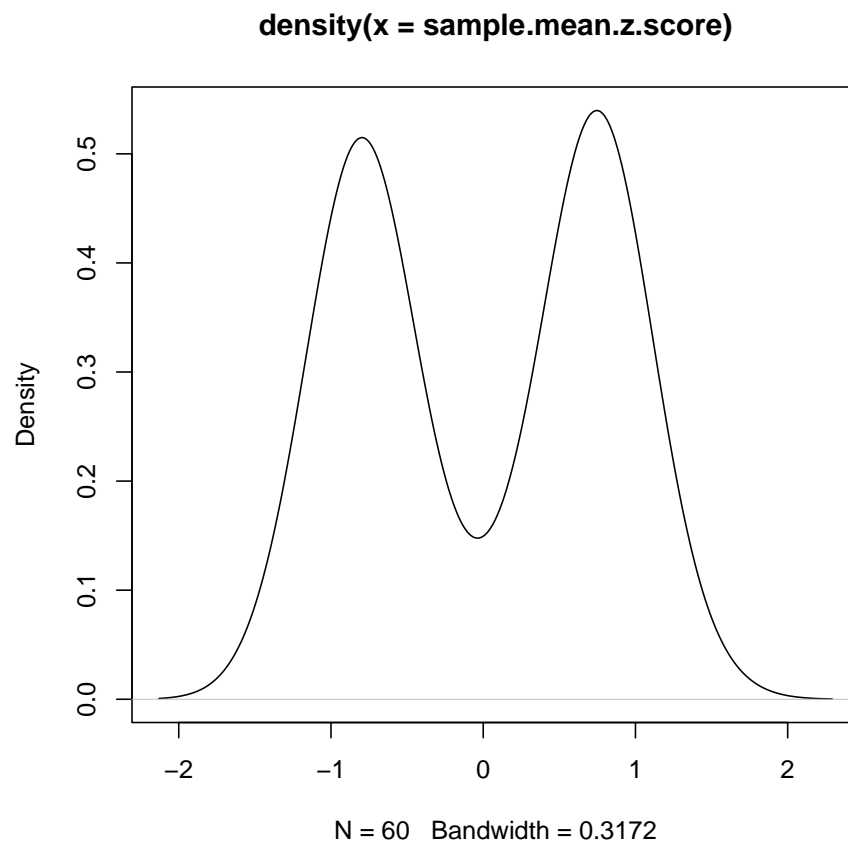


Figure 5: Density plot of mean y chromosome probe z-scores

```
> dip.result <- massi_dip(massi.select.out)
> hist(x=dip.result[[2]], breaks=20)
```

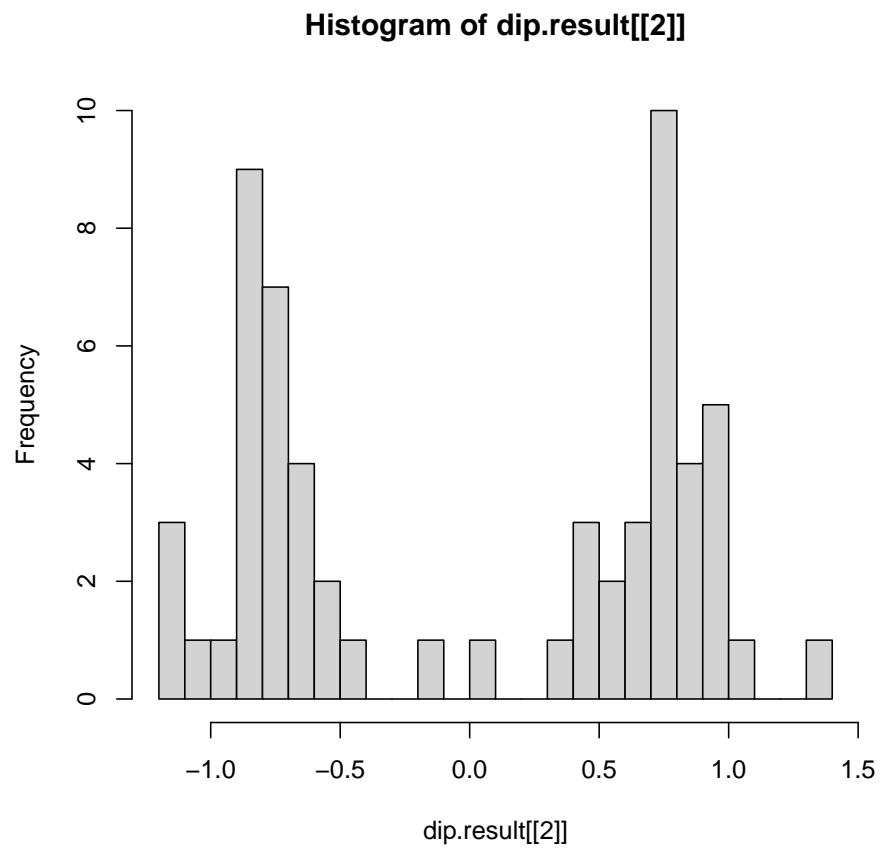


Figure 6: Histogram of mean y chromosome probe z-scores

If the data set has a sex bias that may influence the accuracy of the massiR sex prediction, then the `massi_dip` function is likely to return a dip statistic of <0.08 . For example, if we are to use the massiR test data set to generate a subset to 20 samples composed of 10% males, we will see that the dip statistic returned is <0.08 .

To create this female skewed bias:

get the sample id's for the male and female samples:

```
> male.ids <-
+   subset(sample.results$ID,
+           subset=sample.results$sex=="male")
> female.ids <-
+   subset(sample.results$ID,
+           subset=sample.results$sex=="female")
```

Create a data subset of 20 samples with 10% males:

```
> bias.subset.ids <- c(female.ids[1:18], male.ids[1:2])
> bias.subset <- massi.select.out[bias.subset.ids]
```

Use the `massi.dip` function to test for sex-biased data set:

```
> bias.dip <- massi_dip(bias.subset)
```

Please note that a dip >0.08 is a good indication that there is not a sex bias present that will affect the accuracy of the massiR method. However, a dip statistic <0.08 may still be returned for data sets with $>15\%$ males or female or data sets that are suitable for massiR analysis, therefore the results of the `massi_dip` function should be interpreted with caution and in light of the `massi_cluster` results.

7 Performing massiR analysis with an ExpressionSet object

The massiR pipeline allows the input of expression data in the class `ExpressionSet`. Here is an example of how to use data in the `ExpressionSet` class in a massiR analysis and how to put the results back into the `ExpressionSet`:

Load the example `ExpressionSet` data included with the massiR package:

```
> data(massi.eset, massi.test.probes)
```

Using massiR with an `ExpressionSet` is the same as using a `data.frame` as in the above example:

```
> eset.select.out <-
+   massi_select(massi.eset, massi.test.probes)
> eset.results <-
+   massi_cluster(eset.select.out)
```

Now to get the `massi.cluster` results and add them to the `ExpressionSet`:

```
> # Get the sex for each sample from the massi_cluster results
> eset.sample.results <-
+   data.frame(eset.results[[2]])
> sexData <-
+   data.frame(eset.sample.results[c("ID", "sex")])
> # Extract the order of samples in the ExpressionSet and match with results
> eset.names <-
+   colnames(exprs(massi.eset))
> # match the sample order in massiR results to the same as the ExpressionSet object
> sexData <- sexData[match(eset.names, sexData$ID),]
> # create an annotatedDataFrame to add to ExpressionSet
> pData <- new("AnnotatedDataFrame", data = sexData)
> # add the annotatedDataFrame to the Expressionset as phenoData
> phenoData(massi.eset) <- pData
```

Check the `phenoData` is in the `ExpressionSet` and double check that all sample id's from the `massiR` analysis match the sample identifiers in the `ExpressionSet`.

```
> # check the phenodata is now within the ExpressionSet
> phenoData(massi.eset)

An object of class 'AnnotatedDataFrame'
 rowNames: 1 12 ... 57 (60 total)
 varLabels: ID sex
 varMetadata: labelDescription

> # check that all phenodata id's match expressionSet column names.
> # This must return "TRUE"
> all(massi.eset$ID == colnames(exprs(massi.eset)))

[1] TRUE
```

8 Using the included `massiR` Y chromosome probe lists

The `massiR` package includes lists of Y chromosome probes for widely used Illumina and Affymetrix human gene expression platforms. If you wish to use one of the included probe lists, for example the Illumina human v2 probes:

Load the `massiR` included probe lists:

```
> data(y.probes)
```

Check the names of the platforms for the probe lists.

```
> names(y.probes)
```

```
[1] "illumina_humanwg_6_v1" "illumina_humanwg_6_v2" "illumina_humanwg_6_v1"
[4] "illumina_humanht_12"   "affy_hugene_1_0_st_v1" "affy_hg_u133_plus_2"
```

To get probe list into format for massiR analysis:

```
> illumina.v2.probes <- data.frame(y.probes["illumina_humanwg_6_v2"])
```

The names of the probe lists correspond to Ensembl biomart attribute names. For instructions on obtaining probe identifiers for other platforms, see the section "Using biomaRt to obtain y chromosome probe lists"

9 Using biomaRt to obtain y chromosome probe lists

Obtaining y chromosome probes lists for many microarray platforms is relatively easy using the biomaRt package (Durinik et al. 2005 and Durinik et al. 2009). This method is recommended because Ensembl have mapped probe sequences to reference genomes for many platforms and this allows ambiguous and non-specific probes to be removed. For details on probe mapping methods, see http://jan2013.archive.ensembl.org/info/docs/microarray_probe_set_mapping.html

For example, you can download the probes corresponding to the massiR test data set and obtain the Entrez gene id and genomic positions and convert these into a format for a massiR analysis:

Use the biomaRt package to download genomic regions and Entrez gene id's for Illumina v2 probes:

```
> library(biomaRt)
> mart <- useMart('ensembl', dataset="hsapiens_gene_ensembl")
> filters <- listFilters(mart)
> attributes <- listAttributes(mart)
> gene.attributes <-
+   getBM(mart=mart, values=TRUE,
+         filters=c("with_illumina_humanwg_6_v2"),
+         attributes= c("illumina_humanwg_6_v2", "entrezgene",
+                       "chromosome_name", "start_position",
+                       "end_position", "strand"))
```

Remove the probes mapped to multiple genomic regions:

```
> unique.probe <-
+   subset(gene.attributes, subset=!duplicated(gene.attributes[,1]))
```

Select the probes that correspond to y chromosome genes:

```
> y.unique <-
+   subset(unique.probe, subset=unique.probe$chromosome_name == "Y")
```

Get the probe id's as row.names in the format for massiR analysis:

```
> illumina.v2.probes <-  
+   data.frame(row.names=y.unique$illumina_humanwg_6_v2)
```

This is a straightforwd way of obtaining Y chromosome probes for many microarray platforms that is independent of platform manufacturer annotations and is highly reccomended.

10 References

Christian Hennig (2013) fpc: Flexible procedures for clustering. R package version 2.1-6. <http://CRAN.R-project.org/package=fpc>

Martin Maechler (2013) diptest: Hartigan's dip test statistic for unimodality - corrected code. R package version 0.75-5. <http://CRAN.R-project.org/package=diptest>

Gregory R. Warnes, Ben Bolker, Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber, Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz and Bill Venables (2013). gplots: Various R programming tools for plotting data. R package version 2.12.1. <http://CRAN.R-project.org/package=gplots>

Steffen Durinck, Paul T. Spellman, Ewan Birney and Wolfgang Huber (2009) Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nature Protocols* 4, 1184-1191.

Steffen Durinck, Yves Moreau, Arek Kasprzyk, Sean Davis, Bart De Moor, Alvis Brazma and Wolfgang Huber (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics* 21, 3439-3440.