

deltaRpkm

An R package for a rapid detection of differential gene presence
between related genomes

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***deltaRpkm** is an R package whose main purpose is to quickly identify genes potentially involved in a trait of interest by performing a [differential analysis of genes coverage between two sets of closely related bacterial genomes](#). The package provides functions to compute the RPKM, the δ RPKM, candidate genes filtering and heatmap plot. It also includes methods to perform some batch effect controls and diagnostic plots.*

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1 Prerequisites

1.1 Input files

The deltaRpkM package requires 2 user input files:

1. a [metadata table](#) that provides parameter information for inter-group comparisons, with the following mandatory fields:

```
<sample> <phenotype_1> <phenotype_2> <genome_length> <mapped_reads> <...>
```

- **<sample>** the column containing the sample names
- **<phenotype_1>** the column containing the name of the trait being investigated for the gene differential presence analysis
- **<phenotype_2>** the column containing the 2nd trait that can be added in the heatmap for comparison
- **<genome_length>** the column containing the reference genome length
- **<mapped_reads>** the column containing the total number of mapped reads in each sample
- **<...>**

These are the minimum required elements to be given to the pipeline; more factors can be included for alternative analyses if desired.

2. a [coverage table](#) that combines the mapped read counts per gene and per sample, as this:

```
<chr> <start> <end> <geneID> <sample1_readCounts> <sample2_readCounts> <...>
```

- **<chr>** the column containing the name of reference genome
- **<start>** the column containing the gene start coordinate
- **<end>** the column containing the gene end coordinate
- **<geneID>** the column containing the gene identifier
- **<sample1>** the column named by the sample 1 and containing its mapped read counts
- **<sample2>** the column named by the sample 2 and containing its mapped read counts
- **<...>**

Please make sure that the input tables follow as much as possible those formats (column order and names for the minimum required information). For instance, the sample names in the <sample> column of the metadata table MUST be the same as <sample_readCounts> in the coverage table.

The working examples provided by the package correspond to datasets of different sizes from *Listeria monocytogenes* ([Aguilar-Bultet et al., 2018](#)).

1.2 Minimum of R knowledge

Although deltaRpkM is very simple/user-friendly and comes with an extensive documentation (<https://github.com/frihaka/deltaRpkM/tree/master/doc>), it assumes a minimum familiarity with R, e.g installing libraries from CRAN and Bioconductor, awareness of working environment, basic R commands and objects etc.

1.3 R dependencies

The R package `deltaRpkm`, like any R package, is built upon common R **CRAN** libraries that should be already installed in your system if you are an R user (`ggplot2`, `ggfortify`, `dplyr`, `data.table`...).

It also requires some **Bioconductor** R packages. If not already in your system, please make sure that the following R packages from Bioconductor - `sva` and `Biostrings` - are installed:

```
# for R >= 3.5
> if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install("sva", version = "3.8")
BiocManager::install("Biostrings", version = "3.8")

# for R <= 3.4
source("https://bioconductor.org/biocLite.R")
biocLite("sva")
biocLite("Biostrings")
```

1.4 Download and install deltaRpkm

From GitHub <https://github.com/frihaka/deltaRpkm/>, download the compressed binary file on a local directory.

If you are familiar with R and have already most of the common R packages (`ggplot2`, `ggfortify`, `dplyr`...), you can install from the terminal with the **R CMD INSTALL** command:

```
R CMD INSTALL deltaRpkm_0.1.0_R_x86_64-pc-linux-gnu.tar.gz
```

and then just install the Bioconductor packages as shown above.

If you are not very familiar with R environment, try rather to install `deltaRpkm` with the function `install.packages()`, from inside R/RStudio:

```
> install.packages("path/2/deltaRpkm_0.1.0_R_x86_64-pc-linux-gnu.tar.gz",
  repos = NULL,
  dependencies = TRUE)
```

This will download your missing CRAN libraries that are required by `deltaRpkm`. You will still need to install the Bioconductor packages, as shown above.

2 Overview of the pipeline

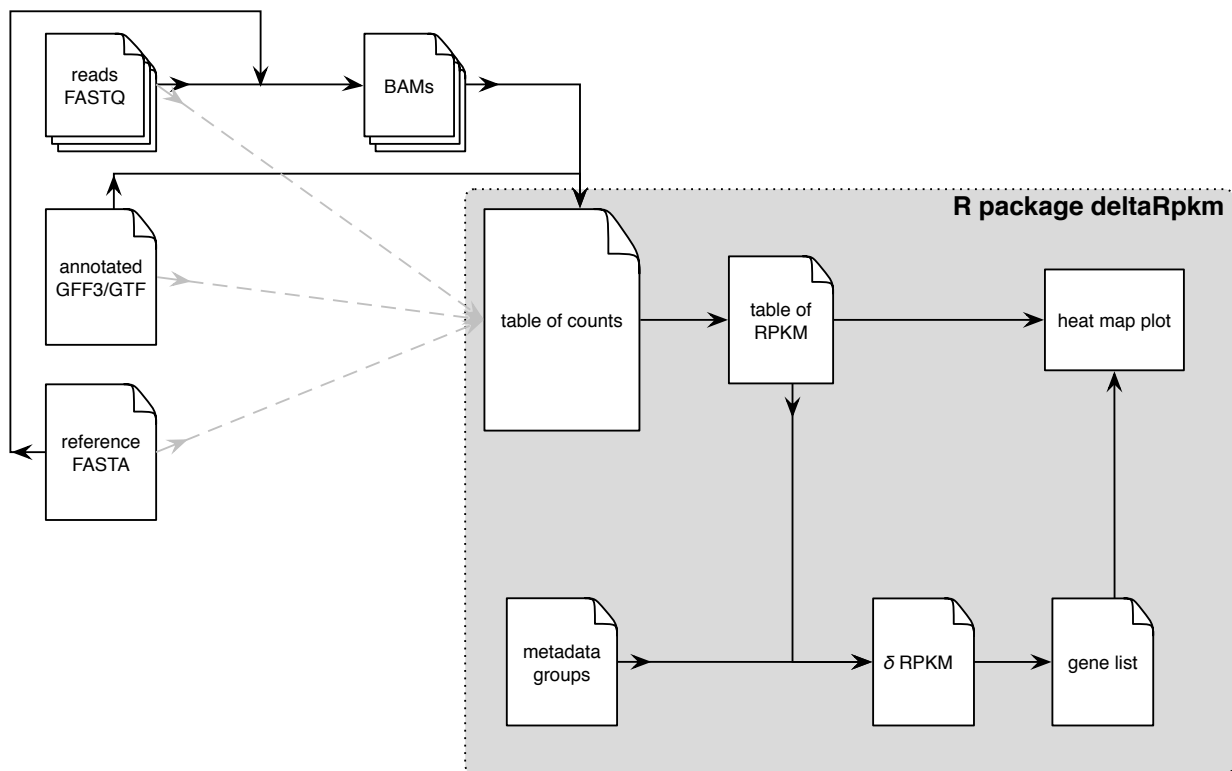


Figure 1: Overview of the deltaRpkm pipeline.

3 Building and loading the coverage table

The read counts per gene table must be pre-computed and provided by the user. Below is the command used to build it with `bedtools multicov` on a terminal:

```
bedtools multicov -bams aln.1.bam ... aln.n.bam -bed <bed/gff/vcf> > coverage_table.csv
```

Notes on `bedtools multicov`:

- any number of bam files can be run together in a batch mode, thus allowing all the samples of the dataset to be included in the coverage table
- do not forget to redirect/output the results into a coverage table

The user can build the read count table with other methods, like the RNA-seq aligner called STAR that maps and produces the coverage table at once.

Please do not forget to ensure that your custom coverage table (either produced by `bedtools multicov` or by STAR etc) follows the following required format:

- `<chr>`
- `<start>`
- `<end>`
- `<geneID>`
- `<sample1_readCounts>`
- `<sample2_readCounts>`
- `<...>`

Alternatively, example datasets derived from [Aguilar-Bultet *et al.*, 2018](#) are available in the `deltaRpkM` package:

```
> data("coverage_table_N51") # this creates coverage_table df in the
  environment
> head(coverage_table_N51[, 1:8])
```

	chr	start	end	geneID	JF4931	JF5172	JF5761
				JF5827			
1	JF5203_chromosome	318	1674	LMJF5203_00001	3109	1466	5582
	5761						
2	JF5203_chromosome	1867	3013	LMJF5203_00002	2778	1099	4737
	4882						
3	JF5203_chromosome	3120	4464	LMJF5203_00003	3218	1473	4914
	5365						
4	JF5203_chromosome	4577	4865	LMJF5203_00004	947	358	1568
	1546						
5	JF5203_chromosome	4868	5981	LMJF5203_00005	2578	932	4415
	4572						
6	JF5203_chromosome	6029	7970	LMJF5203_00006	4125	1853	7018
	7681						

4 Loading the metadata table

The user must provide a metadata table with some minimum informations/columns about the samples:

- a column containing the trait or characteristic 1 data, that will be used for the RPKM comparisons. This is the main characteristic of interest being studied, *i.e* the trait of group 1 with the reference genome. This trait is the criteria of categorizing the datasets into 2 distinct groups and the basis of the whole comparison. For example: "lineage_type" which can include different values but only 2 will be compared. That is why we advise to categorize samples into groups; in the example: Lineage_I and Lineage_II
- a column containing the trait 2 data, that will be used as a colored sidebar of the heatmap: this corresponds to a 2nd characteristic that the user can add to check whether the clustering in the heatmap correlates with this 2nd trait. For example: "infection_origin"

- reference genome length
- total number of mapped reads

The metadata/design table must be a data frame that looks like this:

Table 1: Example of input metadata table.

sample	platform	lineage_type	infection	genome_length	mapped_reads
JF4839	HiSeq2000	Lineage_II	ENV	2900890	8288011
JF4899	HiSeq4000	Lineage_I	CNS	2900890	9797440
JF4901	HiSeq2000	Lineage_I	CNS	2900890	1926369
JF4902	HiSeq2000	Lineage_I	CNS	2900890	1750981
JF4904	HiSeq2000	Lineage_I	CNS	2900890	1469430

A working example metadata dataset from deltaRpkM package is shown below:

```
> data("metadata_table_N51") # this creates metadata_table df in the env
> head(metadata_table_N51)
  sample platform lineage_type infection genome_length mapped_reads
1 JF4906 HiSeq2000 Lineage_I      CNS      2900890      2042865
2 JF4929 HiSeq4000 Lineage_I      CNS      2900890      9469100
3 JF4931 HiSeq3000 Lineage_II     CNS      2900890      5285534
```

Format the metadata information with `deltaRpkM::loadMetadata` function, giving in as arguments:

- **user_metadata** = <data frame of user input design table>
- **delta_phenotype_colname** = <phenotype 1 column name used to build the 2 categories and perform the comparison>
- **heatmapbar_phenotype_colname** = <phenotype 2 column name used to build the extra bar in the heatmap>
- **samples_colname** = <column name containing the sample IDs>
- **genome_length_colname** = <genomic length (in bp) of the reference genome used for mapping>
- **mapped_reads_colname** = <total number of mapped reads, for each sample ID>

```
> design_table <- loadMetadata(user_metadata = metadata_table_N51,
                               delta_phenotype_colname = "lineage_type",
                               heatmapbar_phenotype_colname = "infection",
                               samples_colname = "sample",
```

```

genome_length_colname = "genome_length"
mapped_reads_colname = "mapped_reads")

> head(design_table)
  sample lineage_type infection genome_length mapped_reads
1 JF4906 Lineage_I      CNS      2900890      2042865
2 JF4929 Lineage_I      CNS      2900890      9469100
3 JF4931 Lineage_II     CNS      2900890      5285534

```

5 Convert read counts to RPKM values

5.1 RPKM formula

deltaRpk uses the **Reads Per Kilobase Million RPKM** - a standard RNA-seq metrics that normalizes the read counts per gene for **Sequencing Depth** and **Gene Length**:

with N_s being the total number of read counts in the sample,

$$scalingFactor = \frac{N_s}{10^6} \quad (1)$$

$$RPM = \frac{readCountPerGene}{scalingFactor} \quad (2)$$

$$RPKM = \frac{RPM}{geneLength.10^{-3}} \quad (3)$$

The equation (2) corresponds to the normalization of the read counts by the sample sequencing depth; and equation (3) corresponds to the normalization by the gene length.

5.2 Run `deltaRpk::rpkm`

Run the following `deltaRpk::rpkm` function to compute the RPKM values of each gene, in each sample:

```

> rpkmtable <- rpkm(user_metadata = design_table,
                    coverage_table = coverage_table_N51,
                    delta_phenotype_colname = "lineage_type",
                    heatmapbar_phenotype_colname = "infection")

> head(rpkmtable)
  sample      geneID lineage_type infection reads rpkm
1 JF4906 LMJF5203_00001 Lineage_I      CNS    1177  425
2 JF4906 LMJF5203_00002 Lineage_I      CNS     952  406
3 JF4906 LMJF5203_00003 Lineage_I      CNS    1080  393

```


6 $\delta RPKM$ values

6.1 $\delta RPKM$ formula

The analysis is centered around a pairwise comparison of gene presence/absence between genomes categorized into two different groups following the selected trait or feature:

- a group 1 that shares the trait A of the reference genome
- a group 2 that does not have the reference trait A

For each pairwise comparison of a gene j between a genome x from group 1 and a genome y from group 2, `deltaRpkm::deltarpkm` function computes the difference of their RPKM values at gene j ($\delta RPKM_{jxy}$) as:

$$\delta RPKM_{jxy} = RPKM_{jx} - RPKM_{jy} \quad (4)$$

6.2 Run `deltaRpkm::deltarpkm`

```
> deltarpm_table <- deltarpm(rpkmtable = rpkmtable,
                             genes_names = unique(rpkmtable$genelD),
                             samples_colname = "sample",
                             delta_phenotype_colname = "lineage_type",
                             reference_sample = "JF5203",
                             nonref_delta_phenotype = "Lineage_II")
> head(deltarpkm_table)
genelD sample.group1 lineage_type.group1 infection.group1 reads.group1
rpkmt.group1 sample.group2 lineage_type.group2 infection.group2
reads.group2 rpkmt.group2 deltarpm
1 LMJF5203_00001 JF4906 Lineage_I CNS
1177 425 JF4931 Lineage_II
CNS 3109 433 -8
2 LMJF5203_00001 JF4906 Lineage_I CNS
1177 425 JF5172 Lineage_II
CNS 1466 520 -95
3 LMJF5203_00001 JF4906 Lineage_I CNS
1177 425 JF5761 Lineage_II
ENV 5582 465 -40
```

This run might take a few minutes, depending on the size of the datasets.

7 Differential gene presence

7.1 Strategy

The `deltaRpkm` package main feature is to screen for *the preferential presence of genes in the reference genome group, versus a comparison group*.

We use the method `deltaRpkm::deltaRPKMStats` to infer this set of genes, since they could potentially be involved in the reference genome group trait (`<lineage_type> = "Lineage_type_I"`). This function:

1. computes for each gene j the **median value of all its $\delta RPKM$ (m_j)** derived from the sample pairwise comparisons. Note: a negative median value of all $\delta RPKM$ of a given gene would mean that this gene is "preferentially present" in the comparison samples of group 2 than in the reference genome group 1
2. calculates the **standard deviation s** of all the m_j values in the analysis
3. selects genes as present in the reference genome group 1 based on an **arbitrary threshold of $2.s$** :

$$selectedGene : m_j \geq 2.s \quad (5)$$

In other words, a gene j having a median $\delta RPKM$ value greater than $2.s$ will be considered as "preferentially present" in the reference genome group 1 (with `<lineage_type> = "Lineage_type_I"`) than in the comparison group 2 (with `<lineage_type> = "Lineage_type_II"`).

7.2 Run `deltaRpkM::deltaRPKMStats`

```
> stats_table <- deltaRPKMStats(deltarpkm_table = deltarpm_table)
> head(stats_table)
```

	geneID	sample.group1	lineage_type.group1	infection.group1	reads.group1	rpkM.group1	sample.group2	lineage_type.group2	reads.group2	rpkM.group2	deltarpkm	deltarpkm_median	deltarpkm_medianSD	thres_SD	median_value	selected_gene
1	LMJF5203_00001	JF4906	Lineage_I													
	1177	425	JF4931	Lineage_II												
2	LMJF5203_00001	JF4906	Lineage_I													
	1177	425	JF5172	Lineage_II												
3	LMJF5203_00001	JF4906	Lineage_I													
	1177	425	JF5761	Lineage_II												
					infection.group2	reads.group2	rpkM.group2	deltarpkm	deltarpkm_median	deltarpkm_medianSD	thres_SD	median_value	selected_gene			
1		CNS			3109	433	-8									-31
		114.24				228.48		-								
2		CNS			1466	520	-95									-31
		114.24				228.48		-								
3		ENV			5582	465	-40									-31
		114.24				228.48		-								

The default threshold value to select genes is based on $2.s$. But this threshold can be changed in the `deltaRpkM::deltaRPKMStats` parameter `min_SD_foldChange`, e.g:

```
> stats_table_fcnew <- deltaRPKMStats(min_SD_foldChange = 1.5,
                                       deltarpm_table = deltarpm_table)
```

Note that the column **selected_gene** contains information about whether a given gene should be selected as present preferentially in the reference genome group (noted as "+") or not (noted as "-"). This column will be used later to filter the relevant genes.

7.3 Visual check of the m_j distribution

With the function `deltaRpk::median_plot` it is possible to check visually the median $\delta RPKM$ value of every gene (one dot per gene).

```
> median_plot(data_table = stats_table ,  
               gene_annotation_table = coverage_table_N51) # genes  
               annotation info
```

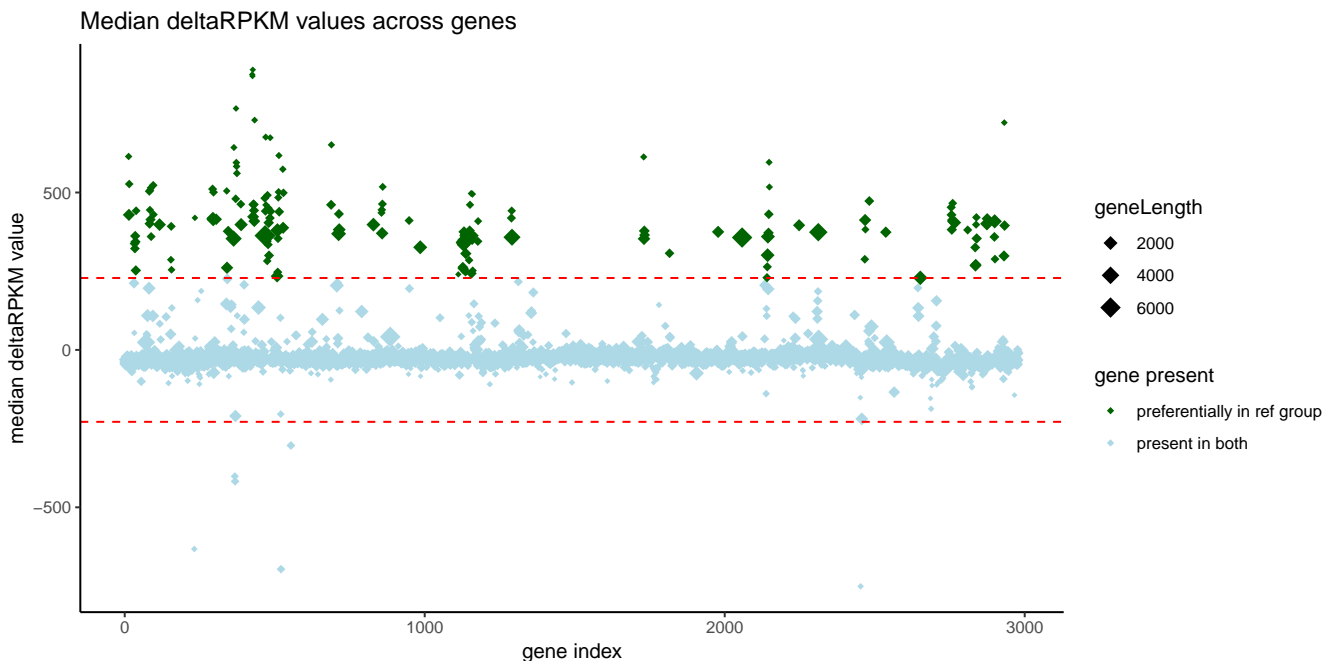


Figure 2: Median $\delta RPKM$ values for all genes. Plot output from `deltaRpk::median_plot`. The negative median $\delta RPKM$ values correspond to genes that appear as better covered in the comparison group 2 than in the reference genome group 1. Note: the gene index value reflects the genomic coordinates since they are ordered as the gene names (these later being themselves given during *de novo* annotation based on the genomic coordinates, roughly speaking).

The genes in dark green in **Figure 2** correspond to the set of genes present in the reference genome group 1 and potentially linked to the trait of interest ("lineage_type").

7.4 Selected gene set

For a given threshold value of median $\delta RPKM$ (by default 2.s of all median $\delta RPKM$ values), we can simply extract the genes appearing as differentially present in the reference genome group 1 (green dots in the **Figure 2**):

```
> differential_present_genes <- unique(stats_table[stats_table$  
  selected_gene %in% "+", ]$geneID)  
> length(differential_present_genes)  
> [1] 173  
> head(differential_present_genes)  
> [1] "LMJF5203_00013" "LMJF5203_00014" "LMJF5203_00015" "LMJF5203_
```

```
00033" "LMJF5203_00034" "LMJF5203_00035"
```

This gene set can be used to perform various functional clustering analysis. We propose in the package a method to build a summary heatmap of their RPKM values and how they relate (or not) to a second trait of interest.

8 Heatmap

8.1 Rational

The idea is to analyse how the RPKM values of the genes specific to the reference genome group 1 distribute across all samples of group 1 and group 2. The aim is:

- to confirm (or infirm) that the heatmap clustering of the samples into two distinct categories is coherent with the initial group 1 and group 2 definition. Typically, the selected genes should present in overall higher RPKM values in the reference genome group 1 than in group 2. But it is not always the case.
- to investigate at a higher resolution the homogeneity of each group

Thus `deltaRpkM` allows to investigate the clustering of the selected genes based on their RPKM values computed earlier. A putative correlation with the second trait can be visualized by adding a color bar corresponding to this second trait given in the metadata table - which is "infection" in the working example dataset.

The heatmap plot is made with the `deltaRpkM::rpkmHeatmap` function, derived from the `gplots::heatmap.2` method (Warnes et al., 2018).

8.2 Preparing the RPKM values for the heatmap

The heatmap will focus only on the *RPKM values of the set of genes that are relevant, i.e* the ones that appear as differentially present in the reference genome group 1 (see the darkgreen dots in **Figure 2**).

For this, we first subset the RPKM data table and keep only the rows/genes that were selected using the `deltaRpkM::subsetRPKMTable`:

```
# Subset the RPKM table for the selected genes
> heatmap_table <- subsetRPKMTable(rpkM_table = rpkMtable,
                                   user_metadata = design_table,
                                   delta_phenotype_colname = "lineage_type",
                                   heatmapbar_phenotype_colname = "infection",
                                   sd_filtered_genes = differential_present_genes
                                   )
> head(heatmap_table)
  sample lineage_type infection LMJF5203_00013 LMJF5203_00014 LMJF5203_
    _00015 LMJF5203_00033 LMJF5203_00034 LMJF5203_00035 LMJF5203_
    00036
1 JF4906      Lineage_I      CNS           607           450
      630           421           445           521
      553
```

2	JF4929	Lineage_I	CNS	581	397
		498	456	447	427
		423			
3	JF4931	Lineage_II	CNS	0	0
		0	68	1	2
		1			

Then the subsetting RPKM values data frame is converted to a matrix (since this is the required format for the heatmap function) using the `deltaRpk::convertHeatmapToMatrix` function:

```
# Convert the subsetting RPKM table to a matrix
> heatmap_matrix <- convertHeatmapToMatrix(wide_rpk_table = heatmap_
  table ,
                                           delta_phenotype_colname = "lineage_type",
                                           heatmapbar_phenotype_colname = "infection"
                                           )
> head(heatmap_matrix)
      LMJF5203_00013 LMJF5203_00014 LMJF5203_00015 LMJF5203_00033
      LMJF5203_00034 LMJF5203_00035 LMJF5203_00036 LMJF5203_00037
      LMJF5203_00038 LMJF5203_00082 LMJF5203_00083 LMJF5203_00084
JF4906          607          450          630          421
          445          521          553          472
          491          619          352          450
JF4929          581          397          498          456
          447          427          423          412
          484           0           0           0
JF4931           0           0           0           68
          1           2           1           0
          1           0          18          457
```

It is important to note that the heatmap matrix must contain sample names as row names.

8.3 Plot heatmap

Finally, we create a summary plot as a heatmap to highlight difference in the RPKM values of the selected genes between samples of group 1 and group 2, using the `deltaRpk::rpkHeatmap` function:

```
> rpkHeatmap(filtered_rpk_matrix = heatmap_matrix ,
              user_metadata = design_table ,
              heatmapbar_phenotype_colname = "infection")
```

This creates an output heatmap file `deltaRpk_heatmap.tiff` in the working directory. The heatmap for the example dataset (*Listeria monocytogenes*, $N=51$) is shown in **Figure 3**. It confirms the clustering of the samples into the initial two categories: group 1 samples cluster together on the upper part corresponding to high RPKM values, while group 2 samples cluster together in the lower part of the heatmap with lower RPKM values, for the selected gene set. The heatmap colors can be easily changed with the color break parameters:

```
> rpkmHeatmap(filtered_rpkm_matrix = heatmap_matrix ,
               user_metadata = design_table ,
               heatmapbar_phenotype_colname = "infection",
               low_color = "col1", # low RPKM values , default "yellow"
               mid_color = "col2", # mid range RPKM values , default "
                                   green"
               high_color = "col3") # high range RPKM values , default "
                                   blue"
```

See the next section for more on color breaks tuning.

8.4 Tuning heatmap parameters: color breaks

`deltaRpkm::rpkmHeatmap` comes with various parameters options (see `?rpkmHeatmap`), some derived from the original `gplots::heatmap.2`, and some specific to `deltaRpkm` analysis.

In particular, the heatmap **color breaks** can be adjusted with i) the `binsize` (default 200), ii) the `lower_limit` (default value 300) and iii) `upper_limit` (default value 550) arguments. These values are based on the distribution of the RPKM values and correspond to the lower and upper boundary RPKM values of the main peak:

```
> hist(rpkmtable$rpkm, freq = FALSE, breaks = 1000)
```

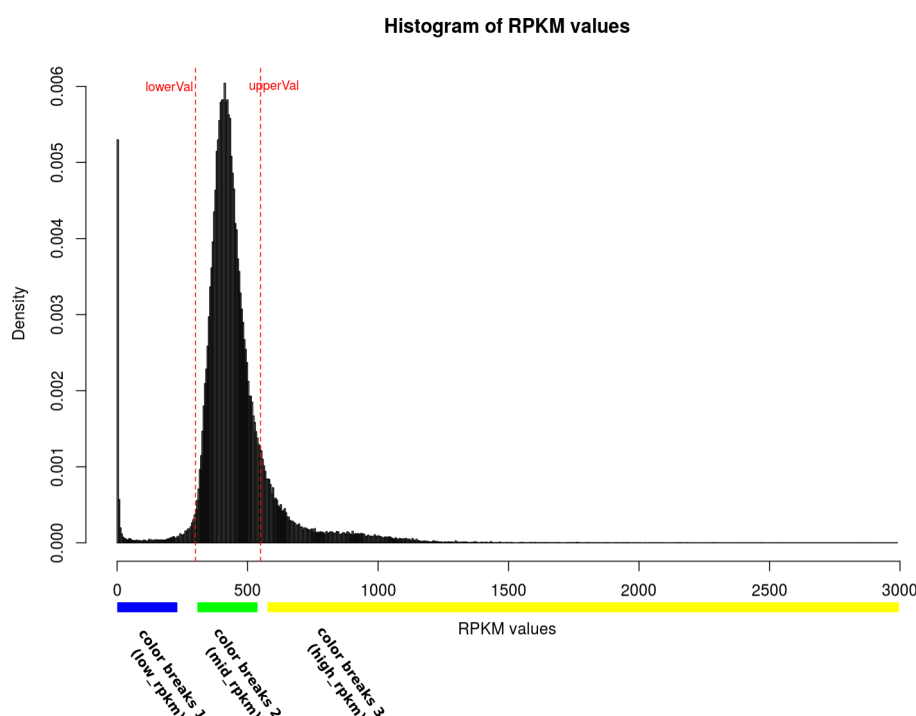


Figure 4: Distribution of RPKM values: inferring the heatmap color breaks from the histogram main peak boundary values. The lower (~ 300) and upper (~ 550) values of RPKM are used in the `deltaRpkm::rpkmHeatmap` to adjust the heatmap color breaks. Working dataset *Listeria monocytogenes*, $N=51$.

Also, `deltaRpkm` proposes some methods to infer these RPKM boundary values with the function `deltaRpkm::boundaries`:

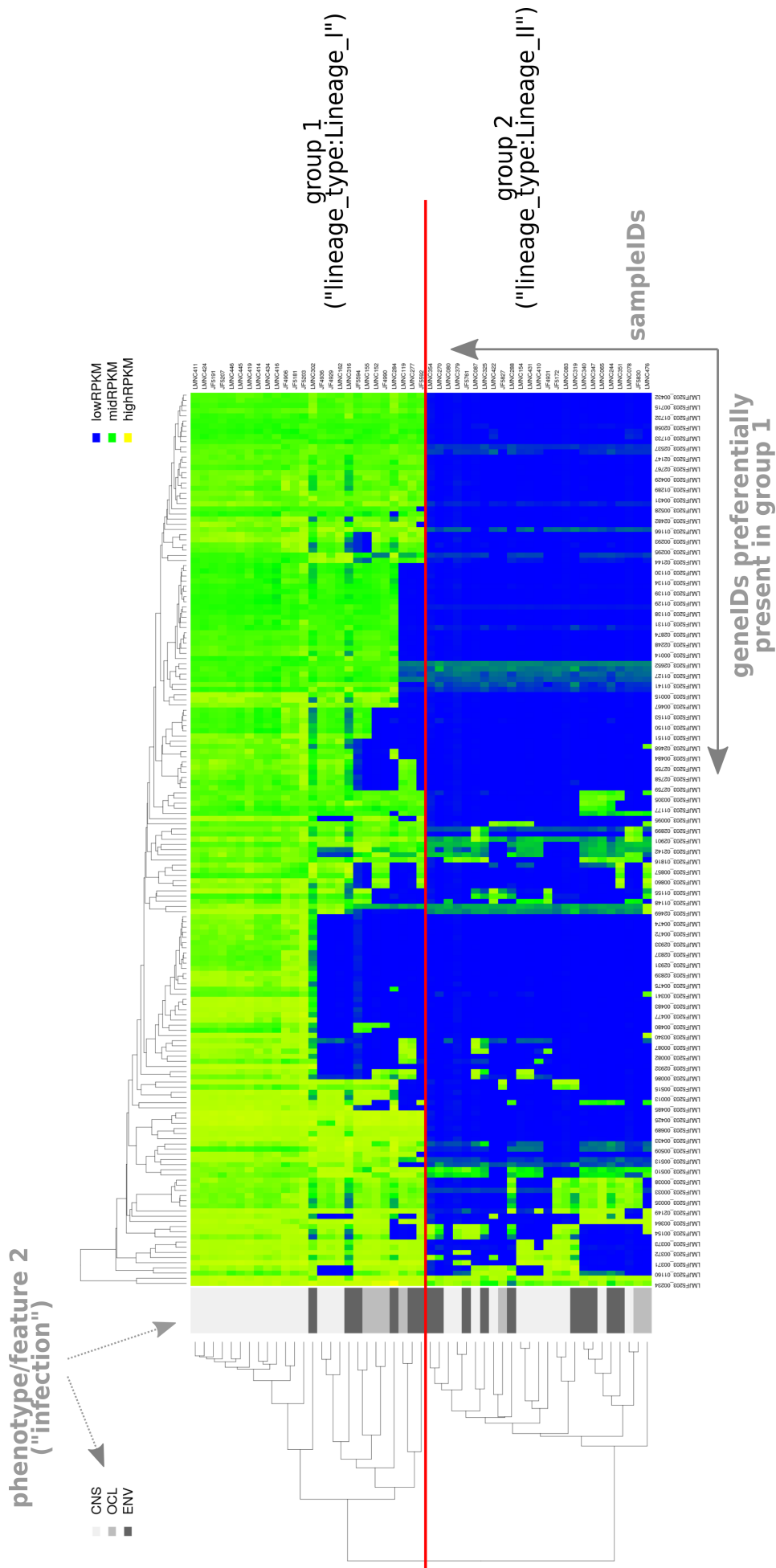


Figure 3: RPKM values distribution of the selected across samples from group 1 and group 2. Plot output from `deltaRpkM::rpkmHeatmap`. The samples cluster following the trait 1 ("lineage_type"), with group 1 annotated as "Lineage_I" and group 2 annotated as "Lineage_II". Most of the selected genes appear with a low RPKM value in group 2 cluster, even though some group 2 samples present high RPKM values (blue pixels) for certain geneIDs, suggesting these genes as potential false positives.

```

# default method mclust
> res <- boundaries(x = rpkmtable$rpkm)
> res$boundaries_df
  boundaries rpkm_values
1 lower_limit      300
2 upper_limit      624

> res <- boundaries(x = rpkmtable$rpkm, strategy = "ratios")
>   boundaries rpkm_values
1 lower_limit      295
2 upper_limit      585

> res <- boundaries(x = rpkmtable$rpkm, strategy = "quartiles")
>   boundaries rpkm_values
1 lower_limit      383
2 upper_limit      487

```

`deltaRpk::boundaries` applies by default the **mclust** parameter, which is derived from the method `mclust::densityMclust`. This can be changed with the parameter `strategy`. The boundary RPKM values can be simply extracted as `res$boundaries_df` containing the RPKM boundary values of interest.

Feel free to play with these RPKM boundary and color break parameter values in `rpkmHeatmap` function and observe the effect(s) on the heatmap readout.

9 deltaRpk performance: downsampling

The initial *Listeria monocytogenes* dataset of N=225 samples is downsampled up to N=7 samples. Each dataset is run through `deltaRpk` pipeline and the different outcomes are compared.

9.1 Dataset size effect on thresholding and gene set selection

The gene differential presence is based on a threshold value defined as 2 times (default value) the standard deviation of the medians of $\delta RPKM$ values. The median plots (**Figure 2**) for all the datasets of different sizes can be summarized in a single boxplot per gene, as shown in **Figure 5**.

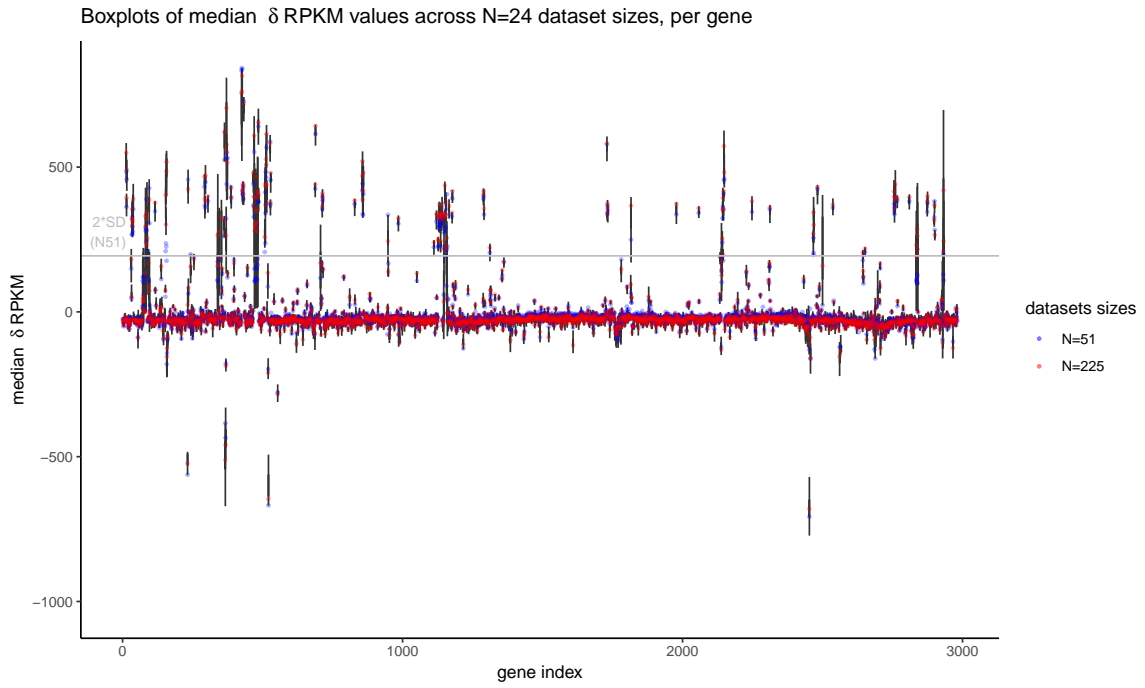


Figure 5: Dataset size effect on the median $\delta RPKM$ distribution (boxplots series). 24 datasets of different sizes ($N=225$ to $N=7$ samples) are plotted as boxplots, one per gene. Datasets with $N=51$ and $N=225$ samples are highlighted to evaluate the degree of $\delta RPKM$ variation between datasets of different sizes.

The boxplot series of **Figure 5** shows that most of the selected genes present a stable median $\delta RPKM$ distribution across all dataset sizes (bars fully above the $2.s$ threshold).

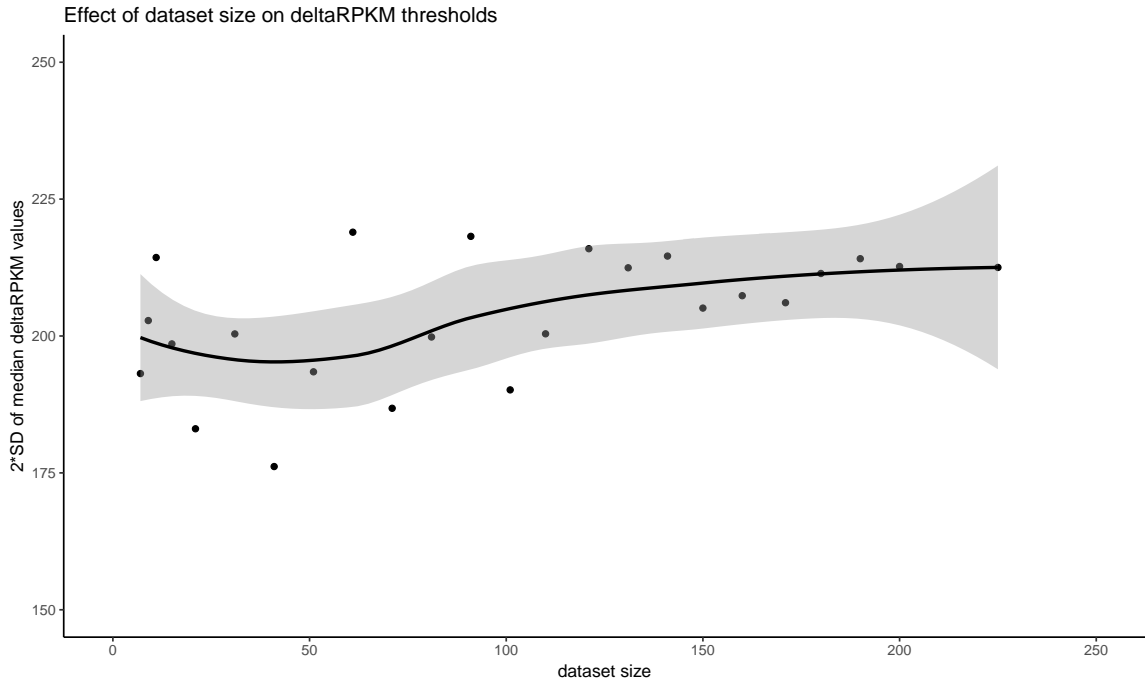


Figure 6: Dataset size effect on the $2*SD(\text{median } \delta RPKM)$ thresholding values. The smooth line is built with `loess()` method; confidence interval in grey.

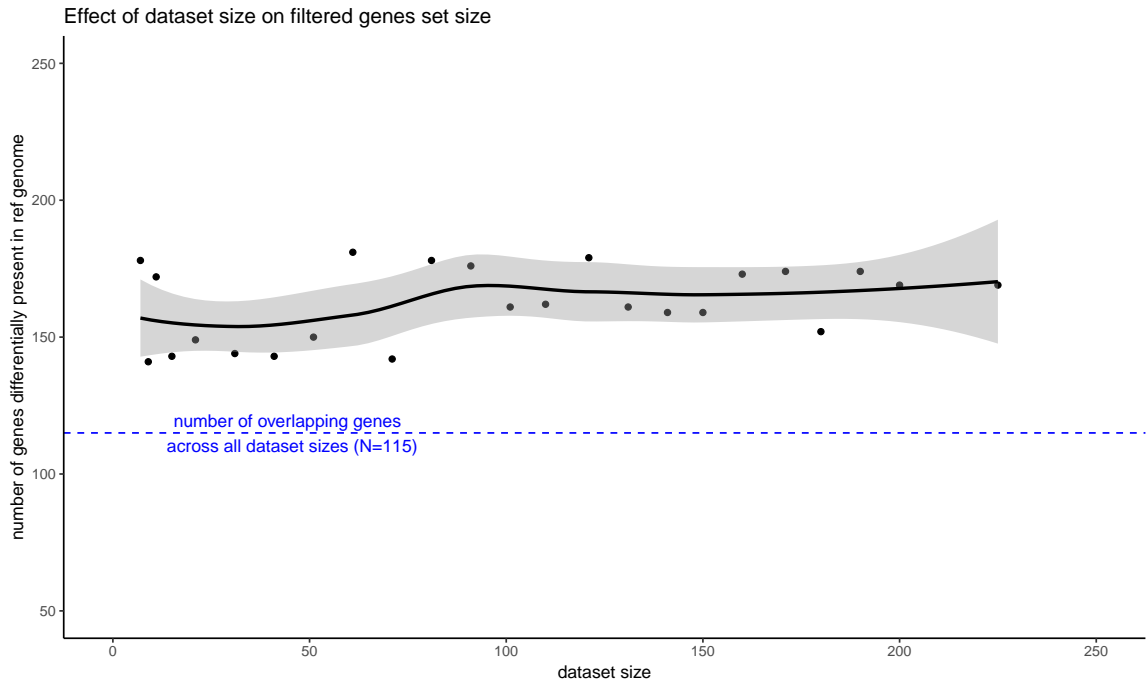


Figure 7: Dataset size effect on genes differentially present in reference genome. The smooth line is built with `loess()` method; confidence interval in grey.

9.2 Dataset size effect on runtime

The runtime increases linearly with the dataset size (**Figure 8**), but it remains reasonably low.

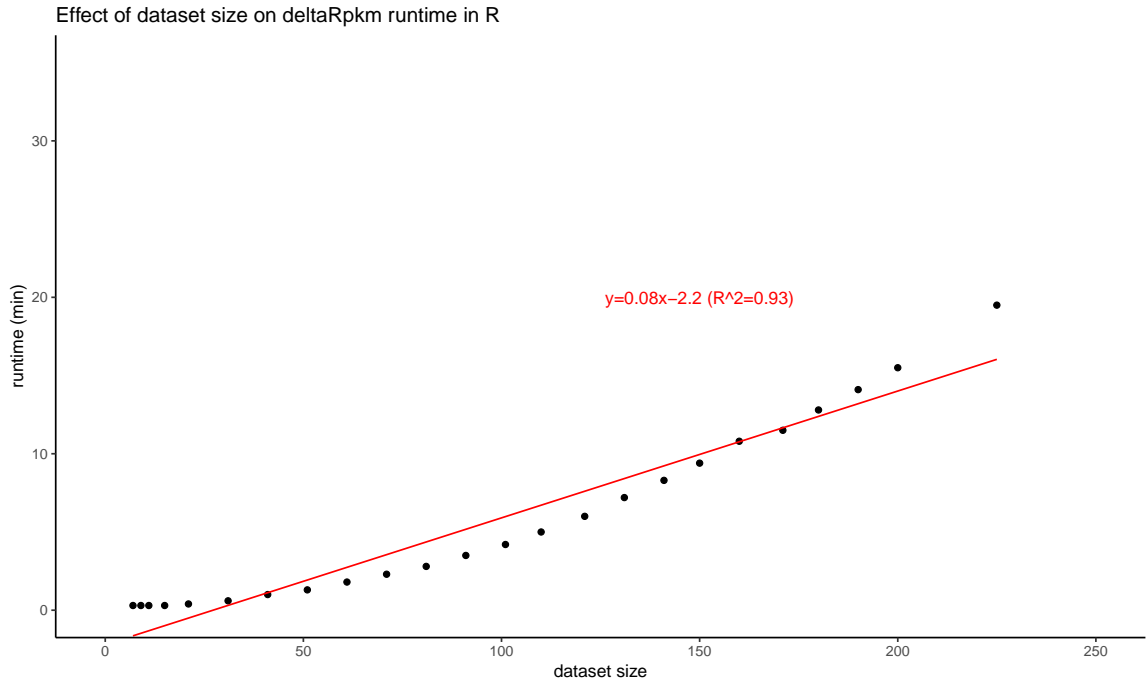


Figure 8: Dataset size and runtime when using deltaRpk. Including the pipeline steps of RPKM computing, batch effect correction, computing $\delta RPKM$ values, statistics, gene selection and plotting heatmap. Analysing with deltaRpk a large dataset of N=225 samples takes < 20min in total in R 3.4.4 (under Ubuntu 14.04.5 LTS).

9.3 Dataset size effect on memory usage

The memory requirement by deltaRpk analysis grows with the sample size, but in a rather linear way: expect ~400M every N~20 samples. So one should be able to run a dataset of up to N~800 samples on a normal desktop machine with 16G of RAM.

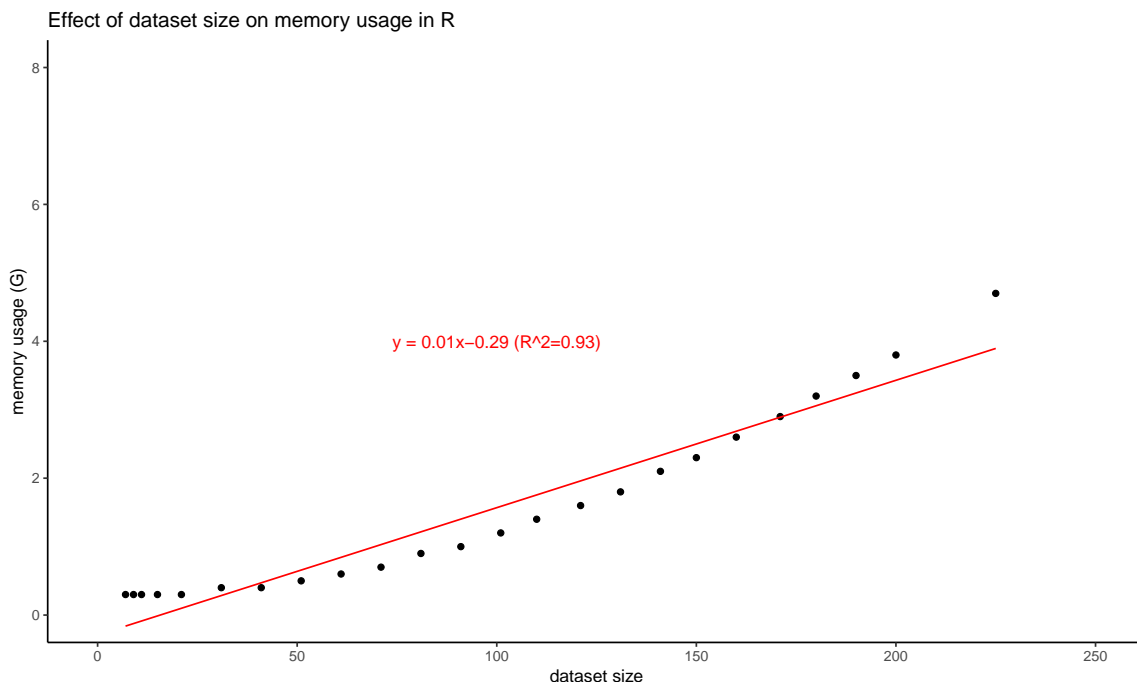
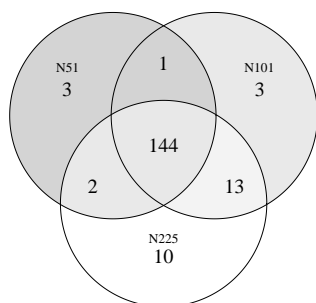


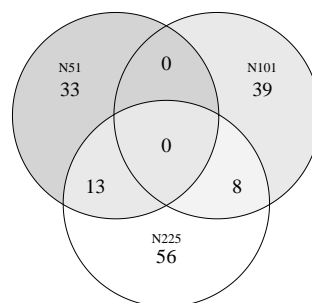
Figure 9: Dataset size and memory usage when using deltaRpk. The pipeline uses < 4G of memory for a dataset of N=225 samples, when ran in R 3.4.4 (under Ubuntu 14.04.5 LTS).

9.4 Random datasets

Random datasets confirm the robustness of selected genes with the deltaRpk method (**Figure 10**). When comparing datasets of different sizes (N=51, N=101, N=225) maintaining the original group classification, most of the genes identified as differentially present in the group 1 are conserved across datasets (**Figure 10(a)**, N=144). While on the other hand, the gene sets derived from random groupings are small and not consistent (**Figure 10(b)**, N=0).



(a) real groups



(b) random groups

Figure 10: Real versus random group classification: random group assignment gives non-robust gene sets across different sizes of datasets. deltaRpk *Listeria monocytogenes* datasets. Batch effect corrected.

10 Binaries and OS platforms

10.1 Ubuntu Trusty Tahr (14.04.5 LTS)

The Linux binary has been built and tested with R 3.4 under Ubuntu 14.04 LTS (Trusty Tahr):

```
R version 3.4.4 (2018-03-15)
Platform: x86_64-pc-linux-gnu (64-bit)
Running under: Ubuntu 14.04.5 LTS

Matrix products: default
BLAS: /usr/lib/libblas/libblas.so.3.0
LAPACK: /usr/lib/lapack/liblapack.so.3.0

locale:
 [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C               LC_TIME=en_GB.UTF-8       LC_COLLATE=en_US.UTF-8
 [5] LC_MONETARY=en_GB.UTF-8   LC_MESSAGES=en_US.UTF-8   LC_PAPER=en_GB.UTF-8      LC_NAME=C
 [9] LC_ADDRESS=C              LC_TELEPHONE=C            LC_MEASUREMENT=en_GB.UTF-8 LC_IDENTIFICATION=C

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

other attached packages:
[1] deltaRpm_0.1.0 ggplot2_3.0.0 bindrcpp_0.2.2 testthat_2.0.0

loaded via a namespace (and not attached):
 [1] colorspace_1.3-2      pryr_0.1.4             colorRamps_2.3          mclust_5.4.1           rprojroot_1.3-2
 [6] XVector_0.16.0        rstudioapi_0.7          roxygen2_6.1.0          bit64_0.9-7            AnnotationDbi_1.38.2
[11] xml2_1.2.0            codetools_0.2-15       splines_3.4.4           annotate_1.54.0         compiler_3.4.4
[16] tictoc_1.0            backports_1.1.2        assertthat_0.2.0       Matrix_1.2-14          lazyeval_0.2.1
[21] limma_3.32.10         cli_1.0.1              tools_3.4.4            gtable_0.2.0           glue_1.3.0
[26] reshape2_1.4.3        dplyr_0.7.6            Rcpp_1.0.0             Biobase_2.36.2         Biostrings_2.44.2
[31] gdata_2.18.0          nlme_3.1-137           stringr_1.4.0          gtools_3.8.1           devtools_1.13.6
[36] XML_3.98-1.16         zlibbioc_1.22.0        scales_1.0.0           parallel_3.4.4         RColorBrewer_1.1-2
[41] memoise_1.1.0         gridExtra_2.3          stringi_1.2.4          RSQLite_2.1.1          genefilter_1.58.1
[46] S4Vectors_0.14.7     desc_1.2.0             caTools_1.17.1.1       BiocGenerics_0.22.1    BiocParallel_1.10.1
[51] rlang_0.3.1           pkgconfig_2.0.2        commonmark_1.6         matrixStats_0.54.0     bitops_1.0-6
[56] lattice_0.20-38       purrr_0.2.5            bindr_0.1.1            labeling_0.3           bit_1.1-14
[61] tidyselect_0.2.4      plyr_1.8.4             magrittr_1.5           R6_2.3.0              IRanges_2.10.5
[66] gplots_3.0.1          DBI_1.0.0              pillar_1.3.0           whisker_0.3-2          withr_2.1.2
[71] mgcv_1.8-27           survival_2.42-6         RCurl_1.95-4.11        ggfortify_0.4.5        tibble_1.4.2
[76] crayon_1.3.4          KernSmooth_2.23-15     grid_3.4.4             sva_3.24.4            data.table_1.11.8
[81] blob_1.1.1            digest_0.6.18          xtable_1.8-3           tidyr_0.8.1           stats4_3.4.4
[86] munsell_0.5.0
```

Figure 11: Session info in R 3.4.4, with RStudio 1.0.143, run under Ubuntu 14.04.5 LTS.

10.2 Ubuntu Bionic Beaver (18.04.2 LTS)

The Linux binary has been built and tested with R 3.4 under Ubuntu 18.04.2 LTS (Bionic Beaver):

```

R version 3.4.4 (2018-03-15)
Platform: x86_64-pc-linux-gnu (64-bit)
Running under: Ubuntu 18.04.2 LTS

Matrix products: default
BLAS: /usr/lib/x86_64-linux-gnu/blas/libblas.so.3.7.1
LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.7.1

locale:
 [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C               LC_TIME=fr_FR.UTF-8
 [4] LC_COLLATE=en_US.UTF-8    LC_MONETARY=fr_FR.UTF-8   LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=fr_FR.UTF-8     LC_NAME=C                 LC_ADDRESS=C
[10] LC_TELEPHONE=C           LC_MEASUREMENT=fr_FR.UTF-8 LC_IDENTIFICATION=C

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

other attached packages:
[1] ggfortify_0.4.5      ggplot2_3.1.0      deltaRpmk_0.1.0      BiocInstaller_1.28.0

loaded via a namespace (and not attached):
 [1] Biobase_2.38.0      pkgload_1.0.2      tidyr_0.8.2         bit64_0.9-7
 [5] splines_3.4.4       gtools_3.8.1       assertthat_0.2.0    stats4_3.4.4
 [9] blob_1.1.1          remotes_2.0.2      sessioninfo_1.1.1   pillar_1.3.1
[13] RSQLite_2.1.1       backports_1.1.3    lattice_0.20-35     glue_1.3.0
[17] limma_3.34.9        digest_0.6.18      RColorBrewer_1.1-2  colorspace_1.4-0
[21] Matrix_1.2-12       plyr_1.8.4         XML_3.98-1.17       pkgconfig_2.0.2
[25] devtools_2.0.1      genefilter_1.60.0  purrr_0.3.0         xtable_1.8-3
[29] scales_1.0.0        gdata_2.18.0       processx_3.2.1      BiocParallel_1.12.0
[33] tibble_2.0.1        annotate_1.56.2     mgcv_1.8-23         IRanges_2.12.0
[37] usethis_1.4.0       withr_2.1.2        BiocGenerics_0.24.0 lazyeval_0.2.1
[41] cli_1.0.1           survival_2.41-3    magrittr_1.5         crayon_1.3.4
[45] memoise_1.1.0       ps_1.3.0           fs_1.2.6            nlme_3.1-131
[49] ggplots_3.0.1.1     pkgbuild_1.0.2     tools_3.4.4         prettyunits_1.0.2
[53] matrixStats_0.54.0  stringr_1.4.0      S4Vectors_0.16.0    munsell_0.5.0
[57] AnnotationDbi_1.40.0 callr_3.1.1         compiler_3.4.4      caTools_1.17.1.1
[61] rlang_0.3.1         grid_3.4.4         RCurl_1.95-4.11     rstudioapi_0.9.0
[65] bitops_1.0-6        labeling_0.3        gtable_0.2.0        DBI_1.0.0
[69] reshape2_1.4.3      R6_2.4.0           gridExtra_2.3        dplyr_0.8.0.1
[73] bit_1.1-14          rprojroot_1.3-2    KernSmooth_2.23-15  desc_1.2.0
[77] stringi_1.3.1       parallel_3.4.4     sva_3.26.0          Rcpp_1.0.0
[81] tidyselect_0.2.5

```

Figure 12: Session info in R 3.4.4, with RStudio 1.1.463, run under Ubuntu 18.04.2 LTS.

10.3 MacOS High Sierra (10.13.6)

The MacOS binary has been built and tested with R 3.4 under MacOS 10.13.6 (High Sierra):

```

R version 3.4.0 (2017-04-21)
Platform: x86_64-apple-darwin15.6.0 (64-bit)
Running under: macOS 10.13.6

Matrix products: default
BLAS: /System/Library/Frameworks/Accelerate.framework/Versions/A/Frameworks/vecLib.framework/Versions/A/libBLAS.dylib
LAPACK: /Library/Frameworks/R.framework/Versions/3.4/Resources/lib/libRlapack.dylib

locale:
[1] en_GB.UTF-8/en_GB.UTF-8/en_GB.UTF-8/C/en_GB.UTF-8/en_GB.UTF-8

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

other attached packages:
[1] ggfortify_0.4.5 ggplot2_3.1.0  deltaRpmk_0.1.0

loaded via a namespace (and not attached):
[1] Rcpp_1.0.0          lattice_0.20-38    tidyr_0.8.2        gtools_3.8.1
[5] assertthat_0.2.0    digest_0.6.18      R6_2.4.0           plyr_1.8.4
[9] stats4_3.4.0        RSQLite_2.1.1     sva_3.26.0         pillar_1.3.1
[13] gplots_3.0.1.1      rlang_0.3.1        lazyeval_0.2.1     gdata_2.18.0
[17] rstudioapi_0.9.0    annotate_1.56.2    blob_1.1.1         S4Vectors_0.16.0
[21] Matrix_1.2-15       labeling_0.3        splines_3.4.0      BiocParallel_1.12.0
[25] stringr_1.4.0       RCurl_1.95-4.11    bit_1.1-14         munsell_0.5.0
[29] compiler_3.4.0      pkgconfig_2.0.2    BiocGenerics_0.24.0 mgcv_1.8-27
[33] tidyselect_0.2.5     tibble_2.0.1       gridExtra_2.3       IRanges_2.12.0
[37] matrixStats_0.54.0  XML_3.98-1.17     crayon_1.3.4       dplyr_0.8.0.1
[41] withr_2.1.2         bitops_1.0-6       grid_3.4.0          nlme_3.1-137
[45] xtable_1.8-3        gtable_0.2.0       DBI_1.0.0           magrittr_1.5
[49] scales_1.0.0        KernSmooth_2.23-15 stringi_1.3.1       reshape2_1.4.3
[53] geneFilter_1.60.0    limma_3.34.9       RColorBrewer_1.1-2 tools_3.4.0
[57] bit64_0.9-7         Biobase_2.38.0     glue_1.3.0          purrr_0.3.0
[61] parallel_3.4.0      survival_2.43-3    AnnotationDbi_1.40.0 colorspace_1.4-0
[65] caTools_1.17.1.1    memoise_1.1.0

```

Figure 13: Session info in R 3.4.0 with RStudio 1.0.143, run under MacOS 10.13.6.

10.4 Windows10

The Windows binary has been built and tested with R 3.6 under Windows10:

```

R version 3.6.1 (2019-07-05)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: windows >= 8 x64 (build 9200)

Matrix products: default

locale:
[1] LC_COLLATE=French_Switzerland.1252 LC_CTYPE=French_Switzerland.1252
[3] LC_MONETARY=French_Switzerland.1252 LC_NUMERIC=C
[5] LC_TIME=French_Switzerland.1252

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

other attached packages:
[1] deltaRpmk_0.1.0 testthat_2.2.1 devtools_2.1.0 usethis_1.5.1

loaded via a namespace (and not attached):
[1] nlme_3.1-140          bitops_1.0-6        matrixStats_0.54.0  fs_1.3.1
[5] bit64_0.9-7           RColorBrewer_1.1-2  rprojroot_1.3-2     tools_3.6.1
[9] backports_1.1.4       R6_2.4.0            KernSmooth_2.23-15 DBI_1.0.0
[13] lazyeval_0.2.2        BiocGenerics_0.30.0 mgcv_1.8-28         colorspace_1.4-1
[17] withr_2.1.2           tictoc_1.0          tidyselect_0.2.5    gridExtra_2.3
[21] prettyunits_1.0.2     processx_3.4.1      bit_1.1-14          compiler_3.6.1
[25] cli_1.1.0             Biobase_2.44.0      desc_1.2.0          labeling_0.3
[29] caTools_1.17.1.2      scales_1.0.0        geneFilter_1.66.0    callr_3.3.1
[33] stringr_1.4.0         digest_0.6.20       colorRamps_2.3       xVector_0.24.0
[37] pkgconfig_2.0.2       sessioninfo_1.1.1   limma_3.40.6        rlang_0.4.0
[41] rstudioapi_0.10       pryr_0.1.4          RSQLite_2.1.2       mclust_5.4.5
[45] BiocParallel_1.18.1   gtools_3.8.1        dplyr_0.8.3         RCurl_1.95-4.12
[49] magrittr_1.5          Matrix_1.2-17       Rcpp_1.0.2          munsell_0.5.0
[53] S4Vectors_0.22.0      stringi_1.4.3       zlibbioc_1.30.0     pkgbuild_1.0.5
[57] gplots_3.0.1.1        plyr_1.8.4          grid_3.6.1          blob_1.2.0
[61] parallel_3.6.1        gdata_2.18.0        crayon_1.3.4        lattice_0.20-38
[65] Biostrings_2.52.0     splines_3.6.1       annotate_1.62.0      zeallot_0.1.0
[69] ps_1.3.0             pillar_1.4.2        reshape2_1.4.3      codetools_0.2-16
[73] stats4_3.6.1          pkgload_1.0.2       XML_3.98-1.20       glue_1.3.1
[77] data.table_1.12.2     remotes_2.1.0       vctrs_0.2.0         gtable_0.3.0
[81] purrr_0.3.2           tidyr_0.8.3         assertthat_0.2.1    ggplot2_3.2.1
[85] xtable_1.8-4          survival_2.44-1.1   tibble_2.1.3        AnnotationDbi_1.46.1
[89] memoise_1.1.0         IRanges_2.18.2     sva_3.32.1         ggfortify_0.4.7

```

Figure 14: Session info in R 3.6.1, with RStudio 1.1.463, run under Windows10.