RTFBSDB package

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Vignette version: 0.4
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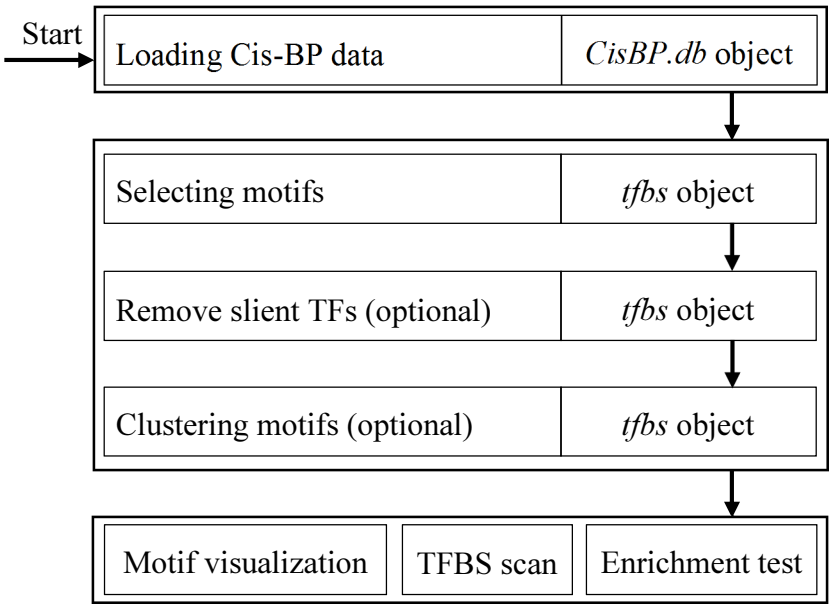
1. Overview

The *rtfbsdb* package provides a convenient platform to find and analyze transcription factor (TF) binding sites in the R environment. Experimentally derived and predicted motifs are imported from the Cis-BP database [1], which contains thousands of motifs in virtually any species of interest. The following instructions will show you how to use the *rtfbsdb* package to search the genome for motif occurrences, how to identify motifs enriched between sets of DNA sequences, and how to visualize motifs in R.

1.1 Flowchart of data analysis



1.2 External dependencies

The *rtfbsdb* package depends not only on other R packages, but also on several UNIX shell commands and other bioinformatics tools. Before you go through the following instructions, please check the requisite commands are available in your operating system $PATH variable and execute normally when run on a command line.

Command	Package	Functions in *rtfbsdb*	Download Link

starch	bedops	*tfbs.scanTFsite()* *tfbs.enrichmentTest()*	http://bedops.readthedocs.org/en/latest/index.html
starchcat	bedops	*tfbs.scanTFsite()*	http://bedops.readthedocs.org/en/latest/index.html
sort-bed	bedops	*tfbs.scanTFsite()* *tfbs.enrichmentTest ()* *tfbs.createFromCisBP()*	http://bedops.readthedocs.org/en/latest/index.html
twoBitInfo	Kent source	*tfbs.createFromCisBP()*	http://hgdownload.cse.ucsc.edu/admin/exe/
samtools	SAMtools	*tfbs.createFromCisBP()*	http://samtools.sourceforge.net
bedtools	bedtools	*tfbs.createFromCisBP()*	http://bedtools.readthedocs.org/en/latest/
awk	Linux/Unix command	*tfbs.createFromCisBP()*	

2. TF site binding identification

2.1 Loading TF information from the Cis-BP database

If you are planning to analyze the human or mouse genome, you don't need to download the Cis-BP dataset because the package includes pre-installed data for human and mouse. Otherwise, you need to download the dataset manually for your target genome or use the function in *rtfbsdb* to download it. The Cis-BP database provides a very nice web page to download the TF information for any species or TF family at http://cisbp.ccbr.utoronto.ca/bulk.php.

In this package, three functions, including "*CisBP.extdata*", "*CisBP.download*" and "*CisBP.ziploa*d", aim to build a CisBP data object. *CisBP.extdata* can load the pre-installed dataset for human and mouse, *CisBP.download* can download and use any species data from the Cis-BP web site, *CisBP.ziploa*d can decompress a zipped dataset from the Cis-BP web site. For example:

```
## Attach the package firstly
library(rtfbsdb);

## Create db from pre-installed dataset
db <- CisBP.extdata("Homo_sapiens");
db1 <- CisBP.extdata("Mus_musculus");
db2 <- CisBP.extdata("Drosophila_melanogaster");

## Create db from downloaded dataset
db3 <- CisBP.download("Mus_musculus");

## Create db from a zipped downloaded dataset
db4 <- CisBP.ziploa("ZIP_FILE_FROM_CISBP.zip", species="Mus_musculus");
```

A zipped file downloaded from Cis-BP includes a database of TF definitions and matched position-specific weight matrices (PWMs), which represent the DNA binding preferences of each TF. Note that Cis-BP downloads omit binding information from the TRANSFAC database, which requires a paid license. If the licensed motifs are available, the function *tfbs.importMotifs*

can be used to merge these motifs into the *tfbs* object after the motif selecting from Cis-BP data file. The Cis-BP zip file includes images representing motif logos which are not used in this package.

2.2 Selecting motif data to create *tfbs* object

Once you have created a Cis-BP object you can load PWM information from a subset of motifs using the function *tfbs.loadFromCisBP*. You can select all motifs in the database (default) or you can select a subset of motifs. For example, selecting motifs binding to TFs in the AP-2 family can be accomplished using:

```
## Select all motifs from CisBP dataset
tfs <- tfbs.createFromCisBP(db);

## Query the CisBP dataset and select the motifs for a transcription factor of interest
tfs.ap2 <- tfbs.createFromCisBP(db, family_name="AP-2");
```

The *tfbs* object returned by *tfbs.createFromCisBP* includes each selected PWM, the ENSEMBL IDs of genes encoding TF binding, and other relevant data. You can get an overview by the command *show*. For human, 1,964 valid motifs are loaded from Cis-BP (9/4/2015) after removing the database entries without a freely available PWM.

```
> show(tfs)
Species:  Homo_sapiens
TF number:  1964
Distance Matrix:  NULL
Cluster Matrix:  NULL
Expression:  NULL

Partial list of TFs
    Motif_ID          DBID TF_Name Family_Name Motif_Type MSource_Identifier
2  M5917_1.02 ENSG00000008196  TFAP2B       AP-2     SELEX            Jolma
3  M5918_1.02 ENSG00000008196  TFAP2B       AP-2     SELEX            Jolma
4  M5919_1.02 ENSG00000008196  TFAP2B       AP-2     SELEX            Jolma
……
```

2.3 Selecting motifs recognized by expressed TFs

The *tfbs.loadFromCisBP* function will also select motifs that are recognized by TFs expressed in a cell type or biological system of interest. The expression of each TF is measured using functional data profiling gene expression levels. Currently *rtfbsdb* supports either RNA-seq or PRO-seq. This feature requires arguments specifying: (1) 2-bit formatted genome sequence data (e.g., hg19.2bit), (2) Gencode gene coordinates (GTF-formatted, http://www.gencodegenes.org/releases/19.html), and (3) Gene expression data in the desired format. Gene expression data collected using RNA-seq requires an indexed BAM file to calculate gene expression. For PRO-seq, bigwig files representing read densities on the plus and minus strand are required. The *tfbs.createFromCisBP* function tests regions defined in the Gencode file that are same as the motif's gene ID for read densities that exceed a background null model, as presented by Core, Waterfall, and Lis [2].

Here we extract the 19th chromosome from the RNA-seq and PRO-seq data to demonstrate how to use the function *tfbs.createFromCisBP.* First example is for RNA-seq data.

```
## Specify the BAM file to filter the expressed TFs only (RNA-seq)
file.bam.chr19 <- system.file("extdata","human_PI_fastq_gz_sort_chr19.bam", package="rtfbsdb")

## Get the partial hg19 (chromosome 19 only) file from pre-installed data
file.hg19.twobit.chr19 <- system.file("extdata","hg19.chr19.2bit",
        package="rtfbsdb")

## Get the partial gencode (chromosome 19 only) file from pre-installed data
file.gencode.gtf.chr19 <- system.file("extdata","gencode.v21.annotation.chr19.gtf.gz",
        package="rtfbsdb")

## Specify the bigwig files to filter the expressed TFs only (PRO-seq)
tfs <- tfbs.selectExpressedMotifs(tfs,
        file.hg19.twobit.chr19,
        file.gencode.gtf.chr19,
        file.bam=file.bam.chr19,
        pvalue.threshold=0.001,
        include.DBID.missing=TRUE,
        seq.datatype="RNA-seq");
```

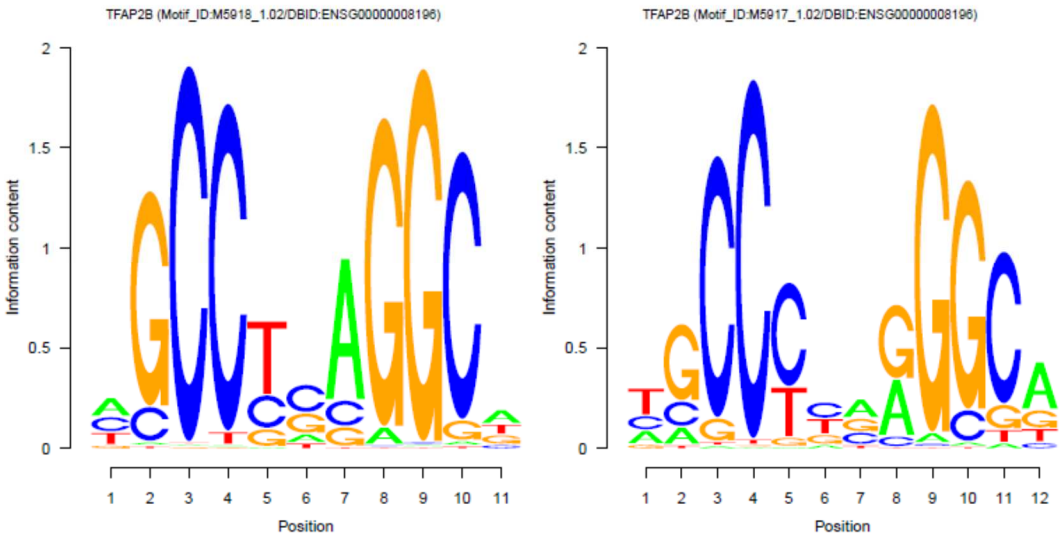
The second example uses PRO-seq experiment data.

```
## Specify the bigwig files to filter the expressed TFs only (PRO-seq)
file.bigwig.minus.chr19 <- system.file("extdata","GSM1480327_K562_PROseq_chr19_minus.bw",
        package="rtfbsdb")
file.bigwig.plus.chr19 <- system.file("extdata","GSM1480327_K562_PROseq_chr19_plus.bw",
        package="rtfbsdb")

## Get the partial hg19 (chromosome 19 only) file from pre-installed data
file.hg19.twobit.chr19 <- system.file("extdata","hg19.chr19.2bit",
        package="rtfbsdb")

## Get the partial gencode (chromosome 19 only) file from pre-installed data
file.gencode.gtf.chr19 <- system.file("extdata","gencode.v21.annotation.chr19.gtf.gz",
        package="rtfbsdb")

## Specify the bigwig files to filter the expressed TFs only (PRO-seq)
tfs <- tfbs.selectExpressedMotifs(tfs,
        file.hg19.twobit.chr19,
        file.gencode.gtf.chr19,
        file.bigwig.plus.chr19,
        file.bigwig.minus.chr19,
        pvalue.threshold=0.001,
        include.DBID.missing=TRUE,
        seq.datatype="PRO-seq");
```

2.4 Visualizing motif logos

Motif logos can be drawn at any point after loading. To draw a single specific motif, use the function *tfbs.drawLogo* to draw a motif logo.

```
## Draw motif logos with only one diagram per page
tfbs.drawLogo(tfs, file.pdf="logos.pdf", motif_id=c("M5917_1.02", "M5918_1.02") )
```

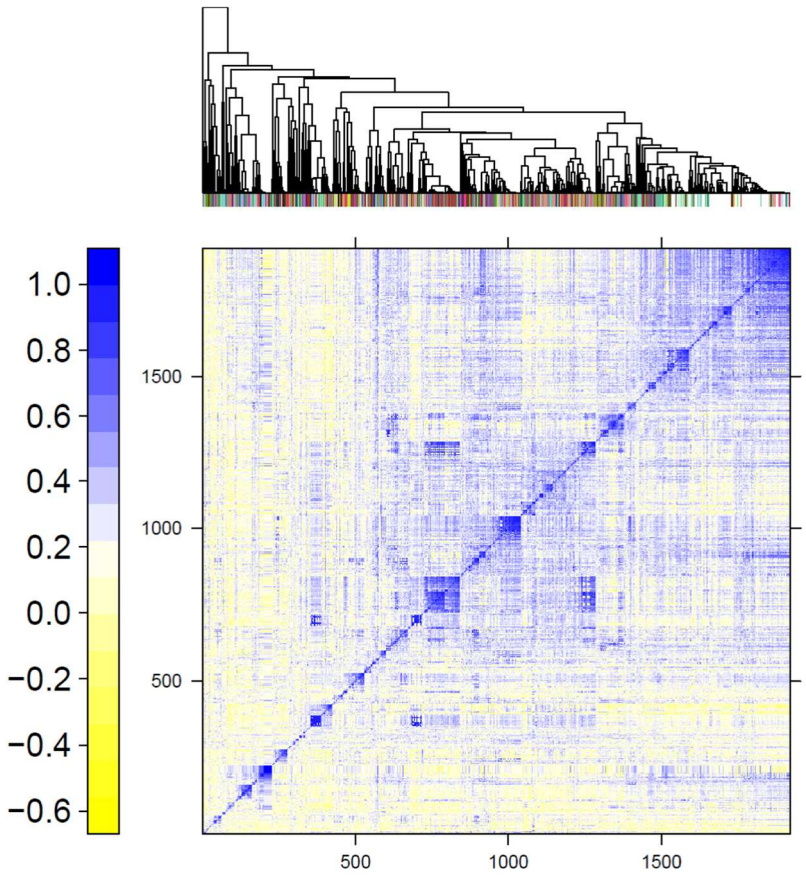


2.5 Clustering (optional)

Many of the motifs indexed in Cis-DB share similar underlying DNA sequence preferences. To increase the power of certain tests, motifs with similar DNA sequence binding preferences can optionally be grouped using hierarchical clustering. The clustering function (*tfbs.clusterMotifs*) can be set to use either of two algorithms: hierarchical agglomerative clustering (*agnes* in the R cluster package) and Affinity Propagation Clustering (*apcluster*[3]). In order to clustering the motifs, the similarity matrix need to be obtained from comparing each combination of motifs, results in a distance matrix with Pearson's R values. This computational procedure can take a long time to execute if the number of motifs is large, so it is not performed in the constructor of the *tfbs* object. To speed it up, it can be run on multiple cores by setting the *ncores* parameter to a value larger than 1. Optionally, *tfbs.clusterMotifs* will output a heat-map representing the similarity between groups of motifs if an output filename is specified in the *pdf.heatmap* parameter.

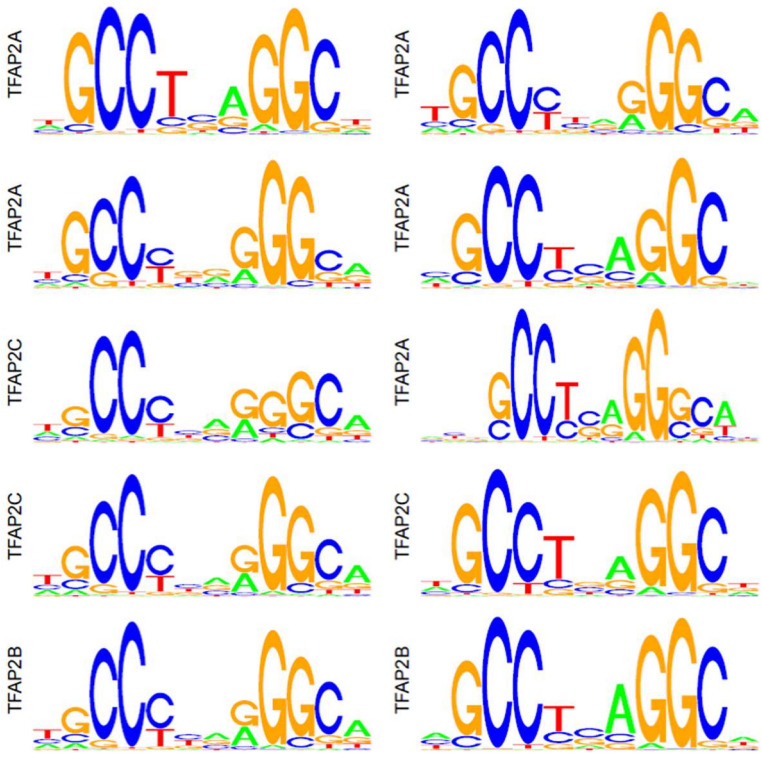
Below is an example of the code to compute the distance matrix, cluster motifs, and generate a figure. The *tfbs.clusterMotifs* function returns a new *tfbs* object with the cluster mapping table.

```
## Generate heatmap and return clustering map
tfs <- tfbs.clusterMotifs(tfs, method="agnes", pdf.heatmap="heatmap.pdf", ncores=25)
```



Besides drawing the heat-map, users can also draw sequence logos for motifs within each cluster using the *tfbs.drawLogosForClusters* function. Each page of the output PDF contains all motif logos that are grouped within a single cluster. This visualization can be useful when checking whether the clustering was conducted using a reasonable number of clusters. The following code demonstrates how to plot group motif logos.

```
## Draw motif logos with one group of TF per page
tfbs.drawLogosForClusters(tfs, "clustering.logos.pdf");
```



2.6 Motif selection and plotting

A single motif representing each cluster is used in most downstream analysis. We provide two methods to select which motifs are used to represent each cluster in downstream analyses. The function *tfbs.selectByRandom* randomly selects one motif from each group of clustering. The function *tfbs.selectByGeneExp* selects one motif with the minimum p-value of gene expression from each group of clustering. The index returned from these two functions can be used in the function call of *tfbs.scanTFsite and tfbs.enrichmentTest*.

2.7 Find TF binding sites across the genome

The first goal of this package is to locate TF binding sites across a genome. The *tfbs.scanTFsite* function matches user selected PWM(s) across the genome given the DNA sequence formatted as a 2 bit file (e.g., hg19.2bit). If desired, users can restrict the motif search to occur within a set of genomic coordinates specified using a BED-formatted data.frame. Below are two examples that show a motif scan across the whole genome and restricted to a specified range.

```
## hg19.2bit is downloaded from http://hgdownload-test.cse.ucsc.edu/goldenPath/hg19/bigZips/
file.twoBit.chr19 <- system.file("extdata","hg19.chr19.2bit", package="rtfbsdb")

## Example 1: Scan the whole genome
## Scan 2bit file within whole genome to find motif binding site
r1.scan <- tfbs.scanTFsite( tfs, file.twoBit.chr19, ncores = 3);

## Example 2: Scan a specified range
## Get a data frame from a plain-text bed file for your range of interest
file.ELF1.chr19 <- system.file("extdata","Chipseq-k562-chr19-ELF1.bed", package="rtfbsdb");
```

```
bed.chipseq.chr19 <- read.table(file.ELF1.chr19);

## build tfbs object by querying motifs in ELF1
tfs.elf1 <- tfbs.createFromCisBP(db, tf_name="ELF1");

## Scan 2bit file within all bed regions to find motif binding sites
r2.scan <- tfbs.scanTFsite(tfs.elf1, file.twoBit.chr19, bed.chipseq.chr19, ncores = 3);
```

The function has two parameters to control how to select binding sites. Specifying a "*threshold*" will return motifs that exceed the log likelihood of the observed N-mer given the PWM minus the log likelihood of the N-mer under a third-order Markov background model. This option controls the specificity of motif discovery very well in most situations. In some cases, the default threshold (6) approximately corresponds to a 10% false discovery rate (FDR), although this varies depending on the DNA sequence composition and motif information content. Using a higher threshold results in a more stringent match to the motif of interest.

Alternatively users can specify a fixed FDR using the "*fdr*" option. This option simulates a set of sequences under a third-order Markov background model and estimates the motif threshold that satisfies the specified FDR. It takes considerably more time to estimate the FDR. If "*fdr"* is specified in the parameter of *threshold.type*, the parameter of *threshold* will be used as the *fdr* threshold.

Optionally, multithreaded processing is supported by setting the *ncores* parameter.

The *tfbs.scanTFsite* function returns a list object consisting of four parts:

1) *$result*: the result of the motif scan. The format can be set using the *return.type* parameter. By default (*return.type*="*matches*") a BED-formatted data frame is returned. The option "*writedb*" writes a starch-compressed (using the bedops package) to disk with matches for each motif, and is useful for large searches resulting in millions of motif matches.

2) *$summary*: a summary of TF scan, including the number of binding sites matched for each motif.

3) *$parm*: the values of control parameters, such as *fdr*, *threshold*, *gc.groups*, *background.order*, *background.length*.

4) *$bed:* the bed-formatted loci information with 6 columns.

The parameters and summary information can be printed out using the *show* command as follows:

```
# return.type="matches"
> r1.scan
Return type:  matches
FDR threshold:  NA
Score threshold:  6
Motifs count:  26
Binding sites:  34518
      TF_Name      Motif_ID Count
7  M2809_1.01   TFAP2C  2504
4  M5963_1.01   TFAP2B  2283
…….

# return.type="writedb "
```

```
> r1.scan
Return type:  writedb
FDR threshold:  NA
Score threshold:  6
Binary Bed file:  scan.db.db.starch
```

The function *tfbs.reportFinding* can export a simple report to a PDF file which shows the motif logos and the number of binding sites discovered for each motif if the "matches" return type is specified.

2.8 Enrichment comparison between two case-control groups

The second goal of *rtfbsdb* is to identify motifs enriched in a user specified set of genomic coordinates compared to a background set. The *tfbs.enrichmentTest* function computes the number of motif occurrences in two sets of genomic coordinates and returns a p-value (Fisher's exact), and other information. Two groups of genomic coordinates are specified as arguments to *tfbs.enrichmentTest* as the arguments 'positive' and 'negative'. To maximize statistical power, sequence sets are typically no less than a few hundred sequences, and backgrounds can often be much larger (i.e., tens of thousands). For example:

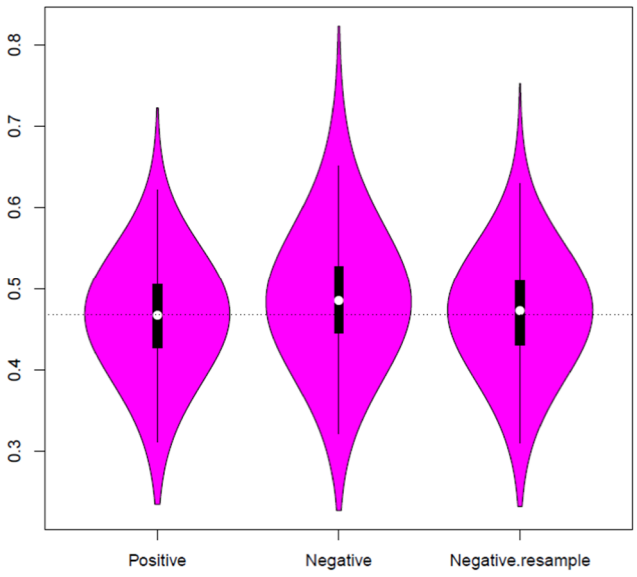
```
## Specify the requisite files from pre-installed data
file.twoBit.chr19 <- system.file("extdata","hg19.chr19.2bit", package="rtfbsdb")
file.ELF1.chr19 <- system.file("extdata","Chipseq-k562-chr19-ELF1.bed",
    package="rtfbsdb");
file.bg.chr19   <- system.file("extdata","k562.chr19.dnase.UW.DUKE.inters.bed",
    package="rtfbsdb");

## Convert the bed file for each condition to a data frame
bed.ELF1.chr19 <- read.table(file.ELF1.chr19 , header=FALSE);
bed.bg.chr19   <- read.table(file.bg.chr19, header=FALSE);

## build tfbs object by querying motifs in ELF1
tfs.elf1 <- tfbs.createFromCisBP(db, tf_name="ELF1");

## Compare motifs between each condition
t.comp <- tfbs.enrichmentTest( tfs.elf1,
    file.twoBit.chr19,
    bed.ELF1.chr19,
    bed.bg.chr19,
    gc.correction=TRUE,
    gc.robust.rep=5,
    file.prefix="comp.db",
    ncores = 3);
```

The *rtfbsdb* package will find motifs that are enriched in a reference set (positive) relative to background (i.e. negative). If the background set can't be specified, the function will stochastically extract the genomic loci adjacent to the positive set, for example:

```
## Compare motifs without background set
```

```
t.comp <- tfbs.enrichmentTest( tfs.elf1,
    file.twoBit.chr19,
    bed.ELF1.chr19,
    gc.correction=TRUE,
    gc.correction.pdf ="elf1.gc.correction.pdf",
    gc.robust.rep=5,
    file.prefix="comp.db",
    ncores = 3);
```

Notably, this type of analysis is often confounded by systematic differences in the GC content between groups. To address this limitation, *tfbs.enrichmentTest* will check whether the mean of the GC content differs significantly between the two groups and shows a p-value (Wilcox test) and a Violin plot. If the p-value is significant, and the parameter of *gc.correction* is set to TRUE (default), the *tfbs.enrichmentTest* function will resample the background (i.e. negative) sequences stochastically to reduce the difference in GC content. The following is a Violin plot to demonstrate the effects of negative correction.



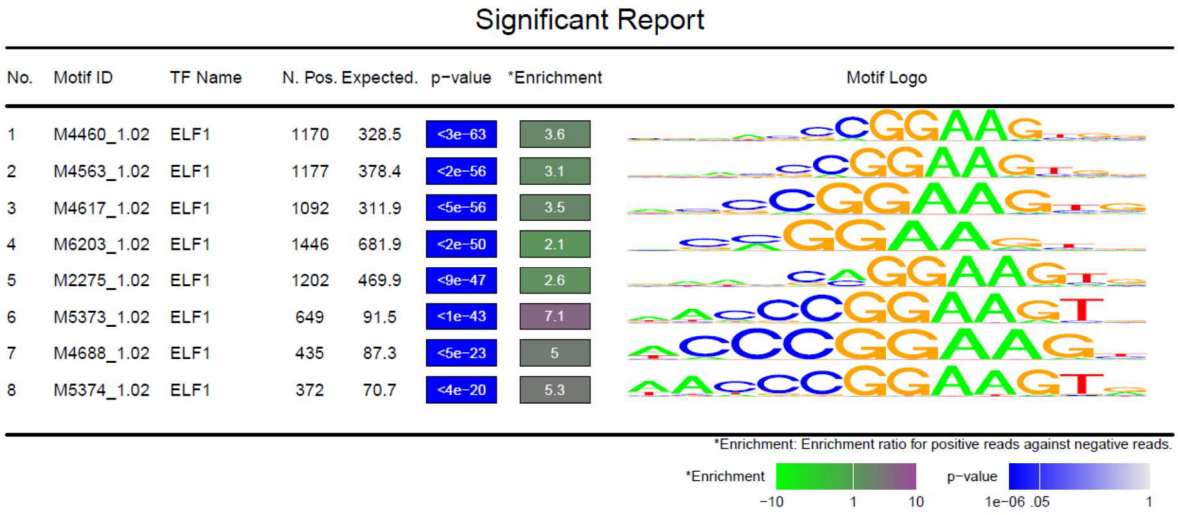
The output of *tfbs.enrichmentTest* includes a data frame with 8 columns, including motif ID, TF name, occurrence in positive group, expectation in negative group, enrichment ratio, p-value of fisher test, and correction value by multiple comparison methods (e.g., Bonferroni or FDR). The *show* command prints significant motifs and other meta data in the result object, as shown below.

```
> show(t.comp)
Negative correction: TRUE
p-value correction: BH
Significant p-value: 0.05
Threshold type : fdr
TF binding threshold: 0.05
```

```
TF binding background.order: 3
TF binding background.length: 1e+05
Total Motif: 336

Significant Motifs(or top 20):
     motif.id          tf.name Npos      Nneg      pv.adj            fold.enrichment
165 M3432_1.01   MEIS1   64        740       1.230174e-05      2.194749
28  M5440_1.01   MEIS3   32        261       1.310005e-05      3.111330
…..
```

This object can be used to write a PDF report for the comparison. The *tfbs.reportEnrichment* function draws a motif list with visual p-value bar, enrichment ratio bar, and motif logos. The following command demonstrates how to print out the significant motifs for which adjusted p-values are less than 0.01. The screen shot of PDF report follows this example.

```
## Output the report for all significant motifs
tfbs.reportEnrichment(tfs, t.comp, file.pdf="test-tfcomp.pdf", report.title="Significant Report",
                      sig.only=T, pv.threshold=0.01, pv.adj="fdr");
```



2.9 SessionInfo()

Session information of the R console used to write this vignette is shown below. It demonstrates the R packages necessary for successful *rtfbsdb* installation.

```
> sessionInfo()
R version 3.1.0 (2014-04-10)
Platform: x86_64-unknown-linux-gnu (64-bit)

locale:
 [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C
 [3] LC_TIME=en_US.UTF-8        LC_COLLATE=en_US.UTF-8
 [5] LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=en_US.UTF-8       LC_NAME=C
```

```
 [9] LC_ADDRESS=C               LC_TELEPHONE=C
[11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C

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

other attached packages:
[1] rtfbsdb_0.1.8 vioplot_0.2   sm_2.2-5.4

loaded via a namespace (and not attached):
[1] apcluster_1.4.1     bigWig_0.2-9        cluster_2.0.3
[4] grid_3.1.0          lattice_0.20-33     latticeExtra_0.6-26
[7] Matrix_1.2-1        parallel_3.1.0      RColorBrewer_1.1-2
[10] Rcpp_0.12.0         rphast_1.6          rtfbs_0.3.4
```

3 Links

(1) Cis-BP database: http://cisbp.ccbr.utoronto.ca/bulk.php

(2) Twobit files: http://hgdownload-test.cse.ucsc.edu/goldenPath/

(3) Gencode files: http://www.gencodegenes.org/

4 References

[1] Weirauch, M. T., Yang, A., Albu, M., Cote, A. G., Montenegro-Montero, A., Drewe, P., ... & Hughes, T. R. (2014). Determination and inference of eukaryotic transcription factor sequence specificity. Cell, 158(6), 1431-1443.

[2] Core, L. J., Waterfall, J. J., & Lis, J. T. (2008). Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science, 322(5909), 1845-1848.

[3] Bodenhofer, U., Kothmeier, A., & Hochreiter, S. (2011). APCluster: an R package for affinity propagation clustering. *Bioinformatics*, *27*(17), 2463-2464.