RTFBSDB package

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 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	Download Link
starch	bedops	http://bedops.readthedocs.org/en/latest/index.html
starchcat	bedops	http://bedops.readthedocs.org/en/latest/index.html
sort-bed	bedops	http://bedops.readthedocs.org/en/latest/index.html
twoBitInfo	Kent source	http://hgdownload.cse.ucsc.edu/admin/exe/
samtools	SAMtools	http://samtools.sourceforge.net
bedtools	bedtools	http://bedtools.readthedocs.org/en/latest/
awk	Linux/Unix command	

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.zipload*, 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.zipload* can decompress a zipped dataset from the Cis-BP web site. For example:

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

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

# Create db from a zipped downloaded dataset
db <- CisBP.zipload("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 databse, which requires a paid license. The Cis-BP zip file includes images representing motif logos which are not used in this package.

2.2 Selecting motif data

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 <- 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, 1,920 valid motifs are loaded from CisBP (6/8/2015) after removing the database entries without a freely available PWM.

```
> show(tfs)
Species:  Homo_sapiens
TF number:  1920
Distance Matrix:  NULL
Expression:  NULL

Partial list of TFs
    Motif_ID           DBID TF_Name Family_Name Motif_Type MSource_Identifier
2  M5736_1.01 ENSG00000008196  TFAP2B       AP-2     SELEX            Jolma
3  M5737_1.01 ENSG00000008196  TFAP2B       AP-2     SELEX            Jolma
4  M5738_1.01 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, $PROVIDE GENCODE URL), 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].

```
# Specify the bigwig files to filter the expressed TFs only (RNA-seq)
tfs <- …

# Specify the bigwig files to filter the expressed TFs only (PRO-seq)
tfs <- tfbs.createFromCisBP( db, file.bigwig.plus="bw.plus", file.bigwig.mins="bw.minus",
              file.twoBit="hg19.2bit",  file.gencode.gtf="hg19.gtf", 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", tf_id=c("M4376_1.01", t"M4440_1.01") )
```

SHOW EXAMPLE

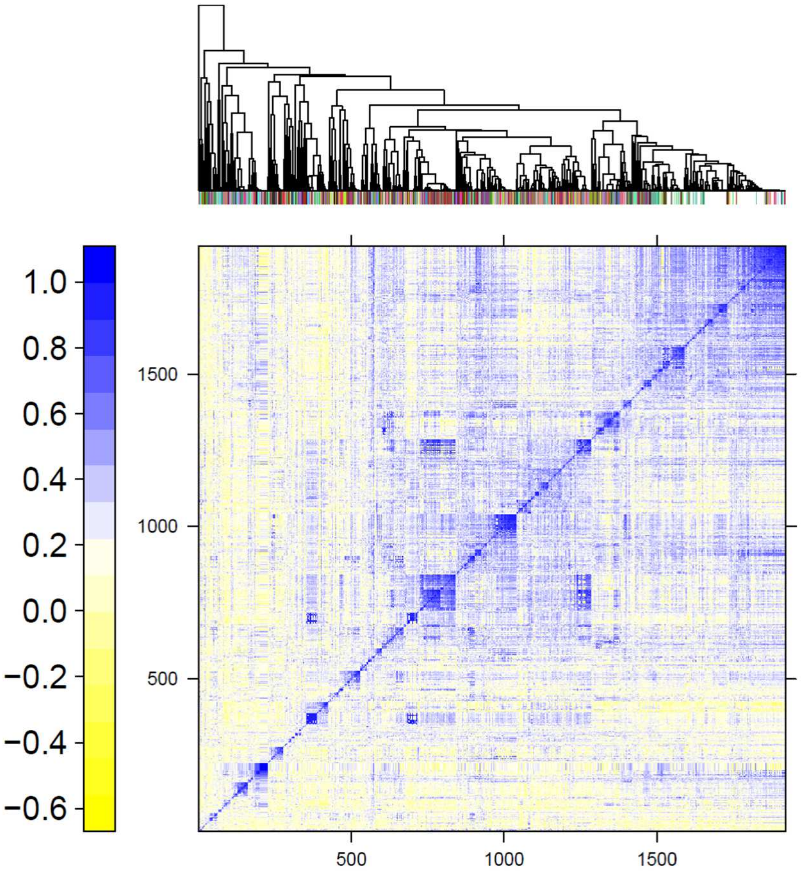
2.5 Clustering (optional)

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 function *tfbs.getDistanceMatrix* can be used to compare each combination of motifs, results in a distance matrix with Pearson's R values. This function 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. The clustering function (*tfbs.clusterMotifs*) can be set to use either of two algorithms: hierarchical agglomerative clustering (*agnes* in the R cluster package) and our own algorithm. Optinally, *tfbs.clusterMotifs* will output a heatmap 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 cluster mapping table for each motif.

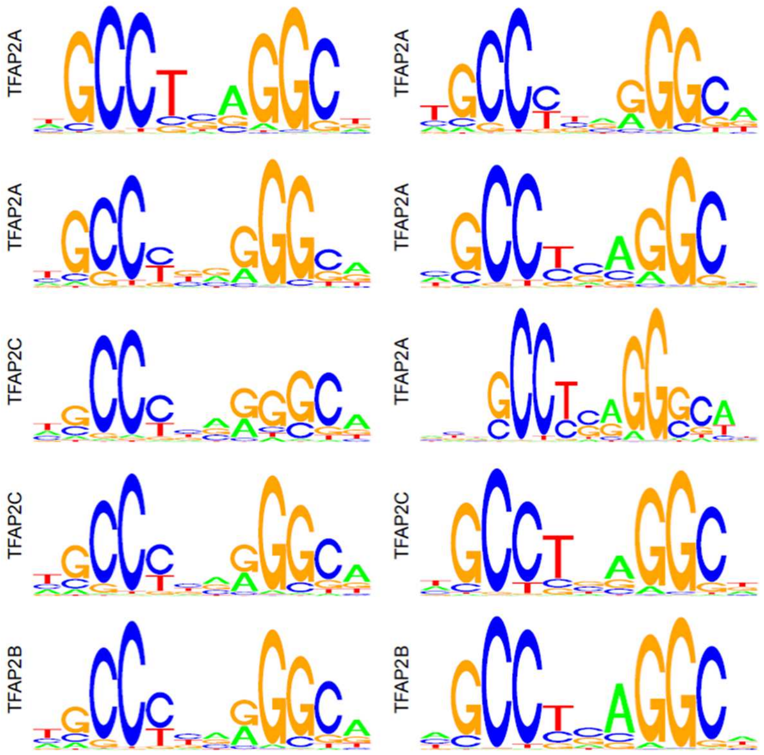
```
# Get the distance matrix
tfs <- tfbs.getDistanceMatrix(tfs, ncores=25)

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



Besides drawing the heatmap, 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, clu, "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.compareTFsite*.

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     <- "hg19.2bit"

Example 1: Scan the whole genome
# Scan 2bit file within whole genome to find motif binding site
r1.scan <- tfbs.scanTFsite( tfs, file.twoBit, ncores = 7);

Example 2: Scan a specified range
# Get a data frame from a plain-text bed file for your range of interest
dREG_H_change_bed <- read.table("./dREG.H.change.bed", header=FALSE);
```

```
# Scan 2bit file within all bed regions to find motif binding site
r2.scan <- tfbs.scanTFsite( tfs, file.twoBit, dREG_H_change_bed, ncores = 7);
```

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. For many motifs, 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 the parameter of *fdr* is specified in the function call, the parameter of *threshold* will be ignored.

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"
> r2.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 "
> r2.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 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.compareTFsite* 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.compareTFsite* 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:

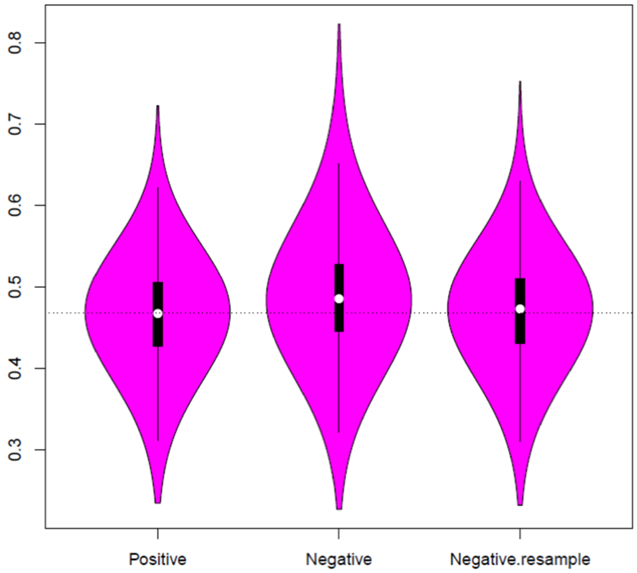
```
file.dREG.H.change.bed <- "/home/zw355/src/rtfbs_db/rtfbsdb/test/dREG.H.change.bed"
file.dREG.all.bed    <- "/home/zw355/src/rtfbs_db/rtfbsdb/test/dREG.all.bed"
file.twoBit     <- "/local/storage/data/hg19/hg19.2bit"

# Convert the bed file for each condition to a data frame
dREG_H_change_bed <- read.table(file.dREG.H.change.bed, header=FALSE);
dREG_all_bed <- read.table(file.dREG.all.bed, header=FALSE);

# Compare motifs between each condition
t.comp <- tfbs.compareTFsite( tfs,
    file.twoBit,
    dREG_H_change_bed,
    dREG_all_bed,
    gc.correction=TRUE,
    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).

Notably, this type of analysis is often confounded by systematic differences in the GC content between groups. To address this limitation, *tfbs.compareTFsite* 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.compareTFsite* 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.



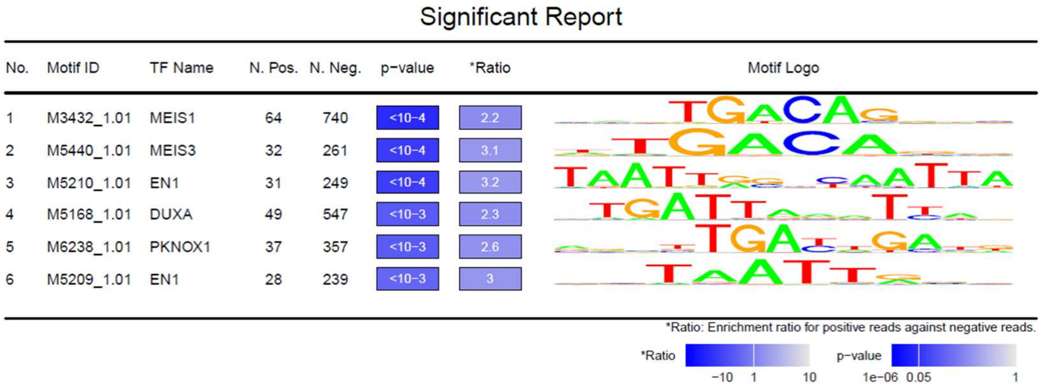
The output of *tfbs.compareTFsite* includes a data frame with 8 columns, including motif ID, TF name, occurrence in positive group, occurrence in negative group, enrichment ratio, p-value of fisher test, and correction value by multiple comparison methods (e.g., Bonferroni or FDR). The *show* command 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
TF binding FDR threshold: 0.05
TF binding score threshold: NA
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            es.ratio
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.reportComparison* 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.

```
tfbs.reportComparson(tfs, t.comp, file.pdf="test-tfcomp.pdf", report.title="Significant Report",
                          sig.only=T, pv.cutoff=0.01, pv.adj="fdr");
```



Significant Report

No.	Motif ID	TF Name	N. Pos.	N. Neg.	p−value	*Ratio	Motif Logo
1	M3432_1.01	MEIS1	64	740	<10−4	2.2	
2	M5440_1.01	MEIS3	32	261	<10−4	3.1	
3	M5210_1.01	EN1	31	249	<10−4	3.2	
4	M5168_1.01	DUXA	49	547	<10−3	2.3	
5	M6238_1.01	PKNOX1	37	357	<10−3	2.6	
6	M5209_1.01	EN1	28	239	<10−3	3	

*Ratio: Enrichment ratio for positive reads against negative reads.

*Ratio
−10
1
10
p−value
1e−06
0.05
1

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] bigWig_0.2-9        cluster_2.0.1       grid_3.1.0
 [4] lattice_0.20-31     latticeExtra_0.6-26 parallel_3.1.0
 [7] RColorBrewer_1.1-2  rphast_1.6          rtfbs_0.3.4
[10] tools_3.1.0
```

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.