diffTF Documentation

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Quick Start and Installation

Welcome to the *diffTF* documentation, and thank you for the interest in our software! These pages provide documentation and additional information for the *diffTF* pipeline.

To get yourself oriented, check the menu on the left or search what you are looking for in the search field in the upper left corner.

This site is organized into the following three parts:

CHAPTER 1

Try it out now!

diffTF runs on Linux and macOS and is even independent on the operating system if combined with `Singularity`. The following quick start briefly summarizes the necessary steps to install and use it.

Principally, there are two ways of installing *diffTF* and the proper tools:

1a. **The "easy" way**: Using `Singularity` and our preconfigured *diffTF* containers that contain all necessary tools, R, and R libraries

> You only need to install Snakemake (see below for details) and `Singularity`. *Snakemake* supports Singularity in Versions >=2.4. You can check whether you already have `Singularity` installed by simply typing
>
> ```
> singularity --version
> ```
>
> Snakemake requires at least version 2.4. If your version is below, please update to the latest `Singularity` version.
>
> **Note:** Make to read the section *Adaptations and notes when running with Singularity* properly!

1b. **The "more complicated" way**: Install the necessary tools (*Snakemake*, *samtools*, *bedtools*, *Subread*, and *R* along with various packages).

> **Note:** Note that all tools require Python 3.
>
> We recommend installing all tools except R via conda, in which case the installation then becomes as easy as
>
> ```
> conda config --add channels defaults
> conda config --add channels conda-forge
> conda config --add channels bioconda
> conda install snakemake bedtools samtools subread
> ```

If conda is not yet installed, follow the installation instructions. Installation is quick and easy. Make sure to open a new terminal after installation, so that *conda* is available.

Note: You do not need to uninstall other Python installations or packages in order to use conda. Even if you already have a system Python, another Python installation from a source such as the macOS Homebrew package manager and globally installed packages from pip such as pandas and NumPy, you do not need to uninstall, remove, or change any of them before using conda.

If you want to install the tools manually and outside of the conda framework, see the following instructions for each of the tools: snakemake, samtools, bedtools, Subread.

In addition, *R* is needed along with various packages (see below for details).

2. **Clone the Git repository:**

```
git clone https://git.embl.de/grp-zaugg/diffTF.git
```

If you receive an error, *Git* may not be installed on your system. If you run Ubuntu, try the following command:

```
sudo apt-get install git
```

For macOS, there are multiple ways of installing it. If you already have *Homebrew* (http://brew.sh) installed, simply type:

```
brew install git
```

Otherwise, consult the internet on how to best install Git for your system.

3. **To run diffTF with an example ATAC-Seq / RNA-seq dataset for 50 TF, simply perform the following steps (see section** *Example dataset* **for dataset details):**

- Change into the `example/input` directory within the Git repository

```
cd diffTF/example/input
```

- Download the data via the download script

```
sh downloadAllData.sh
```

- To test if the setup is correct, start a dryrun via the first helper script

```
sh startAnalysisDryRun.sh
```

- Once the dryrun is successful, start the analysis via the second helper script.

```
sh startAnalysis.sh
```

If you want to include `Singularity` (which we strongly recommend), simply edit the file and add the `--use-singularity` and `--singularity-args` command line arguments in addition to the other arguments (see the Snakemake documentation and the section *Adaptations and notes when running with Singularity* for more details).

Thus, the command you execute should look like this:

```
snakemake --snakefile ../../src/Snakefile --cores 2 --configfile config.
↪json \
 --use-singularity --singularity-args "--bind /your/diffTF/path"
```

Read in section *Adaptations and notes when running with Singularity* about the `--bind` option and what `/your/diffTF/path` means here , it is actually very easy!

You can also run the example analysis with all TF instead of only 50. For this, simply modify the `TF` parameter and set it to the special word `all` that tells *diffTF* to use all recognized TFs instead of a specific list only (see section *TFs* for details).

4. **To run your own analysis**, modify the files `config.json` and `sampleData.tsv`. See the instructions in the section *Run your own analysis* for more details.
5. **If your analysis finished successfully**, take a look into the `FINAL_OUTPUT` folder within your specified output directory, which contains the summary tables and visualization of your analysis. If you received an error, take a look in Section *Handling errors* to troubleshoot.

CHAPTER 2

Prerequisites for the “easy” way

The only prerequisite here is that Snakemake and `Singularity` must be installed on the system you want to run *diffTF*. See above for details with respect to the supported versions etc. For details how to install Snakemake, see below.

CHAPTER 3

Prerequisites for the "manual" way

Note that most of this section is only relevant if you use Snakemake without `Singularity`. This section lists the required software and how to install them. As outlined in Section *Try it out now!*, the easiest way is to install all of them via `conda`. However, it is of course also possible to install the tools separately.

3.1 Snakemake

Please ensure that you have at least version 5.3 installed. Principally, there are multiple ways to install Snakemake. We recommend installing it, along with all the other required software, via conda.

3.2 *samtools*, *bedtools*, *Subread*

In addition, samtools, bedtools and Subread are needed to run *diffTF*. We recommend installing them, along with all the other required software, via conda.

3.3 R and R packages

A working `R` installation is needed and a number of packages from either CRAN or Bioconductor have to be installed. Type the following in `R` to install them:

```
install.packages(c("checkmate", "futile.logger", "tidyverse", "reshape2",
↪"RColorBrewer", "ggrepel", "lsr", "modeest", "boot", "grDevices", "pheatmap",
↪"matrixStats", "locfdr"))

if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")

BiocManager::install(c("limma", "vsn", "csaw", "DESeq2", "DiffBind", "geneplotter",
↪"Rsamtools", "preprocessCore", "apeglm"))
```

CHAPTER 4

Run your own analysis

Running your own analysis is almost as easy as running the example analysis (see section *Example dataset*). Carefully read and follow the following steps and notes:

1. Copy the files `config.json` and `startAnalysis.sh` to a directory of your choice.
2. Modify the file `config.json` accordingly. For example, we strongly recommend running the analysis for all TF instead of just 50 as for the example analysis. For this, simply change the parameter "TFs" to "all". See Section *General configuration file* for details about the meaning of the parameters. Do not delete or rename any parameters or sections.
3. Create a **tab-separated** file that defines the input data, in analogy to the file `sampleData.tsv` from the example analysis, and refer to that in the file `config.json` (parameter `summaryFile`)
4. Adapt the file `startAnalysis.sh` if necessary (the exact command line call to Snakemake and the various Snakemake-related parameters). If you run with Singularity, see the section below for modifications.
5. Since running the pipeline is often computationally demanding, read Section *Executing diffTF - Running times and memory requirements* and decide on which machine to run the pipeline. In most cases, we recommend running *diffTF* in a cluster environment (see Section *Running diffTF in a cluster environment* for details). The pipeline is written in Snakemake, and we strongly suggest to also read Section *Running diffTF* to get a basic understanding of how the pipeline works.

CHAPTER 5

Adaptations and notes when running with Singularity

With `Singularity`, each rule will be executed in pre-configured isolated containers that contain all necessary tools. To enable it, you only have to add the following arguments when you execute Snakemake:

1. `--use-singularity`: Just type it like this!
2. `--singularity-args`: You need to make all directories that contain files that are referenced in the *diffTF* configuration file available within the container also. By default, only the directory and subdirectories from which you start the analysis are automatically mounted inside the container. Since the *diffTF* source code is outside the `input` folder for the example analysis, however, at least the root directory of the Git repository has to be mounted. This is actually quite simple! Just use `--singularity-args "--bind /your/diffTF/path"` and replace `/your/diffTF/path` with the root path in which you cloned the *diffTF* Git repository (the one that has the subfolders `example`, `src` etc.). If you reference additional files, simply add one or multiple directories to the bind path (use the comma to separate them). For example, if you reference the files `/g/group1/user1/mm10.fa` and `/g/group2/user1/files/bla.txt` in the configuration file file, you may add `/g/group1/user1,/g/group2/user1/files` or even just `/g` to the bind path (as all files you reference are within `/g`).

   Note: We note again that within a Singularity container, you cannot access paths outside of the directory from where you started executing Snakemake. If you receive errors in the `checkParameterValidity` rule that a directory does not exist even though you can cd into it, you most likely forgot to include the path this folder or a parent path as part of the `bind` option.

3. `--singularity-prefix /your/directory` (optional): You do not have to, but you may want to add the `--singularity-prefix` argument to store all `Singularity` containers in a central place (here: `/your/directory`) instead of the local `.snakemake` directory. If you intend to run multiple *diffTF* analyses in different folders, you can save space and time because the containers won't have to be downloaded each time and stored in multiple locations.

Please read the following additional notes and warnings related to `Singularity`:

-

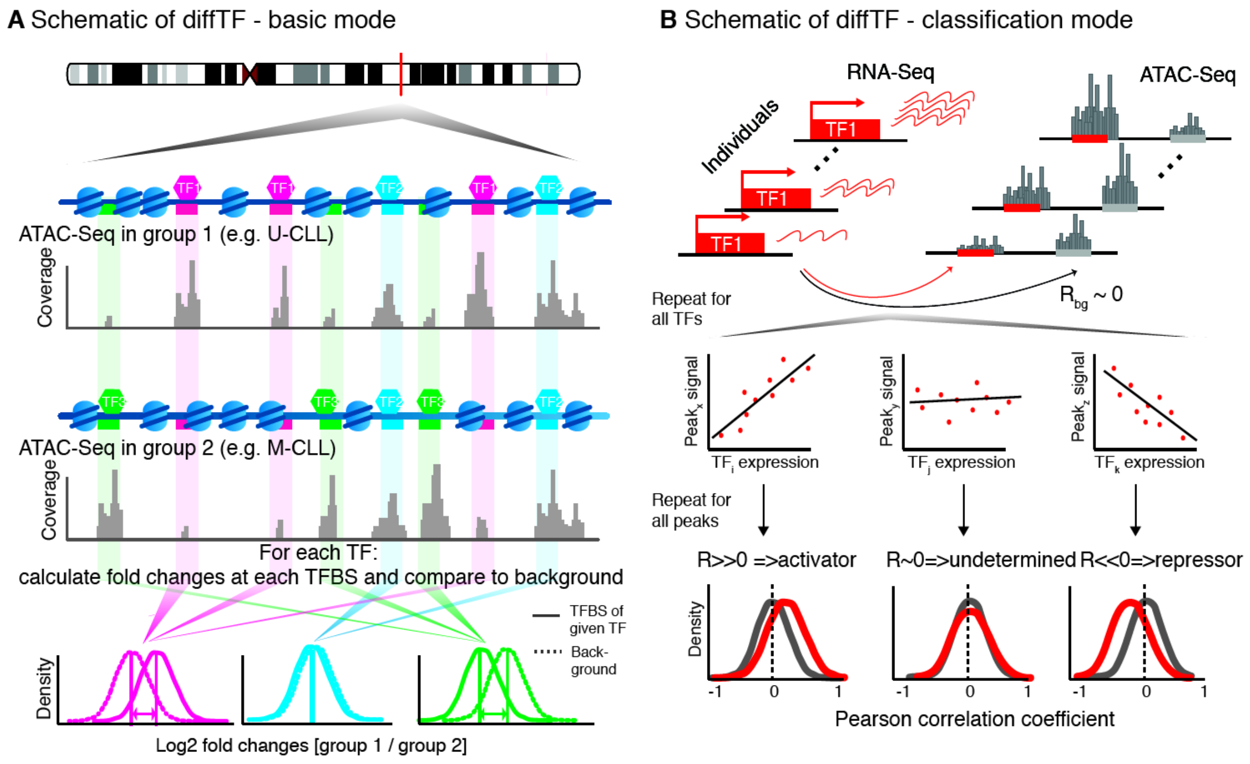
Warning: If you use `Singularity` version 3, make sure you have at least version 3.0.3 installed, as there was an issue with Snakemake and particular `Singularity` versions. For more details, see here.

CHAPTER 6

Workflow

For full information, please see the latest publication (linked here: *Citation*).

The workflow and conceptual idea behind *diffTF* is illustrated by the following three Figures. First, we give a high-level conceptual overview and a biological motivation:



A Schematic of diffTF - basic mode
ATAC-Seq in group 1 (e.g. U-CLL)
Coverage
ATAC-Seq in group 2 (e.g. M-CLL)
For each TF:
calculate fold changes at each TFBS and compare to background
TFBS of given TF
Back-ground
Density
Log2 fold changes [group 1 / group 2]
B Schematic of diffTF - classification mode
RNA-Seq
ATAC-Seq
Individuals
TF1
Repeat for all TFs
R_bg ~ 0
Peak signal
TF expression
Repeat for all peaks
R>>0 =>activator
R~0=>undetermined
R<<0=>repressor
Pearson correlation coefficient

Fig. 1: Conceptual idea and workflow of *diffTF*, the two different modes and the classification

Next, we show a schematic of the *diffTF* workflow from a more technical perspective by showing the actual steps that are performed:

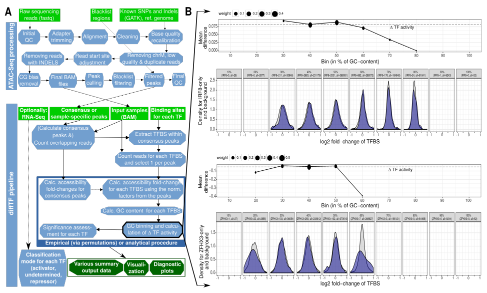


Fig. 2: Summary workflows for data processing and methodological details for *diffTF* (for more details, see Suppl. Figure 1 in the publication).

We now show which rules are executed by *Snakemake* for a specific example (see the caption of the image):

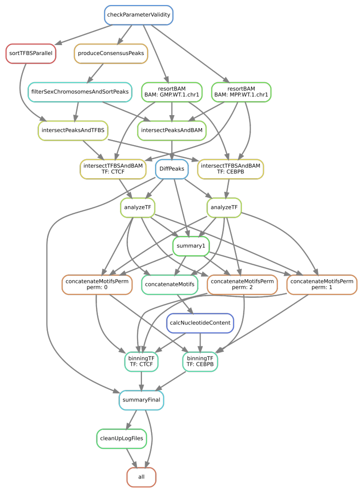


Fig. 3: Exact workflow (a so-called directed acyclic graph, or DAG) that is executed when calling *Snakemake* for an easy of example with two TFs (CEBPB and CTCF) for the two samples GMP.WT1 and MPP.WT1. Each node represents a rule name as defined in the Snakefile, and each arrow a dependency.

diffTF is currently implemented as a *Snakemake* pipeline. For a gentle introduction about *Snakemake*, see Section *Running diffTF*. As you can see, the workflow consists of the following steps or *rules*:

- `checkParameterValidity`: R script that checks whether the specified peak file has the correct format, whether the provided *fasta* file and the *BAM* files are compatible, and other checks
- `produceConsensusPeaks`: R script that generates the consensus peaks using the R package `DiffBind` if none are provided
- `filterSexChromosomesAndSortPeaks`: Filters various chromosomes (sex, unassembled ones, contigs, etc) from the peak file.
- `sortTFBSParallel`: Sort the TFBS lists by position
- `resortBAM`: Sort the *BAM* file for optimized processing (only run if data are paired-end)

- `intersectPeaksAndBAM`: Count all reads for peak regions across all input files
- `intersectPeaksAndTFBS`: Intersect all TFBS with peak regions to retain only TFBS in peak regions
- `intersectTFBSAndBAM`: Count all reads from all TFBS across all input files in a TF-specific manner
- `DiffPeaks`: R script that performs a differential accessibility analysis for the peak regions as well as sample permutations
- `analyzeTF`: R script that performs a TF-specific differential accessibility analysis
- `summary1`: R script that summarizes the previous script for all TFs
- `concatenateMotifs` and `concatenateMotifsPerm`: Concatenates previous results from either real or permuted data (TFBS motives)
- `calcNucleotideContent`: Calculates the GC content for all TFBS
- `binningTF`: R script that performs the binning approach in a TF-specific manner
- `summaryFinal`: R script that summarizes the analysis and calculates final statistics
- `cleanUpLogFiles`: Cleans up the `LOGS_AND_BENCHMARKS` directory (mostly relevant if run in cluster mode)

CHAPTER 7

Input

7.1 Summary

As input for *diffTF* for your own analysis, the following data are needed:

- *BAM* file with aligned reads for each sample (see *summaryFile*)
- genome reference *fasta* that has been used to produce the *BAM* files (see *refGenome_fasta*)
- Optionally: corresponding RNA-Seq data (see *RNASeqCounts*)

In addition, the following files are need, all of which we provide already for human hg19, hg38 and mouse mm10:

- TF-specific list of TFBS (see *dir_TFBS*)
- mapping table (see *HOCOMOCO_mapping*)

Lastly, some metadata files are needed that specify *diffTF*-specific and Snakemake-specific parameters. They are explained in detail in the next sections. If this sounds complicated, don't worry, just take the example analysis, and you will understand within a few minutes what these files are:

- a general configuration file (*General configuration file*)
- a metadata file for the samples (*Input metadata*)
- optionally, if run on a cluster, a cluster configuration file (see in particular the Snakemake documentation for details, but we also provide example cluster files as well as Section *Running diffTF in a cluster environment*)

7.2 General configuration file

To run the pipeline, a configuration file that defines various parameters of the pipeline is required.

Note: Please note the following important points:

- the name of this file is irrelevant, but it must be in the right format (JSON) and it must be referenced correctly when calling *Snakemake* (via the `--configfile` parameter). We recommend naming it `config.json`

- neither section nor parameter names must be changed.
- For parameters that specify a path, both absolute and relative paths are possible. We recommend specifying an absolute path. Relative paths must be specified relative to the *Snakemake* working directory.
- For parameters that specify a directory, there should be no trailing slash.

In the following, we explain all parameters in detail, organized by section names.

7.2.1 SECTION `par_general`

`outdir`

Summary String. Default "output". Root output directory.

Details The root output directory where all output is stored.

`maxCoresPerRule`

Summary Integer > 0. Default 16. Maximum number of cores to use for rules that support multithreading.

Details This affects currently only rules involving *featureCounts* - that is, *intersectPeaksAndBAM* while for rule *intersectTFBSAndBAM*, the number of cores is hard-coded to 4. When running *Snakemake* locally, each rule will use at most this number of cores, while in a cluster setting, this value refers to the maximum number of CPUs an individual job / rule will occupy. If the node the job is executed on has fewer nodes, then the maximum number of cores on the node will be taken.

`regionExtension`

Summary Integer >= 0. Default 100. Target region extension in base pairs.

Details Specifies the number of base pairs each target region (from the peaks file) should be extended in both 5' and 3' direction.

`comparisonType`

Summary String. Default "".

Details This parameter helps to organize complex analysis for which multiple different types of comparisons should be done. Set it to a short but descriptive name that summarizes the type of comparison you are making or the types of cells you compare. The value of this parameter appears as prefix in most output files created by the pipeline. It may also be empty.

`conditionComparison`

Summary String. Default "". Specifies the two conditions you want to compare. Only relevant if *conditionSummary* is specified as a factor.

Details This parameter is only relevant if *conditionSummary* is specified as a factor, in which case it specifies the contrast you are making in *diffTF*. Otherwise, it is ignored. Exactly two conditions have to be specified, comma-separated. For example, if you want to compare GMP and MPP samples, the parameter should be "GMP,MPP". Both conditions have to be present in the column "conditionSummary" in the sample file table (see `summaryFile` (*summaryFile*)).

Note: The order of the two conditions matters. The condition specified first is the reference condition. For the "GMP,MPP" example, all log2 fold-changes will be the log2fc of *MPP* as compared to *GMP*. That means that a positive log2 fold-change means it is higher in *MPP* as compared to *GMP*. Consequently, the final TF activity (denoted *weighted mean difference* in the output tables) will have the same directionality. This is also particularly relevant for the *allMotifs* output file.

`designContrast`

Summary String. Default *conditionSummary*. Design formula for the differential accessibility analysis.

Details This important parameter defines the actual contrast that is done in the differential accessibility analysis. That is, which groups of samples are being compared? Examples include mutant vs wild type, mutated vs. unmutated, etc. The last element in the formula must always be *conditionSummary*, which defines the two groups that are being compared or the continuous variable that is used for inferring negative or positive changes, respectively (see parameter *designVariableTypes*). This name is currently hard-coded and required by the pipeline. Our pipeline allows including additional variables to model potential confounding variables, like gender, batches etc. For each additional variable that is part of the formula, a corresponding and identically named column in the sample summary file must be specified. For example, for an analysis that also includes the batch number of the samples, you may specify this as "~ *Treatment* + *conditionSummary*".

`designContrastRNA`

Summary String. Default *conditionSummary*. Design formula for the RNA-Seq data. Only relevant and needed if parameter (*RNASeqIntegration*) is set to *true*. If missing (to increase compatibility with previous versions of *diffTF*), the default value will be taken.

Details This important parameter defines the actual contrast that is done in the differential accessibility analysis. That is, which groups of samples are being compared? Examples include mutant vs wild type, mutated vs. unmutated, etc. The last element in the formula must always be *conditionSummary*, which defines the two groups that are being compared or the continuous variable that is used for inferring negative or positive changes, respectively (see parameter *designVariableTypes*). This name is currently hard-coded and required by the pipeline. Our pipeline allows including additional variables to model potential confounding variables, like gender, batches etc. For each additional variable that is part of the formula, a corresponding and identically named column in the sample summary file must be specified. For example, for an analysis that also includes the batch number of the samples, you may specify this as "~ *Treatment* + *conditionSummary*".

`designVariableTypes`

Summary String. Default *conditionSummary:factor*. The data types of **all** elements listed in `designContrast` (*designContrast*).

Details Names must be separated by commas, spaces are allowed and will be eliminated automatically. The data type must be specified with a ":", followed by either "numeric", "integer", "logical", or "factor". For example, if `designContrast` (*designContrast*) is specified as "~ *Treatment* + *conditionSummary*", the corresponding types might be "Treatment:factor, conditionSummary:factor". If a data type is specified as either "logical" or "factor", the variable will be treated as a discrete variable with a finite number of distinct possibilities (something like batch, for example).

Note: Importantly, *conditionSummary* can either be specified as "factor" or "numeric"/"integer", which changes the way the results are interpreted and what the log2 fold-changes represent. *conditionSummary* is usually specified as factor because you want to make a pairwise comparison of exactly two conditions. If *conditionSummary* is specified as "integer" or "numeric" (i.e., continuous-valued), however, the variable is treated as continuously-scaled, which changes the interpretation of the results: the reported log2 fold change is then per unit of change of that variable. That is, in the final Volcano plot, TFs displayed in the left side have a negative slope per unit of change of that variable, while TFs at the right side have a positive one.

`nPermutations`

Summary Integer >= 0. Default 50. The number of random sample permutations.

Details If set to a value > 0, in addition to the real data, the sample conditions as specified in the sample table will be randomly permuted *nPermutations* times. This is the recommended way of computing statistical significances for each TF. In this approach, the resulting significance value captures the significance of the effect size (that is, the TF activity) for the real data as compared to permuted one. Note that the maximum number of possible permutations is limited by the number of samples and can be computed with the binomial coefficient *n* over *k*. For example, if you have *n* = 8 samples in total and they split up in the two conditions/groups as *k* = 5 / *k* = 3, the total number of permutations is 8 over 5 or 8 over 3 (they are both identical). We generally recommend setting this value to high values such as 1,000. If the value is set to a number higher than the number of possible permutations, it will be adjusted automatically to the maximum number of permutations as determined by the binomial coefficient.

If set to 0, an alternative way of computing significances that is not based on permutations is performed. First, in the CG normalization step, a Welch Two Sample t-test is performed for each bin and the overall significance by treating the T-statistics as z-scores is calculated, which allows to summarize them across the bins and convert them to one p-value per TF. For this conversion of z-scores per bin to p-value an estimate of the variance of the T-scores is approximated (see the publication for details). This procedure reduces the dependency of the p-value on the sample size (since the number of TFBS can range between a few dozen and multiple tens of thousands depending on the TF).

Note: If set to a value > 0, the `nBootstraps` (*nBootstraps*) is ignored and can be set to any value.

Note: While using permutations is the recommended approach for assessing statistical significance, in some cases it might be more useful to use the analytical approach: (1) If the number of samples is small or the groups show a very uneven distributions, the total number of possible permutations is also very small and therefore also the permutation-based approach might not accurately assess significance. As a rough guideline, we do not recommend running less than 100 permutations. (2) This approach is usually more stringent than the analytical one. If you have only small differences between the two groups and despite the fact there is no strong signal to capture in the first place, you may want to run the analytical approach instead in such a case.

Note: The permutation-based approach is computationally more expensive than the analytical approach. The running time of the pipeline increases with the number of permutations.

Warning: Do not change the value of this parameter after (parts of) the pipeline have been run, some steps may fail due to this change. If you really need to change the value, rerun the pipeline from the *diffPeaks* step onwards.

`nBootstraps`

Summary Integer >= 0. Default 1,000. The number of bootstrap for estimating the variance of the TF-specific T scores in the CG binning step.

Details To properly estimate the variance of the T scores for each TF in the CG binning step, we employ a bootstrap approach using the boot library in R with a user-adjustable number of bootstrap replicates (default 1,000), with resampling the bin-specific data and then performing the t-test against the full sample as described above. We then calculate the variance of the bootstrapped T scores for each bin. For more details, see the methods of the publication.

Note: Only relevant if the `nPermutations` (*nPermutations*) is set to 0. If both are set to 0, an error is thrown.

Warning: If bootstraps are used, it is recommended to use a reasonable large number. We recommend a value 1,000 and found that higher numbers do not add much benefit but instead only increase running time unnecessarily.

`nGCBins`

Summary Integer > 0. Default 10. Number of GC bins for the binning step.

Details

This parameter sets the number of GC bins that are used during the binning step. The default is to split the data into 10 bins (0-10% GC content, 11-20%, ..., 91-100%), for each of which the significance is calculated independently (see Methods). Too many bins may result in bins being skipped due to an insufficient number of TFBS for that particular bin and TF, while too few bins may introduce GC-specific biases when summarizing the signal across all TFBS.

`TFs`

Summary String. Default "all". Either "all" or a comma-separated list of TF names of TFs to include. If set to "all", all TFs that are found in the directory as specified in `dir_TFBS` (*dir_TFBS*) will be used.

Details If the analysis should be restricted to a subset of TFs, list the names of the TF to include in a comma-separated manner here.

Note: For each TF `{TF}`, a corresponding file `{TF}_TFBS.bed` needs to be present in the directory that is specified by `dir_TFBS` (*dir_TFBS*).

Warning: We strongly recommending running *diffTF* with as many TF as possible due to our statistical model that we use that compares against a background model.

`dir_scripts`

Summary String. The path to the directory where the R scripts for running the pipeline are stored.

Details

> **Warning:** The folder name must be `R`, and it has to be located in the same folder as the `Snakefile`.

`RNASeqIntegration`

Summary Logical. true or false. Default false. Should RNA-Seq data be integrated into the pipeline?

Details If set to true, RNA-Seq counts as specified in `RNASeqCounts` (*RNASeqCounts*) will be used to classify each TF into either "activator", "repressor", "unknown", or "not-expressed" for the final Volcano plot visualization and the summary table.

7.2.2 SECTION `samples`

`summaryFile`

Summary String. Default "samples.tsv". Path to the sample metadata file.

Details Path to a tab-separated file that summarizes the input data. See the section *Input metadata* and the example file for how this file should look like.

`pairedEnd`

Summary Logical. true or false. Default true. Is the data paired-end? If single-end, set to false.

Details Both paired-end and single-end data can be run with *diffTF*.

7.2.3 SECTION `peaks`

`consensusPeaks`

Summary String. Default "" (empty). Path to the consensus peak file.

Details If set to the empty string "", the pipeline will generate a consensus peaks out of the peak files from each individual sample using the R package `DiffBind`. For this, you need to provide the following two things:

- a peak file for each sample in the metadata file in the column *peaks*, see the section *Input metadata* for details.
- The format of the peak files, as specified in `peakType` (*peakType*)

If a file is provided, it must be a valid *BED* file with at least 3 columns:

- tab-separated columns
- no column names in the first row
- Columns 1 to 3:
  1. Chromosome
  2. Start position
  3. End position
- Optional (content for each is ignored and not checked for validity):

4. Identifier (will be made unique for each if this is not the case already)
5. Score
6. Strand

> **Warning:** *diffTF* will take a long time to run if the number of peaks is too high. We recommend having less than 100,000 peaks. If the number of peaks is higher for your analysis, we strongly recommend filtering the peaks beforehand to include only the most relevant peaks.

`peakType`

Summary String. Default `narrow`. File format of the individual, sample-specific peak files. Only relevant if no consensus peak file has been provided (i.e., the *consensusPeaks* is empty).

Details Only needed if no consensus peak set has been provided. All individual peak files must be in the same format. We recommend the `narrow` format (files ending in `.narrowPeak`) that is a direct output from *MACS2*, but other formats are supported. See the help for *DiffBind dba* for a full list of supported formats, the most common ones include:

- `bed`: .bed file; peak score is in fifth column
- `narrow`: narrowPeaks file (from *MACS2*)

`minOverlap`

Summary Integer >= 0 or Float between 0 and 1. Default 2. Minimum overlap for peak files for a peak to be considered into the consensus peak set. Corresponds to the `minOverlap` argument in the *dba* function of *DiffBind*. Only relevant if no consensus peak file has been provided (i.e., `consensusPeaks`, *consensusPeaks*, is empty).

Details Only include peaks in at least this many peak sets in the main binding matrix. If set to a value between zero and one, peak will be included from at least this proportion of peak sets. For more information, see the `minOverlap` argument in the *dba* function of *DiffBind* (see here).

7.2.4 SECTION `additionalInputFiles`

`refGenome_fasta`

Summary String. Default 'hg19.fasta'. Path to the reference genome *fasta* file.

Details

> **Warning:** You need write access to the directory in which the *fasta* file is stored, make sure this is the case or copy the *fasta* file to a different directory. The reason is that the pipeline produces a *fasta* index file, which is put in the same directory as the corresponding *fasta* file. This is a limitation of *samtools faidx* and not our pipeline.

> **Note:** This file has to be in concordance with the input data; that is, the exact same genome assembly version must be used. In the first step of the pipeline, this is checked explicitly, and any mismatches will

result in an error.

`dir_TFBS`

Summary String. Path to the directory where the TF-specific files for TFBS results are stored.

Details Each TF *{TF}* has to have one *BED* file, in the format *{TF}.bed*. Each file must be a valid *BED6* file with 6 columns, as follows:

1. chromosome
2. start
3. end
4. ID (or sequence)
5. score or any other numeric column
6. strand

For user convenience, we provide such sorted files as described in the publication as a separate download:

- hg19: For a pre-compiled list of 638 human TF with in-silico predicted TFBS based on the *HOCOMOCO 10* database and *PWMScan* for hg19, download this file:
- hg38: For a pre-compiled list of 767 human TF with in-silico predicted TFBS based on the *HOCOMOCO 11* database and *FIMO* from the MEME suite for hg38, download this file:. For a pre-compiled list of 768 human TF with in-silico predicted TFBS based on the *HOCOMOCO 11* database and *PWMScan* for hg38, download this file:
- mm10: For a pre-compiled list of 422 mouse TF with in-silico predicted TFBS based on the *HOCOMOCO 10* database and *PWMScan* for mm10, download this file:

However, you may also manually create these files to include additional TF of your choice or to be more or less stringent with the predicted TFBS. For this, you only need PWMs for the TF of interest and then a motif prediction tool such as *FIMO* or *MOODS*.

`RNASeqCounts`

Summary String. Default “”. Path to the file with RNA-Seq counts.

Details If no RNA-Seq data is included, set to the empty string “”. Otherwise, if `RNASeqIntegration` (*RNASeqIntegration*) is set to true, specify the path to a tab-separated file with *raw* RNA-Seq counts. We apply some basic filtering for lowly expressed genes and exclude genes with small counts, so there is principally no need for manual filtering unless you want to do so. For guidance, you may want to read Question 4 here.

The first line must be used for labeling the samples, with column names being identical to the sample names as specific in the sample summary table (`summaryFile`, *summaryFile*). If you have RNA-Seq data for only a subset of the input samples, this is no problem - the classification will then naturally only be based on the subset. The first column must be named ENSEMBL and it must contain ENSEMBL IDs (e.g., *ENSG00000028277*) without dots. The IDs are then matched to the IDs from the file as specified in `HOCOMOCO_mapping` (*HOCOMOCO_mapping*).

`HOCOMOCO_mapping`

Summary String. Path to the TF-Gene translation table.

Details If RNA-Seq integration shall be used, a translation table to associate TFs and ENSEMBL genes is needed. For convenience, we provide such a translation table compatible with the pre-provided TFBS lists. Specifically, for each of the currently three TFBS lists, we provide corresponding translation tables for:

1. hg19 with HOCOMOCO 10
2. hg38 with HOCOMOCO 11
3. mm10 with HOCOMOCO 10

If you want to create your own version, check the example translation tables and construct one with an identical structure.

7.3 Input metadata

This file summarizes the data and corresponding available metadata that should be used for the analysis. The format is flexible and may contain additional columns that are ignored by the pipeline, so it can be used to capture all available information in a single place. Importantly, the file must be saved as tab-separated, the exact name does not matter as long as it is correctly specified in the configuration file.

> **Warning:** Make sure that the line endings are correct. Different operating systems use different characters to mark the end of line, and the line ending character must be compatible with the operating system in which you run *diffTF*. For example, if you created the file in MAC, but you run it in a Linux environment (e.g., a cluster system), you may have to convert line endings to make them compatible with Linux. For more information, see here .

It must contain at least contain the following columns (the exact names do matter):

- `sampleID`: The ID of the sample.

> **Note:** Note that each sample ID must be unique! If you want to include replicate samples, rename them, for example by adding "_1", "_2" etc at the end. All that *diffTF* cares about is the correct group assignment as defined by the column *conditionSummary*.

- `bamReads`: path to the *BAM* file corresponding to the sample.

> **Warning:** All *BAM* files must meet *SAM* format specifications. You may use the program *ValidateSamFile* from the *Picard tools* to check and identify problems with your file. Chromosome names must have a "*chr*" as prefix, otherwise *diffTF* may crash.

- `peaks`: absolute path to the sample-specific peak file, in the format as given by `peakType` (*peakType*). Only needed if no consensus peak file is provided.
- `conditionSummary`: String with an arbitrary condition name that defines which condition the sample belongs to. There must be only exactly two different conditions across all samples (e.g., *mutated and unmutated*, *day0 and day10*, . . .). In addition, the two conditions must match the ones specified in the `conditionComparison` (*conditionComparison*).
- if applicable, all additional variables from the design formula except `conditionSummary` must also be present as a separate column.

Warning: Do not change the samples data after you started an analysis. You may introduce inconsistencies that will result in error messages. If you need to alter the sample data, we strongly advise to recalculate all steps in the pipeline.

CHAPTER 8

Output

The pipeline produces quite a large number of output files, only some of which are however relevant for the regular user.

Note: In the following, the directory structure and the files are briefly outlined. As some directory or file names depend on specific parameters in the configuration file, curly brackets will be used to denote that the filename depends on a particular parameter or name. For example, `{comparisonType}` and `{regionExtension}` refer to `comparisonType` (*comparisonType*) and `regionExtension` (*regionExtension*) as specified in the configuration file.

Most files have one of the following file formats:

- .bed.gz (gzipped bed file)
- .tsv.gz (tab-separated value, text file with tab as column separators, gzipped)
- .rds (binary R format, read into with the function `readRDS`)
- .pdf (PDF format)
- .log (text format)

8.1 FOLDER `FINAL_OUTPUT`

In this folder, the final output files are stored. Most users want to examine the files in here for further analysis.

8.1.1 Sub-folder `extension{regionExtension}`

Stores results related to the user-specified extension size (`regionExtension`, *regionExtension*). In the following, the files are ordered by significance or relevance for interpretation an downstream analyses.

Note: In all output files, in the column `permutation`, 0 always refers to the non-permuted, real data, while permutations > 0 reflect real permutations.

FILES `{comparisonType}.summary.volcano.pdf` and `{comparisonType}.summary.volcano.q*.pdf`

Summary A visual summary of the results in the form of a Volcano plot. If you run the classification mode, multiple files are created, as follows:

- `{comparisonType}.summary.volcano.pdf`. This file intentionally empty, see the other files below
- `{comparisonType}.summary.volcano.q{X}.pdf`, with {X} being 0.001, 0.01, 0.05, and 0.1, corresponding to different stringencies of the classification. Thus, only the classification (i.e, coloring of the data points) differs among the 4 PDF files.

If you run only the basic mode, only the file `{comparisonType}.summary.volcano.pdf` is created.

Each PDF contains multiple pages, essentially showing the same data but with different filters, and the structure is as follows:

- Basic mode (10 pages in total)
  - Pages 1-5: Volcano plot for different values for the adjusted p-value, starting from the most stringent, 0.001, to 0.01, 0.05, 0.1 and finally the least stringent 0.2
  - Pages 6-10: Same as pages 1-5, just with the raw p-value
- Classification mode (30 pages in total)
  - Pages 1:15: Volcano plot for different values for the adjusted p-value, starting from the most stringent, 0.001, to 0.01, 0.05, 0.1 and finally the least stringent 0.2. For each of these values, 3 pages are shown: 1: all four classes, 2: excluding not-expressed TFs, 3: only showing activator and repressor TFs (see also the legend)
  - Pages 16-30: Same as pages 1-15, just with the raw p-value

Generally, each page shows a Volcano plot of the differential TF activity (labeled as *weighted mean difference*) between the two conditions you run the analysis for (x-axis) and the corresponding significance (y-axis, adjusted for multiple testing and -log10 transformed). Each point is a TF. The significance threshold is indicated with a dotted line. TFBS is the number of predicted TF binding site that overlap the peak regions and upon which the weighted mean difference is based on. If the classification mode was run, the lgend also shows the TF classification, and points are colored accordingly. Note that different sets of classification classes are shown on each page, see above.

FILE `{comparisonType}.summary.tsv.gz`

Summary The final summary table with all *diffTF* results. This table is also used for the final Volcano plot visualization. The number of columns may vary and depends on the mode you run *diffTF* for (i.e., only basic mode or also classification mode, analytical or permutation-based approach).

Details *The following columns are always present and relevant:*

- *TF*: name of the TF
- *weighted_meanDifference*: This is the TF activity value that captures the difference in accessibility between the two conditions. More precisely, it is the difference of the real and background distribution,

calculated as the weighted mean across all CG bins (see the publication or *Workflow* for a graphical depiction of how this works put plot how this is calculated). In the Volcano plot, this is the x-axis. Higher values in either positive and negative direction indicate a larger TF activity in one of the two conditions (i.e., the predicted TF binding sites for this TFs are more accessible). Positive and negative values denote whether the value was bigger in one or the other condition, see the Volcano plot for easier interpretation as well as the notes for :ref:`conditionComparison`.

- *weighted_CD*: An alternative measure for the effect size that can be seen as alternative for the *weighted_meanDifference* but that we provide nevertheless. It is calculated in a similar fashion as the *weighted_meanDifference*, but instead of taking the difference in the means of the log2 fold-change values from foreground and background, it represents the Cohen's d measure of effect size (as calculated by the `cohensD` function from the *lsr* package), weighted by CG bin as for the *weighted_meanDifference*.
- *TFBS*: The number of predicted TF binding sites for the particular TF that overlap with the peaks and that the analysis was based on.
- *pvalue*: The p-value assesses the significance of the obtained *weighted_meanDifference*. The exact calculation depends on whether permutations are used (permutation-based approach) or not (analytical approach) and is fully described in the *STAR* methods of the publication, section "Estimation of significance for differential activity for each TF"
- *pvalueAdj*: adjusted p-values using Benjamini-Hochberg

The following columns are only relevant if you run the analytical mode:

- *weighted_Tstat* and *variance*: These columns are only relevant for the analytical version. See the section "Estimation of significance for differential activity for each TF" in the *STAR* methods for details. The resulting p-value is based on these columns and we provide them for the sake of completeness.

The following columns are only relevant if you run the classification mode:

- *median.cor.tfs*: The median value for the RNA-ATAC correlations from the foreground (i.e., peaks with a predicted TFBS for the particular TF)
- *classification_**: The columns are explained below, but for each of them, a TF is either classified as *activator*, *undetermined*, *repressor* or *not-expressed*). For details how TFs are classified, see the *STAR* methods, section "Classification of TFs into activator and repressors". Note that the current implementation uses a two-step process to classify TFs. We provide the classifications for both steps for clarity, and they are further subdivided into different classification stringencies (e.g., for more stringent classifications, i.e. smaller values, more TFs are classified as undetermined and only the strongest activators and repressors will be classified as such). These values denote the particular percentiles of the background distribution across the background values for all TF as a threshold for activators and repressors and are used to distinguish real correlations from noise (i.e., activator/repressor from undetermined). The classification stringency goes from 0.001 (most stringent), 0.01, 0.05 to 0.1 (least stringent).
  - classification_q0.* (without final): TF classifications after step 1
  - classification_distr_rawP: The raw p-value of the one-sided Wilcoxon rank sum test for step 2. For TFs that were classified as either repressor or activator after step 1 but for which the raw p value of the Wilcoxon rank sum test was not significant, we changed their classification to undetermined, thereby removing TF classifications with weak support
  - classification_q0.*_final*: TF classifications after step 2 (final, this is what is shown in the Volcano plot)

FILES `{comparisonType}.diagnosticPlotsClassification1.pdf` and `{comparisonType}.diagnosticPlotsClassification2.pdf`

Summary Diagnostic plots related to the classification mode.

File `{comparisonType}.diagnosticPlotsClassification1.pdf`:

- Pages 1-4: Median Pearson correlation for all TFs, ordered from bottom (lowest) to top (highest). Each dot is one TF, and the color of the dot indicates the TF classification (red: repressor, black/gray: undetermined, green: activator). Each page shows the stringency on which the classification is based for this particular threshold as annotated vertical lines, inside of which TFs are classified as undetermined and outside of it as either repressor (left) or activator (right). The more stringent (i.e., smaller values, see the title), the more the two lines move towards the outside, thereby increasing the width of the "undetermined" area.
- Page 5: Summary density heatmap for each TF and for all classifications across stringencies, sorted by the median Pearson correlation (from the most negative one at the bottom to the most positive one at the top). The heatmap visualizes the correlation across all TFBS, in an alternative representation as compared to the previous pages, summarized in one plot. Colors in or closer to red indicate higher densities and therefore an accumulation of values, while ble or close to blue colors indicate the opposite. Thus, repressors will typically have an enrichment of red colors for negative correlation values, while activators have an enrichment for positive values. TFs will low or conflicting signal will be placed in the middle, classified as undetermined. The left part shows the classification of the TFs for all classification stringencies, sorted from left to right by stringency. The first number refers to the stringency as in other plots and files, but here depicted as per cent (i.e., 0.1% refers to the 0.001 stringency as referred to elsewhere). For each stringency, there are two classifications, referring to the two-step procedure as explained above (columns *classification* for the file `{comparisonType}.summary.tsv.gz`). If the signal is strong, the difference between the final and non-final column should be small, while for low-signal classifications, pseudo-significant results will not be significant for the *final* column.

File `{comparisonType}.diagnosticPlotsClassification2.pdf`:

- Pages 1-12: Correlation plots of the TF activity (weighted mean differences, x-axis) from the ATAC-Seq for all TF and the log2 foldchanges of the corresponding TF genes from the RNAseq data (y-axis). Each TF is a point, the size of the point reflects the normalized base mean of the TF gene according to the RNA-Seq data. In addition, the *glm* regression line is shown, colored by the classification. The correlation plots are shown for different classification stringencies. The title also gives the correlation value for both Pearson and Spearman correlation as well as the corresponding p-values along with the classification stringency
- Page 13-14: Regular (13) and MA plot based shrunken log2 fold-changes (14) of the RNA-Seq counts based on the `DESeq2` analysis. Both show the log2 fold changes attributable to a given variable over the mean of normalized counts for all samples, while the latter removes the noise associated with log2 fold changes from low count genes without requiring arbitrary filtering thresholds. Points are colored red if the adjusted p-value is less than 0.1. Points which fall out of the window are plotted as open triangles pointing either up or down. For more information, see here.
- Pages 15-18: Densities of nonnormalized (15) and normalized (16) mean log counts for the different samples of the RNA-Seq data, as well their respective empircal cummulative distribution functions (ECDF, pages 17 and 18 for nonnormalized and normalized mean log counts, respectively). Since most of the genes are (heavily) affected by the experimental conditions, a successful normalization will lead to overlapping densities. The ECDFs can be thought of as integrals of the densities and give the probability of observing a certain number of counts equal to x or less given the data. For more information, see here.
- Page 19: Mean SD plot (row standard deviations versus row means)
- Page 20-end: Density plots for each TF, with one TF per page. The density plot shows the foreground (red, labeled as *Motif*) comprising of the log2 fold-changes of all predicted TF binding sites (TFBS, see title) for the particular TF only, while the background (gray, labeled as *Non-Motif*) shows the log2 fold-changes of all predicted TF binding sites for all other TF.

For the other plots, already documented? To further assess systematic differences between the samples, we can also plot pairwise mean–average plots: We plot the average of the log–transformed counts vs the fold change per gene for each of the sample pairs.

FILE `{comparisonType}.diagnosticPlots.pdf`

Summary Various diagnostic plots for the final TF activity values, mostly related to the permutation-based approach.

Details If the permutation-based approach has been used, the structure is as follows:

- Page 1: Density plot of the weighted mean difference (TF activity) values from the permutations (black) and the real values (red) across all TF. Note that the number of points in the permuted data contains more values - if 1000 permutations have been used for 640 TF, it contains 640 * 1000 values, while the red distribution only contains 640 values. This plot summaries the overall signal: if the red and black curve show little difference, it generally indicates that the observed weighted mean difference (TF activity) values across all TF are very similar to permuted values and therefore, noise. Importantly, however, there might well be individual TFs that show a large signal, which should be visible also in the red line by having outlier values towards the more extreme values. Permuted values, however, usually cluster strongly around 0, which is the expected difference between the conditions if the data are permuted.
- Page 2 onward: Density plot for the weighted mean difference (TF activity) values from the permutations (one value per permutation, black) vs the single real value (red vertical line). The significance that is shown in the Volcano plot is based on the comparison of the permuted vs the real value (see methods for details). In brief, it is calculated as an empirical two-sided p-value per TF by comparing the real value with the distribution from the permutations and calculating the proportion of permutations for which the absolute differential TF activity is larger. For example, the p-value is small if the real value (i.e., the red line) is outside of the distribution or close to the corner of the permuted values. The p-value is consequently large, however, if the real value falls well within the distribution of the permuted values.
- Rest: Various summary plots for different variables

If the analytical mode has been run, the plots related to the permutations are missing from the PDF.

FILE `{comparisonType}.allMotifs.tsv.gz`

Summary Summary table for each TFBS. This file contains summary data for each TF and each TFBS and allows a more in-depth investigation.

Details Columns are as follows:

- *permutation*: Permutation number. This is always 0 and can therefore be ignored
- *TF*: name of the TF
- *chr*, *MSS*, *MES*, *strand*, *TFBSID*: Genomic location and identifier of the (extended) TFBS
- *peakID*: Genomic location and annotation of the overlapping peak region
- *l2FC*, *pval*, *pval_adj*: Results from the *limma* or *DESeq2* analysis, see the respective documentation for details (see below for links and further explanation). These column names are shared between *limma* and *DESeq2*. l2FC are interpreted as described in the `conditionComparison` (*conditionComparison*)
- *DESeq_baseMean*, *DESeq_ldcSE*, *DESeq_stat*: Results from the *DESeq2* analysis, see the *DESeq2* documentation for details (e.g., *?DESeq2::results*). If *DESeq2* was not run for calculating log2 fold-changes (i.e., if the value for the `nPermutations` (*regionExtension*) is >0), these columns are set to NA.
- *limma_avgExpr*, *limma_B*, *limma_t_stat*: Results from the *limma* analysis, see the *limma* documentation for details (e.g., *??topTable*). If *limma* was not run (i.e., if the value for the `nPermutations` (*regionExtension*) is 0), these columns are set to NA.

FILE `{comparisonType}.TF_vs_peak_distribution.tsv.gz`

Summary This summary table contains various results regarding TFs, their log2 fold change distribution across all TFBS and differences between all TFBS and the peaks

Details See the description of the file `{TF}.{comparisonType}.summary.rds`. This file aggregates the data for all TF and adds the following additional columns: - *pvalue_adj*: adjusted (fdr aka BH) p-value (based on *pvalue_raw*) - *Diff_mean*, *Diff_median*, *Diff_mode*, *Diff_skew*: Difference of the mean, median, mode, and skewness between the log2 fold-change distribution across all TFBS and the peaks, respectively

8.2 FOLDER `PEAKS`

Stores peak-associated files.

8.2.1 FILES `{comparisonType}.consensusPeaks.filtered.sorted.bed`

Summary Produced in rule `filterSexChromosomesAndSortPeaks`. Filtered and sorted consensus peaks (see below). the *diffTF* analysis is based on this set of peaks.

Details Filtered consensus peaks (removal of peaks from one of the following chromosomes: chrX, chrY, chrM, chrUn*, and all contig names that do not start with "chr" such as *random* or *hapl_gl*

8.2.2 FILE `{comparisonType}.allBams.peaks.overlaps.bed.gz`

Summary Produced in rule `intersectPeaksAndBAM`. Counts for each consensus peak with each of the input *BAM* files.

Details No further details provided yet. Please let us know if you need more details.

8.2.3 FILE `{comparisonType}.sampleMetadata.rds`

Summary Produced in rule `DiffPeaks`. Stores data for the input data (similar to the input sample table), for both the real data and the permutations.

Details No further details provided yet. Please let us know if you need more details.

8.2.4 FILE `{comparisonType}.peaks.rds`

Summary Produced in rule `DiffPeaks`. Internal file. Stores all peaks that will be used in the analysis in rds format.

Details No further details provided yet. Please let us know if you need more details.

8.2.5 FILE `{comparisonType}.peaks.tsv.gz`

Summary Produced in rule `DiffPeaks`. Stores the results of the differential accessibility analysis for the peaks.

Details No further details provided yet. Please let us know if you need more details.

8.2.6 FILE `{comparisonType}.normFacs.rds`

Summary Produced in rule `DiffPeaks`. Gene-specific normalization factors for each sample and peak.

Details This file is produces after the differential accessibility analysis for the peaks. The normalization factors are used for the TF-specific differential accessibility analysis.

8.2.7 FILES `{comparisonType}.diagnosticPlots.peaks.pdf`

Summary Produced in rule `DiffPeaks`. Various diagnostic plots for the differential accessibility peak analysis for the real data.

Details The pages are as follows:

(1) Density plots of non-normalized (page 1) and normalized (page 2) mean log counts as well their respective empirical cumulative distribution functions (ECDF, pages 3 and 4 for nonnormalized and normalized mean log counts, respectively)

(2) pairwise mean-average plots (average of the log-transformed counts vs the fold-change per peak) for each of the sample pairs. This can be useful to further assess systematic differences between the samples. Note that only a maximum of 20 different pairwise plots are shown for time and efficacy reasons.

(3) mean SD plots (row standard deviations versus row means, last page)

8.2.8 FILE `{comparisonType}.DESeq.object.rds`

Summary Produced in rule `DiffPeaks`. The *DESeq2* object from the differential accessibility peak analysis.

Details No further details provided yet. Please let us know if you need more details.

8.3 FOLDER `TF-SPECIFIC`

Stores TF-specific files. For each TF `{TF}`, a separate sub-folder `{TF}` is created by the pipeline. Within this folder, the following structure is created:

8.3.1 Sub-folder `extension{regionExtension}`

FILES `{TF}.{comparisonType}.allBAMs.overlaps.bed.gz` and `{TF}.{comparisonType}.allBAMs.overlaps.bed.summary`

Summary Overlap and *featureCounts* summary file of read counts across all TFBS for all input *BAM* files.

Details For more details, see the documentation of *featureCounts*.

FILE `{TF}.{comparisonType}.output.tsv.gz`

Summary Produced in rule `analyzeTF`. A summary table for the differential accessibility analysis.

Details See the file `{comparisonType}.allMotifs.tsv.gz` in the `FINAL_OUTPUT` folder for a column description.

FILE `{TF}.{comparisonType}.outputPerm.tsv.gz`

Summary Produced in rule `analyzeTF`. A subset of the file `{TF}.{comparisonType}.output.tsv.gz` that stores only the necessary permutation-specific results for subsequent steps.

Details This file has the following columns (see the description for the file `{TF}.{comparisonType}.output.tsv.gz` for details): - *TF* - *TFBSID* - *log2fc_perm* columns, which store the permutation-specific log2 fold-changes of the particular TFBS. Permutation 0 refers to the real data

FILE `{TF}.{comparisonType}.summary.rds`

Summary Produced in rule `analyzeTF`. A summary table for the log2 fold-changes across all TFBS *limma* results.

Details This file summarizes the TF-specific results for the differential accessibility analysis and has the following columns: - *TF*: name of the TF - *permutation*: The number of the permutation. - *Pos_l2FC*, *Mean_l2FC*, *Median_l2FC*, *sd_l2FC*, *Mode_l2FC*, *skewness_l2FC*: fraction of positive values, mean, median, standard deviation, mode value and Bickel's measure of skewness of the log2 fold change distribution across all TFBS - *pvalue_raw* and *pvalue_adj*: raw and adjusted (fdr aka BH) p-value of the t-test - *T_statistic*: the value of the T statistic from the t-test - *TFBS_num*: number of TFBS

FILES `{TF}.{comparisonType}.diagnosticPlots.pdf`

Summary Produced in rule `analyzeTF`. Various diagnostic plots for the differential accessibility TFBS analysis for the real data.

Details See the description of the file `{comparisonType}.diagnosticPlots.peaks.pdf` in the `PEAKS` folder, which has an identical structure. Here, the second last page shows two density plots of the log2 fold-changes for the specific pairwise comparson that *diffTF* run for, one for the peak log2 fold-changes (independent of any TF) and one for the TF-specific one (i.e., across all TFBS from the subset of peaks with a TFBS for this TF). The last page shows the same but in a cumulative representation.

FILE `{TF}.{comparisonType}.permutationResults.rds`

Summary Produced in rule `binningTF`. Contains a data frame that stores the results of bin-specific results.

Details No further details provided yet. Please let us know if you need more details.

FILE `{TF}.{comparisonType}.permutationSummary.tsv.gz`

Summary Produced in rule `binningTF`. A final summary table that summarizes the results across bins by calculating weighted means.

Details The data of this table are used for the final visualization.

FILE `{TF}.{comparisonType}.covarianceResults.rds`

Summary Produced in rule `binningTF`. Contains a data frame that stores the results of the pairwise bin covariances and the bin-specific weights.

Details

Note: Covariances are only computed for the real data but not the permuted ones.

8.4 FOLDER `LOGS_AND_BENCHMARKS`

Stores various log and error files.

- `*.log` files from R scripts: Each log file is produced by the corresponding R script and contains debugging information as well as warnings and errors:
  - `checkParameterValidity.R.log`
  - `produceConsensusPeaks.R.log`
  - `diffPeaks.R.log`
  - `analyzeTF.{TF}.R.log` for each TF `{TF}`
  - `summary1.R.log`
  - `binningTF.{TF}.log` for each TF `{TF}`
  - `summaryFinal.R.log`
- `*.log` summary files: Summary logs for user convenience, produced at very end of the pipeline only. They should contain all errors and warnings from the pipeline run.
  - `all.errors.log`
  - `all.warnings.log`

8.5 FOLDER `TEMP`

Stores temporary and intermediate files. Since they are usually not relevant for the user, they are explained only very briefly here.

8.5.1 Sub-folder `SortedBAM`

Stores sorted versions of the original *BAMs* that are optimized for fast count retrieval using *featureCounts*. Only present if data are paired-end.

- `{basenameBAM}.bam` for each input *BAM* file: Produced in rule `resortBAM`. Resorted *BAM* file

8.5.2 Sub-folder `extension{regionExtension}`

Stores results related to the user-specified extension size (`regionExtension`, *regionExtension*)

- `{comparisonType}.allTFBS.peaks.bed.gz`: Produced in rule `intersectPeaksAndTFBS`. *BED* file containing all TFBS from all TF that overlap with the peaks after motif extension
- `conditionComparison.rds`: Produced in rule `DiffPeaks`. Stores the condition comparison as a string. Some steps in *diffTF* need this file as input.
- `{comparisonType}.motifs.coord.permutation{perm}.bed.gz` and `{comparisonType}.motifs.coord.nucContent.permutation{perm}.bed.gz` for each permutation `{perm}`: Produced in rule `calcNucleotideContent`, and needed subsequently for the binning. Temporary and result file of *bedtools nuc*, respectively. The latter contains the GC content for all TFBS.
- `{comparisonType}.checkParameterValidity.done`: temporary flag file

- `{TF}_TFBS.sorted.bed` for each TF `{TF}`: Produced in rule `sortTFBSParallel`. Coordinate-sorted version of the input TFBS. Only "regular" chromosomes starting with "chr" are kept, while sex chromosomes (chrX, chrY), chrM and unassembled contigs such as chrUn are additionally removed.
- `{comparisonType}.allTFBS.peaks.bed.gz`: Produced in rule `intersectPeaksAndTFBS`. *BED* file containing all TFBS from all TF that overlap with the peaks before motif extension

CHAPTER 9

Running *diffTF*

9.1 General remarks

diffTF is programmed as a *Snakemake* pipeline. *Snakemake* is a bioinformatics workflow manager that uses workflows that are described via a human readable, Python based language. It offers many advantages to the user because each step can easily be modified, parts of the pipeline can be rerun, and workflows can be seamlessly scaled to server, cluster, grid and cloud environments, without the need to modify the workflow definition or only minimal modifications. However, with great flexibility comes a price: the learning curve to work with the pipeline might be a bit higher, especially if you have no *Snakemake* experience. For a deeper understanding and troubleshooting errors, some knowledge of *Snakemake* is invaluable.

Simply put, *Snakemake* executes various *rules*. Each *rule* can be thought of as a single *recipe* or task such as sorting a file, running an R script, etc. Each rule has, among other features, a name, an input, an output, and the command that is executed. You can see in the `Snakefile` what these rules are and what they do. During the execution, the rule name is displayed, so you know exactly at which step the pipeline is at the given moment. Different rules are connected through their input and output files, so that the output of one rule becomes the input for a subsequent rule, thereby creating *dependencies*, which ultimately leads to the directed acyclic graph (*DAG*) that describes the whole workflow. You have seen such a graph in Section *Workflow*.

In *diffTF*, a rule is typically executed separately for each TF. One example for a particular rule is sorting the TFBS list for the TF CTCF.

In *diffTF*, the total number of *jobs* or rules to execute can roughly be approximated as 3 * `nTF`, where `nTF` stands for the number of TFs that are included in the analysis. For each TF, three sets of rules are executed:

1. Calculating read counts for each TFBS within the peak regions (rule `intersectTFBSAndBAM`)
2. Differential accessibility analysis (rule `analyzeTF`)
3. Binning step (rule `binningTF`)

In addition, one rule per permuation is executed, so an additional `nPermutations` rules are performed. Lastly, a few other rules are executed that however do not add up much more to the overall rule count.

9.2 Executing *diffTF* - Running times and memory requirements

diffTF can be computationally demanding depending on the sample size and the number of peaks. In the following, we discuss various issues related to time and memory requirements and we provide some general guidelines that worked well for us.

Warning: We generally advise to run *diffTF* in a cluster environment. For small analysis, a local analysis on your machine might work just fine (see the example analysis in the Git repository), but running time increases substantially due to limited amount of available cores.

9.2.1 Analysis size

We now provide a *very rough* classification into small, medium and large with respect to the sample size and the number of peaks:

- Small: Fewer than 10-15 samples, number of peaks not exceeding 50,000-80,000, normal read depth per sample
- Large: Number of samples larger than say 20 or number of peaks clearly exceeds 100,000, or very high read depth per sample
- Medium: Anything between small and large

9.2.2 Memory

Some notes regarding memory:

- Disk space: Make sure you have enough space left on your device. As a guideline, analysis with 8 samples need around 12 GB of disk space, while a large analysis with 84 samples needs around 45 GB. The number of permutations also has an influence on the (temporary) required storage and a high number of permutations (> 500) may substantially increase the memory footprint. Note that most space is occupied in the *TEMP* folder, which can be deleted after an analysis has been run successfully. We note, however, that rerunning (parts of) the analysis will require regenerating files from the TEMP folder, so only delete the folder or files if you are sure that you do not need them anymore.
- Machine memory: Although most steps of the pipeline have a modest memory footprint of less than 4 GB or so, depending on the analysis size, some may need 10+ GB of RAM during execution. We therefore recommend having at least 10 GB available for large analysis (see above).

9.2.3 Number of cores

Some notes regarding the number of available cores:

- *diffTF* can be invoked in a highly parallelized manner, so the more CPUs are available, the better.
- you can use the `--cores` option when invoking *Snakemake* to specify the number of cores that are available for the analysis. If you specify 4 cores, for example, up to 4 rules can be run in parallel (if each of them occupies only 1 core), or 1 rule can use up to 4 cores.
- we strongly recommend running *diffTF* in a cluster environment due to the massive parallelization. With *Snakemake*, it is easy to run *diffTF* in a cluster setting. Simply do the following:
  - write a cluster configuration file that specifies which resources each rule needs. For guidance and user convenience, we provide different cluster configuration files for a small and large analysis. See the folder

src/clusterConfigurationTemplates for examples. Note that these are rough estimates only. See the *Snakemake* documentation for details for how to use cluster configuration files.

– invoke *Snakemake* with one of the available cluster modes, which will depend on your cluster system. We used `--cluster` and tested the pipeline extensively with *LSF/BSUB* and *SLURM*. For more details, see the *Snakemake* documentation

9.2.4 Total running time

Some notes regarding the total running time:

- the total running time is very difficult to estimate beforehand and depends on many parameters, most importantly the number of samples, their read depth, the number of peaks, and the number of TF included in the analysis.
- for small analysis such as the example analysis in the Git repository, running times are roughly 30 minutes with 2 cores for 50 TF and a few hours with all 640 TF.
- for large analysis, running time will be up to a day or so when executed on a cluster machine

9.3 Running *diffTF* in a cluster environment

If *diffTF* should be run in a cluster environment, the changes are minimal due to the flexibility of *Snakemake*. You only need to change the following:

- create a cluster configuration file in JSON format. See the files in the `clusterConfigurationTemplates` folder for examples. In a nutshell, this file specifies the computational requirements and job details for each job that is run via *Snakemake*.
- invoke *Snakemake* with a cluster parameter. As an example, you may use the following for a *SLURM* cluster:

```
snakemake -s path/to/Snakefile \
--configfile path/to/configfile --latency-wait 30 \
--notemp --rerun-incomplete --reason --keep-going \
--cores 16 --local-cores 1 --jobs 400 \
--cluster-config path/to/clusterconfigfile \
--cluster " sbatch -p {cluster.queue} -J {cluster.name} \
    --cpus-per-task {cluster.nCPUs} \
   --mem {cluster.memory} --time {cluster.maxTime} -o \"{cluster.output}\" \
   -e \"{cluster.error}\"  --mail-type=None --parsable "
```

- the corresponding cluster configuration file might look like this:

```
{
  "__default__": {
    "queue": "htc",
    "nCPUs": "{threads}",
    "memory": 2000,
    "maxTime": "1:00:00",
    "name": "{rule}.{wildcards}",
    "output": "{rule}.{wildcards}.out",
    "error": "{rule}.{wildcards}.err"
  },
  "resortBAM": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
```

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```
  "intersectPeaksAndPWM": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "intersectPeaksAndBAM": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "intersectTFBSAndBAM": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "DiffPeaks": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "analyzeTF": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "binningTF": {
    "memory": 5000,
    "maxTime": "1:00:00"
  },
  "summaryFinal": {
    "memory": 5000,
    "maxTime": "0:30:00"
  },
  "cleanUpLogFiles": {
    "memory": 1000,
    "maxTime": "0:30:00"
  }
}
```

A few motes might help you to get started:

- **each** name in the `--cluster` argument string from the command line (here: `queue`, `name` `nCPUs`, `memory`, `maxTime`, `output`, and `error`) must appear also in the __`default`__ section of the referenced cluster configuration file (via `--cluster-config`)
- for brevity here, only rules with requirements different from the specified default have been included here in the online version, while the templates in the repository contain all rules, even if they have the same requirements as the default. The latter makes it easier for practical purposes to change requirements later on
- the `--cluster` argument is the only part that has to be adjusted for your cluster system. It is quite simple really, you essentially just link the content of the configuration file to the cluster system you want to submit the jobs to. More specifically, you refer to the cluster configuration file via the `cluster.` string, followed by the name of the parameter in the cluster configuration. For parameters that refer to filenames, an extra escaped quotation mark `\"` has been added so that the command also works in case of spaces in filenames (which should *always* be avoided at all costs)
- the cluster configuration file has multiple sections defined that correspond to the names of the rules as defined in the Snakefile, plus the special section __`default`__ at the very top, the latter of which specifies the default cluster options that apply to all rules unless overwritten via its own rule-specific section
- **each** name (e.g., here: `queue`, `name` `nCPUs`, ``` ` ```memory, `maxTime`, `output`, and `error`) **must be defined** in the __`default`__ section of the cluster configuration file

- note that in this example, we provided some extra parameters for convenience such as `name` (so the cluster job will have a reasonable name and can be recognized) that are not strictly necessary
- the `{threads}` syntax of the `nCPUs` name can be generally used and is a placeholder for the specified number of threads for the particular rule, as specified in the corresponding `Snakefile`
- in our example, memory is given in Megabytes, so 5000 refers to roughly 5 GB. Queue names are either `htc` or `1day`. Adjust this accordingly to your cluster system.
- for more details, see the Snakemake documentation
- **Note:** From a practical point of view, just try to mimic the parameters that you usually use for your cluster system, and modify the cluster configuration file accordingly. For example, if you need an additional argument such as `-A` (which stands for the *group* you are in for a SLURM-based system), simply add `-A {cluster.group}` to the command line call and add a `group` parameter to the `__default__` section (see also the note below).

CHAPTER 10

Frequently asked questions (FAQs)

Here a few typical use cases, which we will extend regularly in the future if the need arises:

1. I received an error, and the pipeline did not finish.

   As explained in Section *Handling errors*, you first have to identify and fix the error. Rerunning then becomes trivially easy: just restart *Snakemake*, it will start off where it left off: at the step that produced that error.

2. I received an error, and I do not see any error message.

   First, check the cluster output and error files if you run *diffTF* in cluster mode. They mostly contain an actual error message or at least the print the exact command that resulted in an error. If you executed locally or still cannot find the error message, see below for guidelines.

3. I want to rerun a specific part of the pipeline only.

   This common scenario is also easy to solve: Just invoke *Snakemake* with `--forcerun {rulename}`, where `{rulename}` is the name of the rule as defined in the Snakefile. *Snakemake* will then rerun the specified run and all parts downstream of the rule. If you want to avoid rerunning downstream parts (think carefully about it, as there might be changes from the rerunning that might have consequences for downstream parts also), you can combine `--forcerun` with `--until` and specify the same rule name for both.

4. I want to modify the workflow.

   Simply add or modify rules to the Snakefile, it is as easy as that.

5. *diffTF* finished successfully, but nothing is significant.

   This can and will happen, depending on the analysis. The following list provides some potential reasons for this:

   - The two conditions are in fact very similar and there is no signal that surpasses the significance threshold. You could, for example, check in a PCA plot based on the peaks that are used as input for *diffTF* whether they show a clear signal and separation.

- There is a confounding factor (like age) that dilutes the signal. One solution is to add the confounding variable into the design model, see above fo details. Again, check in a PCA plot whether samples cluster also according to another variable.
- You have a small number of samples or one of the groups contains a small number of samples. In both cases, if you run the permutation-based approach, the number of permutations is small, and there might not be enough permutations to achieve significance. For example, if you run an analysis with only 10 permutations, you cannot surpass the 0.05 significance threshold. As a solution, you may switch to the analytical version. Be aware that this requires to rerun large parts of the pipeline from the *diffPeaks* step onwards.
- You have a very small number of peaks and therefore also a small number of TF binding sites within the peaks, resulting in many TFs to be skipped in the analysis due to an insufficient number of binding sites. As a solution, try increasing the number of peaks or verify that the predicted binding sites are not too stringent (if done independently, therefore not using our TFBS collection that was produced with *PWMScan* and *HOCOMOCO*). We recommend having at least a few thousand peaks, but this can hardly be generalized and depends too much on the biology, the size of the peaks etc.
- You run the (usually more stringent) permutation-based approach. If the number of permutations is too low, p-values may not be able to reach significance. For more details, see *nPermutations*. You may want to rerun the analysis using the analytical approach or using more permutations (if the number of samples makes this possible at all); however, the problems raised above may still apply.

6. *diffTF* finished successfully, but almost everything is significant.

   This can also happen and is usually a good sign. The following list provides some potential reasons for this:

   - If you run the analytical mode, consider running the permutation-based approach in addition. The permutation-based approach tends to be more stringent and usually results in fewer TFs being significant. However, as explained in the paper and here, it can only be used if the number of samples is sufficiently high.
   - If too many TFs are significant, you have multiple choices that can of course also be combined: First, you may use a more stringent adjusted p-value threshold. Keep in mind that the Volcano plot PDF shows only a few selected thresholds, and you can always be even more stringent when working with the final result table that is also written to the `FINAL_OUTPUT` folder. Second, you may further filter them by additional criteria such as the number of binding sites (e.g., filtering TFs with a very small number of binding sites, a TF activity that is not large enough, or by their predicted mode of action if you used the classification mode). Third, you may further subdivide them into families and subsequently focus, for example, only one particular TF family. Alternatively, you can classify the TFs into "known" and "novel" for the particular comparison.

7. I want to change the value of a parameter.

   If you want to do this, please contact us, and we help and then update the FAQ here.

If you feel that a particular use case is missing, let us know and we will add it here!

CHAPTER 11

Handling errors

11.1 Error types

Errors occur during the *Snakemake* run can principally be divided into:

- Temporary errors (often when running in a cluster setting)
  - might occur due to temporary problems such as bad nodes, file system issues or latencies
  - rerunning usually fixes the problem already. Consider using the option `--restart-times` in *Snakemake*.
- Permanent errors
  - indicates a real error related to the specific command that is executed
  - rerunning does not fix the problem as they are systematic (such as a missing tool, a library problem in R)

From our experience, most errors occur due to the following issues:

- Software-related problems such as R library issues, non-working conda installation etc. Consider using the Singularity-enhanced version of *diffTF* (version 1.2 and above) that immediately solves these issues.
- issues arising from the data itself. Here, it is more difficult to find the cause. We tried to cover all cases for which *diffTF* may fail, so please post an issue on our Bitbucket Issue Tracker if you believe you found a new problem.

11.2 Identify the cause

To troubleshoot errors, you have to first locate the exact error. Depending on how you run *Snakemake* (i.e., in a cluster setting or not), check the following places:

- in locale mode: the *Snakemake* output appears on the console. Check the output before the line "Error in rule", and try to identify what went wrong. Errors from R script should in addition be written to the corresponding R log files in the in the `LOGS_AND_BENCHMARKS` directory. Sometimes, no error message might be displayed, and the output may look like this:

```
Error in rule intersectTFBSAndBAM:
        jobid: 1287
        output: output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TF-SPECIFIC/HXA10/
↪extension100/FL-WTvsFL-EKO.all.HXA10.allBAMs.overlaps.bed, output-FL-WT-vs-EKO-
↪ATAC-distal-Linj-activ/TF-SPECIFIC/HXA10/extension100/FL-WTvsFL-EKO.all.HXA10.
↪allBAMs.overlaps.bed.gz, output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TEMP/
↪extension100/FL-WTvsFL-EKO.all.HXA10.allTFBS.peaks.extension.saf
RuleException:
CalledProcessError in line 493 of /mnt/data/bioinfo_tools_and_refs/bioinfo_tools/
↪diffTF/src/Snakefile:
Command ' set -euo pipefail;   ulimit -n 4096 &&
            zgrep "HXA10_TFBS\." output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TEMP/
↪extension100/FL-WTvsFL-EKO.all.allTFBS.peaks.extension.bed.gz | awk 'BEGIN {␣
↪OFS = "\t" } {print $4"_"$2"-"$3,$1,$2,$3,$6}' | sort -u -k1,1  >output-FL-WT-
↪vs-EKO-ATAC-distal-Linj-activ/TEMP/extension100/FL-WTvsFL-EKO.all.HXA10.allTFBS.
↪peaks.extension.saf &&
            featureCounts             -F SAF            -T 4             -Q 10  ␣
↪                        -a output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TEMP/
↪extension100/FL-WTvsFL-EKO.all.HXA10.allTFBS.peaks.extension.saf            -s␣
↪0            -O             -o output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TF-
↪SPECIFIC/HXA10/extension100/FL-WTvsFL-EKO.all.HXA10.allBAMs.overlaps.bed       ␣
↪      /mnt/data/common/tobias/diffTF/ATAC-bam-files/FL-WT-ProB-1.bam /mnt/data/
↪common/tobias/diffTF/ATAC-bam-files/FL-WT-ProB-2.bam /mnt/data/common/tobias/
↪diffTF/ATAC-bam-files/FL-WT-ProB-3.bam /mnt/data/common/tobias/diffTF/ATAC-bam-
↪files/FL-Ebf1-KO-ProB-1.bam /mnt/data/common/tobias/diffTF/ATAC-bam-files/FL-
↪Ebf1-KO-ProB-2.bam /mnt/data/common/tobias/diffTF/ATAC-bam-files/FL-Ebf1-KO-
↪ProB-3.bam &&
            gzip -f < output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TF-SPECIFIC/
↪HXA10/extension100/FL-WTvsFL-EKO.all.HXA10.allBAMs.overlaps.bed > output-FL-WT-
↪vs-EKO-ATAC-distal-Linj-activ/TF-SPECIFIC/HXA10/extension100/FL-WTvsFL-EKO.all.
↪HXA10.allBAMs.overlaps.bed.gz ' returned non-zero exit status 1.
  File "/mnt/data/bioinfo_tools_and_refs/bioinfo_tools/diffTF/src/Snakefile",␣
↪line 493, in __rule_intersectTFBSAndBAM
  File "/opt/anaconda3/lib/python3.6/concurrent/futures/thread.py", line 56, in␣
↪run
Removing output files of failed job intersectTFBSAndBAM since they might be␣
↪corrupted:
output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TEMP/extension100/FL-WTvsFL-EKO.all.
↪HXA10.allTFBS.peaks.extension.saf
Removing temporary output file output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TF-
↪SPECIFIC/FLI1/extension100/FL-WTvsFL-EKO.all.FLI1.allBAMs.overlaps.bed.
Removing temporary output file output-FL-WT-vs-EKO-ATAC-distal-Linj-activ/TEMP/
↪extension100/FL-WTvsFL-EKO.all.FLI1.allTFBS.peaks.extension.saf.
```

Finding the exact error can be troublesome, and we recommend the following:

  – execute the exact command as pasted above in a stepwise fashion. The command above consists of several commands that are chained together with *&&*, so copy and paste the individual parts, starting with the first part, execute it locally, and see if you receive any error message.

  – once you have an error message, you can start troubleshooting it. The first step is always to actually see and understand the error.

- in cluster mode: either error, output or log file of the corresponding rule that threw the error in the `LOGS_AND_BENCHMARKS` directory. If you are unsure in which file to look, identify the rule name that caused the error and search for files that contain the rule name in it.

In both cases, you can check the log file that is located in `.snakemake/log`. Identify the latest log file (check the date), and then either open the file or use something along the lines of:

```
grep -C 5 "Error in rule" .snakemake/log/2018-07-25T095519.371892.snakemake.log
```

This is particularly helpful if the *Snakemake* output is long and you have troubles identifying the exact step in which an error occurred.

11.3 Common errors

We here provide a list of some of the errors that can happen and that users reported to us. This list will be extended whenever a new problem has been reported.

1. R related problems

   Many errors are R related. R and *Bioconductor* use a quite complex system of libraries and dependencies, and you may receive errors that are related to R, *Bioconductor*, or specific libraries.

   ```
   *** caught segfault ***
   ...
   Segmentation fault
   ...
   ```

   Note: This particular message may also be related to an incompatibility of the *DiffBind* and *DESeq2* libraries. See the *Change log* for details, as this has been addressed in version 1.1.5.

   More generally, however, such messages point to a problem with your R and R libraries installation and have per se nothing to do with *diffTF*. In such cases, we advise to reinstall the latest version of *Bioconductor* and ask someone who is experienced with this to help you. Unfortunately, this issue is so general that we cannot provide any specific solutions. To troubleshoot and identify exactly which library or function causes this, you may run the R script that failed in debug mode and go through it line by line. See the next section for more details.

   Note: We strongly recommend running the *Singularity* version of *diffTF* (version 1.2 and above) that immediately solves these issues. See the *Change log* for more details and the section *Try it out now!*

2. Singularity-related errors

   Although *Singularity* errors are rare (up until now), it might happen that you receive an error that is related to it. Up until now, these were either of temporary nature (so trying again a while after fixes it) or related to the system you are running *Singularity* on (e.g., a misconfiguration of some sort), among others.

   For example, in July 2019, SingularityHub was down for a few days due to a single user misusing the service, which had to be shutdown because of that. When trying to download the *diffTF* Singularity images in that time period, the error message was:

   ```
   FATAL: Failed to get manifest from Shub: No response received from␣
   ↪singularity hub
   ```

   Another common error is related to not including paths for the `bind` option, resulting in "Directory not found" errors, see *Adaptations and notes when running with Singularity* for details!

   If you do not know what the error is, post an Issue in the Bitbucket Issue Tracker tracker and we are hopefully able to help you quickly.

3. Data-specific errors

Errors can also be due to incompatible data. For example, if a BAM file contains both single-end and paired-end reads (which is unusual, lots of programs may exit with errors for such data) and in the configuration file the parameter *pairedEnd* is set to true, the *repair* step from *Subread* will fail with an error message. In such a case, the single-end reads should either be removed from the BAM file (this is the preferred option, unless the majority of reads are single-end) or *pairedEnd* is set to false, the latter of which then treats all reads to be single-end (with the consequence that then, not fragments are counted, but just individual reads, which may result in different results due to altered number of counts).

11.4 Fixing the error

11.4.1 General guidelines

After locating the error, fix it accordingly. We here provide some guidelines of different error types that may help you fixing the errors you receive:

- Errors related to erroneous input: These errors are easy to fix, and the error message should be indicative. If not, please let us know, and we improve the error message in the pipeline.
- Errors of technical nature: Errors related to memory, missing programs, R libraries etc can be fixed easily by making sure the necessary tools are installed and by executing the pipeline in an environment that provides the required technical requirements. For example, if you receive a memory-related error, try to increase the available memory. In a cluster setting, adjust the cluster configuration file accordingly by either increasing the default memory or (preferably) or by overriding the default values for the specific rule.
- Errors related to *Snakemake*: In rare cases, the error can be due to *Snakemake* (corrupt metadata, missing files, etc). If you suspect this to be the case, you may delete the hidden `.snakemake` directory in the folder from which you started the analysis. *Snakemake* will regenerate it the next time you invoke it then.
- Errors related to the input data: Error messages that indicate the problem might be located in the data are more difficult to fix, and we cannot provide guidelines here. Feel free to contact us.

11.4.2 Debugging R scripts to identify the cause of an error

If an R script fails with a technical error such as `caught segfault` (a segmentation fault), you may want to identify the library or function call that causes the message in order to figure out which library to reinstall. To do so, open the R script that fails in *RStudio*, and execute the script line by line until you identify the line that causes the issue. Importantly, read the instructions in the section at the beginning of the script that is called `SAVE SNAKEMAKE S4 OBJECT THAT IS PASSED ALONG FOR DEBUGGING PURPOSES`. Briefly, you simply have to make the *snakemake* object available in your R workspace, which contains all necessary information to execute the R script properly. Normally, *Snakemake* automatically loads that when executing a script. To do so, simply execute the line that is pasted there in R, it is something like this:

```
snakemake = readRDS("{outputFolder}/LOGS_AND_BENCHMARKS/checkParameters.R.rds")
```

Replace `{outputFolder}` by the folder you used for the analysis, and adjust the `checkParameters` part also accordingly. Essentially, you just have to provide the path to the corresponding file that is located in the `LOGS_AND_BENCHMARKS` subdirectly within the specified output directory.

11.4.3 Rerunning *Snakemake*

After fixing the error, rerun *Snakemake*. *Snakemake* will continue at the point at which the error message occurred, without rerunning already successfully computed previous steps (unless specified otherwise).

CHAPTER 12

Understanding and interpreting results

Having results is exciting; however, as with most software, now the maybe even harder part starts: Understanding and interpreting the results. Let's first remind ourselves: The main goal of *diffTF* is to aid in formulating testable hypotheses and ultimately improve the understanding of regulatory mechanisms that are driving the differences on a system-wide scale.

12.1 General notes

- Irrespective of whether or not you also used the classification mode, we recommend that the first thing to check is the Volcano plot PDF.
- If a specific question is not addressed here, feel free to contact us. We ill then add it here.
- Note that *diffTF* captures differential accessibility, which does not necessarily imply a functional difference. See the publication for more discussion and details.
- the significance as calculated by the empirical or analytical approach should not be over-interpreted from our point of view. We find the TF activity to be the more important measure.

12.2 Specifics for the basic mode

The following procedure may be useful as a rough guideline:

- Start with the most stringent adjusted p-value threshold (0.01)
- Categorize into one of the 3 following cases: - (a) There are no or almost none TFs significant: You may simply use a less stringent adjusted p-value threshold. If the least stringent adjusted p-value threshold (0.2) does also not have any or only very few significant TFs, see the *Frequently asked questions (FAQs)* for possible explanations. In such a (rare) case, it might be worthwhile then to check the raw p-values instead of the adjusted ones. - (b) A few TFs are significant: You hit the sweet spot! Try to characterize and understand the TFs and whether they make biological sense for you. See also the notes for (c) below.

- (c) A lot or the majority of TFs are significant (say more than 50 to 100): See the *Frequently asked questions (FAQs)* for possible explanations and how to best proceed.

12.3 Specifics for the classification mode

- be aware of the limitations, see below

12.3.1 Limitations

As written in the publication, we note that *diffTF* is prone to mis-classifying TFs that (1) act bifunctionally as activators and repressors in different genomic contexts or along with different co-factors, (2) are heavily regulated post-translationally, or (3) show little variation in RNA expression across the samples. Some of these mis-classifications may represent interesting subjects for future investigations. Furthermore, if two TFs have similar motifs, which makes it difficult to distinguish them, *diffTF* may have difficulties in classifying them correctly. Thus, for distinguishing the functional roles of TFs from the same motif-family, further biochemical experiments are needed.

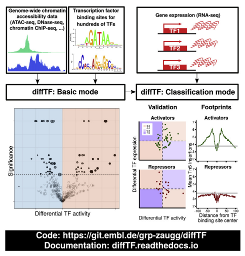
CHAPTER 13

Biological motivation

Transcription factors (TFs) regulate many cellular processes and can therefore serve as readouts of the signaling and regulatory state. Yet for many TFs, the mode of action—repressing or activating transcription of target genes—is unclear. Here, we present diffTF (https://git.embl.de/grp-zaugg/diffTF) to calculate differential TF activity (basic mode) and classify TFs into putative transcriptional activators or repressors (classification mode). In basic mode, it combines genome-wide chromatin accessibility/activity with putative TF binding sites that, in classification mode, are integrated with RNA-seq. We apply diffTF to compare (1) mutated and unmutated chronic lymphocytic leukemia patients and (2) two hematopoietic progenitor cell types. In both datasets, diffTF recovers most known biology and finds many previously unreported TFs. It classifies almost 40% of TFs based on their mode of action, which we validate experimentally. Overall, we demonstrate that diffTF recovers known biology, identifies less well-characterized TFs, and classifies TFs into transcriptional activators or repressors.

For a graphical summary of the idea, see the section *Workflow*

This is the graphical abstract:


Genome-wide chromatin accessibility data (ATAC-seq, DNase-seq, chromatin ChIP-seq, ...)
Transcription factor binding sites for hundreds of TFs
Gene expression (RNA-seq)
TF1
TF2
TF3
diffTF: Basic mode
diffTF: Classification mode
Significance
Differential TF activity
Validation
Footprints
Activators
Repressors
Differential TF expression
Differential TF activity
Mean Tn5 insertions
Distance from TF binding site center
Code: https://git.embl.de/grp-zaugg/diffTF
Documentation: diffTF.readthedocs.io

The paper is open access and available online, please see the section *Citation* for details.

CHAPTER 14

Example dataset

We provide a the toy dataset that is included in the Git repository to test diffTF. It is a small ATAC-Seq/RNA-Seq dataset comparing two cell types along the hematopoietic differentiation trajectory in mouse (multipotent progenitors - MPP - versus granulocyte-macrophage progenitors - GMP) and comes from Rasmussen et al. 2018. Generally, hematopoiesis is organized in a hierarchical manner, and the following Figure shows the hematopoietic hierarchy in more detail and also places GMP and MPP cells:

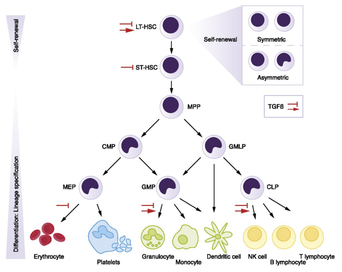


Fig. 1: U.Blank et. al., Blood 2015 (http://www.bloodjournal.org/content/bloodjournal/125/23/3542/F1.large.jpg)

The data consists of ATAC-Seq data of 4 replicates for each of the two cell types (4 GMP vs. 4 MPP), and is limited to chr1 only to reduce running times and complexity. RNA-seq data are also available, which allows using the TF classification within the diffTF framework. As mentioned in the paper, the small number of samples makes the correlation-based classification of the TFs into activators and repressors unreliable, but we nevertheless include it here for the small dataset to show how to principally enable our AR classification in diffTF.

In the example analysis, you can investigate the differential TF activity of a set of 50 (or even all of the 400+) TFs to identify the known drivers of the well-studied mouse hematopoietic differentiation system. Overall, we expect to see TFs that more specific for stem cells renewal being more active in the MPPs, while in GMPs, TFs responsible for the further myeloid cell differentiation (CEBP family, NFIL3) should be enriched.

CHAPTER 15

Help, contribute and contact

If you have questions or comments, feel free to contact us. We will be happy to answer any questions related to this project as well as questions related to the software implementation. For method-related questions, contact Judith B. Zaugg (judith.zaugg@embl.de) or Ivan Berest (berest@embl.de). For technical questions, contact Christian Arnold (christian.arnold@embl.de).

If you have questions, doubts, ideas or problems, please use the Bitbucket Issue Tracker. We will respond in a timely manner.

CHAPTER 16

Citation

If you use this software, please cite the following reference:

Ivan Berest*, Christian Arnold*, Armando Reyes-Palomares, Giovanni Palla, Kasper Dindler Rasmussen, Holly Giles, Peter-Martin Bruch, Wolfgang Huber, Sascha Dietrich, Kristian Helin, Judith B. Zaugg. *Quantification of Differential Transcription Factor Activity and Multiomics-Based Classification into Activators and Repressors: diffTF*. 2019. Cell Reports 29(10), P3147-3159.E12.

Open Access. DOI: https://doi.org/10.1016/j.celrep.2019.10.106

CHAPTER 17

Change log

Version 1.6 (2020-01-22)

- The documentation received a major update, in particular the section output files. In addition, a few new methodological figures have been added as well as an interpretation section.

Version 1.5 (2019-12-03)

- *diffTF* has been published in Cell Reports! See the section *Citation* for details.
- raw and adjusted p-values for the permutation-based approach can now not be 0 anymore. We now use the approach described here. In a nutshell, the smallest p-value is now 1/(*nPermutations* + 1), with *nPermutations* denoting the number of permutations and thereby depends on the number of permutations - having more permutations makes the minimum p-value smaller.
- for plotting TF densities, a fixed bandwidth of 0.1 was used before. We now removed this and bandwidth is determined automatically. We noticed that in some cases, the fixed bandwidth may lead to a smoothing of the density curves while without fixing it, the densities look more rugged. As we do not want to introduce visual artifacts, we decided to remove it.
- various minor code improvements, particularly related to the AR classification
- small fixes in the Snakefile

Version 1.4 (2019-09-24)

- Various small improvements for increasing user experience
  - For the peaks and TF-specific diagnostic plots, the pairwise mean-average plots between pairs of samples has been disabled by default. It was set to a maximum of 5 previously, but due to its time consuming nature and limited usage, we feel this is not needed for most users. We might add a parameter in the future to adjust this more flexibly, contact us if you want to have them back.
  - various small improvements for the various diagnostic plots from the last step (*summaryFinal*), most of which concern classification-related plots and plots for the permutation-based approach
- For all output tables, *diffTF* now outputs only 2-3 significant digits, which reduces the size of some output tables significantly. The memory footprint is thus overall decreased. More digits are not needed in our opinion.

- added diagnostic plots for the RNA-Seq data that is used for the classification. These include MA plots (based on original and shrunken log2 fold changes, (non)normalized log count density plots across all samples, and a mean-sd plot),
- added preliminary support for interaction terms in the design formula. This was not possible before but should now work without *diffTF* failing. if you continue having issues, please let us know.
- the Singularity image for R has been updated. If you stored a previous version of it on disk, please delete it so it is downloaded anew the next time you run *diffTF*. The image also updates R to version 3.6.1 in the container and corresponding package versions.

Version 1.3.3 (2019-07-25)

- Fixed a bug that caused erroneously small p-values for the permutation-based approach in cases when the number of permutations that was actually done was smaller than what was specified in the configuration file (e.g., if 1000 permutations have been specified in the configuration file, but only 70 were actually done such as for a typical 4 vs 4 analysis). This is now corrected, and the last step of the pipeline (*summaryFinal*) should be rerun to correct for it. Although this happened in the special circumstances as described above (small number of samples and yet using the permutation-based approach), we apologize for this oversight. Thanks to Frauke Huth for noticing!

Version 1.3.2 (2019-07-24)

- Fixed the error "unable to find an inherited method for function 'assay' for signature '"matrix", "character"'" that arises due to a new implementation of the *normOffsets* function from the *csaw* package in versions above 1.14.1. The Singularity image that comes with *diffTF* still uses csaw version 1.14.1, for which the original implementation works fine, but for newer R installations (that is, *csaw* >= 1.16) the above error is thrown. In the code, the function call is now dependent on the version of the *csaw* package. This was the most common error that was thrown when running *diffTF* without Singularity, and should therefore increase compatibility.
- Minor changes to make the *diffTF* code more compatible for future releases of R (e.g., replacing the deprecated *data_frame* by *tibble*, specifying the package for functions that are defined in different packages)
- updated the *startAnalysis* scripts due to a changed folder structure in the *examples* subfolder

Version 1.3 and 1.3.1 (2019-07-17)

- Various minor changes and small bug fixes as reported by users
- improved the RNA-Seq classification, further information will follow soon.
- updated Documentation for errors that users reported and why they occur
- improved error messages in the pipeline for various cases

Version 1.2.5 (2019-03-13)

- Updated the TFBS_hg38_FIMO_HOCOMOCOv11 archive one more time to exclude non-assembled contigs such as HLA*. To make the pipeline more stable for such edge cases, the parameter `dir_TFBS_sorted` has been removed, and sorting and filtering of chromosomes is now always performed. Only chromosomes are kept in both the consensus peak files and the TFBS bed files that start with `chr` and are neither sex chromosomes (`chrX` or `chrY`) nor `chrM`. If you want to keep sex chromosomes in your analysis (although we think this is not recommended), simply edit the Snakefile and remove the "chrX" and "chrY" occurences in the two filtering rules.

Version 1.2.4 (2019-03-04)

- Fixed an issue with `checkParameterValidity.R` that caused an error message when loading TFBS files with a numeric score. Thanks to Scott Berry for pointing it out.
- Updated the TFBS_hg38_FIMO_HOCOMOCOv11 archive. The bed files are now properly pre-sorted

Version 1.2.3 (2019-02-27)

- Added a pre-compiled list of 768 human TF with in-silico predicted TFBS based on the *HOCOMOCO 11* database and *PWMScan* for hg38 as well as updating the other pre-compiled lists to account for recent changes and retractions in the *HOCOMOCO* database. See section *dir_TFBS* for details.
- added an additional filtering in the binning step for a rare corner case due to changes in the number of samples during an analysis

Version 1.2.2 (2019-02-01)

- Minor code fixed. Removed the creation of the circular plot, which has been replaced with the Volcano plot over time. Fixed a bug that could have led to wrong log2 fold-change values for the RNA-Seq data under special circumstances. We recommend rerunning the `summaryFinal` rule. Ask us for more details if you are concerned about this.

Version 1.2.1 (2019-01-22)

- Increased the value for `expressions` in R from 5000 (the R default) to 10000. Some users reported that they receive a "Error: evaluation nested too deeply: infinite recursion / options(expressions=)?" error message. Thanks to Benedict Man Hung Choi!

Version 1.2 (2018-12-10)

- The Snakemake / *diffTF* pipeline can now be combined with **Singularity**. Singularity is similar to Docker and provides a containerization approach. This has significant implications for users: Except for Snakemake and Singularity, no other tool, R or R package has to be installed prior to using *diffTF* anymore, which makes installing *diffTF* much easier and completely independent of the underlying operating system. We now provide two Singularity containers with all necessary tools and packages that are automatically integrated into the workflow. See the section *Adaptations and notes when running with Singularity* and *Try it out now!* for more details. **Please note that for this to work reliably, Snakemake must be updated to at least version 5.3.1**.
- added two validity checks in the `checkParameterValidity.R` script for the TFBS files. Start and end coordinates are now asserted whether they are non-negative.

Version 1.1.8 (2018-11-07)

- changed the call to the `mlv` function from the `modeest` package due to a breaking implementation change in version 2.3.2 that was published end of October 2018. *diffTF* now checks the package version for `modeest` and calls the functions in dependence of the specific version.

Version 1.1.7 (2018-10-25)

- the default value of the minimum number of data points for a CG bin to be included has been raised from 5 to 20 to make the variance calculation more reliable
- various small updates to the `summaryFinal.R` script

Version 1.1.6 (2018-10-11)

- fixed small issue in `checkParameterValidity.R` when not having sufficient permissions for the folder in which the fasta file is located
- updated the `summaryFinal.R` script. Now, for the Volcano plot PDF, in addition to adj. p-values, also the raw p-values are plotted in the end. This might be helpful for datasets with small signal when no adj. p-value is significant. In addition, labeling of TFs is now skipped when the number of TFs to label exceeds 150. THis makes the step faster and the PDF smaller and less crowded.
- small updates to the translation table for mm10
- adding two local rules to the Snakefile for potential minor speed improvements when running in cluster mode

Version 1.1.5 (2018-08-14)

- optimized `checkParameterValidity.R` script, only TFBS files for TFs included in the analysis are now checked
- addressed an R library compatibility issue independent of *diffTF* that users reported. In some cases, for particular versions of R and Bioconductor, R exited with a *segfault* (memory not mapped) error in the `checkParameterValidity.R` that seems to be caused by the combination of *DiffBind* and *DESeq2*. Specifically, when *DiffBind* is loaded *before DESeq2*, R crashes with a segmentation fault upon exiting, whereas loading *DiffBind after DESeq2* causes no issue. If there are further issues, please let us know. Thanks to Gyan Prakash Mishra, who first reported this.
- fixed an issue when the number of peaks is very small so that some TFs have no overlapping TFBS at all in the peak regions. This caused the rule `intersectTFBSAndBAM` to exit with an error due to grep's policy of returning exit code 1 if no matches are returned (thanks to Jonas Ungerbeck, again).
- removed the `--timestamp` option in the helper script `startAnalysis.sh` because this option has been removed for Snakemake >5.2.1
- Documentation updates

Version 1.1.4 (2018-08-09)

- minor, updated the `checkParameterValidity.R` script and the documentation (one package was not mentioned)

Version 1.1.3 (2018-08-06)

- minor, fixed a small issue in the Volcano plot (legends wrong and background color in the plot was not colored properly)

Version 1.1.2 (2018-08-03)

- fixed a bug that made the `3.analyzeTF.R` script fail in case when the number of permutations has been changed throughout the analysis or when the value is higher than the actual maximum number (thanks to Jonas Ungerbeck)

Version 1.1.1 (2018-08-01)

- Documentation updates (referenced the bioRxiv paper, extended the section about errors)
- updated the information on how to load the snakemake object into the R workspace in the corresponding R scripts
- fixed a bug that made the labels in the Volcano plot switch sides (thanks to Jonas Ungerbeck)
- merged some diagnostic plots for the AR classification in the last step
- renamed R scripts and R log files to make them consistent with the cluster output and error files

Version 1.1 (2018-07-27)

- added a new parameter `dir_TFBS_sorted` in the config file to specify that the TFBS input files are already sorted, which saves some computation time by not resorting them
- updated the TFBS files that are available via download (some files were not presorted correctly)
- added support for single-end BAM files. There is a new parameter `pairedEnd` in the config file that specifies whether reads are paired-end or not.
- restructured some of the permutation-related output files to save space and computation time. The rule `concatenateMotifsPerm` should now be much faster, and the TF-specific `...outputPerm.tsv.gz` files are now much smaller due to an improved column structure

Version 1.0.1 (2018-07-25)

- fixed a bug in `2.DiffPeaks.R` that sometimes caused the step to fail, thanks to Jonas Ungerbeck for letting us know
- fixed a bug in `3.analyzeTF` for rare corner cases when *DESeq* fails

Version 1.0 (2018-07-01)

- released stable version

CHAPTER 18

License

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