

HTSanalyzeR2: An R package for GSEA and network analysis of high-throughput data

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Abstract

This package provides gene set over-representation, enrichment analysis and enriched sub-network analyses for various preprocessed high-throughput data as well as time series data generated by either CRISPR, RNA-seq, micro-array or RNAi in a unified workflow. More importantly, it could generate an interactive shiny report encompassing all the results and visualizations, facilitating the users maximally for downloading, modifying the visualization parts with personal preference and sharing with others by publishing the report to [Shinyapps.io](https://shinyapps.io).

Package

HTSanalyzeR2 0.99.13

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1 An overview of HTSanalyzeR2

While high-throughput experiments are no longer bottlenecks for biologists to dissect the functional mechanisms genome-widely, how to efficiently interpret different kinds of data and visualize them remains a challenge. There is also no software so far claimed to be able to perform functional annotation for time series data with interactive visualization. Here, we have implemented a versatile R package, **HTSanalyzeR2**, which has several advantages as below:

- **HTSanalyzeR2** can perform gene set over-representation, enrichment and network analyses for pre-processed data generated by various popular high-throughput experiments including RNA-seq, CRISPR, micro-array and RNAi in a unified workflow.
- For time series data or a similar experiment coming from different groups, **HTSanalyzeR2** can perform time series analysis and comparative analysis for mutual comparison.
- **HTSanalyzeR2** could generate an interactive report for users downloading, modifying the visualizations as well as sharing with others.

1.1 Supported analysis

- Over-representation analysis
- Gene set enrichment analysis
- Enriched subnetwork analysis
- Time series analysis

1.2 Supported data types

- Gene list
- Named phenotypes preprocessed from either **RNA-seq**, **micro-array**, **RNAi** or **CRISPR**
- **'Time Series'** data

1.3 Supported ontologies/pathways

- Gene Ontology: [GO](#)
 - Molecular function (MF)
 - Biological process (BP)
 - Cellular component (CC)
- Kyoto Encyclopedia of Genes and Genomes pathways: [KEGG](#)
- Molecular Signatures Database: [MSigDB v6.1](#)
 - h: hallmark gene sets
 - c1: positional gene sets
 - c2: curated gene sets
 - c3: motif gene sets
 - c4: computational gene sets
 - c5: GO gene sets
 - c6: oncogenic signatures
 - c7: immunologic signatures

- Customized gene sets

1.4 Supported species

- Gene Ontology and KEGG gene sets support any species that have an **OrgDb** object in [Bioconductor](#).
- MSigDB gene sets only support all 8 gene set collections for **Homo Sapiens** and three gene set collections: 'c2', 'c6' and 'c7' for **Mus musculus**.

1.5 Visualization

- GSEA plot
- Enrichment map
- Enriched subnetwork
- Interactive report for all results

Before starting the demonstration, you need to install and load the following packages:

```
library(HTSanalyzeR2)
library(org.Hs.eg.db)
library(KEGGREST)
library(GO.db)
library(igraph)
library(TxDb.Hsapiens.UCSC.hg19.knownGene)
```

2 Case study1: Single dataset analysis for gene expression data

This case study uses **HTSanalyzeR2** to perform gene set over-representation analysis, enrichment analysis and enriched subnetwork analyses on a common gene expression profile. Basically, this dataset is from a micro-array experiment on 90 colon cancer patients with GEO number named [GSE33113](#). Using the Colon Cancer Consensus Molecular Subtyping classifier generated by Guinney J et al. in 2015 (Guinney J (2015)), we can easily get the subtype label of each patient. Motivated by the poorest prognosis of CMS4 patients, we want to detect the enriched pathways of CMS4 patients compared to non-CMS4 patients. To this end, first we need to do the differential expression analysis using the most popular R package 'limma' tailored for micro-array data. However, to make this vignette simple enough, we skip to explain this step and start from the result gotten by 'limma'.

2.1 Hypergeometric test and gene set enrichment analysis

2.1.1 Prepare the input data

To perform gene set enrichment analysis for single dataset, you must prepare the following inputs:

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1. a named numeric vector of phenotypes (normally this would be a vector of genes with log2 fold change).
2. a list of gene set collections (could be generated by **HTSanalyzeR2** or use customized gene sets).

First you need to prepare a named phenotype.

```
data(GSE33113_limma)
phenotype <- as.vector(GSE33113_limma$logFC)
names(phenotype) <- rownames(GSE33113_limma)
```

Then, if you want to do hypergeometric test on a list of interested genes simultaneously, you need to define the 'hits' as your interested genes. For example, here we define the hits as genes with absolute log2 fold change greater than 1 and adjust p value less than 0.05. **In this case, the names of phenotype, namely all the input genes, would be taken as the background gene list to perform hypergeometric test.**

Note: In cases if you want to do hypergeometric test with only a list of hits and no phenotype, **HTSanalyzeR2** can also realize it. For details please go to Part5: Special usage of HTSanalyzeR2.

```
## define hits if you want to do hypergeometric test
hits <- rownames(GSE33113_limma[abs(GSE33113_limma$logFC) > 1 &
                                   GSE33113_limma$adj.P.Val < 0.05, ])
```

Then we must define the gene set collections. A gene set collection is a list of gene sets, each of which consists of a group of genes with a similar known function. **HTSanalyzeR2** provides facilities which greatly simplify the creation of up-to-date gene set collections including three Gene Ontology terms: Molecular Function (MF), Biological Process (BP), Cellular Component (CC) and KEGG pathways. Gene sets in a comprehensive molecular signatures database, **MSigDB** (Arthur Liberzon (2011)), for Homo Sapiens and Mus musculus are also provided. Here to simplify the demonstration, we will only use one GO, KEGG and one MSigDB gene set collection. To work properly, you need to choose the right species for your input genes. Besides, these gene set collections must be provided as a named list as below:

```
## generate gene set collection
GO_BP <- GOGeneSets(species="Hs", ontologies=c("BP"))
PW_KEGG <- KeggGeneSets(species="Hs")
MSig_C2 <- MSigDBGeneSets(collection = "c2", species = "Hs")

## combine all needed gene set collections into a named list for further analysis
ListGSC <- list(GO_BP=GO_BP, PW_KEGG=PW_KEGG, MSig_C2=MSig_C2)
```

2.1.2 Initialize and preprocess

An S4 class named 'GSCA' is developed to perform hypergeometric test in order to find the gene sets sharing significant overlapping with hits. Gene set enrichment analysis, as described by Subramanian et al. (Subramanian A (2005)), can also be conducted.

To initialize a new 'GSCA' object, the previous prepared phenotype and a named list of gene sets collections are needed. In addition, as said before, if you also want to do hypergeometric test, 'hits' is needed.

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```
gsca <- GSCA(listOfGeneSetCollections=ListGSC,  
             geneList=phenotype, hits=hits)
```

Then a preprocess step including invalid input data removing, duplication removing by different methods, initial gene identifiers converting to Entrez ID and phenotype ordering needs to be performed to fit for the next analysis. See the help documentation of function *preprocess* for more details.

```
gsca1 <- preprocess(gsca, species="Hs", initialIDs="SYMBOL",  
                   keepMultipleMappings=TRUE, duplicateRemoverMethod="max",  
                   orderAbsValue=FALSE)
```

2.1.3 Perform analysis

After getting a preprocessed 'GSCA' object, you can perform hypergeometric test and gene set enrichment analysis using the function named *analyze*. This function needs an argument called *para*, which is a list of parameters including:

- *pValueCutoff*: a single numeric value specifying the cutoff for adjusted p-values considered significant.
- *pAdjustMethod*: a single character value specifying the pvalue adjustment method.
- *nPermutations*: a single numeric value specifying the number of permutations for deriving p-values of GSEA.
- *minGeneSetSize*: a single numeric value specifying the minimum number of genes shared by a gene set and the background genes, namely the phenotype. Gene sets with fewer than this number are removed from both hypergeometric test and GSEA.
- *exponent*: a single integer or numeric value used in weighting phenotypes in GSEA, as described by Subramanian et al. (Subramanian A (2005)).

```
gsca2 <- analyze(gsca1,  
                para=list(pValueCutoff=0.05, pAdjustMethod="BH",  
                          nPermutations=100, minGeneSetSize=150,  
                          exponent=1),  
                doGSEA = TRUE, doGSEA = TRUE)
```

In this case study, we only use 100 permutations and set a very large *minGeneSetSize* just for a fast compilation of this vignette. In real applications, you may want a much smaller threshold (e.g. 10) and more permutations (e.g. 1000) to get a more robust GSEA result.

During the enrichment analysis of gene sets, the function evaluates the statistical significance of the gene set scores by performing a large number of permutations. To perform it more efficiently, our package allows parallel calculation based on the *doParallel* package. To do this, the user simply needs to register and claim to use multiple cores **before** running *analyze*.

```
## analyze using 4 cores  
if (requireNamespace("doParallel", quietly=TRUE)) {  
  doParallel::registerDoParallel(cores=4)  
} else {  
}  
  
gsca2 <- analyze(gsca1,  
                para=list(pValueCutoff=0.05, pAdjustMethod="BH",
```

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```
nPermutations=100, minGeneSetSize=150,
exponent=1),
doGSOA = TRUE, doGSEA = TRUE)
```

After analyzing, all the results are stored in slot *result* and can be easily accessed using a function named *getResult*. If hypergeometric test and GSEA are both performed, gene sets which are both significant in this two kinds of analysis based on either pvalue or adjusted pvalue can be accessed.

```
## 1. hypergeometric test result using BP gene sets from GO
head(getResult(gsca2)$HyperGeo.results$G0_BP, 3)
##           Universe Size Gene Set Size Total Hits Expected Hits
## G0:0030198          18865           192         450      4.579910
## G0:0007155          18865           415         450      9.899284
## G0:0044267          18865           188         450      4.484495
##           Observed Hits           Pvalue Adjusted.Pvalue
## G0:0030198             43 1.436797e-29    4.998542e-28
## G0:0007155             50 2.486029e-21    5.135204e-20
## G0:0044267             22 8.440134e-10    7.539093e-09

## 2. GSEA result using KEGG gene sets
head(getResult(gsca2)$GSEA.results$PW_KEGG, 3)
##           Observed.score Pvalue Adjusted.Pvalue
## hsa04022      0.4977871      0              0
## hsa00230     -0.3699180      0              0
## hsa05152      0.5278489      0              0

## 3. result both significant regarding to pvalues in hypergeometric test
## and GSEA using 'c2' gene sets from MSigDB
head(getResult(gsca2)$Sig.pvals.in.both$MSig_C2, 3)
##                                     HyperGeo.Pvalue
## TAKEDA_TARGETS_OF_NUP98_H0XA9_FUSION_8D_UP      1.590818e-05
## TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ERYTHROCYTE_UP 1.590818e-05
## BIDUS_METASTASIS_DN      1.039777e-02
##                                     GSEA.Pvalue
## TAKEDA_TARGETS_OF_NUP98_H0XA9_FUSION_8D_UP      0
## TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ERYTHROCYTE_UP 0
## BIDUS_METASTASIS_DN      0

## 4. result both significant regarding to adjust pvalues in hypergeometric test
## and GSEA using 'c2' gene sets from MSigDB
head(getResult(gsca2)$Sig.adj.pvals.in.both$MSig_C2, 3)
##                                     HyperGeo.Adj.Pvalue
## TAKEDA_TARGETS_OF_NUP98_H0XA9_FUSION_8D_UP      8.412245e-05
## TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ERYTHROCYTE_UP 8.412245e-05
## BIDUS_METASTASIS_DN      3.041117e-02
##                                     GSEA.Adj.Pvalue
## TAKEDA_TARGETS_OF_NUP98_H0XA9_FUSION_8D_UP      0
## TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ERYTHROCYTE_UP 0
## BIDUS_METASTASIS_DN      0
```

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In addition, to make the results more understandable, users are highly recommended to annotate the gene sets ID to names by function *appendGSTerms*. As a result, an additional column named 'Gene.Set.Term' would appear.

```
gsca3 <- appendGSTerms(gsca2, goGSCs=c("GO_BP"),
                      keggGSCs=c("PW_KEGG"),
                      msigdbGSCs = c("MSig_C2"))
head(getResult(gsca3)$GSEA.results$PW_KEGG, 3)
##               Gene.Set.Term Observed.score Pvalue Adjusted.Pvalue
## hsa04022 cGMP-PKG signaling pathway    0.4977871      0           0
## hsa00230 Purine metabolism    -0.3699180      0           0
## hsa05152 Tuberculosis      0.5278489      0           0
```

2.1.4 Summarize results

A *summarize* method could be performed to get a general summary for an analyzed 'GSCA' object including the gene set collections, genelist, hits, parameters for analysis and the summary of result.

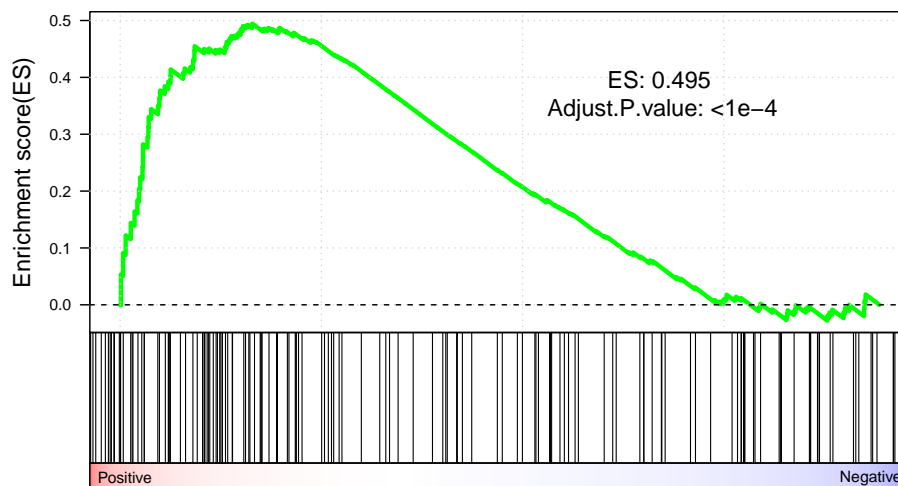
```
summarize(gsca3)
##
## -No of genes in Gene set collections:
##      input above min size
## GO_BP  11857           90
## PW_KEGG  329           38
## MSig_C2 3762          533
##
##
## -No of genes in Gene List:
##      input valid duplicate removed converted to entrez
## Gene List 21656 21655           21655           18865
##
##
## -No of hits:
##      input preprocessed
## Hits  469           450
##
##
## -Parameters for analysis:
##      minGeneSetSize pValueCutoff pAdjustMethod
## HyperGeo Test 150           0.05           BH
##
##      minGeneSetSize pValueCutoff pAdjustMethod nPermutations exponent
## GSEA 150           0.05           BH           100           1
##
##
## -Significant gene sets (adjusted p-value< 0.05 ):
##      GO_BP PW_KEGG MSig_C2
## HyperGeo  26      5      224
## GSEA      54     21      402
## Both      25      5      202
```


2.1.5 Plot gene sets

To better view the GSEA result for a single gene set, you can use *viewGSEA* to plot the positions of the genes of the gene set in the ranked phenotypes and the location of the enrichment score. To this end, you must first get the gene set ID by *getTopGeneSets*, which can return all or the top significant gene sets from GSEA results. Basically, the user needs to specify the type of results – “HyperGeo.results” or “GSEA.results”, the name(s) of the gene set collection(s) as well as the type of selection– all (by parameter ‘allSig’) or top (by parameter ‘ntop’) significant gene sets.

```
topGS <- getTopGeneSets(gsca3, resultName="GSEA.results",
                        gscs=c("G0_BP", "PW_KEGG"), allSig=TRUE)

topGS
## $G0_BP
##      G0:0006397      G0:0006412      G0:0043123      G0:0008360      G0:0008380
## "G0:0006397" "G0:0006412" "G0:0043123" "G0:0008360" "G0:0008380"
##      G0:0007568      G0:0001666      G0:0090090      G0:0007596      G0:0051607
## "G0:0007568" "G0:0001666" "G0:0090090" "G0:0007596" "G0:0051607"
##      G0:0032496      G0:0006364      G0:0007411      G0:0030335      G0:0007507
## "G0:0032496" "G0:0006364" "G0:0007411" "G0:0030335" "G0:0007507"
##      G0:0044267      G0:0006974      G0:0030198      G0:0006281      G0:0016477
## "G0:0044267" "G0:0006974" "G0:0030198" "G0:0006281" "G0:0016477"
##      G0:0070374      G0:0001525      G0:0000398      G0:0007049      G0:0007267
## "G0:0070374" "G0:0001525" "G0:0000398" "G0:0007049" "G0:0007267"
##      G0:0010628      G0:0019221      G0:0043547      G0:0007399      G0:0043065
## "G0:0010628" "G0:0019221" "G0:0043547" "G0:0007399" "G0:0043065"
##      G0:0006955      G0:0051301      G0:0043687      G0:0006954      G0:0008285
## "G0:0006955" "G0:0051301" "G0:0043687" "G0:0006954" "G0:0008285"
##      G0:0010469      G0:0045087      G0:0007155      G0:0008284      G0:0043312
## "G0:0010469" "G0:0045087" "G0:0007155" "G0:0008284" "G0:0043312"
##      G0:0043066      G0:0007275      G0:0030154      G0:0007186      G0:0045944
## "G0:0043066" "G0:0007275" "G0:0030154" "G0:0007186" "G0:0045944"
##      G0:0007165      G0:0001701      G0:0016055      G0:0006511      G0:0007601
## "G0:0007165" "G0:0001701" "G0:0016055" "G0:0006511" "G0:0007601"
##      G0:0016579      G0:0015031      G0:0055114      G0:0000122
## "G0:0016579" "G0:0015031" "G0:0055114" "G0:0000122"
##
## $PW_KEGG
##      hsa04022      hsa00230      hsa05152      hsa05016      hsa05202      hsa04360      hsa04020
## "hsa04022" "hsa00230" "hsa05152" "hsa05016" "hsa05202" "hsa04360" "hsa04020"
##      hsa04510      hsa05205      hsa04015      hsa04714      hsa04810      hsa04014      hsa04060
## "hsa04510" "hsa05205" "hsa04015" "hsa04714" "hsa04810" "hsa04014" "hsa04060"
##      hsa04010      hsa05165      hsa04151      hsa05200      hsa01100      hsa05206      hsa04062
## "hsa04010" "hsa05165" "hsa04151" "hsa05200" "hsa01100" "hsa05206" "hsa04062"
viewGSEA(gsca3, gscName="G0_BP", gsName=topGS[["G0_BP"]][3])
```



You can also plot all or the top significant gene sets in batch and store them as png or pdf format into a specified path by using `plotGSEA`.

```
plotGSEA(gsca3, gscs=c("GO_BP", "PW_KEGG"), ntop=3, filepath=".")
```

2.1.6 Enrichment Map

To get a comprehensive view of the hypergeometric test result or GSEA result instead of a list of significant gene sets with no relations, our package provides `viewEnrichMap` function to draw an enrichment map for better interpretation (Merico D (2010)). More specifically, in the enrichment map, nodes represent significant gene sets sized by the genes it contains and the edge represents the Jaccard similarity coefficient between two gene sets. Nodes color are scaled according to the adjusted pvalues (the darker, the more significant). For hypergeometric test, there is only one color for nodes whereas for GSEA enrichment map, the default color is set by the sign of enrichment scores (red:+, blue:-). You can also set your favourite format by changing the parameter named 'options'.

However, users are always highly recommended to use function `report` to visualize and modify the enrichment map with personal preference in an interactive report, such as different layout, color and size of nodes, types of labels and etc. More details please go to Part4: An interactive Shiny report of results.

```
## the enrichment map for top 5 significant gene sets in 'PW_KEGG' and 'GO_MF'
viewEnrichMap(gsca3, gscs=c("PW_KEGG", "GO_BP"),
  allSig = FALSE, gsNameType = "term", ntop = 5)
```

2.1.7 Enrichment Map with specific gene sets

It is often the case that the enrichment map would be of large size due to the huge number of enriched gene sets. However, you may only be interested in a small part of them. A big size of enrichment map would also be in a mess and lose the information it can offer. In that way, **HTSanalyzeR2** provides an interface allowing users to draw the enrichment map on their interested gene sets. More details please see the help documentation of function `viewEnrichMap`.

Figure 1:

```
## specificGeneset needs to be a subset of all analyzed gene sets
## which can be roughly gotten by:
tmp <- getTopGeneSets(gscs3, resultName = "GSEA.results", gscs=c("PW_KEGG"),
                     ntop = 200, allSig = FALSE)

## In that case, we can define specificGeneset such as below:
PW_KEGG_geneset <- tmp$PW_KEGG[c(2, 3, 6, 7, 10, 11)]

## the name of specificGenesets also needs to match with the names of tmp
specificGeneset <- list("PW_KEGG"=PW_KEGG_geneset)
viewEnrichMap(gscs3, resultName = "GSEA.results", gscs=c("PW_KEGG"),
              allSig = FALSE, gsNameType = "term",
              ntop = NULL, specificGeneset = specificGeneset)
```

2.2 Enriched subnetwork analysis

You can also perform subnetwork analysis (Beisser (2010), Dittrich MT (2008)) to extract the subnetwork enriched with nodes which are associated with a significant phenotype using **HTSanalyzeR2**. The network can either be fetched by our package to download specific species network from BioGRID database or defined by users.

2.2.1 Prepare input, initialize and preprocess

An S4 class named 'NWA' is developed to perform subnetwork analysis. To initiate an 'NWA' object, you need to prepare a named numeric vector called 'pvalues'. If phenotypes for genes are also available, they can be inputted in the initialization step and used to highlight nodes with different colors in the identified subnetwork. In that case, the nodes are colored by the sign of phenotypes (red:+, blue:-).

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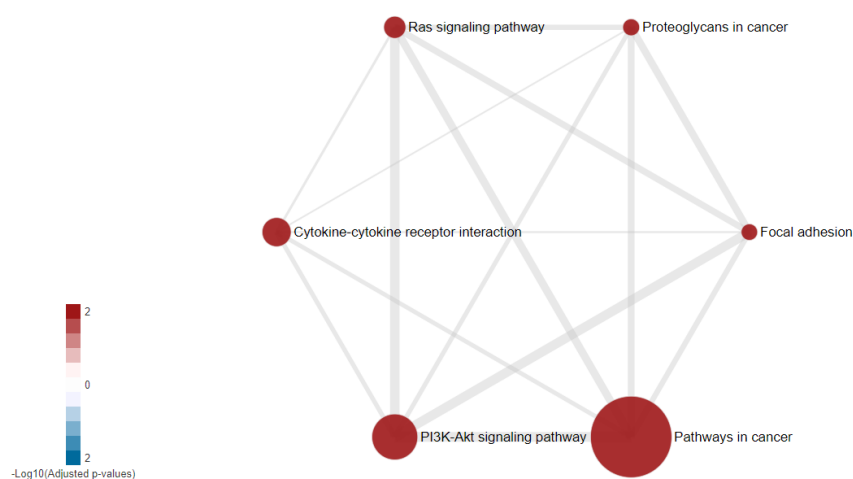


Figure 2:

When creating a new object of class 'NWA', the user also has the possibility to specify the parameter 'interactome' which should be an object of class 'igraph'. If it is not available, the interactome can also be set up later.

```
pvalues <- GSE33113_limma$adj.P.Val
names(pvalues) <- rownames(GSE33113_limma)
nwa <- NWA(pvalues=pvalues, phenotypes=phenotype)
```

The next step is to preprocess the inputs. Similar to 'GSCA' class, the function *preprocess* can conduct invalid input data removing, duplication removing by different methods and initial gene identifiers converting to Entrez ID.

```
nwa1 <- preprocess(nwa, species="Hs", initialIDs="SYMBOL",
  keepMultipleMappings=TRUE, duplicateRemoverMethod="max")
```

Then, you need to create an interactome for the network analysis using method *interactome* if you have not inputted your own interactome in the initial step. To this end, you can either specify the species and fetch the corresponding network from BioGRID database, or input an interaction matrix if it is in right format: a matrix with a row for each interaction, and at least contains the three columns "InteractorA", "InteractorB" and "InteractionType", where the interactors are specified by Entrez ID. For more details please see *help(interactome)*.

Here, we just use *interactome* to download an interactome from BioGRID, which would meet user's requirements in most cases.

```
nwa2 <- interactome(nwa1, species="Hs", genetic=FALSE)

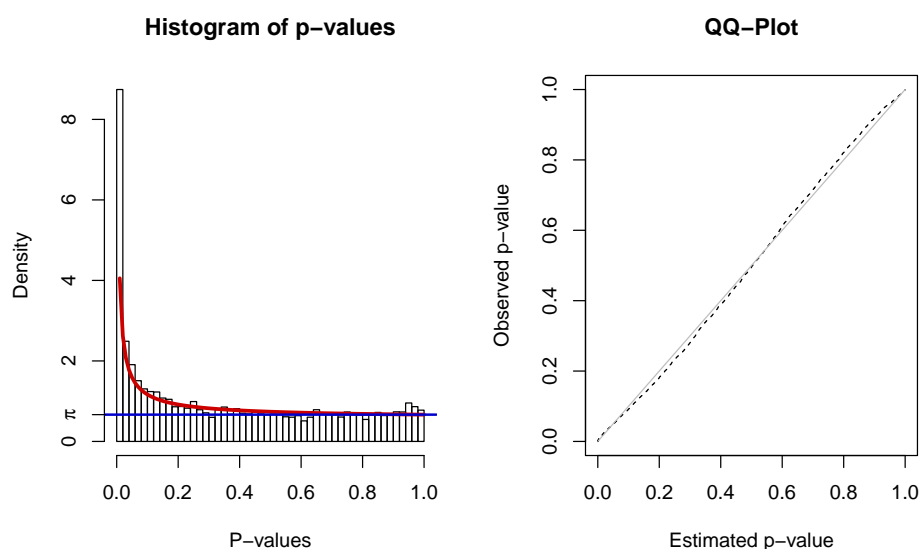
getInteractome(nwa2)
```

2.2.2 Perform analysis and view the identified subnetwork

Having preprocessed the input data and created the interactome, the subnetwork analysis could be performed by using the *analyze* method. This function will plot a figure showing the fitting of the BioNet model to your distribution of pvalues (Beisser (2010)), which is a good way to check the choice of statistics used in this function. The argument *fdr* of the method *analyze* is the false discovery rate for BioNet to fit the beta-uniform mixture (BUM) model. The parameters of the fitted model will then be used for the scoring function, which subsequently enables the BioNet package to search the optimal scoring subnetwork. See BioNet for more details (Beisser (2010)).

Here, to simplify this vignette, we set a very strict 'fdr' as 1e-07. In practice, you may want to set a less strict one (e.g. 0.01)

```
nwa3 <- analyze(nwa2, fdr=1e-07, species="Hs")
```



Similar to 'GSCA', you can also view the subnetwork by *viewSubNet*. Again, for better visualization, modification and downloading, users are highly recommended to view the result in an interactive Shiny report by function *report*.

```
viewSubNet(nwa3)
```

2.2.3 Summarize results

Like 'GSCA', the method *summarize* could also be used to get a general summary of an analyzed 'NWA' object including inputs, interactome, parameters for analysis and the size of identified subnetwork.

```
summarize(nwa3)
##
## -p-values:
##          input          valid  duplicate removed
##          21656          21655          21655
## converted to entrez      in interactome
```

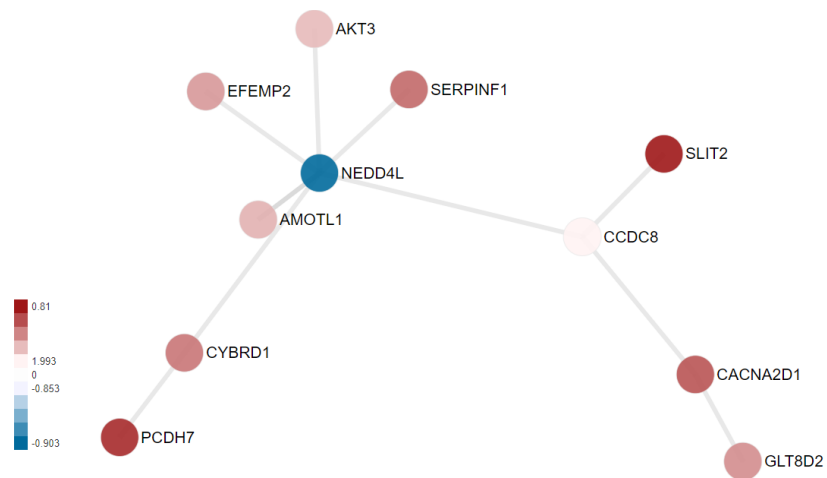


Figure 3:

```
##          18865          14287
##
##
## -Phenotypes:
##          input          valid  duplicate removed
##          21656          21655          21655
## converted to entrez      in interactome
##          18865          14287
##
##
## -Interactome:
##          name    species genetic node No edge No
## Interaction dataset Biogrid Hs      FALSE  20223  258975
##
##
## -Parameters for analysis:
##          FDR
## Parameter 1e-07
##
##
## -Subnetwork identified:
##          node No edge No
## Subnetwork    11    11
```

3 Case study2: Time series analysis for CRISPR data

This case study uses a time series CRISPR genome-wide drop-out data as a demonstration to perform time series analysis. Data 'd7', 'd13' and 'd25' are three gRNA sequencing data after transducing the CRISPR system into a human cancer cellline on day 7, day 13 and day 25 separately (Tzelepis K (2016)), they are further preprocessed by [MAGECK] (<https://sourceforge.net/p/mageck/wiki/Home/>) to identify significant essential genes from genome-scale CRISPR knockout screens. Again, here to simply the compilation of this vignette, we skip the preprocessed steps and start from the results gotten by MAGECK.

3.1 Hypergeometric test and gene set enrichment analysis

3.1.1 Prepare the input data

To perform analysis for time series data, one must prepare the following inputs:

1. A character matrix contains experiment information with each experiment in row and descriptions in column. Specifically, it should at least contain two columns named as 'ID' and 'Description'.
2. A list of phenotypes, each element of this list is a phenotype of that experiment. **An important thing here needs to be noted is the order of each element of this list must match the order of 'ID' in the experiment information matrix.**
3. A list of gene set collections which can either be gotten by HTSanalyzeR2 or defined by users using customized gene sets.

To make it easy to compile this vignette, here we only use Biological Process (BP) in Gene Ontology to make a demonstration.

```
data(d7, d13, d25)
expInfor <- matrix(c("d7", "d13", "d25"), nrow = 3, ncol = 2, byrow = FALSE,
                  dimnames = list(NULL, c("ID", "Description")))
datalist <- list(d7, d13, d25)
phenotypeTS <- lapply(datalist, function(x) {
  tmp <- as.vector(x$neg.lfc)
  names(tmp) <- x$id
  tmp
})

GO_BP <- GOGeneSets(species="Hs", ontologies=c("BP"))
ListGSC <- list(GO_BP=GO_BP)
```

Similar as single dataset analysis, if you also want to do hypergeometric test, a list of hits is needed. Here, each element of this list is a hits of that experiment. Also, **the order of each element of this list must match the order of 'ID' in the experiment information matrix.** Here, for each data set, we define genes with *pvalue* less than 0.01 as hits.

```
hitsTS <- lapply(datalist, function(x){
  tmp <- x[x$neg.p.value < 0.01, "id"]
})
```

```
tmp
})
```

3.1.2 Initialize and preprocess

To perform gene set enrichment analysis and hypermetric test for time-series data, an S4 class 'GSCABatch' which can pack the time series data to do further analysis is developed. First, you need to create a new 'GSCABatch' object using the prepared inputs.

```
gscaTS <- GSCABatch(expInfor = expInfor,
                    phenotypeTS = phenotypeTS, listOfGeneSetCollections = ListGSC,
                    hitsTS = hitsTS)
```

Then, the 'GSCABatch' object need to be preprocessed using *preprocessGscaTS* method. The preprocess procedure here is the same as single data set. This step would return a list of preprocessed 'GSCA' object.

```
gscaTS1 <- preprocessGscaTS(gascaTS, species="Hs", initialIDs="SYMBOL",
                             keepMultipleMappings=TRUE,
                             duplicateRemoverMethod="max",
                             orderAbsValue=FALSE)
```

3.1.3 Perform analysis

After getting a list of preprocessed 'GSCA' object, you can use *analyzeGscaTS* to perform hypergeometric test as well as GSEA on it. The parameters' function here is the same as in single data set. Similarly, to speed up you can use multiple cores via *doParallel* package. This step would return a list of analyzed 'GSCA' object.

```
## analyze using 4 cores
if (requireNamespace("doParallel", quietly=TRUE)) {
  doParallel::registerDoParallel(cores=4)
} else {
}

gscaTS2 <- analyzeGscaTS(gascaTS1, para=list(pValueCutoff=0.05,
                                             pAdjustMethod="BH",
                                             nPermutations=100,
                                             minGeneSetSize=100,
                                             exponent=1),
                        doGSOA = TRUE, doGSEA = TRUE)

## GSEA result of the first experiment usingBP gene sets
head(getResult(gascaTS2[[1]])$GSEA.results$G0_BP, 3)
```

To make the result more understandable, users are highly recommended to annotate the gene sets ID to names by function *appendGSTermsTS*. As a result, an additional column named 'Gene.Set.Term' would appear.


```
gscaTS3 <- appendGSTermsTS(gscaTS2, goGSCs=c("GO_BP"))
head(getResult(gscaTS3[[1]])$GSEA.results$GO_BP, 3)
##                               Gene.Set.Term Observed.score Pvalue
## GO:0006281                DNA repair      -0.6898409      0
## GO:0001525                angiogenesis    -0.1840823      0
## GO:0000398 mRNA splicing, via spliceosome -0.8693455      0
##                               Adjusted.Pvalue
## GO:0006281                0
## GO:0001525                0
## GO:0000398                0
```

You can then use *reportAll* to generate an interactive Shiny report to visualize a union enrichment map for this time series data. To put it more specific, a union enrichment map is generated by taking the union of significant gene sets in each experiment and then form an enrichment map as illustrated before. Thus there maybe be some gene sets not significant in one time point but in others. More details please see Part4: An interactive Shiny report of results.

3.2 Enriched subnetwork analysis

3.2.1 Prepare input, initialize and preprocess

An S4 class named 'NWABatch' is developed to pack time series data for further subnetwork analysis. You need first to create a new object of class 'NWABatch'. To this end, a list of pvalues is needed. Each element of this list is a vector of pvalues of that experiment. **Again, an important thing needs to be noted is the order of each element of this list must match the order of 'ID' in the experiment information matrix.** If a list of phenotypes is also available, they can be inputted during the initialization stage and used to highlight nodes with different colors in the identified subnetwork. Also, the order of each element of this phenotypes list must match the order of 'ID' in the experiment information matrix.

```
pvalueTS <- lapply(datalist, function(x){
  tmp <- as.vector(x$neg.p.value)
  names(tmp) <- x$id
  tmp
})

## generate a new 'NWABatch' object for further analysis
nwaTS <- NWABatch(expInfor = expInfor,
  pvalueTS = pvalueTS, phenotypeTS = phenotypeTS)
```

After creating an object of 'NWABatch', a preprocessing step needs to be performed which will return a list of preprocessed 'NWA' objects.

```
nwaTS1 <- preprocessNwaTS(nwaTS, species="Hs", initialIDs="SYMBOL",
  keepMultipleMappings=TRUE,
  duplicateRemoverMethod="max")
```

3.2.2 Perform analysis

Similarly, an interactome needs to be created before performing subnetwork analysis using *interactomeNwaTS* if you have not inputted your own interactome in the initial step. You can either specify the species and fetch the corresponding network from BioGRID database, or input an interaction matrix if it is in right format. More details please see *help(interactomeNwaTS)*.

Then, *analyzeNwaTS* could perform the subnetwork analysis for a list of 'NWA' object, which would take a few minutes. Finally, this step would return a list of analyzed 'NWA' objects.

```
nwaTS2 <- interactomeNwaTS(nwaTS1, species="Hs",  
                           reportDir="HTSanalyzerReport", genetic=FALSE)  
nwaTS3 <- analyzeNwaTS(nwaTS2, fdr=0.0001, species="Hs")
```

```
## get a general summary for the first experiment  
summarize(nwaTS3[[1]])
```

You can then use *reportAll* to generate an interactive Shiny report to visualize a union subnetwork for this time series data. To put it more specific, a union subnetwork is generated by taking the union of identified subnetwork in each experiment. Thus there maybe be some genes not identified in the subnetwork of one time point but in others. More details please see Part4: An interactive Shiny report of results.

4 An interactive Shiny report of results

To better visualize all the results, our package could generate an interactive Shiny report containing all the results in. For single data set result such as 'gsca3' and 'nwa3' generated by the above analysis, you can either use *report* or *reportAll* as below:

```
## 1. generate an interactive shiny report for gsca object using report  
report(gsca=gsca3)  
  
## 2. generate an interactive shiny report for nwa object using report  
report(nwa=nwa3)  
  
## 3. generate an interactive shiny report for gsca object using reportAll  
reportAll(gsca=gsca3)  
  
## 4. generate an interactive shiny report for both gsca  
##    and nwa object using reportAll  
reportAll(gsca=gsca3, nwa=nwa3)
```

For time series data, you should use *reportAll* to generate the report. In addition, you can reset the order of time series data for visualization by setting the argument 'TSOrder'.

```
## 1. generate an interactive shiny report for  
##    a list of gsca objects using reportAll  
reportAll(gsca=gscaTS3)  
  
## 2. generate an interactive shiny report for  
##    a list of nwa objects using reportAll
```

```
reportAll(nwa=nwaTS3)

## 3. generate an interactive shiny report for a list of
##   gsca objects by customized order using reportAll
reportAll(gsca=gscaTS3, TSOrder=names(gscaTS3)[c(3, 1, 2)])
```

In the interactive report, for hypergeometric test and GSEA results of single data set, you can download the table of different gene set collection in different format such as 'csv' or 'pdf'. On the right of this interface, there is the summary information about this analysis. For the dynamic enrichment map, you can change the layout, set node size and color, label types, edge thickness and download it as 'svg' format. For subnetwork analysis result, you can also change the above mentioned items to fit your requirements.

Intriguingly, for time series data result, you can see a dynamic change for each 'time point' in either the union enrichment map or the union subnetwork, which could give you a general view about the difference among each time point.

Similar with single data set analysis, you can also see the enrichment map of specific genesets for time series data by specifying the argument 'specificGeneset' in *reportAll*.

```
## As told previously, specificGeneset needs to be a subset of all
## analyzed gene sets which can be roughly gotten by:
tmp <- getTopGeneSets(gscaTS3[[1]], resultName = "GSEA.results",
                     gscs=c("G0_BP"), ntop = 20000, allSig = FALSE)

## In that case, we can define specificGeneset as below:
G0_BP_geneset <- tmp$G0_BP[c(4,2,6,9,12)]

## the name of specificGenesets also needs to match with the names of tmp
specificGeneset <- list("G0_BP"=G0_BP_geneset)
reportAll(gsca=gscaTS3, specificGeneset=specificGeneset)
```

After calling *report* or *reportAll*, it would automatically generate a directory with names starting with "GSCARReport", "NWARReport" or "AnalysisReport" which includes the result named as "results.RData" and an R script named as "app.R". Open "app.R" in RStudio, users can publish and share the report with others via [Shinyapps.io](https://shinyapps.io), details please go to [Shinyapps.io](https://shinyapps.io).

5 Special usage of HTSanalyzeR2

5.1 Hypergeometric test with no phenotype

In case if you only have a list of genes and want to do hypergeometric test with gene sets having known functions, **HTSanalyzeR2** provides an interface to realize it. Since phenotype is only used as background genes in hypergeometric test, you can manually set all the genes of that species as phenotype and give them a pseudo value to fit **HTSanalyzeR2** as below:

```
data(d7)
hits1 <- d7$id[1:200]

## set all the coding genes of Homo sapiens as phenotype
```

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```
allgenes <- keys(TxDb.Hsapiens.UCSC.hg19.knownGene, keytype = "GENEID")
## change Entrez ID to gene name to keep consistent with hits
allgenes <- mapIds(org.Hs.eg.db, keys = allgenes, keytype = "ENTREZID", column = "SYMBOL")

## give phenotype a pseudo value to fit for HTSanalyzeR2
phenotype <- rep(1, length(allgenes))
names(phenotype) <- allgenes
```

Then, you can use the artificial phenotype as gene background and your hits to perform hypergeometric test.

```
gsca <- GSCA(listOfGeneSetCollections=ListGSC,
             geneList=phenotype, hits=hits1)
## the following analysis is the same as before
```

5.2 Customized gene set

When you have your own gene sets with specific functions, but they do not belong to any GO terms, KEGG or MSigDB. In that case, you can set your customized gene set collection and follow the format of GO, KEGG and MSigDB gene set collections. An important thing here you need pay attention to is the ID of genes in the gene set collection must be Entrez ID.

```
## Suppose your own gene sets is geneset1 and geneset2
allgenes <- keys(org.Hs.eg.db, "ENTREZID")
geneset1 <- allgenes[sample(length(allgenes), 100)]
geneset2 <- allgenes[sample(length(allgenes), 60)]

## Set your custom gene set collection and make the format to fit HTSanalyzeR2
CustomGS <- list("geneset1" = geneset1, "geneset2" = geneset2)

## then the gene set collections would be as below:
ListGSC <- list(CustomGS=CustomGS)
## other part is the same as before
```

5.3 An interface to fgsea package

HTSanalyzeR2 also provides an interface to [fgsea](#) package which proposes a novel algorithm for fast preranked gene set enrichment analysis using cumulative statistic calculation. Details please see [fgsea](#) (Sergushichev (2016)).

To perform GSEA by [fgsea](#) instead of HTSanalyzeR2, users need to specify the parameter *GSEA.by* in *analyze* (for single data set) or *analyzeGscaTS* (for time series analysis) with *fgsea*. Otherwise, it will use **HTSanalyzeR2** by default.

```
gsca4 <- analyze(gsca1,
                para=list(pValueCutoff=0.05, pAdjustMethod="BH",
                          nPermutations=100, minGeneSetSize=150,
                          exponent=1),
```

```
doGSOA = TRUE, doGSEA = TRUE,
GSEA.by = "fgsea")
```

5.4 Extract intersected genes between enriched pathways and input gene list

Once you've finished the GSEA or hypergeometric test, you may be interested in some pathways and wonder which genes are shared by that pathway and your input gene list.

```
## Suppose you are interested in "cell adhesion" in Biological processes in Gene Ontology,
## We can retrieve the GO ID of this pathway:
GO_BPresult <- getResult(gsca3)$HyperGeo.results$GO_BP
cell_adhesion_GOID <- rownames(GO_BPresult[GO_BPresult$Gene.Set.Term == "cell adhesion", ])

## Then get the genes in this pathway
cell_adhesion_gene <- GO_BP[[cell_adhesion_GOID]]

## change Entrez ID to gene symbol
cell_adhesion_gene <- mapIds(org.Hs.eg.db, keys = cell_adhesion_gene,
                             keytype = "ENTREZID", column = "SYMBOL")
## 'select()' returned 1:1 mapping between keys and columns

## get the shared genes between this pathway and your input gene list
intersect(cell_adhesion_gene, hits)
## [1] "CD36" "CDH2" "CDH11" "COL5A1" "COL6A2" "COL6A3" "COL8A1"
## [8] "COL12A1" "COL16A1" "COMP" "VCAN" "CTGF" "CYP1B1" "DPT"
## [15] "EPA3" "FAP" "FN1" "CYR61" "ISLR" "ITGAM" "LSAMP"
## [22] "MYH10" "NOV" "DDR2" "OMD" "OLR1" "CXCL12" "SPOCK1"
## [29] "SPP1" "THBS2" "TNFAIP6" "VCAM1" "ADAM12" "SSPN" "SRPX"
## [36] "AOC3" "MPDZ" "WISP1" "ITGBL1" "FEZ1" "NUAK1" "EDIL3"
## [43] "SPON1" "GPNMB" "POSTN" "CHL1" "EMILIN1" "ITGA11" "CERCAM"
## [50] "BOC"
```

6 A pipeline function for CRISPR data pre-processed by MAGeCK

For the CRISPR data pre-processed by MAGeCK, we also provide a pipeline function to do a comprehensive analysis including GSEA and subnetwork analysis, which would be seamless linking with MAGeCK and provides great convenience to the users. Finally, it would automatically generate a dynamic shiny report containing all the results.

```
ListGSC = list(GO_BP=GO_BP, PW_KEGG=PW_KEGG)
HTSanalyzeR4MAGeCK(MAGeCKdata = d7,
                    selectDirection = "negative",
                    doGSOA = FALSE,
                    doGSEA = TRUE,
```

```
listOfGeneSetCollections = ListGSC,  
species = "Hs",  
initialIDs = "SYMBOL",  
pValueCutoff = 0.05,  
pAdjustMethod = "BH",  
nPermutations = 100,  
minGeneSetSize = 100,  
exponent = 1,  
keggGSCs=c("PW_KEGG"),  
goGSCs = c("GO_BP"),  
msigdbGSCs = NULL,  
doNWA = TRUE,  
reportDir = "HTSanalyzerReport",  
nwAnalysisGenetic = FALSE,  
nwAnalysisFdr = 0.001)
```

7 Session Info

```
## R version 3.5.0 (2018-04-23)  
## Platform: x86_64-pc-linux-gnu (64-bit)  
## Running under: Ubuntu 18.04 LTS  
##  
## Matrix products: default  
## BLAS: /usr/lib/x86_64-linux-gnu/openblas/libblas.so.3  
## LAPACK: /usr/lib/x86_64-linux-gnu/libopenblas-p-r0.2.20.so  
##  
## locale:  
##  [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C  
##  [3] LC_TIME=en_US.UTF-8      LC_COLLATE=en_US.UTF-8  
##  [5] LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8  
##  [7] LC_PAPER=en_US.UTF-8     LC_NAME=C  
##  [9] LC_ADDRESS=C             LC_TELEPHONE=C  
## [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C  
##  
## attached base packages:  
## [1] parallel stats4      stats      graphics  grDevices  utils      datasets  
## [8] methods   base  
##  
## other attached packages:  
##  [1] TxDb.Hsapiens.UCSC.hg19.knownGene_3.2.2  
##  [2] GenomicFeatures_1.32.0  
##  [3] GenomicRanges_1.32.3  
##  [4] GenomeInfoDb_1.16.0  
##  [5] igraph_1.2.1  
##  [6] GO.db_3.6.0  
##  [7] KEGGREST_1.20.0  
##  [8] org.Hs.eg.db_3.6.0  
##  [9] AnnotationDbi_1.42.1
```

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```
## [10] IRanges_2.14.10
## [11] S4Vectors_0.18.3
## [12] Biobase_2.40.0
## [13] BiocGenerics_0.26.0
## [14] HTSanalyzeR2_0.99.13
## [15] BiocStyle_2.8.2
##
## loaded via a namespace (and not attached):
## [1] fgsea_1.6.0           colorspace_1.3-2
## [3] hwriter_1.3.2         rprojroot_1.3-2
## [5] XVector_0.20.0        affyio_1.50.0
## [7] DT_0.4                bit64_0.9-7
## [9] mvtnorm_1.0-8         codetools_0.2-15
## [11] splines_3.5.0         doParallel_1.0.11
## [13] robustbase_0.93-1     knitr_1.20
## [15] splots_1.46.0         Rsamtools_1.32.0
## [17] prada_1.56.0          annotate_1.58.0
## [19] cluster_2.0.7-1       Rmpfr_0.7-0
## [21] vsn_3.48.1            png_0.1-7
## [23] shinydashboard_0.7.0  graph_1.58.0
## [25] shiny_1.1.0           rrcov_1.4-4
## [27] compiler_3.5.0        httr_1.3.1
## [29] backports_1.1.2       assertthat_0.2.0
## [31] Matrix_1.2-14         lazyeval_0.2.1
## [33] limma_3.36.2          later_0.7.3
## [35] prettyunits_1.0.2     htmltools_0.3.6
## [37] tools_3.5.0           gmp_0.5-13.1
## [39] gtable_0.2.0          GenomeInfoDbData_1.1.0
## [41] affy_1.58.0           Category_2.46.0
## [43] fastmatch_1.1-0       Rcpp_0.12.17
## [45] Biostrings_2.48.0     preprocessCore_1.42.0
## [47] rtracklayer_1.40.3    iterators_1.0.9
## [49] xfun_0.2              stringr_1.3.1
## [51] mime_0.5              miniUI_0.1.1.1
## [53] XML_3.98-1.11         BioNet_1.40.0
## [55] DEoptimR_1.0-8        zlibbioc_1.26.0
## [57] MASS_7.3-50           scales_0.5.0
## [59] RankProd_3.6.0        BiocInstaller_1.30.0
## [61] colourpicker_1.0      hms_0.4.2
## [63] promises_1.0.1        SummarizedExperiment_1.10.1
## [65] RBGL_1.56.0           RColorBrewer_1.1-2
## [67] curl_3.2              yaml_2.1.19
## [69] memoise_1.1.0         gridExtra_2.3
## [71] ggplot2_2.2.1         biomaRt_2.36.1
## [73] stringi_1.2.3         RSQLite_2.1.1
## [75] genefilter_1.62.0     cellHTS2_2.44.0
## [77] pcaPP_1.9-73          foreach_1.4.4
## [79] BiocParallel_1.14.1   matrixStats_0.53.1
## [81] rlang_0.2.1           pkgconfig_2.0.1
## [83] bitops_1.0-6          evaluate_0.10.1
## [85] lattice_0.20-35       GenomicAlignments_1.16.0
```

```
## [87] htmlwidgets_1.2      bit_1.1-14
## [89] GSEABase_1.42.0      plyr_1.8.4
## [91] magrittr_1.5         bookdown_0.7
## [93] R6_2.2.2             DelayedArray_0.6.1
## [95] DBI_1.0.0            pillar_1.2.3
## [97] survival_2.42-3      RCurl_1.95-4.10
## [99] tibble_1.4.2         crayon_1.3.4
## [101] rmarkdown_1.10       progress_1.2.0
## [103] locfit_1.5-9.1       grid_3.5.0
## [105] data.table_1.11.4    blob_1.1.1
## [107] digest_0.6.15        xtable_1.8-2
## [109] httpuv_1.4.4.1       munsell_0.5.0
```

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